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# *IN VITRO* CULTURE ESTABLISHMENT AND CALLUS PROLIFERATION IN *SIMAROUBA GLAUCA L.*

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## Abstract

*Simarouba glauca*, a multipurpose, evergreen tree capable of withstanding diverse agro climatic conditions is indigenous to South Florida, West Indies and Brazil. Now it is cultivated in few regions of India like Maharashtra, Orissa, Rajasthan, Tamil Nadu, Karnataka and Andhra Pradesh. It has oil composition similar to popular oil feedstock such as *Jatropha curcas* that are being used to produce biodiesel. Poor seed germination, delayed flowering and failure of vegetative cutting paved way for the necessity to develop micro propagation techniques of this plant through organogenesis and callus cultures. An efficient regeneration protocol of *Simarouba glauca* was undertaken from nodal segment, shoot tip, leaf tips and leaf petiole explants to see the possibility of developing faster and reliable growth. Callus was induced under various medium compositions. The maximum callus induction frequency is reported with the MS basal media supplemented with 2.5mg/L 2, 4-D (CIM-4) in shoot tip explants and required least days for culture establishment. Nodal segments under similar medium concentration also resulted in better establishment of cultures as compared to the leaf segments whereas BAP and NAA along with MS basal medium were helpful in callus induction but failed to initiate further regeneration. Thus, results from present investigation clearly indicated the possibility of multiplying *Simarouba glauca* through micro propagation using above medium compositions and would help in increasing its population rapidly in India.

***Key words:*** *Simarouba glauca*, media, callus, micropropagation, sterilization etc.

## Introduction

The increasing demand for the forest products and the continuous deterioration of natural resources means that we cannot rely only upon the exploitation of natural forests (Jain *et al.*, 1997; Tzfira *et al.*, 1998). Thus, there will be an increasing pressure on existing forest lands because of limited expansion of arable land, rapid growth of human population, improvement of economic status, environmental degradation, deforestation, water shortages and global warming (Jain, 1998). Due to diminishing rate of fossil fuels and their higher emission capacity, the demand for alternative or non-conventional fuels increases and biofuel can be one of the alternative resources (Chhetri and Islam, 2008; Berktay and Nas, 2008; Demirbas, 2007; Konwer *et al.*, 2007; Demirbas A., 2007; Najafpour *et al.*, 2006; Demirbas, 2007; Alkeptin and Mustafa, 2008; Demirbas, 2008). Biofuels can be obtained or generated from biomass or biological material collected from living material or living resources. Recommending a low-cost input technology for cultivating woody plants that can grow well even with erratic and low rainfall, in turn giving assured returns is of great significance.

*Simarouba glauca DC*, paradise tree or Lakshmi taru, a multipurpose, evergreen tree capable of withstanding diverse agro climatic conditions, indigenous to South Florida, West Indies and Brazil. (Joshi and Joshi *et al.*, 2002) can be a feasible alternative. In India it is cultivated in Maharashtra, Orissa and is at the introductory stage of plantation in other regions like Rajasthan, Gujarat andhra Pradesh, Tamil Nadu, West Bengal, Karnataka and Orissa. It can even be grown on waste and marginal land and is not grazed up by cattles and can be used for the reclamation of wastelands in India (Sharma and Dwivedi, 2016). It has oil composition similar to popular oil feedstock such as *Jatropha curcas* that are being used for the production of biodiesel (Dash *et al.*, 2008). Its seeds can also be used as a coagulant for water treatment after the extraction of oil from it. The oil cake

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left after oil production is rich in nitrogen, potash and phosphorous which works as a good organic manure. The oil extracted from *Simarouba* can be processed to form edible oil because its seeds are rich in edible fat and can be used for cooking. It also has a wide range of ethanobotanical and pharmaceutical properties. *Simarouba* bark is a very effective medicine in dysentery, malaria and fever. *Simarouba* wood is light, attractively grained, moderately strong, generally less preferred by wood eating insects and hence best suited for timber, fuel and paper industry. *Simarouba glauca* takes 5-6 years to grow into a complete flowering tree and it is polygamo-dioecious. Poor seed germination, delayed flowering and poor growth of vegetative cuttings paved way for the necessity to develop micro propagation techniques in *Simarouba glauca* through organogenesis and callus cultures (Heller, 1996; Purkayastha *et al.*, 2010; Openshaw, 2000). Mass multiplication techniques for Simarouba are not yet standardized, as evident from the available literature. However, attempts have been made for its *in vitro* shoot multiplication (Rout and Das, 1994; Rout *et al.*, 1999). Present study is designed to develop an efficient regeneration protocol for callus induction using nodal segments, shoot tips, leaf tips and leaf petioles as explants.

