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Efficient plant regeneration protocol for *Simarouba glauca* DC: A medicinal tree and an alternative source of edible oil - using explants derived from physiologically mature trees

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Abstract
An efficient and reproducible plant regeneration protocol was developed from petiole and axillary bud explants derived from physiologically mature trees of *Simarouba glauca* DC, a promising medicinal tree that has potential to offer an alternative source of edible oil. This is the first ever report of plant regeneration protocol for mature trees of *Simarouba glauca* DC. PVP (1.5 g/l), ascorbic acid (100 mg/l) and citric acid (25 mg/l) was used to effectively counter the phenolics released from the explants of the mature tree. Petiole explants appeared to have better regeneration capacity than axillary bud explants. The petiole explants produced 88.47 % embryogenic calli on MS + BA (2.5 mg/l) + Kinetin (1.5 mg/l) + IBA (0.5 mg/l) + NAA (0.1 mg/l). The calli produced were green and friable. The maximum number of shoots (09.63±0.11) with a shoot length of 05.41±0.12 cm were produced from theses embryogenic calli derived from the petiole explants on the MS medium supplemented with 2.0 mg/l BA + 1.0 mg/l 2ip + 0.3 mg/l GA_3. The petiole explants showed a shoot induction per cent of 92.34±0.12 which was higher than that produced from the axillary bud explants. Profuse rooting was obtained from the shoots regenerated from the petiole explants on the MS medium supplemented with IBA (0.1 mg/l) + NAA (0.5 mg/l) having a root induction response of 92.50±0.06 % with the highest number of roots (06.72±0.13) and root length (05.74±0.16 cm). The survival percentage of the plants in the field after acclimatization was 85.5%.

Keywords: *Simarouba glauca*, regeneration, cytokinins, acclimatization, additives, shoot multiplication

Introduction
The edible oil scenario in India has undergone a magnanimous change in the past few decades. India has an annual edible oil requirement of 21 mt. The edible oil consumption is following a rising trend in India having doubled in the last ten years. Attaining self sufficiency in edible oil seems to be a mirage. Ultimately there is no alternative to boosting domestic production rather than to explore and propagate new oilseed stocks that are perennial and that hold a long term promise to reduce the dependence on imports along with ensuring health and economic security. Because India is perennially deficit in edible oils, there is a ready market for simarouba oil. The tree-borne oilseed, *Simarouba glauca* DC. is a versatile evergreen tree attaining immense attention as a prospective energy crop and medicinal plant. Every segment of the plant is beneficial. Simarouba seed includes 35– 40% kernel. Simarouba fat is edible and is at par with other edible oils. Simarouba was introduced in India by National Bureau of Plant Genetic Resources as a potential source of vegetable oil during late sixties in sub-humid climate of Orissa. Soon after, the cultivation of simarouba extended to the semi arid, dry and saline land areas of Gujarat, Maharashtra, Tamilnadu, Karnataka and Andhra Pradesh (Rath, 1987) [10]. Simarouba tree is capable of growing competently in marginal wasteland with degraded soil. Seeds contain 55–70% edible oil and so are economically important. Additionally, Simarouba oil can also be exploited for the manufacture of soap, lubricant, paint, polishes and pharmaceuticals. Shells can be used in hard board industry. Beverage industry can proficiently use the fruit pulp, composing 11–12% sugars. Productive manure can be concocted from leaf litter. Bark and leaf have pharmaceutical value. This multipurpose tree crop with wide range of adaptability to different agroclimatic regions, easy maintenance and with an inbuilt genetic potential to give high oil yield, is going to become one of the very important oilseed crops in the country. The said tree has got the potential of plantation in the waste land area, as it requires very less water and does not require any high quality supervision (Patil and Gaikwad. 2011) [7]. In general, *Simarouba* is propagated by seed, but the seeds generate variation and it is a polygammodioecious tree and also require 5-6 years for bearing

of fruits, therefore the genetic uniformity is also compromised through seed propagation. Availability of planting material is very less. Very few studies have been carried out in tissue culture of *Simarouba glauca*. High multiplication ratio in such cases can be achieved by tissue culture techniques, which enables to manifold increase of desired plants in a short time.

