

In vitro micropropagation of *Simarouba glauca* DC.

A R Lavanya^{1,2}, M Muthukumar¹, S Muthukrishnan¹, V Kumaresan^{1,3}, T Senthil Kumar^{4*}, A S Rao⁵ and M V Rao¹

¹Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, India

²Department of Botany, Periyar E V R Government Arts College, Tiruchirappalli 620 023, India

³Department of Botany, Aringar Anna Government Arts College, Attur, Salem 636 121, India

⁴Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli 620 024, India

⁵Department of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, India

Received 17 November 2014; revised 10 February 2015; accepted 23 March 2015

An efficient and improved micropropagation protocol was developed from different explants of 2-month-old seedlings of *Simarouba glauca* DC. The highest number of shoots (55.3) with 100% response was obtained from cotyledonary-node explant on MS medium fortified with 6-benzyl adenine (BA; 3.0 mg/L), kinetin (KN; 0.5 mg/L), α -naphthalene acetic acid (NAA; 0.5 mg/L) and glutamine (10 mg/L). Further, the highest rooting response (95%) with 15 roots of 5.8 cm root length was observed on MS medium supplemented with NAA (3.0 mg/L). The rooted plantlets were hardened by transferring to paper containers with soil and sand (2:1) and kept in shade for acclimatization. These plants were further transferred to the field with a survival percentage of 81.5%.

Keywords: Additives, growth regulators, micropropagation, *Simarouba glauca*, shoot multiplication

Introduction

Simarouba glauca DC. (Family: Simaroubaceae), known as Paradise tree, is a fast growing, multipurpose, ecofriendly tree. It grows up to 20 m height with a trunk 50-80 cm in diam (Fig. 1a). The tree flowers annually during December-February and bears fruits during March-April. The Paradise tree is well suited for reclamation of marginal/wastelands and for watershed management. It is native of El Salvador in the Central America¹. It was first introduced to India by National Bureau of Plant Genetic Resources at its Research Station at Amravati, Maharashtra in 1966 and to the University of Agricultural Sciences, Bangalore in 1986. Now, in India, it is cultivated in Andhra Pradesh, Gujarat, Rajasthan, Karnataka, Tamil Nadu, Orissa and West Bengal².

The seeds of *S. glauca* contain 60-70% edible oil, which can be used in the manufacture of vanaspati, and/or margarine, soaps, lubricants, paints, polishes, pharmaceuticals etc. The cake is rich in nitrogen (8%), phosphorous (1.1%) and potassium (1.2%). The fruit pulp is rich in sugars (11%) and can be used in the beverage and fermentation industry. The shells

can be used in the manufacture of particle board, activated charcoal or as a fuel. The wood is used in the preparation of quality furniture, toys, in match industry and as pulp in paper industry. The plant is also used in the treatment of amoebiasis, malaria, diarrhoea, snake bite, anthelmintic, colitis, intestinal parasites, dyspepsia, haemostatic, viral diseases and cancer. The tree contains 'quassinoids, a triterpene, and fatty acids (oleic acid, 52-54%; stearic acid, 27-33%; palmitic acid, 11-12%)³.

The tree is mainly propagated through seeds and also partly by grafting. Major constraints in large scale planting are the short viability (2-3 month) of seeds and polygamodioecious nature of the plant¹. About 5% of seed population produces exclusively male flowers (staminate) and 40-50% of population mainly male flowers with a few bisexual flowers (andromonoecious), while the remaining 40-50% population produces only the female flowers (pistillate)⁴. Hence, an effective approach is essential for rapid mass multiplication of *S. glauca* by tissue culture.