## Material and Methods

Healthy seeds and plant samples for the micropropagation and regeneration of *Simarouba glauca* were procured from the experimental farm of Central Agro-Forestry Research Institute (CAFRI), Jhansi (U.P) and Forest Research and Development Department (TAPOVAN), Gwalior (M.P).

### Selection of Explants

The different parts of plant like leaf, petiole, shoot tips, nodal segment, axillary buds and stem tips were used as explants Different plant parts were excised and 0.3-0.4 cm long pieces were cultured on the callusing media.

**Table 1:** Different types of media composition for callus induction.

| S. No. | Media | Basal media | Auxin (mg/L) | | | | Cytokinin (mg/L) |
|---|---|---|---|---|---|---|---|
| | | M.S | NAA | 2,4-D | IAA | IBA | BAP |
| 1 | CIM-1 | M.S | 0.0008 | | | | 0.150 |
| 2 | CIM-2 | M.S | - | 0.5 | - | - | - |
| 3 | CIM-3 | M.S | - | 1.0 | - | - | - |
| 4 | CIM-4 | M.S | - | 2.5 | - | - | - |
| 5 | CIM-5 | M.S | - | 5.0 | - | - | - |

### Sterilization of Explants for Callus Culture

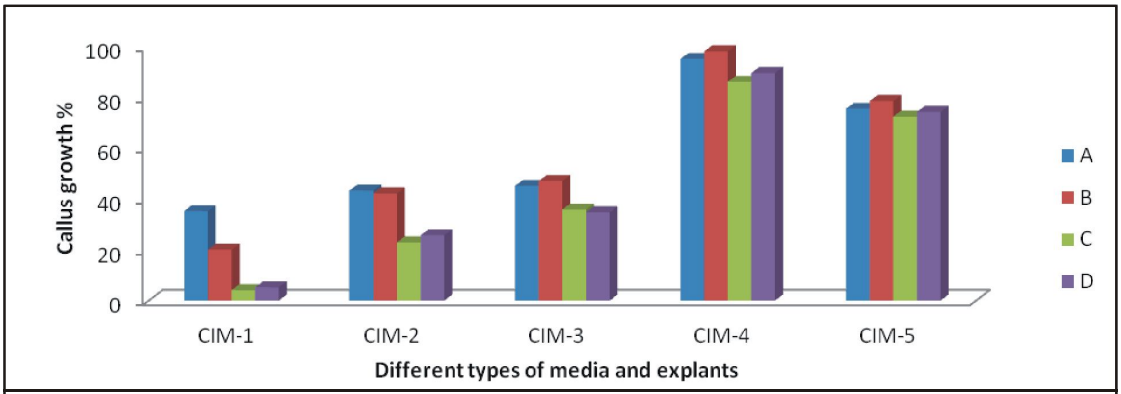
Fresh, healthy and young explants were collected and washed properly under running tap water for half an hour. Explants were then treated with 2.5% Ascorbic acid for 10 minutes followed by 0.1% $HgCl_2$ for 3 minutes and sterilized explants were properly washed with double distilled water 4-5 times to remove the traces of sterilizing chemicals and explants were kept in autoclaved distilled water prior to the inoculation (Sharma and Dwivedi, 2016). All the experiments were repeated thrice and had 10 replicates each of a single explant.

### Media Preparation

Total of five types of different media combinations (using NAA, BAP and 2, 4-D) were used for the *in vitro* regeneration of *Simarouba glauca* as given in the table 1. Charcoal was also used to enhance regeneration, but it was found ineffective for growth.

## Results and Discussion

In the present study four explants were selected and after several rounds of preliminary studies, experimentation by trial and error, callus induction media were selected. The selected media combinations include a wide range of different levels of auxins and cytokinins in various combination and ratio as given in the table 1.




(CIM- Callus induction media, A- Nodal Segment, B-Shoot tip, C-Leaf Tip, D-Leaf petiole)

