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Research Article

IN VITRO REGENERATION FROM SHOOT TIP AND NODAL EXPLANTS OF *SIMAROUBA GLAUCA* DC, A PROMISING BIODIESEL TREE

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ABSTRACT: An efficient regeneration protocol was developed from shoot tip and nodal explants of *Simarouba glauca* DC, *a* promising biodiesel plant. Nodal explants appeared to have better regeneration capacity than shoot tip explants (40%) in the tested media. The highest regeneration frequency (90%) and shoot number (7.00 ± 1.00 shoots per explants) were obtained in nodal explants in Murashige and Skoog's (MS) medium supplemented with 6-benzylaminopurine (BAP) 4.43 µM and α-naphthalene acetic acid (NAA) 5.36 µM.Induced shoot buds were multiplied and elongated on the MS medium supplemented with BAP (4.44 µM), NAA (5.36 µM) and TDZ (Thidiazuron) 2.27 µM with 9.66±0.33 (mean length 5.35±0.32 cm) and 9.00±0.57 (mean length 4.51±0.15cm) shoots using nodal segments and shoot tip explants, respectively. Half-strength woody plant medium (WPM) containing 2.46µM indole-3-butyric acid (IBA) produced the maximum number of roots (6.00±1.15). The rooted plantlets were hardened on MS basal liquid medium and subsequently in polycups containing sterile soil and vermiculite (1:1) and successfully established in pots.

Keywords: *Simarouba gluca*, regeneration, biodiesl tree, acclimatization

Abbreviations: BAP -6-benzyl amino purine, $CaCl_2$ –Calcium chloride, IAA - Indole-3-acetic acid, IBA - Indole-3-butyric acid, KNO_3 –Potassium nitrate, MS- Murashige & Skoog medium, NAA –α-naphthalene acetic acid, NH_3NO_3–Ammonium nitrate, TDZ –Thidiazuron, WPM- Woody plant medium

INTRODUCTION

Simarouba glauca DC. commonly known as paradise tree or aceituno is a versatile fast growing multipurpose oilseed tree belonging to the Simaroubaceae family. It is considered as a potential source of edible biodiesel and also known for various medicinal properties having ability to check the soil erosion and improvement in the ground water balance.

It grows well in tropical and subtropical climates and widely distributed in South Florida, Central America and Kenya. In India it is cultivated in Orissa, Andhra Pradesh, Karnataka, Maharashtra and Tamilnadu. The seeds of Simarouba have edible oil content (60-75%) which can be used in manufacture of vegetable fat or margarine (Joshi and Hiremath, 2001).Simarouba oil is also used in industrial manufacture of soap, lubricant, paint, polishes and pharmaceuticals, etc. (Joshi and Hiremath, 2000). It is estimated that 10 million hectares of wasteland or marginal land has to be covered with oil trees in all to cater the needs of oil shortage in the nation (Joshi and Joshi, 2002). In India, out of 100 bio-diesel producing species, *S.glauca* is identified as a promising biodiesel tree having high yield potential (Neelakantan, 2004; Armour, 1959; Shantha *et al*; 1996). It has the potential (1000-2000 kg oil $ha^{-1}yr^{-1}$) to become one of the world's key energy crops in the coming years. The fruit pulp of this species contain 11-12% sugars (Rath *et al*; 1987) and useful for fermentation/beverage industry. This species is valued for its wood which is light and termite resistant, hence useful in making light furniture, toys, packing material, pulp (for paper industry) and matches. Tree is also used for medicinal purposes because bark and leaf containing triterpenes 'Simarubin' useful in curing amoebiasis, diarrhoea and malaria (Armour, 1959; Wright *et al*.1988; Franssen *et al*.1997; Rivero-Cruz *et al*; 2005). This ecofriendly tree can be planted even on marginal lands under water stress conditions.

