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In Vitro Somatic Embryogenesis in Some Oil Yielding Tropical Tree Species

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ABSTRACT

Somatic embryogenesis was achieved in two oil yielding tropical tree species i.e. Simarouba glauca & *Azadirachta indica using immature zygotic embryos as explants on Murashige and Skoog* (*MS*) *medium supplemented with* 0.5 - 1.5 *mg/l benzylaminopurine* (*BA*) *and* 2.0 - 3.0 *mg/l NAA* (1-*napthaleneacetic acid*) *or* 2,4-*D* (2,4-*dichlorophenoxyacetic acid*) *and* 3% *sucrose. MS medium containing* 1.0 *mg/l BA and* 2.0 *mg/l NAA was noted to be the most effective in inducing friable embryogenic callus* (*FEC*) *in Simarouba glauca*; *the number of somatic embryos per culture varied in MS medium supplemented with* 1.0 - 1.5 *mg/l BA and* 1.0 *mg/l NAA. In Azadirachta indica, somatic embryos developed on MS medium supplemented with* 0.5 *mg/l BA and* 1.5 - 2.0 *mg/l* 2,4-*D which were in various shapes and sizes after the first subculture on MS medium supplemented with* 0.25 *mg/l abscisic acid. The somatic embryos which developed shoots were isolated and rooted in* 1/2 *strength MS medium supplemented with* 0.25 *mg/l abscisic acid and* 2% *sucrose. About* 25% *of embryos germinated within* 20 *days of culture in case of Simarouba glauca and* 62% *in Azadirachta indica. The somatic embryo-derived plantlets were transferred to the field after being hardened in the climate controlled hardening chamber*.

Keywords**:** *Somatic Embryogenesis, Immature Zygotic Embryos, Growth Regulators, Oil Yielding Tropical Tree*

1. Introduction

Simarouba glauca Linn. (Simaroubaceae) a multipurpose fast growing tree from Amazon rainforest and other tropical areas in Mexico, Cuba, Haita, Jamaica and Central America is grown for seed oil, medicines, firewood and revegetation of barren wastelands even under moisture and nutrient stress. *Azadirachta indica* A.Juss. (Meliaceae), popularly called the neem tree, is distributed in the tropical and subtropical regions of the world [1]. A native of India and Myanmar, *Azadirachta indica* was introduced to Africa, the Middle East, South America and Australia. All parts of these plants including fruit, seed, leaf, root and bark are used for their medicinal properties. The neem tree reportedly contains more than 100 bioactive compounds. The most important bioactive compound is azadirachtin, other compounds are gedunin, nimbin and sodium nimbinate. Some plants of *Simarouba glauca* and *Azadirachta indica* bear fruits in profusion, hence were considered as elite candidates for cloning.

Regeneration of plants via somatic embryogenesis has been preferred as a method for multiplication of valuable germplasm in many woody species. Somatic embryogenesis was reported for a number of dicotyledonous and monocotyledonous angiosperms but fewer woody species [2-4]. Woody species were recalcitrant to *in vitro* culture and regeneration and most of those reports focused on propagation or multiplication through organogenesis using various explants. There are number of reports on somatic embryogenesis of woody species [5-11]. It was thought that plants must coordinate the growth of root and shoot meristems to maintain an appropriate balance of root and shoot organs, respond and adapt to various environmental conditions to achieve an inter-meristem coordination for growth and development involving the interplay of several long-range signals [12,13]. In this study the requirements of culture media, including environmental conditions for induction of somatic embryogenesis, maturation and germination of the somatic embryos in economically important oil yielding tropical tree species for their adaptability to harsh conditions were investigated.

2. Materials and Methods

2.1. Plant Material

Immature fruits (drupe) of *Simarouba glauca* and *Aza-*

dirachta indica were collected from elite trees growing in dry deciduous forests 50 - 60 days after fruit set. The fruits were washed with 2% (w/v) detergent solution (Teepol) for 10 min, further ringed with 70% ethanol for 1 min, surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride for 15 min, followed by three 5 min rinses in sterile distilled water. Embryonic axis along with cotyledons were aseptically cultured on Murashige and Skoog [14] medium supplemented with various concentrations of BA or Kn (0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/l), NAA or 2,4-D (0, 0.5, 1.0, 1.5, 2.0, 3.0 mg/l) alone or in combinations for callus induction. The pH of the media was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to addition of 0.8% (w/v) agar (Qualigen, India). Routinely, 20 ml of molten medium was dispensed into 25 × 150 mm glass tubes (Borosil, India), capped with non-absorbent cotton plugs wrapped in one layer of cheesecloth. The cultures were sterilized at 121˚C and 104 kPa for 15 min.