Earlier reports on micropropagation of *S. glauca* have recorded only 5.83 and 9.66 shoots per nodal explant, respectively by Rout and Das⁵ and Shukla and Padmaja⁶. Others have reported somatic embryogenesis from callus cultures of cotyledons⁷ and embryonic axil with cotyledons⁸. While Rout *et al*⁹

*Author for Correspondence:

Tel: +91-4312407071 Ext. 130, Fax: +91-431-2407045.
senthil2551964@yahoo.co.in

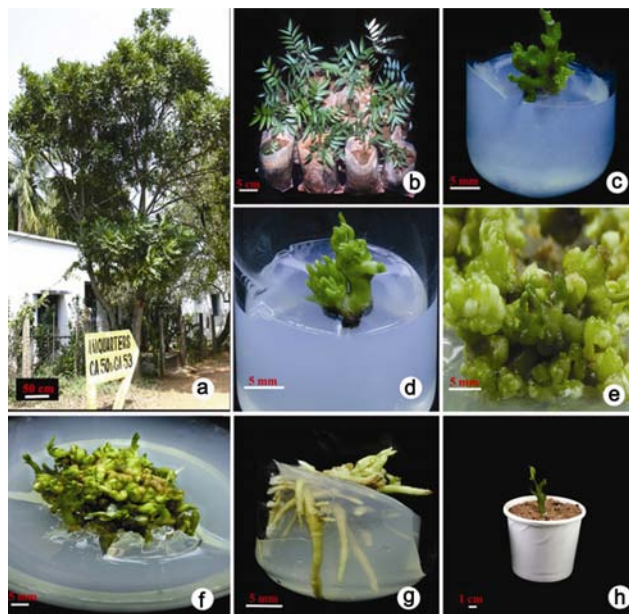


Fig. 1—*In vitro* micropropagation of *S. glauca*: (a) Habit of *S. glauca* tree; (b) 2-month-old seedlings *via in vivo* germination; (c & d) Multiple shoot initiation from cotyledonary node and axillary bud explant on MS medium with BA (3.0 mg/L), KN (0.5 mg/L) and NAA (0.5 mg/L), within 35 d; (e & f) Multiple Shoot proliferation of cotyledonary node and axillary bud explants on MS medium with BA (3.0 mg/L), KN (0.5 mg/L), NAA (0.5 mg/L) and glutamine (10 mg/L), within 35 d; (g) Root initiation from an *in vitro* raised microshoot on MS medium with NAA 3.0 mg/L, within 35 d; & (h) Hardened *in vitro*-regenerated plant of *S. glauca* after acclimatization, after 8 wk.

have demonstrated the role of peroxidase in rooting of microshoots. In the present study, an improved and efficient micropropagation protocol from different explants of *S. glauca* has been reported.

Materials and Methods

Plant Material

Seeds of *S. glauca* were procured from the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India. The seeds were treated with conc. H_2SO_4 for 1 h. The seed coat was softened by scrubbing the surface and washed thoroughly in running tap water. Then the seeds were rinsed with sterilized distilled water and placed in rotary shaker for uniform imbibition for 2 d. On 3rd d, seeds were thoroughly washed with sterile distilled water, then sowed in the polythene covers containing soil and sand (1:1) and maintained under shade in the garden.

Selection of Explants and Sterilization

Explants (1-2 cm in size), such as, cotyledonary node, apical bud and axillary bud, were collected from 2-month-old seedlings. They were initially washed with few drops of liquid detergent (Teepol-

Sigma Aldrich-86350) for 5 min, followed by rinsing in tap water for 10-15 min. The washed explants were treated with 70% alcohol for 1 min, followed by sterile distilled water and then with 0.1% (w/v) mercuric chloride for 3 min. Finally, the explants were rinsed 4-5 times with sterile distilled water and cut into small pieces, each 0.5-1.0 cm long.

Shoot Bud Induction and Multiplication

The sterilized explants were cultured on Murashige and Skoog (MS)¹⁰ medium containing cytokinins, such as, 6-benzyl adenine (BA) and kinetin (KN) (0.5-4.0 mg/L), individually or in combination for shoot bud initiation. For further multiplication, these shoots were cultured on the medium supplemented with BA (3.0 mg/L), KN (0.5 mg/L) and different concentrations of auxins, indole-3-acetic acid (IAA), α -naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) (0.05-1.0 mg/L).