S. glauca is conventionally propagated by seeds but major constraints in the large scale planting are the short viability of the seeds for 2-3 months, its polygamodioecious nature and difficulty in the identification of the sex of the saplings at early stage. In certain instances, 60% of the seedlings are males and out of the remaining females only few are high bearers (Sudhakar Babu *et al*, 2007), therefore softwood grafting of productive females is preferred for getting higher returns. Conventional methods of grafting has limitations as it is labour intensive, season dependent and requires sufficient number of root stocks. Therefore, an effective approach is needed for rapid and mass propagation of *S.glauca* by which cloning and multiplication of elite plants of desired sex (female plants) is possible within short time and space.

A few studies on *in vitro* culture of Simarouba have been reported using nodal and leaf explants (Rout &Das, 1995&1999), somatic embryogenesis from callus cultures of cotyledons (Rout &Das, 1994a&b, Das, 2011) and role of peroxidase in rooting of microshoots (Rout *et al*.1999b) however, yet there is no efficient *in vitro* protocol is available for the mass multiplication of this promising biodiesel plant.

Direct regeneration, without an intervening callus stage, is preferred since extensive callus formation and long-term callus culture can lead to somaclonal variations (Evans and Bravo 1986). Direct shoot regeneration from explants would maintain genotype fidelity, which could be lost in shoots arising from callus (Srivatanakul *et al*. 2000).Keeping in mind the economical importance of *S. glauca* as a potential biodiesel plant, critical analysis of the earlier protocol, in the present study we report an efficient system of *in vitro* direct shoot regeneration system for *S. glauca* using both shoot tip and nodal segment as explants. The influence of the age of donor plant for the explants and regeneration response *in vitro* was also studied.

MATERIALS AND METHODS

Plant resources, explant preparation and sterilisation

Shoot tip and nodal explants were collected from two-year-old seedlings which were procured from Andhra Pradesh Forest Academy, Dulapally, Hyderabad and maintained in the glasshouse of Department of Plant Sciences, University of Hyderabad, Hyderabad. *In vitro* studies were also conducted with the shoot tips and nodal explants obtained from ten-year-old mature tree of *S. glauca* available at Acharya N.G.Ranga Agriculture University, Rajendra Nagar, Hyderabad, Andhra Pradesh. Leaves were removed from the branches and cut into five to six segments, having one node in each segment. The explants were placed in a beaker containing tap water and washed thoroughly with running tap water for 30 min and for another 5 min with 1% aqueous solution of Tween 20.Explants were repeatedly washed with single distilled water and disinfected with 70% ethanol for 5 sec and subsequently surface sterilized with mercuric chloride solution (0.1% w/v) for 5 min followed by repeated rinsing with sterile double distilled water under aseptic conditions. Explants of 1.5 -2.0 cm were trimmed (~0.5 cm.) at the base and cultured on MS and WPM media with different concentrations and combinations of BAP (2.21-8.87 µM) and NAA (5.36µM) with 3% sucrose for shoot regeneration and multiplication. The multiplied shoots and axillary shoot buds, formed under optimal condition, were transferred for proliferation on MS and WPM media fortified with BAP (2.21-8.87 µM), NAA (5.36µM) and TDZ (2.27µM). The number of the proliferated shoots was determined after 30 days of culture.

Rooting

Normal and healthy regenerated shoots (2–2.5 cm in length) with at least two expanded leaves were transferred to the half and full-strength of WPM media with IBA (2.46µM and 4.92µM) and IAA (2.85 and 5.70 µM) for root induction. Nine explants were cultured in each treatment and all the experiments were repeated three times. The number and length of roots/shoot were recorded 4 weeks after shoots were placed on the root induction medium.

All experiments were carried out in culture tubes (150×25 mm) containing 20 ml of culture medium. The pH of the media was adjusted to 5.8 before gelling with 0.8%agar (w/v) (Hi-media, India). Cultures were incubated under 16h/8h light/dark cycles (artificial light of 75.7 µmol m^{-2} s^{-1}) at 24±2^{0}C.

Data collection and analysis

All experiments were carried out with three replicates in each treatment. Significant differences between the means, SE, SD, CV and ANOVA was done using the statistical software (CIMAP State version 0.4) available in the Division of Genetics and Plant Breeding of the Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India, that is based on the standard methods described in Panse and Sukhatme(1967).The results are expressed as mean ± standard error (SE).Standard error is given to indicate the variation among the means of six experiments based on three replicates for each treatment. The experiments were set up in a completely randomized design.