2.2. Induction of Somatic Embryogenesis

Callus mass (500 ± 20 mg) was transferred to MS medium supplemented with different concentrations of BA, kinetin and 2,4-D or NAA (0, 0.25, 0.5, 1.0, 1.5, 2.0 , 2.5 and 3.0 mg/l) singly or in combinations for induction of somatic embryogenesis. L-proline or L-glutamine was added to the culture medium to enhance the embryogenic potential. The cultures were incubated under 16h photoperiod with light intensity of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool, white fluorescent lamps (Phillips, India) at 25˚C ± 2˚C. Subculturing was made every 4 week intervals. The media were solidified with 0.8% agar-agar. Morphological changes were recorded through visual observations at 3-week intervals. The embryogenic response and number of somatic embryos per culture were recorded. Each treatment had 20 replicates and the experiment was repeated three times. Data were also recorded in respect to embryogenic frequency, number of embryos and frequency of normal embryos per culture.

2.3. Maturation and Germination of Somatic Embryos

The somatic embryos were transferred to various culture media with (0.1 - 0.25 mg/l abscisic acid and 2% sucrose) or without growth regulators for maturation and germination. The cultures were routinely sub-cultured at 4-week intervals. In another experiment, the cultures were kept in the dark for 2 weeks which were then transferred to the light for germination of somatic embryos. In all the experiments, each treatment consisted of 10 replications and the experiments were repeated three times. The somatic embryos were transferred to 1/2 MS medium supplemented with 2% sucrose and abscisic acid (0.1 - 0.25 mg/l) for germination.