**Fig. 1:** Percentage of callus growth under different medium conditions.

**Table 2:** Response of callus under different culture conditions.

| Media | Explant | Response in % | | | | | Callus Characteristics | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of callus | 1 -7 Days | 7 - 14 Days | 14 - 21 Days | 21 - 28 Days | Nature | Colour | Callus size (cm) |
| CIM-1 | A | 8 | 10.5 | 26.8 | 39.4 | 35.1 | Compact, Green | PG | 0.56 ± 0.31 |
| | B | 4 | 8.2 | 10.4 | 15.3 | 20 | Compact, Green | PG | 0.52 ± 0.30 |
| | C | 8 | 5.3 | 2 | 3.2 | 4 | Fragile | PY | 0.48 ± 0.34 |
| | D | 6 | 6 | 5.2 | 7.4 | 5.1 | Globular, Fragile | PY, PG | 0.35 ± 0.19 |
| CIM-2 | A | 13 | 10 | 25.3 | 35.4 | 43.2 | Fragile, Green | PG, PY | 0.55 ± 0.38 |
| | B | 14 | 12 | 26.2 | 34.3 | 42.1 | Fragile, Compact | PY | 0.51 ± 0.36 |
| | C | 9 | 8 | 13.5 | 20.4 | 22.8 | Globular, Compact | PG, Y | 0.48 ± 0.29 |
| | D | 8 | 10.1 | 14.2 | 23.4 | 25.6 | Fragile Green | PY, G | 0.41 ± 0.28 |
| CIM-3 | A | 14 | 13.5 | 26.4 | 36.5 | 45.0 | Green, Globular | PY, PG | 0.56 ± 0.42 |
| | B | 13 | 15.0 | 30.5 | 39.8 | 47.0 | Compact, Green. | PG | 0.59 ± 0.47 |
| | C | 11 | 12.2 | 22.3 | 30.5 | 35.7 | Green, Compact | PG | 0.55 ± 0.41 |
| | D | 09 | 12.3 | 21.4 | 29.3 | 34.6 | Compact | PYG | 0.56 ± 0.39 |
| CIM-4 | A | 21 | 28.0 | 59.2 | 78.0 | 95.0 | Compact | PY, G | 0.80 ± 0.48 |
| | B | 25 | 30.0 | 63.4 | 85.3 | 98.0 | Globular, Compact | PYG | 0.86 ± 0.54 |
| | C | 18 | 23.3 | 60.8 | 75.0 | 86.0 | Compact | PY, PG | 0.68 ± 0.54 |
| | D | 17 | 24.0 | 62.0 | 78.4 | 89.5 | Globular, Compact | PY, PG | 0.71 ± 0.59 |
| CIM-5 | A | 16 | 25.2 | 40.3 | 69.6 | 75.4 | Globular Compact | PG | 0.55 ± 0.39 |
| | B | 18 | 29.1 | 45.4 | 70.3 | 78.5 | Fragile Compact | PY | 0.56 ± 0.40 |
| | C | 13 | 23.0 | 39.8 | 65.4 | 72.3 | Green Compact | PGY | 0.50 ± 0.30 |
| | D | 10 | 24.0 | 40.5 | 67.3 | 74.1 | Green Globular | PG | 0.52 ± 0.35 |

CIM- Callus induction media, A- Nodal Segment, B-Shoot tip, C-Leaf Tip, D-Leaf petiole

Only 35% callogenesis was noticed in the explants from nodal segments having satisfactory growth along with yellowish green callus having size of 0.56±0.31cm while rest of the explants responded poorly under CIM-1 (MS+ 0.0008mg/L NAA +0.150mg/L BAP) as shown in the table 2, fig 1 and 2 and this is in contradiction to the results of Verma, (2013) with the 60% response under MS medium supplemented with NAA (0.125ppm) and BAP (1.5ppm) and also found enhanced growth of callus with 50-60% induction frequency in apical buds as compared to the leaf segments under MS medium supplemented with NAA (0.5 mg/L) + BAP(2.0 mg/L). Callus turned yellow from white in color within 60 days which confirms the findings of Rout and Das, (1994), as they also observed yellow coloured callus. In the present study nodal segments responded more positively towards the callus proliferation rather than shoot tip explants and similar results have been observed by Shrivastava and Banerjee *et al.,* (2008) that nodal explants appeared to have 40% more regeneration capacity as compared to the shoot tip. Rout and Das, (1994) observed maximum callus proliferation with MS+ 13.42 µM NAA and BA 11.1 µM but the present investigation does not confirm their findings as we achieved maximum callus induction with MS medium supplemented with 2, 4-D. Leaf segments showed poor induction of callus which is supported by Verma (2013) whereas Rajore and Batra, (2007) reported callus induced from leaf explants on MS