Shoot Proliferation, Elongation and Rooting

For further enhancement in shoot multiplication, the additives like adenine sulphate, glutamine and sodium citrate (5-20 mg/L) were added to the medium containing BA (3.0 mg/L), KN (0.5 mg/L) and NAA (0.5 mg/L). The microshoots were transferred to MS medium supplemented with gibberellic acid (GA_3) (0.5-5.0 mg/L) individually or in combination with cytokinin for shoot elongation.

The microshoots were excised and subcultured on MS medium containing IAA, IBA, NAA (0.5-4.0 mg/L) individually and in combination with KN (0.1-2.0 mg/L) for root induction.

Acclimatization and Hardening

In vitro raised plantlets (4-wk-old) with well-developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transferred to plastic containers containing mixture of sterilized sand and soil (1:2). The plantlets were maintained inside the culture room. Around the plantlets, 70-80% relative humidity was maintained by covering them with polythene cover. The plantlets were watered with $\frac{1}{4}$ strength MS basal solution devoid of sucrose and meso-inositol at 3 d interval for a period of 3 wk. The acclimatized plantlets were then transferred to earthen pots containing soil and kept under shade for another 8 wk before transferring to the field.

Culture Vessels and Culture Conditions

Shoot initiation, elongation and rooting were carried out in culture tubes (Borosil, 150×25 mm²)

with 10 mL of medium for single explant. Shoot multiplication and proliferation were performed in conical flasks (Borosil, 250 mL) with 75 mL of medium for 5 explants. All the utensils used were thoroughly washed with running tap water then rinsed with distilled water. Thereafter all the glass wares were sterilized in hot air oven at 220°C for 1 h. The cultures were incubated in culture room maintained at 25±2°C, under a 16 h photoperiod with a light intensity of 35 µΣ m⁻² s⁻¹ from Philips cool white fluorescent tubes with 55-60% relative humidity.

Statistical Analysis

A minimum of 25 replicates were taken for each treatment and all the experiments were repeated thrice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The experimental design was random and factorial. The data pertaining to shoot proliferation frequencies and shoot number, shoot elongation and rooting were subjected to mean and mean separation analysis by using Duncan's Multiple Range Test (DMRT)¹¹.

Results and Discussion

Multiple Shoot Formation

Although several media were available for the culture of tree species, MS medium was selected for

the present study because of its suitability reported in many cases like *Wrightia tinctoria*¹², *Pterocarpus marsupium*¹³ and *Bauhinia vahlii*¹⁴. Besides media, type of explants can influence the rate of explant sprouting and shoot multiplication in woody plants. The choice of explant is a critical factor that determines the success of most tissue culture experiments. Further, explants from the mature trees are not chosen for *in vitro* propagation because of phenolic exudation and high risk of contamination¹⁵⁻¹⁷. In the present study, explants were selected from 2-month-old seedling of *S. glauca* (Fig. 1b). Among the various explants, cotyledonary node showed best response compared to other explants. Individual cytokinin treatment results only less number of shoots, whereas the combination of BA (3.0 mg/L) and KN (0.5 mg/L) increased the shoot multiplication frequency (100, 100 & 90 %) and shoot numbers (25.8, 15.0 & 13.2) in cotyledonary node, axillary bud and apical bud, respectively (Table 1; Figs 1c & d).

Role of Auxins and Additives

For further improvement in multiple shoot induction, the shoots were tested against various concentrations of IAA, IBA and NAA along with BA (3.0 mg/L) plus KN (0.5 mg/L). During the proliferation stage, a high cytokinin to auxin

Table 1—Multiple shoot induction of cotyledonary node, axillary bud and apical bud explants of *S. glauca* grown on MS medium supplemented with cytokinins, auxins and organic additives (after 35 d)

