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Callus induction and RAPD analysis of *Simarouba glauca* DC

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Callus induction for somatic embryogenesis from *Simarouba glauca* DC leaf explants of three genotypes (*S. glauca* 5, *S. glauca* 19 and *S. glauca* 21) was studied. Leaf explants (leaf segments from basal, middle and tip of the leaves) were cultured on two types of nutrient media; SGC1 and SGC2. Both media contained Murashige and Skoog (MS) medium with vitamins: 100 mg/L ascorbic acid, 0.5 mg/L 6-benzylaminopurine (BAP), 0.5 to 5.0 mg/L NAA (1-napthaleneacetic acid), and 3.0 g/L sucrose. The SGC2 media additionally contained 0.5 to 5.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). SGC2 media generated better callusing response compared to SGC1, thus displaying the importance of using 2,4-D in combination with NAA for callus induction. MS medium containing 2.5 mg/L NAA (SGC1.5) was noted to be the most effective in the initiation of friable embryogenic callus. On the other hand, MS medium containing a combination of 2.0 mg/L NAA and 2.0 mg/L 2,4-D was effective in the early initiation of friable embryogenic callus. In addition, a higher frequency of callus formation was observed from basal leaf segment as compared to that from middle and apical leaf segments. A random amplified polymorphic DNA (RAPD) analysis was also performed to see the genetic differences between the three *S. glauca* genotypes used in this study. The performance of *S. glauca* 5 and *S. glauca* 19 for higher callus frequency over the *S. glauca* 21 could be attributed to the genotypic differences between these genotypes. Overall, our protocol using SGC 2.4 media yielded optimal results and is suitable for large scale micropropagation of *S. glauca*.

Key words: *Simarouba glauca*, somatic embryogenesis, callus, plant growth regulators, RAPD and biofuel.

INTRODUCTION

Simarouba glauca DC, commonly known as “paradise tree”, is an emerging and promising plant for biofuel production. At approximately 65% oil seed content, when compared to current sources of biofuels, such as *Camelina*’s 43% oil seed content, the *S. glauca* tree produces a much higher oil yield (Zubr, 1997). Each fully grown tree yields 15 to 30 kg nutlets, which is equivalent to 2.5 to 5.0 kg of oil. Although, *S. glauca* has the potential to be an effective second generation biofuel crop, it requires a long pre-bearing period (5 to 6 years) to

mature and bear seeds (Mansai and Gaikwad, 2011). The tissue culture technique, micropropagation, can rapidly multiply elite *S. glauca* germplasm. Somatic embryogenesis via callus route is preferred to the *in vitro* regeneration method because it is a useful tool for recovery of large number of plants with little somaclonal variation and also to recover genetically modified plants (Manasi and Gaikwad, 2011). Despite its promise as an effective biofuel source, little scientific research has been performed on *in vitro* propagation of *S. glauca*. Rout and Das (1994) reported somatic embryogenesis from immature cotyledon of *S. glauca* using 6-benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA). Of these, 20 to 25% of somatic embryos were regenerated into plants. It is well known in tissue culture that different genotypes of a species respond differently to a combination of hormones due to their underlying genomic variation (Rutkowska-Krause et al., 2003; Bregitzer et al., 1998). However, most *Simarouba* genotypes respond quite well to different combinations of auxins and cytokinins for induction of callus (Das, 2011).

Using a similar technique, Das (2011) showed that 2,4-D was effective for callus induction from cotyledons. *S. glauca* is an outcrossing species, thus, the genotype of cotyledon explants are ambiguous. Hence, to clone an elite *S. glauca* tree, regeneration from a vegetative part of the tree (shoot, leaf explants) is essential. A callus based regeneration system is necessary to assist genetic transformation of *S. glauca* to achieve quick genetic improvement. Therefore, we studied regeneration of *S. glauca* from leaf explants through a callus based, somatic embryogenesis system.There was a significant difference in the *in vitro* responses (callus induction and subsequent regeneration) of the three *S. glauca* genotypes we studied. Seeds were obtained from an open pollinated *Simarouba* nursery in Florida, USA. Each seed was grown individually as a plant in a greenhouse under controlled conditions and assigned a number. Three plants with promising physiological characteristics were chosen for the current study (SIM 5, SIM 9 and SIM 21). For this reason, we used a Random Amplified Polymorphic DNA (RAPD) analysis, which uses random primers to detect DNA polymorphisms in order to determine if there was identifiable genetic variability between the genotypes studied. Endogenously present PGRs in leaves are known to play an important role in tissue culture, by interacting with PGRs added to the nutrient media. For example, different segments of leaves have different levels of auxin (Davies, 2004). For a clearer understanding, each leaf blades were cut into four segments: the tip, two middle segments and the base (near the petiole).