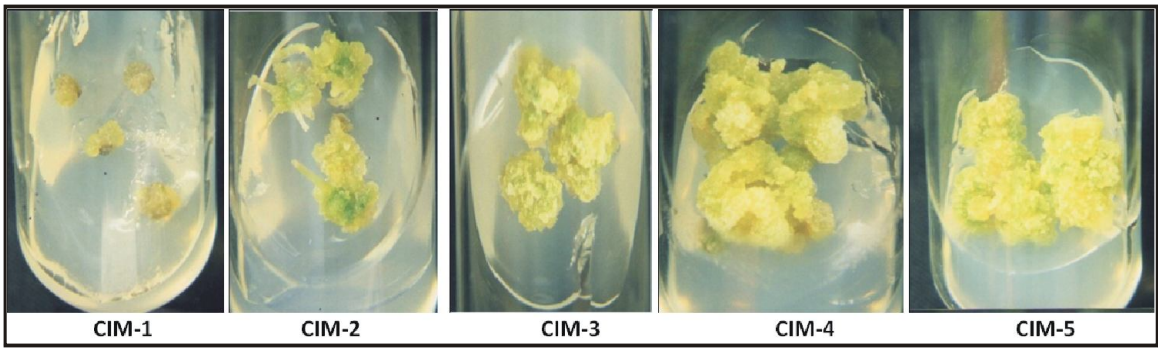



**Fig. 2:** Response of callus under different medium.

basal medium supplemented with BAP (5.0mg/L) + NAA (1.0mg/L) and also concluded that these hormones are best for the callus induction in *Jatropha curcas*. No significant response was seen using charcoal as an additive (Warakagoda and Subhasinghe, 2009) similarly in our study use of charcoal as an additive in the CIM-1 media did not favour the growth of callus. Dudhare *et al.,* (2008) reported callus induction under MS medium supplemented with different combinations of BAP and 2, 4-D while the present investigation confirms the proliferation of callus in the presence of 2, 4-D (CIM-2, 0.5mg/L) alone from all types of explants within 10-15 days. However, frequency of callus induction was found to be higher under CIM-3 during sub culturing (MS + 0.5 mg/L 2, 4-D) as shoot tips and nodal segments having 42-43% callus formation having pale yellowish green color. Leaf segments also responded positively under this combination of media and 22-25% induction of callus with the average size of 0.45±0.25cm was observed similarly.

Healthy, globular, green and compact callus was reported with nodal segments and shoot tip explants showing comparatively better response with continuous increment in callus growth under CIM-3 (MS+1.0mg/L 2, 4-D) media whereas leaf tip explants were found to have moderate response and slow growth was observed with leaf petiole explants. However, Kakuturu *et al.,* (2014) observed 77% callus induction from leaf explants under M.S medium supplemented with various combinations of NAA and 2, 4-D (0.5 to 5mg/l) & a fixed amount of BAP. When we used MS, basal media supplemented with 2, 4-D (2.5mg/L) results obtained were highly significant. When callus was sub cultured it showed comparatively higher induction frequency in nodal segments with 45% response in 28 days with 0.56 ± 0.42cm size of callus having pale yellow green color whereas increased callus size of 0.59 ± 0.47cm with 47% response was observed in shoot tips while Meena *et al.,* (2008) reported only 25% induction of callus from nodal segments under similar concentration of 2, 4-D. However, 34-35% callus formation was reported with leaf segments in the present study. Highest callus induction was reported with shoot tip explants under CIM-4 (MS + 2.5mg/L 2, 4-D) and the callus was pale yellowish in color with maximum survival while nodal segments gave optimum growth within 6-7 weeks. Dudhare *et al.,* (2014) reported that callus proliferation was observed in different concentrations of 2, 4-D and BAP however the present study revealed better callogenesis with higher concentration of both 2, 4-D and BAP and required fewer days for establishment from shoot tips, but in case of leaf explants the results were not satisfactory as the leaf tip and leaf petiole explants demonstrated moderate response under similar conditions. Kakuturu *et al.,* (2014) has concluded that 2, 4-D to be an essential part of the medium for callus induction in case of *Simarouba glauca* and our study showed that 2, 4-D when used alone proved to be highly effective for induction of callus. Results obtained with CIM-4 were highly significant.

Maximum callus of 0.86±0.54cm with 98% callus induction was reported with the shoot tip while nodal segments had 95% response with compact green callus of 0.80±0.48cm size whereas 86% to 89% callus induction was observed with leaf tip and leaf petioles explants. Therefore 2, 4-D proved to be an important factor for callus induction in *Simarouba glauca* however Verma *et al.,* (2008) observed only 35% callus induction with 2mg/L of 2, 4-D and 95% with 4 mg/L 2, 4-D. In CIM-5 (MS+5mg/L 2, 4-D) media callus induction frequency was 76-78% with explants of nodal segments and shoot tip while explants of leaf tip and leaf petiole did not respond positively whereas Dudhare *et al.,* (2014) observed maximum callus proliferation by using MS+0.5 mg/L 2, 4-D and required least days for further growth of callus. While 70-75% callus induction frequency having pale yellowish green color was reported under CIM-5 in almost all the explants. Addition of charcoal to the medium had no effective response on callus induction.

## Conclusion

An effective protocol for *in vitro* regeneration of *Simarouba glauca* in a short period of time can be developed through the encouraging results of the present study. Shoot tip explants proved to be best suited for callus induction under 2, 4-D treatment. Establishment of shoots and roots can be possible after the successful callus induction. The results from this study provides the template for increasing the plantation of *Simarouba glauca* and this may prove beneficial in utilization of large stretch of wastelands in India.

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