Earlier reports on micropropagation of *S. glauca* have recorded only 5.83 and 9.66 shoots per nodal explant, respectively by Rout and Das (1999) [14] and Shukla and Padmaja (2013) [15]. Others have reported somatic embryogenesis from callus cultures of cotyledons and embryonic axil with cotyledons. While Rout *et al*., (1994) [12] have demonstrated the role of peroxidase in rooting of microshoots. A standard protocol is not available for rapid multiplication of these plants.

In the present study, an efficient micropropagation protocol from different explants of physiologically mature tree of *S. glauca* has been reported. This research paper is the first successful report of regeneration of *Simarouba glauca* from the explants derived from physiologically mature trees. The role of different plant growth regulators on the callus induction, shoot and root multiplication have been studied.

Material and Methods

Plant Material

Young leaves and axillary buds of *Simarouba glauca* were collected from 8-9 year old trees grown in the Centre for Crop Improvement, SDAU, S.K. Nagar, Gujarat, India.

Selection of explants and sterilization

The young leaves and axillary buds collected were washed in running water and were rinsed with distilled water 2-3 times in the laboratory. Leaf petioles (1.0 cm) and axillary buds, used as explants were excised in the laminar air flow hood. The explants were treated for 5 minutes with 0.1% (w/v) mercuric chloride with 2 drops of tween-20 per 100 ml solution and rinsed once with sterile distilled water followed by a short treatment with 70% alcohol for 30 seconds. Finally, the explants were rinsed 4-5 times with sterile distilled water.

Callus induction and somatic embryogenesis

Callus induction and somatic embryogenesis was carried out on the Murashige and Skoog (MS) [6] supplemented with BA (2.5 mg/L), KIN (1.5 mg/L), naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) (0.1-1.0 mg/L) along with adenine sulphate (50 mg/l), glutamine (100 mg/l), ascorbic acid (100 mg/l), citric acid (25 mg/l), PVP (1.5 g /l), sucrose (35 g/l), agar (7g/l).

Shoot bud induction and proliferation

Murashige and Skoog (MS) [6] was used along with various concentrations of plant growth regulators like 6-benzyl adenine (BA) and kinetin (KIN) (0.5-3.5 mg/L), individually or in combination for shoot bud induction along with adenine sulphate (50 mg/l), glutamine (100 mg/l), ascorbic acid (100 mg/l), citric acid (25 mg/l), PVP (1.5 g /l), sucrose (35 g/l), agar (7g/l).

Shoot multiplication and elongation

The shoot buds were transferred to the multiplication medium supplemented with BA (0.5-3.0 mg/L) and 2ip (0.5-2 mg/L) along with GA_3 (0.3 mg/l) individually or in combination for shoot multiplication. All other additives like adenine sulphate (25 mg/l), glutamine (100 mg/l), ascorbic acid (75 mg/l), citric acid (25 mg/l), PVP (1.0 g /l), sucrose (30 g/l), agar (7g/l) were used to supplement the multiplication medium.

In-vitro rooting

The elongated shoots were subcultured on ½ strength MS medium supplemented with NAA, IBA (0.1, 0.5 and 1.0 mg/l) for rooting.

Culture conditions

The cultures were incubated in culture room maintained at 25±2° C, under a 16 h photoperiod with a light intensity of 40 $\mu\ m^{-2}\ s^{-1}$ from Philips cool white fluorescent tubes with 100% relative humidity.

Acclimatization and Hardening

In vitro raised plantlets (5 month old) with well developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transferred to plastic cups containing potting-mixture of sterilized sand, soil and coco peat (1:1:1). The plantlets were maintained inside the culture room covering them with polythene cover to maintain the relative humidity. The plantlets were watered with 1/2 strength MS basal salt solution devoid of sucrose and myo-inositol supplemented with 1mg/l IBA at 2 d interval for a period of 15 days. The regenerated plantlets were shifted to polyhouse, the polythene cover was removed and the plantlets were transferred to big polythene bags containing soil and kept under shade for another 2 months before transferring to the field.

Statistical analysis

A minimum of 30 cultures 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. All experiments were performed in triplicate and were submitted to the Student's t-test which was used to indicate significant differences ($p < 0.001$ or $p < 0.05$). Data represent means ± standard error for three replicates.