Cytokinins (mg/L)	Cotyledonary node		Axillary bud		Apical bud	
	Shoot bud induction (%)	Shoot number ^a	Shoot bud induction (%)	Shoot number ^a	Shoot bud induction (%)	Shoot number ^a
BA + KN						
3.0 0.1	94.6 ^{bc}	15.4 ^{de}	92.0 ^c	9.8 ^{de}	81.3 ^d	8.5 ^d
3.0 0.25	97.3 ^b	18.5 ^{bc}	98.6 ^{ab}	12.3 ^b	85.3 ^{bc}	10.1 ^b
3.0 0.5	100.0 ^a	25.8 ^a	100.0 ^a	15.0 ^a	90.0 ^a	13.2 ^a
3.0 1.0	89.3 ^{de}	20.0 ^b	93.3 ^{bc}	11.8 ^{bc}	86.6 ^b	9.7 ^{bc}
BA + KN + NAA						
3.0 0.5 0.05	89.0 ^c	19.3 ^d	85.3 ^c	13.4 ^f	74.6 ^d	11.5 ^e
3.0 0.5 0.1	97.3 ^{ab}	25.4 ^c	92.0 ^{ab}	17.8 ^c	81.3 ^{bc}	16.2 ^b
3.0 0.5 0.5	100.0 ^a	33.0 ^a	95.0 ^a	22.0 ^a	90.0 ^a	18.0 ^a
3.0 0.5 1.0	94.6 ^{ab}	28.6 ^b	89.3 ^b	19.7 ^b	84.0 ^b	15.4 ^{bc}
BA + KN + NAA + GLU						
3.0 0.5 0.5 05	93.3 ^c	41.7 ^{cd}	85.3 ^b	35.0 ^b	74.6 ^{ab}	31.3 ^b
3.0 0.5 0.5 10	100.0 ^a	55.3 ^a	90.0 ^a	40.1 ^a	80.0 ^a	38.0 ^a
3.0 0.5 0.5 15	98.6 ^{ab}	49.2 ^b	78.6 ^{cd}	31.8 ^c	69.3 ^{bc}	27.8 ^{cd}
3.0 0.5 0.5 20	90.0 ^{cd}	38.5 ^d	72.0 ^{ef}	27.4 ^{de}	61.0 ^e	21.0 ^{ef}

^aValues are mean of 25 replicates per treatment and repeated thrice. In each subset values with the same superscript are not significantly different at 5% probability level according to DMRT

ratio is used to reduce apical dominance of the main shoot and to enhance axillary buds growth. Among the three auxins tested, NAA (along with BA+KN) was the most effective in inducing multiple shoot formation. Cotyledonary node explants showed the highest percentage of shoot multiplication (100%) and shoot number (33.0) at 0.5 mg/L NAA (Table 1). Similarly the combination of BA, KN and NAA was found effective in cotyledon explant of *Pterocarpus santalinus*¹⁸.

Many reports specify that organic nitrogen source like amino acids instead of inorganic source like nitrates or ammonium sulphates can improve the cell proliferation as well as regeneration in specific genotypes¹⁹. Additives like adenine sulphate, sodium citrate and glutamine (5-20 mg/L) were used in multiple shoot formation. Glutamine (10 mg/L) showed the high multiplication rate, *i.e.*, shoot number (55.3, 40.1 & 38.0) and frequency (100, 100 & 90%), in the following order, cotyledonary node, axillary bud and apical bud (Table 1; Figs 1e & f). Glutamine is reported to promote the shoot proliferation in many plant species like *Vitex negundo*²⁰ and *Ficus religiosa*²¹. This is the first ever report to produce maximum yield of 55.3 shoots per cotyledon explant, whereas Rout and Das⁵ had recorded only 5.83 shoots as their maximum yield per axillary bud explant and Shukla and Padmaja⁶ 9.66±0.33 shoots per nodal explant. Our results showed remarkable increase in the means of shoot numbers because of the selection of appropriate explant type and age. We had chosen 2-month-old seedlings, whereas Rout and Das⁵ and Shukla and Padmaja⁶ had selected explants from 2-yr-old trees.

Shoot Elongation, Rooting and Acclimatization

The formation of microshoots (0.5-1.0 cm) was observed in all the treatments. These microshoots were excised and transferred to MS medium supplemented with GA₃ (0.5-5.0 mg/L) individually or in combination with cytokinin for shoot elongation. However, no further elongation in the microshoots was observed even at higher concentrations (data not shown). It has been earlier reported that microshoots (1-2 cm) produced in this tree species were characterized by abnormal leaf morphology, short and compact shoots and difficulty in elongation and root regeneration⁹.