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Acclimatization

The *in vitro*-raised plantlets with well-developed roots were washed gently in running tap water to remove the attached medium and then transferred to plastic pots containing autoclaved soil, sand, farmyard manure and cocopeat (3:1:1:1),and soaked with1/2 strength WPM salts. Each pot was covered with polythene bags to maintain a high humidity (~ 90%) and kept in the culture room (26±2^{0}C). After 2 weeks, the humidity in the sealed pots was reduced by punching holes in the bags and then moved to the glass house (temperature 25^{0}C - 30^{0}C, photoperiod 12h, light intensity 500-1700 µmol m^{-2} s^{-1}).After two weeks, the polythene covers were removed and *in vitro*-raised plantlets were gradually exposed under glass house condition and successfully established in the pots containing soil and farmyard manure (3:1) and survival rate of *in vitro*-raised plantlets was recorded.

RESULTS

Culture establishment

The establishment of shoot tips and nodal explants was adversely affected by the browning of the media due to the leaching of phenolic compounds into the media from the cut ends of explants taken from mature trees limiting both the growth and differentiation of the shoots (Fig.1-a).Incorporation of 100 mgl^{-1} ascorbic acid and 0.02% activated charcoal in culture medium decrease the extent of browning but did not favour shoot initiation.

Axillary bud initiation at high frequency from nodal explants than shoot tip was observed within 30 days after inoculation. In case of shoot tip explants, shoot induction (5.00±1.00) was achieved on the same composition (Fig.1-b, c&d, Table -1).High frequency (90%) of multiple shoot induction (7.00±1.00) from nodal segments was observed on the MS medium augmented with 4.43µM BAP and 5.36µM NAA. On the other hand, the lowest percentage of multiple shoots induction was found to be 10% on the MS medium supplemented with 4.43µM BAP (Fig.1-e, f &g, Table -1&2). Out of two types of basal salts tested, MS medium supplemented with BAP and NAA led to a higher frequency of regeneration. These differences in frequency of shoot regeneration could be attributed to differences in basal salts composition.

Table-1: Effect of BAP and NAA on multiple shoot induction from shoot tip and nodal explants of *S. glauca*

Media and combinations of growth regulator (µM)			Shoot tip		Nodal segments		
NAA	BAP	% shoot formation	Mean number of shoot/ explant ± SE	Mean shoot length ± SE (cm)	% shoot formation	Mean number of shoot/ explant ± SE	Mean shoot length ± SE (cm)
MS + 0.0	2.21	00	00	00	00	00	00
	4.43	10	2.33±0.88	1.95±0.11	10	1.66±0.33	0.78±0.11
	8.86	15	2.66±0.66	1.67±0.11	20	1.33±0.33	0.87±0.23
MS + 5.36	2.21	25	4.00±0.57	1.99±0.20	65	1.66±0.33	1.24±0.31
	4.43	40	5.00±1.00	2.23±0.17	90	7.00±1.00	3.29±0.15
	8.86	30	3.00±0.57	1.56±0.21	80	1.33±0.33	1.25±0.32
WPM + 0.0	2.21	35	3.66±0.66	1.62±0.13	75	2.00±0.57	1.26±0.28
	4.43	30	3.33±0.33	1.46±0.08	50	3.66±1.20	1.51±0.11
	8.86	25	2.00±1.15	1.32±0.27	40	1.33±0.88	1.16±0.38
WPM +5.36	2.21	20	2.66±0.33	1.16±0.10	30	2.00±0.57	1.05±0.36
	4.43	20	2.33±0.66	1.70±0.18	35	2.33±0.66	1.34±0.14
	8.86	25	3.66±0.33	2.02±0.24	25	3.00±0.57	1.25±0.15
gm			3.05	1.69	2.30		1.16
sem			0.69	0.22	0.66		0.31
cd at 1%			2.74	0.88	2.63		1.25
cd at 5%			2.02	0.65	1.94		0.92

Data were recorded after 4 weeks of culture. Values represent mean ± SE of three replicates in three repeated experiments.