MATERIALS AND METHODS

Plant materials and culture method

The three different genotypes (two year old seedlings) of *S. glauca* used in this study were maintained in the climate controlled greenhouse at Penn State University Harrisburg. Young, fully developed leaves (1st to 3rd leaf) from the tip were examined. The leaves were washed with 5% Tween 20® for 10 min and moved to the laminar flow cabinet for surface sterilization and inoculation. Washed leaves were surface sterilized first with 10% Clorox® bleach for 5 min, then followed by 100 mg/l mercuric chloride for 4 min, which were then rinsed several times with sterile distilled water.

After surface sterilization, the leaves were cut into 4 equal size pieces; the apex, two middle parts (mid_1, mid_2), and the basal segment. All explants were inoculated on different media's (SGC1.1- SGC1.10 and SGC21.1- SGC21-10). The explants were placed with their adaxial or abaxial side touching the media in equal numbers, all of which were incubated in a TC60 growth chamber (Conviron®) at 25 ± 2°C in the dark. Sixteen explants were cultured into each media type and of the 16 explants, four were tip, four were mid_1, four were mid_2, and four basal explants. The explants were sub-cultured into fresh media every four weeks. The experiment was repeated with a similar number of replicates.

Media preparation

To assess the effect of NAA alone and in combination with 2,4-D on callus induction from *S. glauca* leaf explants, ten levels of NAA and 2,4-D (0.5 to 5 mg/L) were used in combination with a fixed amount of BAP as described in Table 1. The composition of SGC1 was MS medium (Murashige and Skoog, 1962) + 30 g/L of sucrose, 0.5 mg BAP, 100 mg of ascorbic acid, varying concentrations of NAA (0.5 to 5.0 mg/L), 0.7% agar, and pH adjusted to 5.8 before autoclaving at 121°C and 1.2 Kg/cm^2 pressure for 20 min to sterilize the medium. The composition of SGC2 was the same as SGC1 except that, in addition to NAA, another cytokinin, 2,4-D (0. 5 to 5.0 mg/L) was added to the medium. SGC Control media only contained MS nutrients, 30 g/L sucrose, 7.0 g/L Agar and pH was adjusted to 5.8. Shoot induction media composed of MS nutrients, 30 g/L sucrose, 0.1 mg IBA, 0.5 mg/L adenine sulfate ($AdSO_4$) and 100 mg/L ascorbic acid, with varying levels of BAP (0.5 to 5 mg/L) and pH was adjusted to pH 5.8 (Table 1).

Observations

Observations were taken every three to four weeks. For each type of media, the number of calli growing from each of the 16 explants was noted and the percent of callus was obtained. Letters "L" for Loose and "F" for Friable were assigned to the type of morphology the callus exhibited. The callusing frequency was calculated as:

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Abbreviations: BAP, 6-Benzylaminopurine; **NAA,** 1-napthaleneacetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **MS,** Murashige and Skoog; **PCR,** polymerase chain reaction.

Table 1. Composition of growth media for callus and shoot induction.

Callus induction media					Shoot regeneration media	
SGC1*		SGC2*			SGS**	
Media #	NAA (mg/L)	Media #	NAA (mg/L)	2,4-D (mg/L)	Media #	BAP (mg/L)
Control	--	Control	--	--	Control	--
SGC1.1	0.5	SGC2.1	0.5	0.5	SGS1.1	0.5
SGC1.2	1.0	SGC2.2	1.0	1.0	SGS1.2	1.0
SGC1.3	1.5	SGC2.3	1.5	1.5	SGS1.3	1.5
SGC1.4	2.0	SGC2.4	2.0	2.0	SGS1.4	2.0
SGC1.5	2.5	SGC2.5	2.5	2.5	SGS1.5	2.5
SGC1.6	3.0	SGC2.6	3.0	3.0	SGS1.6	3.0
SGC1.7	3.5	SGC2.7	3.5	3.5	SGS1.7	3.5
SGC1.8	4.0	SGC2.8	4.0	4.0	SGS1.8	4.0
SGC1.9	4.5	SGC2.9	4.5	4.5	SGS1.9	4.5
SGC1.10	5.0	SGC2.10	5.0	5.0	SGS1.10	5.0

*****The composition of SGC1 and SGC2 was 4.43 g/L MS-Vit. + 30 g/L Sucrose + 0.5 mg/L BAP + 100 mg/L ascorbate with the indicated levels of NAA and 2,4-D. **The composition of SGS was 4.43 g/L MS-Vit. + 30 g/L Sucrose + 0.1 mg/L IBA + 0.5 mg/L $AdSO_4$ + 100 mg/L ascorbate with the indicated levels of BAP.

Table 2. Primer sequences used for random amplified polymorphic DNA analysis.