Results and Discussions

Extensive sterilization of explants was carried out to establish aseptic cultures as the explants were collected directly from the field grown physiologically mature trees of *Simarouba glauca* DC. Being a medicinal tree, *Simarouba glauca* DC produces a large amount of phenolic substances which are released from the cut ends of the explants. This result in browning of the culture media and the explants which fail to produce any response under *in vitro* conditions. Anti-phenolic agents like polyvinyl pyrrolidone (PVP), ascorbic acid and citric acid were used in this study to minimize browning of the explants and media. PVP (1.5 g/l), ascorbic acid (100 mg/l) and citric acid (25 mg/l) effectively controlled the browning of the cultures and enhanced the culture establishment.

Callus induction and somatic embryogenesis

The petioles and the axillary bud explants were cultured on the solid MS medium supplemented with adenine sulphate (50 mg/l), glutamine (100 mg/l), ascorbic acid (100 mg/l), citric acid (25 mg/l), PVP (1.5 g /l), sucrose (35 g/l) and various concentrations of cytokinins and auxins. It was observed that the petiole explants started expanding within a week of culture while the axillary buds responded after 20 days of inoculation. The callus initiation from petiole explants started

after two weeks of culture while it took four weeks for the axillary bud explants to form callus. The petioles developed into green friable calli while the axillary buds formed white and compact calli. The callus induction percentage was higher in the petiole explants (98.23 – 0.00 %) as ccompared to the axillary bud explants (68.49 – 0.00 %) (Fig.1a). The development off embryogenic calli was higher in the petiole explants (88.47%) than the axillary bud explants (65.32%). The MS medium supplemented with BA (2.5 mg/l) + Kinetin (1.5 mg/l) + IBA (0.5 mg/l) + NAA (1.0 mg/l) produced the highest calli in petiole as well as in axillary bud explants (Fig 1b). The petiole explants produced the highest embryogenic calli when cultured on the MS medium supplemented with BA (2.5 mg/l) + Kinetin (1.5 mg/l) + IBA (0.5 mg/l) + NAA (0.1 mg/l) while the axillary bud explants developed highest number of embryogenic calli on the same medium on which it produced highest calli (Fig. 2). The development of embryogenic calli was found to be dependent on the auxin concentration and NAA was found to be the major auxin to promote the development of embryogenic calli (Fig. 1). According to Rout and Das, (1994) [12], the maximum callus induction was found in MS + BA and MS + NAA but our investigation does not confirm their findings as we achieved maximum callus induction with MS + BA + KIN +IBA + NAA. Kakuturu *et al*., 2014 [4] also obtained friable embryogenic calli from leaf explants on MS medium supplemented with NAA and 2, 4-D. The efficiency of embryogenesis was strongly dependent on the composition of auxin and cytokinin concentrations. Our findings agree well with the results of several species of woody plants (James *et al*. 1984) [3]; (Rout *et al*., 1991) [13]; (Radojevic, 1988) [9]. Our findings are in contradiction to Dudhare *et al*., 2014 [2] who obtained yellow compact calli from axillary bud explants, however we obtained friable green calli from the petiole explants and white compact calli from the axillary bud explants.

Shoot bud induction and proliferation

The shoot regeneration frequency was found to be dependent on the concentrations of the different cytokinins used in the culture media. Out of the sixteen combinations of media tested, petioles showed the highest shoot induction frequency of 92.34±0.12 per cent observed on MS medium supplemented with 2.5 mg/l BA + 2.0 mg/l Kinetin followed by 88.75±0.13 observed in MS medium supplemented with 3.5 mg/l BA and 1.0 mg/l Kinetin. As the concentration of BA and Kinetin was decreased, the shoot induction frequency drastically reduced. The same sixteen combinations of media were tested for axillary bud explants and the highest shoot induction frequency of 83.33±0.23 per cent was obtained on MS medium supplemented with 1.5 mg/l BA and 1.0 mg/l Kinetin followed by 76.90±0.17 per cent on MS medium supplemented with 1.5 mg/l BA and 2.0 mg/l Kinetin. As the concentration of BA and Kinetin was increased, the shoot induction frequency drastically reduced. (Table 1)

The average number of shoot buds induced per calli was higher for petiole explants (29.54±0.28) obtained in the MS medium supplemented with 3.5 mg/l BA + 1.0 mg/l Kinetin; as compared to that produced by axillary bud explants (14.29±0.15) obtained on the MS medium supplemented with 1.5 mg/l BA + 2.0 mg/l Kinetin (Fig. 1c-d). In corroboration to our results combination of BA and KIN has been also reported to induce shoots in many woody tree species like *Wrightia tinctoria* (Purohit and Kukda, 2004) [8], *Pterocarpus marsupium* (Tiwari *et al*., 2004) [16] and *Bauhinia vahlii* (Dhar and Upreti, 1999) [1].