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Table-2: Analysis of variance (ANOVA) mean sum of square (M.S.S.) among 4 treatments in *S. glauca*

Sources of variation	d.f.	Shoot tip		Nodal segments	
		Number of shoots induced/ explants	Length of shoots (cm.)	Number of shoots induced/ explant	Length of shoots (cm.)
Treatments	11	2.47	0.562**	9.058**	0.841*
Error	24	1.44	0.149	1.333	0.304
Total	35				

*, **=P□0.05 and P□0.01, respectively

Shoot proliferation

Multiple shoot formation from shoot tip and nodal explants of *S.glauca* was evaluated on MS medium containing different concentrations and combinations of auxin and cytokinin. Out of two explants tested in our study, nodal explants produced greater number of shoots (9.66±0.33) after one subculture on MS medium with 5.36 µM NAA, 4.43µM BAP and 2.27 µM TDZ whereas the produced multiple shoot number was up to 9 in Shoot tip explants on the same multiplication medium (Table-2). In the present study BAP alone was proved less effective comparatively in the bud break and proliferation of the shoots. Multiplication of the shoots achieved with a high frequency from both explants on full-strength of MS medium with 4.43µM BAP and 5.36µM NAA but the same composition was not found suitable for good proliferation of the shoots and failed to elongate. Nine to ten elongated shoots (9.66±0.33) were achieved on the same multiplication medium fortified with 2.27µM TDZ after one subcultures in nodal explants (Table -3).

Table-3: Effect of BAP, NAA and TDZ on proliferation of shoot from shoot tip and nodal explants of *S. glauca*

Media and combinations of growth regulator (µM)			Shoot tip		Nodal segments	
NAA	BAP	TDZ	Mean number of axillary buds with multiple shoot ± SE	Mean shoot length ± SE(cm)	Mean number of axillary buds with multiple shoot ± SE	Mean shoot length ± SE(cm)
MS + 0.0	2.21	00	4.66±0.88	1.76±0.22	00	00
	4.43	00	5.66±1.45	1.91±0.20	5.33±0.88	2.20±0.16
	8.86	00	4.33±0.88	1.97±0.33	5.33±0.33	1.97±0.19
MS + 5.36	2.21	2.27	6.00±0.57	2.78±0.31	6.00±0.57	2.93±0.36
	4.43	2.27	7.00±0.57	4.51±0.15	9.66±0.33	7.35±0.32
	8.86	2.27	4.66±0.33	2.64±0.44	6.66±0.88	3.78±0.30
WPM + 0.0	2.21	2.27	3.66±1.20	2.00±0.50	5.00±1.00	3.89±0.16
	4.43	2.27	3.00±1.00	1.74±0.11	4.66±0.33	2.77±0.17
	8.86	2.27	3.33±0.88	2.02±0.31	4.33±2.18	3.10±0.37
WPM + 5.36	2.21	2.27	3.66±0.33	1.97±0.15	5.36±0.33	3.71±0.33
	4.43	2.27	2.33±1.45	1.15±0.25	4.66±0.33	2.54±0.37
	8.86	2.27	3.33±1.33	1.73±0.15	3.86±0.33	2.66±0.19
gm			4.44	2.23	6.36	2.23
sem			0.99	0.61	0.83	0.61
cd at 1%			3.93	2.42	3.29	2.42
cd at 5%			2.90	1.79	2.43	1.79
cv			38.79	47.50	22.69	38.11

Rooting and acclimatization

Well developed shoots of 1.0-2.0 cm length were isolated and cultured on full and half-strength of MS and WPM media having different concentrations of IBA and IAA for root induction. Microshoots raised *in vitro* failed to develop roots on GRs free basal media of WPM and MS. Out of the two media evaluated for rooting response the maximum number of roots (6.00±1.15) with 90 % root induction having an average length of 3.20±0.19 cm. were obtained on half-strength of WPM medium supplemented with 2.46µM IBA after 35 days of inoculation (P□0.01), whereas it was minimum (20%) on half-strength WPM medium supplemented with 5.70µM IAA (Fig.1-h &i, Table -5&6). Rooting efficiency was poor on IAA supplemented medium compared to IBA as reported in several plant species (Thomas, 2007; Yadav *et al*.1990). Moreover, presence of IAA resulted in the formation of basal callus with a low number of roots. After 35 days, well rooted plantlets were obtained. Subsequently, the plantlets were removed from the culture tubes, washed gently under running tap water and planted in pots containing soil and farmyard manure in 3:1(v/v) ratio (Fig.1-j&k).The potted plantlets were covered by transparent polythene sheet to maintain suitable humidity. After sufficient acclimatization, the plantlets were transplanted in the natural condition, where 80% plants were survived (Fig.1-l).