Primer #	Primer sequence
OPG-1	5' AAGACGACGG 3'
OPG-2	5' AATCCGCTGG 3'
OPG-3	5' AGTCGGCCCA 3'
OPG-4	5' AACAGGGCAG 3'
OPG-5	5' TGGAAGCACC 3'
OPG-6	5' AGGCAGCCTG 3'
OPG-7	5' GATGCGACGG 3'
OPG-8	5' CCAGATGGGG 3'
OPG-9	5' GGGGGCTTGA 3'
OPG-10	5' TCCCGGTCTC 3'
OPG-11	5' GAAGGCTCCC 3'
OPG-12	5' GGGAGCGCTT 3'
OPG-13	5' GTTCTCGGAC 3'
OPG-14	5' AACTGGCCCC 3'
OPG-15	5' TGGTCATCCC 3'

$$Callusing\ Frequency = \frac{Number\ of\ explants\ callusing}{Total\ number\ of\ explants\ cultured} \times 100\%$$

The callus induction frequency of a particular leaf segment (tip, mid1, mid_2, or base) was calculated by taking the average callus induction frequencies from genotype *S. glauca* 5, *S. glauca* 19 and *S. glauca* 21 on a particular medium using the above formula. The callusing frequency and callus type were also noted individually of the three different genotypes. Friable callus frequency was calculated as:

$$Friable\ Callus\ Frequency = \frac{Number\ of\ friable\ callus}{Total\ number\ of\ explants\ callusing} \times 100\%$$

RAPD analysis

Total genomic DNA was extracted from young leaves using DNAzol (Life Technologies). The DNA quality and quantity was determined by Nanovue spectrophotometer and the comparison was done with standard DNA electrophoresis on 0.8% agarose gel. Initially, a total of 15 primers were screened to achieve reproducible PCR product (Table 2). All primers were analyzed for understanding polymorphic relationships between SIM 5, 19 and 21 and to build the UPGWA tree in Figure 4. The primer OPG-3 was selected for PCR amplification and the subsequent RAPD analysis. PCR amplifications were performed in triplicate using a PCR master mix from Promega in a 25 µL reactions containing DNA template. The amplification program was as follows: initial denaturation at 94°C for 1 min followed by 40 cycles of 94°C for 1 min; 36°C for 45 s; 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated on 1.2% agarose gel and photographed. For RAPD analysis reproducible, unambiguous DNA bands were scored as 1 for the presence or 0 for the absence of bands in each lane. The RAPD data obtained was analyzed using the online tool http://genomes.urv.cat/UPGMA/index.php?entrada.

RESULTS

The genotypes in the present study showed a wide range of response for callus induction on SGC1 and SGC2 media with different levels of NAA and 2,4-D. The frequencies of callus induction of leaf tissues from genotypes *S. glauca* 5, *S. glauca* 19, and *S. glauca* 21 ranged from 27.08 to 77.08% after 30 days, which lead to the production of friable callus. The friable callus frequency of 41.67 to 100% was observed within 120 days of incubation on SGC1 and SGC2 media (Figure 1, Table 3). Further sub-culturing calli lead to the formation of a friable callus in all genotypes on SGC1 and SGC2 media with varying frequencies (Figure 1).

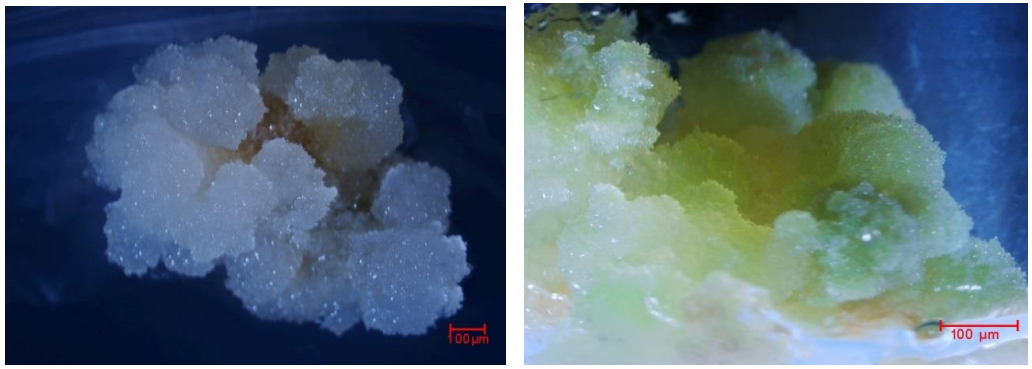

Figure 1. *Simarouba glauca* callus (a) after 30 days of culture and (b) after 120 days of culture.

Table 3. Effect of auxins on callus initiation of *Simarouba glauca*.