The microshoots were transferred to rooting medium consisting of MS medium with different concentrations of auxins (IAA, IBA and NAA) and KN. NAA (3.0 mg/L) showed the highest rooting response (95%) with the highest number of roots (15) and root length (5.8 cm) for cotyledonary explant (Table 2; Fig. 1g). The present observation was contrary to earlier studies on *S. glauca* where IBA gave the best rooting response^{6,9}. Microshoots obtained from axillary bud explants recorded 90% response with 9.0 roots and 5.1 cm root length, whereas microshoots from apical bud explants gave 80% response with 7.0 roots and 4.8 cm root length.

The microshoots with the lengthy roots were transferred to plastic containers containing mixture of sterilized soil and sand (2:1) and covered with transparent polythene cover punctured for holes to lower down the high atmospheric humidity gradually. After 4 wk of acclimatization, the plantlets were transferred to earthen pots containing soil and kept under shade condition for 8 wk before transplanting to the field (Fig. 1h). The survival percentage of the acclimatized plants in the field was 81.5%.

Table 2—Rooting response of *in vitro* raised shoots of *S. glauca* grown on MS medium supplemented with auxins and KN (after 35 d)

Auxin (mg/L)	Cotyledonary node			Axillary bud			Apical bud		
	% response	Root number ^a	Root length (cm) ^a	% response	Root number ^a	Root length (cm) ^a	% response	Root number ^a	Root length (cm) ^a
NAA									
0.5	78.6 ^d	6.3 ^e	2.9 ^e	69.3 ^e	5.9 ^e	2.5 ^e	61.0 ^e	3.8 ^{de}	2.5 ^e
1.0	84.0 ^c	8.5 ^d	3.4 ^d	74.6 ^d	6.4 ^d	3.0 ^d	69.3 ^d	4.9 ^d	3.0 ^d
2.0	92.0 ^{ab}	12.1 ^b	4.3 ^c	81.3 ^c	7.3 ^c	3.8 ^c	74.6 ^{bc}	6.1 ^b	3.9 ^{bc}
3.0	95.0 ^a	15.0 ^a	5.8 ^a	90.0 ^a	9.0 ^a	5.2 ^a	80.0 ^a	7.0 ^a	4.8 ^a
4.0	89.3 ^b	10.5 ^c	5.0 ^b	86.6 ^{ab}	8.5 ^{ab}	4.5 ^b	76.0 ^b	5.8 ^{bc}	4.0 ^b

^aValues are mean of 25 replicates per treatment and repeated thrice

Values with the same superscript are not significantly different at 5% probability level according to DMRT

Conclusion

The present study was undertaken to develop an improved and efficient protocol for large scale production of *S. glauca* plants. Explants, namely, cotyledonary node, axillary bud and apical bud were collected from 2-month-old seedlings. The highest number of shoots (55.3) from cotyledonary node was obtained in MS medium supplemented with BA (3.0 mg/L), KN (0.5 mg/L), NAA (0.5 mg/l) and glutamine (10 mg/L). This was 5-10 folds higher compared to previous reports. The highest rhizogenesis was observed on MS medium supplemented with 3.0 mg/L NAA within 35 d of culture. The survival percentage of the plants in the field after acclimatization was 81.5%.