Table-4: Analysis of variance (ANOVA) mean sum of square (M.S.S.) among 4 treatments in proliferation and elongation of shoots in *S. glauca*

Sources of variation	d.f.	Shoot tip		Nodal segments	
		Number of shoots produced/culture	Length of shoots (cm.)	Number of shoots produced/culture	Length of shoots (cm.)
Treatments	11	9.59**	25.8**	2.10	5.02**
Error	24	2.97	2.08	1.12	1.18
Total	35				

**=P□0.01

Table-5: Effect of different concentrations of auxins on rooting of *in vitro*-raised shoots in *S.glauca* after 4 weeks in culture

Media and growth regulator (µM)		Rooting			
		Percentage rooting	Mean number of roots/shoot ± SE	Mean root length ± SE(cm.)	Callusing
WPM+IBA	2.46	40	2.66±0.33	2.03±0.32	No
	4.92	45	4.00±1.15	2.29±0.24	No
$^{1/2}$WPM+IBA	2.46	90	6.00±1.15	3.20±0.19	No
	4.92	80	1.33±0.88	2.20±0.68	No
$^{1/2}$WPM+IAA	2.85	30	1.33±0.33	2.08±2.06	Yes
	5.70	20	1.33±0.33	2.12±0.62	Yes
gm			2.77	2.26	
sem			0.79	0.59	
cd at 1%			3.42	2.58	
cd at 5%			2.44	1.84	
cv			49.47	45.83	

Table-6: Analysis of variance (ANOVA) mean sum of square (M.S.S.) among 4 treatments in *in vitro* rooting in *S. glauca*

Sources of variation	d.f.	Rooting	
		Number of roots/ shoots	Length of roots (cm.)
Treatments	5.0	10.8**	1.16
Error	12.0	1.88	1.07
Total	17.0		

Each value represents the mean ± SE of three independent experiments with 30 explants per treatment. Data with the same letter in a column were not significantly different according to Duncan's multiple comparison test**=P□0.01

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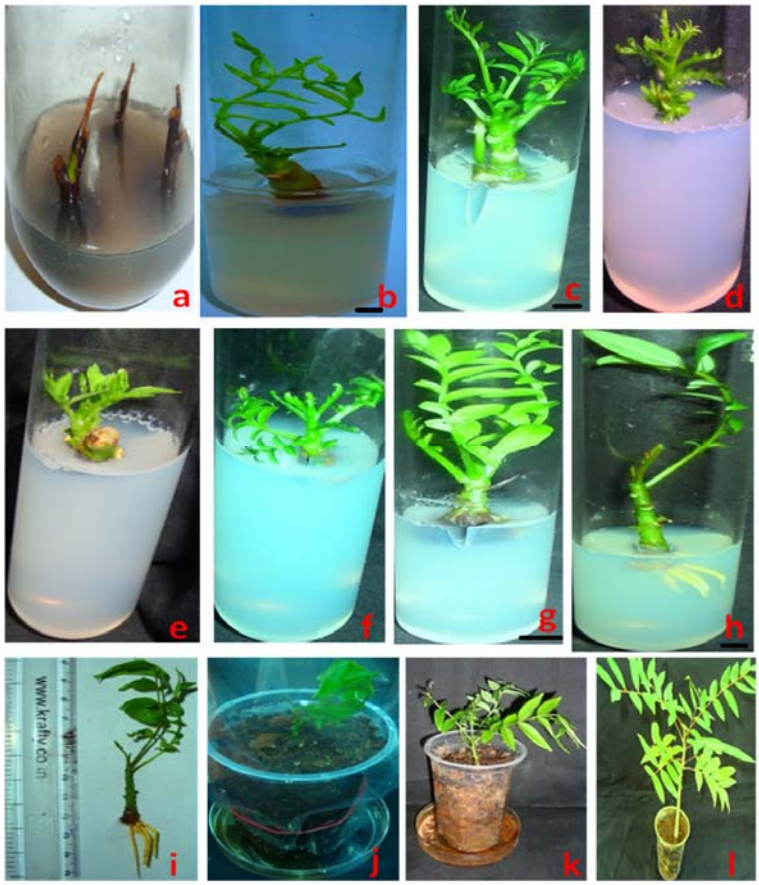