Media	SIM 5*			SIM 19*			SIM 21*		
	30 Day	90 Day	120 Day	30 Day	90 Day	120 Day	30 Day	90 Day	120 Day
Control	0.00 ± 0.00	0.00±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SGC1.1	47.92±3.65	72.92±3.42	50.00±7.22	31.25±2.71	60.42±1.88	0.00 ± 0.00	50.00± .77	68.75±4.69	41.67±7.51
SGC1.2	50.00±0.90	89.58±2.60	66.67±4.17	43.75±4.77	70.83±3.93	66.67±8.33	52.08±4.97	70.83±3.42	50.00±6.25
SGC1.3	54.17±2.76	89.58±1.38	91.67±2.08	29.17±3.76	58.33±4.77	66.67±8.33	43.75±4.13	75.00±3.93	83.33±4.17
SGC1.4	56.25±3.25	85.42±2.27	100.00±0.00	54.17±3.76	91.67±2.76	75.00±6.25	52.08±4.97	77.08±4.97	100.00±0.00
SGC1.5	41.67±5.21	81.25±3.25	83.33±2.08	70.83±5.13	100.00±2.90	100.00±0.00	54.17±4.45	83.33±3.42	91.67 ± 2.08
SGC1.6	64.58±2.27	95.83±1.04	100.00±0.00	41.67±5.51	68.75 ± 5.80	100.00±0.00	43.75±3.93	62.50±6.25	91.67 ± 2.08
SGC1.7	68.75±1.56	95.83±1.04	100.00±0.00	52.08±4.26	83.33 ± 4.07	100.00±0.00	41.67±4.13	77.08±3.17	100.00±0.00
SGC1.8	58.33±0.52	93.75±0.90	75.00 ± 6.25	52.08±6.89	64.58 ± 7.57	100.00±0.00	50.00±4.26	79.17±3.76	91.67 ± 2.08
SGC1.9	52.08±3.65	87.50±3.13	66.67 ± 5.51	29.17±4.54	64.58 ± 5.63	100.00±0.00	47.92±6.01	60.42±7.67	50.00 ± 6.25
SGC1.10	25.00±4.13	56.25±5.63	58.33 ± 7.51	39.58±2.76	81.25 ± 4.26	91.67±2.08	27.08±5.29	54.17±5.73	41.67 ± 7.51
SGC2.1	31.25±4.51	87.50±2.27	41.67 ± 7.51	43.75±2.39	68.75 ± 0.00	41.67±2.08	37.50±3.61	72.92±6.77	50.00 ± 7.22
SGC2.2	58.33±2.76	83.33±1.38	75.00 ± 6.25	47.92±1.38	68.75 ± 0.90	75.00 ± 0.00	50.00±4.69	64.58±6.65	75.00 ± 6.25
SGC2.3	62.50±3.25	93.75±2.60	75.00 ± 6.25	60.42±1.38	91.67 ± 1.04	91.67 ± 2.08	45.83±5.73	60.42±7.67	91.67 ± 2.08
SGC2.4	66.67±1.38	100.00±2.39	100.00±0.00	66.67±1.04	89.58 ± 2.27	91.67 ± 2.08	56.25±5.63	70.83±5.13	100.00±0.00
SGC2.5	66.67±0.52	93.75±2.27	100.00±0.00	70.83±1.38	75.00 ± 1.88	100.00±0.00	50.00±3.61	75.00±3.61	100.00±0.00
SGC2.6	64.58±1.38	87.50±3.17	100.00±0.00	72.92±2.90	79.17 ± 1.04	100.00±0.00	54.17±4.63	56.25±5.49	100.00±0.00
SGC2.7	72.92±2.27	89.58±0.90	100.00±0.00	77.08±3.42	83.33 ± 2.27	100.00±0.00	70.83±5.80	79.17±2.76	100.00±0.00
SGC2.8	72.92±2.27	91.67±1.38	100.00±0.00	70.83±4.97	75.00 ± 3.65	100.00±0.00	66.67±6.01	66.67±6.14	100.00±0.00
SGC2.9	62.50±0.90	81.25±1.88	100.00±0.00	70.83±1.88	87.50 ± 3.13	100.00±0.00	68.75±5.63	83.33±3.42	100.00±0.00
SGC2.10	56.25±0.90	95.83±1.04	91.67 ± 2.08	64.58±1.38	83.33 ± 4.17	91.67 ± 2.08	54.17±5.29	60.42±4.26	100.00±0.00

*Percent of explants with calli (Mean of three replications ± SE).

Table 4. Friable callus frequency after 120 days of culturing.