Shoot multiplication and elongation

Although several plant growth regulator combinations was used for shoot multiplication in *Simarouba glauca* DC type of explants can influence the shoot multiplication. The choice of explant is a critical factor that determines the success of most tissue culture experiments.

Among the explants, petioles showed best response compared to axillary bud explants. The petiole explants produced 09.63±0.11 number of shoots per callus on the MS medium supplemented with 2.0 mg/l BA + 1.0 mg/l 2ip + 0.3 mg/l GA_3 while the axillary buds produced 05.87±0.27 average shoots per callus on MS medium supplemented with 1.0 mg/l BA + 2.0 mg/l 2ip + 0.3 mg/l GA_3 (Fig 1e-h). The shoot length obtained was highest for the petiole explants (05.41±0.12 cm) obtained on the MS medium supplemented with 3.0 mg/l BA + 0.5 mg/l 2ip + 0.3 mg/l GA_3 (Table 2). The axillary bud explants produced shoots with shoot length ranging from 04.36±0.12 to 02.35±0.32. Unlike our findings, Lavanya *et al*., (2016) [5] reported maximum shoot multiplication from cotyledonary explants derived from seedlings of *Simarouba glauca.* Many researchers have reported good extent of multiple shoot induction from seedling derived explants, on MS medium supplemented with BA, IAA (Dudhare *et al*., 2014) [2]; MS + BA (Rout *et al*., 1999) [14]; MS + B + KIN + NAA (Lavanya *et al*., 2016) [5]; MS + BA + NN + TDZ (Shukla and Padmaja, 2013) [15], while we report Maximum shoot multiplication on the MS medium supplemented with BA + 2ip + GA_3.

***In vitro* rooting**

The multiple shoots regenerated were excised individually and were transferred to half strength MS medium supplemented with IBA (0.0-1.0 mg/L) and NAA (0.0-1.0 mg/L). The shoots that were transferred to MS medium with IBA (0.1 mg/l) + NAA (0.5 mg/l) showed the highest rooting response (92.50±0.06 %) with the highest number of roots (06.72±0.13) and root length (05.74±0.16 cm) for petiole explants (Table 2; Fig. 1g). Besides, the shoots that were transferred to MS medium with IBA (0.1 mg/l) + NAA (1.0 mg/l) showed the highest rooting response (69.27±0.13 %) with the highest number of roots (04.83±0.16) and root length (04.06±0.16 cm) for axillary bud explants (Table 3). On the contrary, maximum number of roots was obtained on half strength WPM medium + IBA by Shukla and Padmaja, (2013) [15]; full strength MS + IBA (Rout and Das, 1999) [14]; full strength MS + NAA (Lavanya *et al*., 2016) [5]; full strength MS + IAA (Dudhare *et al*., 2014) [2].

Acclimatization and hardening

The sturdy regenerated plantlets with the lengthy and strong roots were transferred to plastic containers containing mixture of sterilized sand: soil: coco peat (1:1:1) and covered with transparent polythene cover punctured with holes to maintain the relative humidity. These plantlets were initially kept in the culture room for 15 days and were later transferred to the poly-house for acclimatization for 2 months before transplanting to the field (Fig. 1h). The survival percentage of the acclimatized plants in the field was 85.5% (Fig 1i).