a : Culture showing leaching of phenols
b : Induction of shoot buds from shoot tip on MS+4.43µM BAP +5.36µM NAA within two weeks (Bar 0.22mm)
c : Multiple shoots induction from shoot tip on MS+4.43µM BAP +5.36µM NAA within two weeks (Bar 0.45mm)
d : Proliferation of the shoots from shoot tip on MS+4.43µM BAP +5.36µM NAA + 2.27µM TDZ within four weeks
e : Induction of axillary buds from nodal segments on MS+4.43µM BAP +5.36µM NAA within two weeks
f : Multiple shoots induction from nodal segments on MS+4.43µM BAP +5.36µM NAA within two weeks
g : Proliferation of the shoots from nodal segments on MS+4.43µM BAP +5.36µM NAA + 2.27µM TDZ within four weeks (Bar 0.73mm)
h&i : Rooting of in vitro- regenerated shoots on half-strength WPM+2.46µM IBA after 35 days (Bar 0.32mm)
j&k : Acclimatization of the in vitro-raised plantlets in culture room l : Acclimatized plant under natural condition

Figure 1: a-l: *In vitro* regeneration of *Simarouba glauca* DC from shoot tip and nodal segments

DISCUSSION

Plant regeneration was achieved from shoot tip and nodal explants tested in this study for the first time. However, a significant difference in the efficiency of shoot regeneration among the explants studied, with nodal segments exhibiting the highest in response.The addition of thidiazuron (TDZ 2.27µM) to the medium had a significant effect (P>0.05) on shoot proliferation (Table -4). The combination of BAP, NAA and TDZ was crucial for shoot proliferation and elongation as reported in several plant species (Thomas, 2007, Matand and Prakash, 2007).

The number of shoots per explants was higher when sub culturing was done on fresh medium at four week intervals on the similar medium. The average number of shoot buds per explants increased up to 2-3 fold (based on the explants shoot tip and nodal segment, respectively) within eight weeks of initial culture which could be maintained for longer periods without any loss in the morphogenetic potential. The type and concentration of basal salts affected shoot proliferation. The highest proliferation of shoots was obtained on WPM medium over MS. In *Litsea cubeba* (Mao *et al*., 2000) and *Gomortega keule* (Concha and Davey, 2011) it was reported that for shoot proliferation, WPM was preferable to MS as a basal medium. Present study contradicts the report of Rout & Das (1999) in Simarouba where they reported the inevitability of high concentration of IBA is necessary with full strength of MS medium for *in vitro* rooting whereas in this case percentage of rooting was enhanced on half-strength WPM supplemented with a very low concentration of IBA, although the number of shoots produced by each shoot was limited compared with the response of other woody species.

CONCLUSION

Nodal segment was the best explants showing maximum shoot regeneration on MS medium supplemented with 4.43µM BAP and 5.36µM NAA as reported in several medicinal and woody species, such as; *Azadirachta indica* (Kabir *et al*.1994), *Adhatoda vasica* (Banu *et al*.1997), *Centella asiatica* (Nath and Buragohain, 2003; Mohapatra *et al*.2008), *Aristolochia indica* (Manjula *et al*.1997). The optimum rooting of multiplied *Simarouba* shoots was achieved on half-strength WPM medium supplemented with IBA or IAA as reported that rooting of several woody plants is facilitated by a reduced (half) concentration of salts in the culture medium (Rout *et al*.,2000). By far, the present study on Simarouba has developed a refined protocol which can be easily adopted for large-scale micropropagation.

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