Media	SIM 5*	SIM 19*	SIM 21*
SGC0 (Control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
SGC1.1	50.00 ± 7.22	0.00 ± 0.00	41.67 ± 7.51
SGC1.2	66.67 ± 4.17	66.67 ± 8.33	50.00 ± 6.25
SGC1.3	91.67 ± 2.08	66.67 ± 8.33	83.33 ± 4.17
SGC1.4	100.00 ± 0.00	75.00 ± 6.25	100.00 ± 0.00
SGC1.5	83.33 ± 2.08	100.00 ± 0.00	91.67 ± 2.08
SGC1.6	100.00 ± 0.00	100.00 ± 0.00	91.67 ± 2.08
SGC1.7	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC1.8	75.00 ± 6.25	100.00 ± 0.00	91.67 ± 2.08
SGC1.9	66.67 ± 5.51	100.00 ± 0.00	50.00 ± 6.25
SGC1.10	58.33 ± 7.51	91.67 ± 2.08	41.67 ± 7.51
SGC2.1	41.67 ± 7.51	41.67 ± 2.08	50.00 ± 7.22
SGC2.2	75.00 ± 6.25	75.00 ± 0.00	75.00 ± 6.25
SGC2.3	75.00 ± 6.25	91.67 ± 2.08	91.67 ± 2.08
SGC2.4	100.00 ± 0.00	91.67 ± 2.08	100.00 ± 0.00
SGC2.5	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.6	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.7	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.8	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
SGC2.9	100.00 ± 0.00	100.00 ± 0.00	100.00 ±0.00
SGC2.10	91.67 ± 2.08	91.67 ± 2.08	100.00 ± 0.00

*Percent of explants with calli (mean of three replications ± SE)

Callus induction on SGC1 media

Callus initiation of genotype *S. glauca* 19 was exception with the callus initiation frequency of 70.83 on SGC1.5 medium. After 90 days of incubation on callus induction media, the highest callusing response of 100% was seen in genotype *S. glauca* 19 on SGC1.5. In genotype *S. glauca* 5, the highest callus induction frequency was found to be 95.83% on day 90 of culturing on SGC1.7. Genotype *S. glauca* 21 yielded the highest callus induction frequency of 83.33% on day 90 of incubation on SGC1.5. After 120 days of sub-culturing, genotype *S. glauca* 19 performed well on SGC1 in comparison to genotypes *S. glauca* 5 and *S. glauca* 21 for friable callus production. It yielded 100% friable callus on SGC1.5, SGC1.6, SGC1.7, SGC1.8, and SGC1.9. 100% friable callus formation was observed in genotype *S. glauca* 5 on SGC1.4, SG 1.6 and SG 1.7 whereas genotype *S. glauca* 21 could produce 100% friable callus from SGC2.4 and SGC1.7 (Table 4).

Callus induction on SGC2 media

The highest callusing response of 100% was seen in genotype *S. glauca* 5 on SGC2.4 after 90 days. In genotype *S. glauca* 19, the highest callus induction frequency was found to be 91.67% on day 90 of culturing on SGC2.3. Genotype *S. glauca* 21 yielded the highest callus induction frequency of 83.33% on day 90 of incubation on SGC2.9. After 120 days of sub-culturing, the three genotypes produced friable callus with different frequencies. An additional number of friable calli was obtained from SGC2 media than from SGC1. 100% friable callus formation was observed in genotype *S. glauca* 5 on SGC2.4, SGC2.5, SGC2.6, SGC2.7, SGC2.8, and SGC2.9. Genotype *S. glauca* 19 yielded 100% friable callus on SGC2.5, SGC2.6, SGC2.7, SGC2.8, and SGC2.9. 100% friable callus was obtained in genotype *S. glauca* 21 on SGC2.4, and SGC2.5, SGC2.6, SGC2.7, SGC2.8, SGC2.9 and SGC2.10 (Table 4). When the selected friable calli were transferred to the shoot regeneration media with BAP, tiny shoots began to appear (Figure 2). Observations were made after 60 days of culturing on shoot regeneration media. Friable calli on SGS1.6 with 3.0 mg/L BAP produced highest number of shoots in genotype *S. glauca* 5 and genotype *S. glauca* 19 (7.5 and 5.5, respectively). SGS2.5 produced the highest number of shoots/callus (5.5 shoots) in genotype *S. glauca* 21 (Table 5).

Callus induction from different leaf segments

Callusing frequency by leaf segments was noted to be highest on base followed by Mid_2, Mid_1, and the tip of the

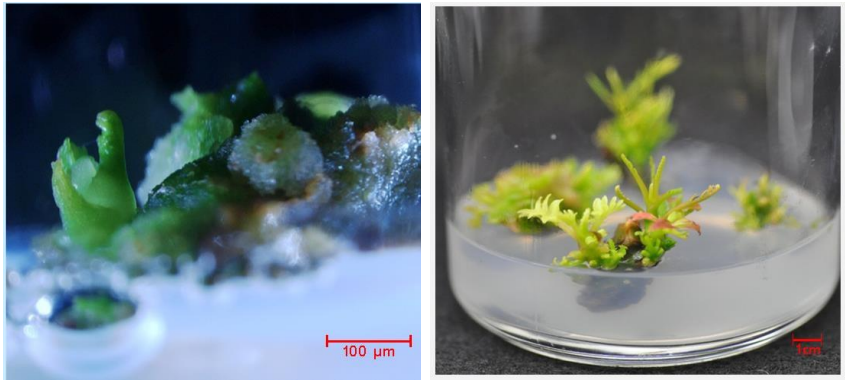

Figure 2. *Simarouba glauca* shoot formation (a) after 150 days of culture and (b) after 180 days of culture.