Table 1: Shoot induction in petiole and axillary bud explants of *Simarouba glauca* DC on MS media supplemented with various concentrations of cytokinins

BA	KIN	Petiole explants		Axillary buds	
		Shoot induction %	Average Number of shoot buds induced per calli	Shoot induction %	Average Number of shoot buds induced per calli
0.50	0.50	42.23±0.17	19.43±0.33	56.67±0.12	07.40±0.34
0.50	1.00	19.43±0.32	02.53±0.26	32.67±0.24	08.24±0.24
0.50	2.00	14.00±0.37	06.02±0.20	59.47±0.19	06.41±0.31
0.50	3.00	51.33±0.27	21.23±0.27	47.90±0.03	09.77±0.28
1.50	0.50	27.33±0.29	21.57±0.13	62.00±0.16	09.68±0.29
1.50	1.00	51.63±0.16	15.84±0.32	83.33±0.23	12.32±0.16
1.50	2.00	42.32±0.22	13.42±0.36	76.90±0.17	14.29±0.15
1.50	3.00	18.45±0.35	11.63±0.31	39.63±0.13	10.32±0.12
2.50	0.50	65.27±0.19	19.35±0.23	71.67±0.14	08.43±0.32
2.50	1.00	85.14±0.18	27.42±0.14	42.97±0.13	10.66±0.27
2.50	2.00	92.34±0.12	26.70±0.23	22.13±0.22	05.49±0.18
2.50	3.00	74.42±0.16	25.36±0.28	24.06±0.31	05.34±0.28
3.50	0.50	83.33±0.23	26.67±0.05	27.67±0.23	09.27±0.32
3.50	1.00	88.75±0.13	29.54±0.28	22.23±0.16	03.22±0.36
3.50	2.00	85.54±0.19	27.61±0.31	35.67±0.24	04.26±0.37
3.50	3.00	65.83±0.08	24.34±0.18	22.31±0.16	05.53±0.33

Table 2: Shoot multiplication and elongation in petiole and axillary bud explants of Simarouba glauca on MS media supplemented with various concentrations of cytokinins and Giberrelic acid

BA	2ip	GA^3	Petiole explants		Axillary buds	
			Average Number of shoots	Shoot length (cm)	Average Number of shoots	Shoot length (cm)
0.50	0.50	0.30	02.36±0.33	02.63±0.36	02.41±0.26	03.11±0.34
0.50	1.00	0.30	02.42±0.31	02.47±0.33	02.79±0.32	02.64±0.28
0.50	2.00	0.30	03.16±0.30	04.38±0.26	02.38±0.34	02.52±0.36
1.00	0.50	0.30	02.85±0.28	02.18±0.19	03.52±0.27	03.52±0.28
1.00	1.00	0.30	03.57±0.18	03.45±0.25	02.54±0.26	03.51±0.17
1.00	2.00	0.30	03.42±0.16	04.26±0.17	05.87±0.27	04.11±0.13
2.00	0.50	0.30	08.75±0.05	05.06±0.12	03.82±0.38	03.28±0.16
2.00	1.00	0.30	09.63±0.11	04.87±0.24	02.59±0.31	04.03±0.08
2.00	2.00	0.30	05.32±0.17	04.52±0.13	04.88±0.18	03.89±0.21
3.00	0.50	0.30	07.46±0.16	05.41±0.12	05.22±0.15	04.34±0.22
3.00	1.00	0.30	06.21±0.13	05.03±0.11	04.53±0.13	04.36±0.12
3.00	2.00	0.30	03.17±0.20	04.33±0.15	02.44±0.28	02.35±0.32

Table 3: *In vitro* rooting in petiole and axillary bud explants of *Simarouba glauca* DC on half-strength MS media supplemented with various concentrations of auxins

IBA	NAA	Petiole explants			Axillary buds		
		Rooting %	Average Number of roots per shoot	Root length (cm)	Rooting %	Average Number of roots per shoot	Root length (cm)
0.00	0.00	25.23±0.42	04.32±0.16	03.88±0.32	23.63±0.24	03.61±0.16	02.73±0.26
0.00	0.50	65.38±0.16	05.21±0.15	04.93±0.14	57.23±0.18	03.82±0.18	02.64±0.26
0.00	1.00	73.47±0.13	05.44±0.14	05.01±0.12	58.54±0.17	04.11±0.14	03.62±0.28
0.10	0.00	44.70±0.27	03.45±0.22	02.38±0.36	52.18±0.19	03.54±0.16	02.78±0.32
0.10	0.50	92.50±0.06	06.72±0.13	05.74±0.16	64.35±0.12	04.47±0.13	03.87±0.24
0.10	1.00	89.95±0.15	06.26±0.12	05.36±0.18	69.27±0.13	04.83±0.16	04.06±0.16
0.50	0.00	42.80±0.12	04.18±0.26	03.42±0.12	43.21±0.23	03.58±0.19	03.99±0.12
0.50	0.50	90.66±0.12	06.04±0.11	05.43±0.13	57.58±0.21	03.61±0.23	03.54±0.14
0.50	1.00	91.24±0.14	05.03±0.13	05.42±0.12	65.26±0.11	03.97±0.22	02.97±0.22
1.00	0.00	29.40±0.38	03.17±0.32	03.57±0.22	41.06±0.16	04.11±0.13	03.84±0.32
1.00	0.50	58.92±0.22	04.38±0.26	02.39±0.32	33.32±0.22	04.05±0.12	03.18±0.12
1.00	1.00	61.24±0.24	05.23±0.18	03.19±0.28	44.03±0.14	03.78±0.14	03.52±0.16