References

- 1 Armour R P, Investigations on *Simarouba glauca* DC. in El Salvador, *Econ Bot*, 13 (1959) 41-66.
- 2 Rath T, Simarouba—An exotic edible oil tree, *Indian For*, 113 (1987) 73-76.
- 3 Patil M S & Gaikwad D K, A critical review on medicinally important oil yielding plant Laxmiataru (*Simarouba glauca* DC.), *J Pharm Sci Res*, 3 (2011) 1195-1213.
- 4 Joshi, S & Hiremath, S, Simarouba—A potential oilseed tree, *Curr Sci*, 78 (2000) 694-697.
- 5 Rout G R & Das P, *In vitro* micropropagation of mature *Simarouba glauca* Linn.—An oil yielding tree, *Bangladesh J Bot*, 24 (1995) 137-141.
- 6 Shukla S P & Padmaja G, *In vitro* regeneration from shoot tip and nodal explants of *Simarouba glauca* DC, a promising biodiesel tree, *Int J Appl Biol Pharm Technol*, 4 (2013) 206-213.
- 7 Rout G R & Das P, Somatic embryogenesis from callus cultures of *Simarouba glauca* Linn., *Indian J Exp Biol*, 32 (1994) 581-583.
- 8 Das P, *In vitro* somatic embryogenesis in some oil yielding tropical tree species, *Am J Plant Sci*, 2 (2011) 217-222.
- 9 Rout G R, Samantary S & Das P, Root induction in microshoots of *Simarouba glauca* L. *in vitro*: Peroxidase as a marker for rooting, *Silvae Genet*, 48 (1999) 14-17.
- 10 Murashige T & Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiol Plant*, 15 (1962) 473-497.
- 11 Gomez K Z & Gomez A A, *Statistical procedure for agricultural research with emphasis of rice* (International Rice Research Institute, Los Bans, Philippines) 1976, p 294.
- 12 Purohit S D & Kukda G, Micropropagation of an adult tree—*Wrightia tinctoria*, *Indian J Biotechnol*, 3 (2004) 216-220.
- 13 Tiwari S, Shah P & Singh K, *In vitro* propagation of *Pterocarpus marsupium* Roxb.: An endangered medicinal tree, *Indian J Biotechnol*, 3 (2004) 422-425.
- 14 Dhar U & Upreti J, *In vitro* regeneration of a mature leguminous liana (*Bauhinia vahlii* Wight & Arnott), *Plant Cell Rep*, 18 (1999) 664-669.
- 15 Brassard N, Richer C, Tousignant D & Rioux, J A, Multiplication végétative de l'*Acer saccharum*: Contribution é la micropropagation, *Can J For Res*, 33 (2003) 682-690.
- 16 Padilla I M G & Encina C L, Micropropagation of adult cherimoya (*Annona cherimola* Mill.) cv. Fino de Jete, *In Vitro Cell Dev Biol-Plant*, 40 (2004) 210-214.
- 17 Bowen-O'Connor C A, Hubstenberger J, Killough C, VanLeeuwen D M & St. Hilaire R, *In vitro* propagation of *Acer grandidentatum* Nutt, *In Vitro Cell Dev Biol-Plant*, 43 (2007) 40-50.
- 18 Arockiasamy S, Ignacimuthu S & Melchias G, Influence of growth regulators and explant type on *in vitro* shoot propagation and rooting of red sandal wood (*Pterocarpus santalinus* L.), *Indian J Exp Biol*, 38 (2000) 1270-1273.
- 19 Komalavalli N & Rao M V, *In vitro* micropropagation of *Gymnema elegans* W & A—A rare medicinal plant, *Indian J Exp Biol*, 35 (1997) 1088-1092.
- 20 Bhatt I D & Dhar U, Micropropagation of Indian wild strawberry, *Plant Cell Tissue Organ Cult*, 60 (2000) 83-88.
- 21 Murch S J, Wierenga E J, El-Demerdash M A & Saxena P K, *In vitro* propagation of the Egyptian medicinal plant, *Echinops spinosissimus* Turra, *Plant Cell Tissue Organ Cult*, 70 (2003) 325-327.
- 22 Vasudevan A, Selveraj N, Ganapathi A, Kasthuriangan S, Anbazhagan V R *et al*, Glutamine: A suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L., *Biol Plant*, 48 (2004) 125-128.
- 23 Steephen M, Nagarajan S & Ganesh D, Phloroglucinol and silver nitrate enhances axillary shoot proliferation in cotyledonary nodal explants of *Vitex negundo* L.—An aromatic medicinal plant, *Iran J Biotechnol*, 8 (2010) 82-89.
- 24 Siwach P & Gill A R, Enhanced shoot multiplication in *Ficus religiosa* L. in the presence of adenine sulphate, glutamine and phloroglucinol, *Physiol Mol Biol Plants*, 17 (2011) 271-280.