Table 5. Callus Induction frequency by leaf segments.

Media	Tip*		Mid 1*		Mid 2*		Base*	
	30 day	120 day	30 day	120 day	30 day	120 day	30 day	120 day
Control	0.00% ± 0.00	0.00% ± 0.00	0.00% ± 0.00	0.00%±0.00	0.00% ± 0.00	0.00%±0.00	0.00% ± 0.00	0.00%± 0.00
SGC 1.1	12.50% ± 6.94	62.50%±5.51	20.83% ± 5.81	54.17%±5.01	37.50% ± 5.81	87.50%±4.86	54.17% ± 3.67	95.83% ± 0.00
SGC 1.2	25.00% ± 5.81	70.83%± 5.81	29.17% ± 5.42	91.67%±2.20	50.00% ± 4.39	100.00%±3.03	54.17% ± 4.66	83.33% ± 0.00
SGC 1.3	16.67% ± 6.91	58.33% ± 5.68	33.33% ± 6.05	75.00%±5.51	41.67% ± 5.15	87.50% ± 4.22	45.83% ± 6.29	83.33% ± 0.00
SGC 1.4	37.50% ± 4.36	87.50% ± 2.08	37.50% ± 4.66	83.33%±3.67	45.83% ± 5.01	100.00%±1.39	54.17% ± 3.61	100.00% ± 0.00
SGC 1.5	33.33% ± 5.68	83.33% ± 1.84	37.50% ± 5.89	95.83%±1.39	45.83% ± 6.51	100.00%±1.39	58.33% ± 5.15	83.33% ± 0.00
SGC 1.6	25.00% ± 5.89	66.67% ± 4.66	37.50% ± 5.29	83.33%±2.95	54.17% ± 4.86	91.67% ± 2.20	62.50% ± 5.89	79.17% ± 0.00
SGC 1.7	37.50% ± 5.10	87.50%± 2.95	50.00% ± 4.55	87.50%±3.03	62.50% ± 4.39	95.83% ± 2.08	70.83% ± 6.59	95.83% ± 0.00
SGC 1.8	29.17% ± 5.68	79.17% ± 3.67	37.50% ± 5.68	91.67%±2.78	50.00% ± 5.01	95.83% ± 1.84	62.50% ± 5.10	100.00% ± 0.00
SGC 1.9	33.33% ± 4.66	79.17% ± 5.51	41.67% ± 4.86	79.17%±5.42	50.00% ± 6.59	79.17% ± 5.51	54.17% ± 6.91	91.67% ± 0.00
SGC 1.10	29.17% ± 3.61	70.83% ± 6.71	33.33% ± 4.05	75.00%±6.29	41.67% ± 5.68	79.17% ± 6.62	45.83% ± 6.17	100.00% ± 0.00
SGC 2.1	25.00% ± 6.17	79.17% ± 2.78	33.33% ± 5.51	79.17%±4.39	50.00% ± 3.87	87.50% ± 5.89	50.00% ± 3.87	91.67% ± 3.61
SGC 2.2	29.17% ± 6.40	83.33% ± 3.03	41.67% ± 4.17	83.33%±2.95	41.67% ± 4.55	87.50% ± 5.68	66.67% ± 2.20	95.83% ± 5.68
SGC 2.3	33.33% ± 5.68	87.50%± 2.94	45.83% ± 4.55	91.67%±3.03	58.33% ± 4.66	87.50%± 4.66	62.50% ± 4.66	95.83% ± 2.08
SGC 2.4	41.67% ± 4.55	95.83% ± 1.84	54.17% ± 4.71	95.83%±1.84	62.50% ± 4.22	95.83% ± 3.61	83.33% ± 4.17	100.00% ± 4.66
SGC 2.5	50.00% ± 5.15	91.67% ± 1.39	62.50% ± 2.95	100.00%±4.17	66.67% ± 4.22	100.00%±5.01	75.00% ± 5.10	95.83% ± 4.55
SGC 2.6	50.00% ± 4.71	100.00%±2.78	54.17% ± 4.71	95.83%± 5.29	62.50% ± 3.67	100.00%±5.42	83.33% ± 5.01	100.00% ± 7.05
SGC 2.7	54.17% ± 4.55	95.83% ± 1.39	62.50% ± 4.05	95.83% ± 2.20	62.50% ± 5.01	95.83% ± 4.17	79.17% ± 4.22	100.00% ± 5.01
SGC 2.8	50.00% ± 5.89	87.50% ± 4.22	45.83% ± 5.93	95.83% ± 3.03	66.67% ± 4.66	100.00%±4.22	75.00% ± 4.17	100.00% ± 3.67
SGC 2.9	45.83% ± 5.15	100.00%±1.84	54.17% ± 4.17	100.00%±2.08	62.50% ± 4.17	100.00%±4.71	79.17% ± 3.61	100.00% ± 4.71
SGC 2.10	37.50% ± 5.29	83.33% ± 2.94	45.83% ± 3.87	87.50% ± 5.68	62.50% ± 3.03	100.00%±4.71	70.83% ± 3.67	95.83% ± 5.42

*Percent of explants with calli (mean of three replications ± SE).