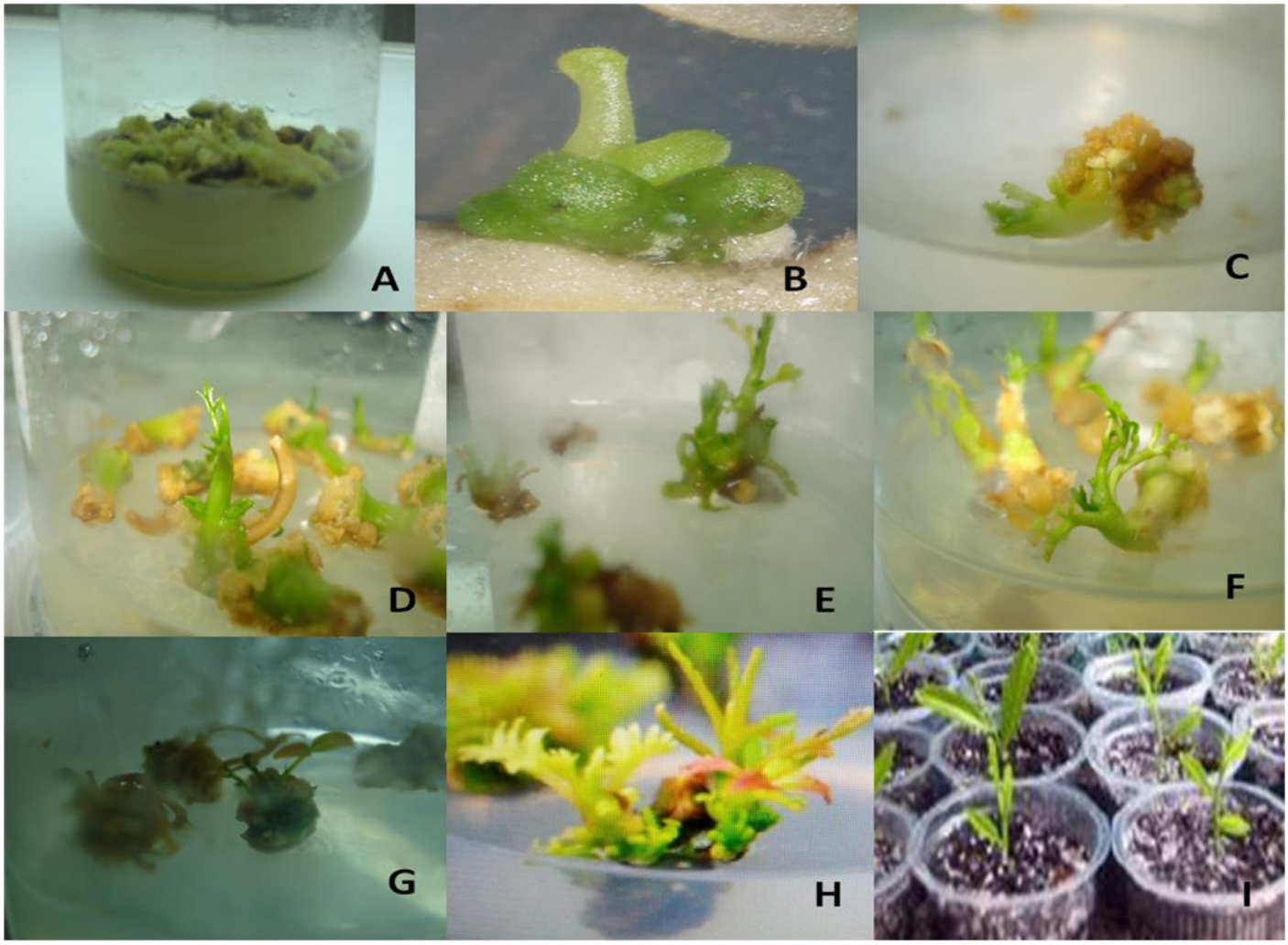

Fig 1: Simarouba glauca DC. Micropropagation: (A) Green compact calli regenerated from petiole explants; (B) Somatic embryogenesis from calli; (C-D) Shoot induction from somatic embryo; (E-H) Multiple shoot induction and shoot elongation; (I) Acclimatization and hardening of regenerated plantlets

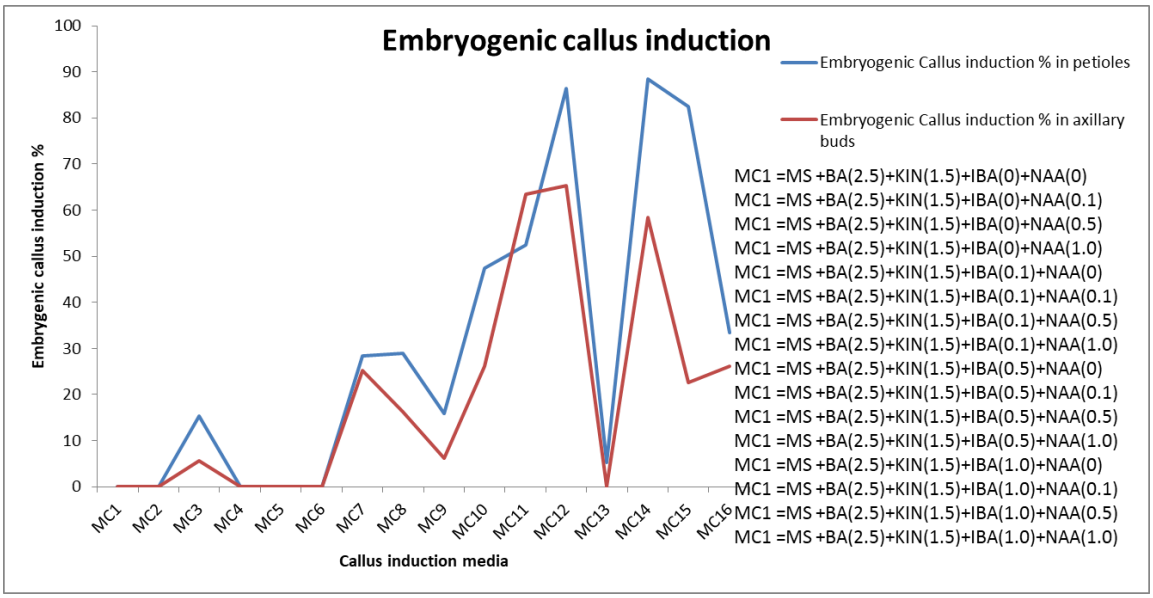

Fig 2: Embryogenic calli induction in petiole and axillary bud explants on MS medium supplemented with different combinations of cytokinins and auxins

Conclusions

We report an efficient and reproducible regeneration protocol for Simarouba glauca explants derived from physiologically mature trees. The leaf petioles proved to be the the most responsive explants that produced a large mass of embryogenic calli over the axillary bud explants. The maximum number of shoots were produced from theses embryogenic calli derived from the petiole explants on the MS medium supplemented with 2.0 mg/l BA + 1.0 mg/l 2ip + 0.3 mg/l GA3. Profuse rooting was obtained on the MS

medium supplemented with IBA (0.1 mg/l) + NAA (0.5 mg/l). The survival percentage of the plants in the field after acclimatization was 85.5%. This is the first report of an efficient plant regeneration protocol from physiologically mature trees of *Simarouba glauca* DC. The explants derived from mature trees produce a lot of phenolic compounds that result in browning of the explants and the medium resulting in tissue death. The present study has effectively reported how to overcome the tissue browning and reduce the release of phenolics by use of anti-phenolic compounds and anti-oxidants. Earlier reports of micropropagation are from the seedling derived explants of *Simarouba glauca* DC. which are highly totipotent and release less phenolics.

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