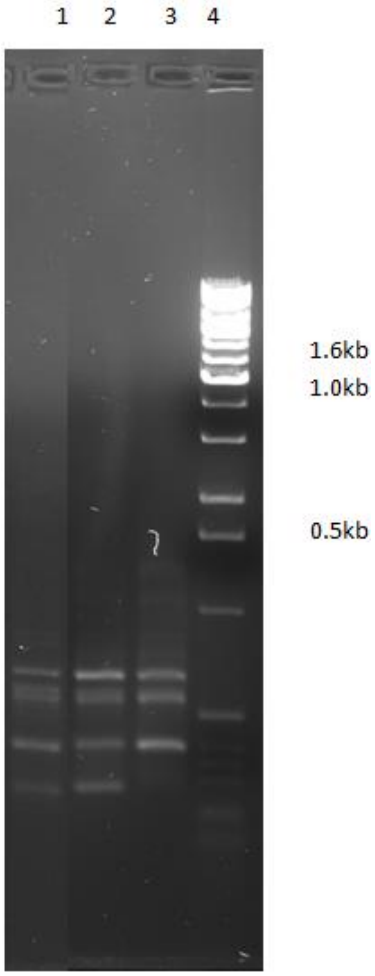

Figure 3. Gel electrophoresis of PCR with Primer 3 of genotype *Simarouba glauca* 5, *S. glauca* 19, and *S. glauca* 21. Lanes 1-4, *S. glauca* genotypes *S. glauca* 5, *S. glauca* 19 and *S. glauca* 21, respectively; lane 4: 1 Kb DNA extension ladder (Invitrogen).

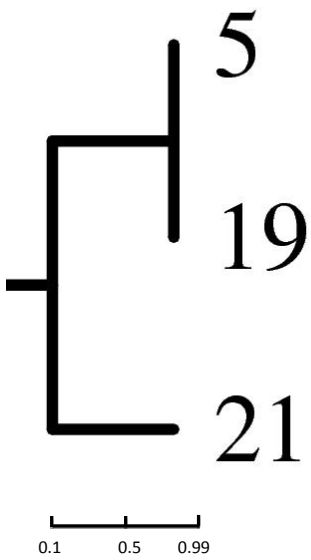

Figure 4. UPGWA tree inferred from the simple match coefficients of *Simarouba glauca* genotypes.

leaf. The more distal the segment was the lower callus induction frequency was observed (Table 5). A trend of increase in callus induction frequency was observed in all the segments with longer sub-culturing on SGC1, as well as on SGC2 media. However, SGC2 produced higher number of calli compared to SGC1 in a shorter time. After 30 days the highest callusing frequency on SGC1 of up to 70.83% was observed on the base segments. Mid_1 and Mid_2 segment of leaves could produce up to 50 and 62.50% callus, respectively. The callus induction frequency reduced in the leaf tip with the highest frequency of 37.50%. Similarly, on SGC2 medium, the base segment had the highest callusing frequency (83.33%). The callus induction frequency from Mid_2 and Mid_1 segments was up to 66.67 and 62.50%, respectively. The leaf tip could produce 54.17% callus.

After 120 days of culturing on SGC1 media, the callusing frequency of up to 100% was noted on the base and Mid_2 segments. A reduction in the callus induction frequency was observed in Mid_1 and tip segments; 91.67 and 87.50%, respectively. Callus induction frequency of 100% was seen on SGC2 media from all the segments used in the study after 120 days.

Genetic variation among *S. glauca* genotypes was studied using random amplified polymorphic DNA method. The genomic polymorphisms of the three genotypes of *S. glauca* produced with primers OPG-4 are presented in Figure 4. All genotypes yielded 1-10 PCR - amplified DNA fragments that ranged between 100 and 2000 bp (Figure 3). Only fragments with a high intensity were taken into account. The DNA polymorphism of SIM 19 and 21 were similar to each other, but different from that of genotype *S. glauca* 5 (Figure 4). Thus, the genotypes with high callus induction frequencies were found to be similar than the genotype with low callus induction rate.

DISCUSSION

Paradise tree, *S. glauca,* is a promising biofuel plant for both its high oil content and its adaptability to grow in a wide range of climate. In the present study, an optimum system of callus generation in *S. glauca* was investigated. Different combinations and concentrations of auxins and cytokinins have been used to induce callus in *S. glauca* (Rout and Das, 1994; 1999). In the present study, we tested two sets of media, SGC1 and SGC2.

Table 6. Effect of BAP on shoot regeneration from callus after 60 days of culturing on shoot regeneration media.

BAP mg/L	SIM 5	SIM 19	SIM 21
Control	0.00	0.00	0.00
0.5	4.50	3.00	2.00
1.0	6.00	3.50	4.50
1.5	4.50	4.50	4.50
2.0	5.00	4.00	4.50
2.5	6.50	5.00	5.50
3.0	7.50	6.00	5.00
3.5	6.00	6.00	5.00
4.0	4.50	3.50	5.00
4.5	3.50	1.50	3.00
5.0	2.50	1.50	1.50

Callus induction media SGC1 had increasing concentrations from 0.5 to 5.0 mg/L, while SGC2 had increasing concentrations of both NAA and 2,4-D from 0.5 to 5.0 mg/L. Previous studies have shown that 2,4-D when combined with NAA has a synergistic effect on the callus formation (Das, 2011). Our study shows that NAA, along with 2,4-D, is more efficient in initiating callus from leaf explants than NAA alone. The leaf explants showed faster response on SGC1 compared to SGC2 at 30, 60, 90 and 120 day time point. After 120 days, SGC2 had comparatively higher callusing frequencies in each media compared to SGC1. The combination of NAA with 2,4-D was also effective in obtaining friable callus from an already induced callus. The observation taken on 120 day time point indicated that 100% friable callus was obtained from media SGC1.5 - 2.9. Moreover, previous reports have indicated that the combination of 2,4-D with other auxins improved callus initiation (Debeaujon and Branchard, 1993; Khan et al., 2011; Qin Mao et al., 2006). Once the callus initiation is started, one auxin is sufficient to produce friable callus as in case of cucumber (Elmeer and Hennerty, 2008). Previous studies showed that high levels of auxins are required for the induction of embryogenic callus; however, cytokinins are critical for somatic embryo formation (Palmer and Keller, 2011; Webster et al., 2006). Shooting was also initiated from friable callus. Our experiments recorded an average number of 7.5 shoots per callus mass on media supplemented with 3.0 mg/L BAP (Table 6).

Callus induction and plant regeneration of many plant species is genotype-specific. Genotype *S. glauca* 19 performed well on the SGC1.5 medium in obtaining both the initial callus and friable callus. Medium type SGC1.5, which contained only one auxin (NAA, 3.5mg/L), initiated 70.83% callus production and lead to the production of 100% friable callus in genotype *S. glauca* 19. Our result supports the previous findings with other plants. Our results indicate that, genotype *S. glauca* 19 could be used in future studies that require the production of friable callus with NAA as a sole source of callus initiator. However, a combination of NAA and 2,4-D could be used on a wider selection of *S. glauca* genotypes for callus initiation leading to the production of friable callus; in this case, all the genotypes performed well in obtaining friable callus on SGC2 media and could be used in future studies of plant regeneration and transformation. Separating the leaf segments revealed that the base of the leaf gave the highest callus responses than the tip of the leaf. This indicated the role of important endogenously present PGRs at the basal level of the *Simarouba* leaf that interact with exogenous substances in the media supplemented. Levels of endogenous growth regulators vary, not only among different plant organs, but also among different tissues of the same organ. Our study indicates that the highest callus response comes from the basal part of the leaf rather than the tip of the leaf. The Ye et al. (2012) study on callus induction in *Zizyphus jujuba* found that the basal section had higher frequency of callus formation compared to the apical and middle sections of the same leaf, underlining the fact that harmonized levels of endogenous and exogenous growth regulators are required to yield better results (Davies, 2004).

Different genotypes can respond to tissue culturing differently. This differential response perhaps is because of the genetic differences among these genotypes (Hadrys et al., 1992). In the present study, genotype *S. glauca* 5 and *S. glauca* 19 showed higher callusing frequencies compared to genotype *S. glauca* 21. RAPD analysis with OPG-3 primer revealed difference in banding patterns of PCR-amplified product. Genotype *S. glauca* 5 and genotype *S. glauca* 19 shared more DNA bands as compared to those with genotype *S. glauca* 21. Our results indicate that genotypic differences noticed through RAPD analysis directly reflect the differences noticed in the callusing frequencies. Previous studies using RAPD analysis have shown a strong association between the genetic diversity and geographical distribution of crops (Li et al., 2012). The genotypic differences that appeared in RAPD also suggest that the genotypes used in the present study came from different geographical locations.

Since, *S. glauca* is highly cross-pollinated, the genetic variation observed in the present study could be due to heterozygosity at some marker loci. However, further studies are required to ascertain if these loci could be associated to the difference in callus induction in these genotypes. These loci could be used as molecular markers to screen the *S. glauca* genotypes for high callus initiation response. Our results indicate that SGC 2.4 media produced optimum results and is economical in the long run for large scale micropropagation of *S. glauca* or for establishing a regeneration system for genetic transformation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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