3. Results and Discussion

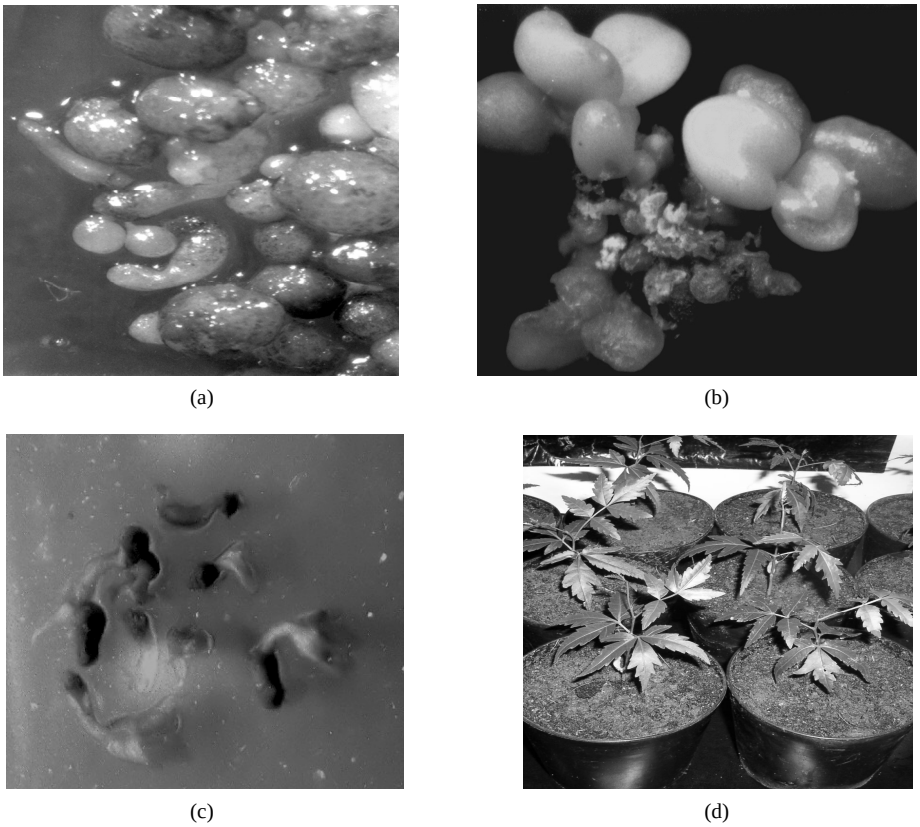
3.1. Induction of Somatic Embryogenesis

The effects of the different growth regulators on embryogenic callus induction are indicated in **Table 1**. Friable calli developed from immature zygotic embryos within 3 - 4 weeks of culture on MS medium supplemented with various concentrations of auxins and cytokinins. The maximum proliferation of callus was noted in the medium containing BA and NAA or 2,4-D. Kinetin was not as effective in callus induction as BA. BA with 2,4-D in the medium was observed to be better for callus proliferation. The proliferated calli were subsequently sub-cultured on various media for embryogenesis. Embryogenic callus mass developed on MS medium supplemented with 0.5 - 2.0 mg/l BA and 2.0 - 3.0 mg/l NAA in *S. glauca* and 0.5 mg/l BA and 1.0 - 2.0 mg/l 2,4-D in case of *A. indica*.. The medium devoid of growth regulators did not help in proliferation of embryogenic calli. The maximum rate of embryogenic callus proliferation was noted on MS medium supplemented with 1.0 - 1.5 mg/l BA and 2.0 mg/l NAA in *Simarouba glauca* and 0.5 - 1.0 mg/l BA and 1.0 - 2.0 mg/l 2,4-D in *Azadirachta indica* (**Figures 1(a)-(b)** and **2(a)**, **Table 1**). Proliferation of friable embryogenic calli in terms of fresh weight was better in the medium having BA as compared to Kn. Similar responses were observed when 2,4-D was replaced with NAA. BA at a concentration of 1.0 mg/l along with 2.0 mg/l NAA improved the rate of embryogenic callus proliferation and improved the production of somatic embryos per culture; though NAA helped in the proliferation of embryogenic callus as good as the 2,4-D, few somatic embryos developed in *Simarouba glauca*. However, the medium supplemented with 0.5 mg/l BA and 1.5 mg/l 2,4-D showed a higher rate of proliferation of embryogenic calli in *A. indica* (**Table 2**). Embryogenic callus induction was faster in *S. glauca* in the media containing NAA as compared to those having 2,4-D; 2,4-D, however, showed better results in *A. indica*. Embryo development in somatic cells was often accompanied with cellular stress. Moreover, NAA, the most frequently used compound for induction of somatic embryogenesis (**Figure 2(b)**), was known to activate many stress related genes supporting the hypothesis that somatic embryogenesis resulted due to extreme stress response of cultured cells. Proline acted as a potential antioxidant, which helped in ameliorating the stress [15]. Moon et al [8] reported that MS medium containing 3% sucrose, 1.0 g/l glutamine along with 2,4-D helped in development of somatic embryos in *Oplopanax elatus*. ABA and activated charcoal appeared to be very important agents for

Table 1. Effect of cytokinins and auxins on induction of embryogenic callus from immature zygotic embryos of *Simarouba glauca* (SG) and *Azadirchta indica* (AI).

MS + growth regulator (mg/l)				Percent of Explant Response(Mean ± SE)*	
Kn	BA	2,4-D	NAA	SG	AI
0	0.25	0.5	0	18.2 ± 0.8 (NE)	29.4 ± 0.6 (NE)
0	0.25	1.0	0	26.4 ± 0.6 (NE)	32.2 ± 0.8 (E)
0	0.25	2.0	0	42.1 ± 0.8 (NE)	45.6 ± 0.7 (E)
0	0.5	2.0	0	49.8 ± 0.7 (NE)	66.8 ± 0.6 (E)
0	0.5	3.0	0	60.2 ± 0.8 (NE)	51.2 ± 0.8 (E)
0.25	0	1.0	0	38.4 ± 0.8 (NE)	34.4 ± 0.9 (NE)
0.50	0	2.0	0	38.2 ± 0.5 (NE)	50.1 ± 1.0 (NE)
1.00	0	3.0	0	54.2 ± 0.7 (NE)	56.6 ± 0.4 (NE)
0	0.25	0	1.0	32.8 ± 0.9(E)	25.6 ± 0.7 (NE)
0	0.25	0	2.0	45.8 ± 0.6 (E)	36.4 ± 0.8 (NE)
0	0.5	0	3.0	68.8 ± 0.8 (E)	58.4 ± 0.8 (NE)
0	1.0	0	2.0	75.2 ± 0.6 (E)	56.2 ± 0.7 (NE)
0	1.5	0	2.0	76.2 ± 0.8 (E)	66.6 ± 1.0 (NE)
0.25	0	0	20	56.6 ± 0.7 (NE)	56.9 ± 1.1 (NE)
0.50	0	0	3.0	64.4 ± 0.6 (NE)	68.4 ± 0.5 (NE)

*20 Replicates per culture; repeated thrice. NE—Non-embryogenic Calli, E—Embryogenic Calli.



(a) (b) (c) (d)

Figure 1. *In vitro* somatic embryogenesis in *Azadirchta indica.* (a)-(b) Development of somatic embryos from immature zygotic embryos after 4 weeks of culture. (c) Somatic embryos with cotyledons and radicle. (d) Embryo derived plantlets established in pots.

Table 2. Development of somatic embryos in embryogenic callus cultures of *Simarouba glauca* (SG) and *Azadirchta indica* (AI) cultured on different induction media after 8 weeks of subculture.

Culture Medium + 3% (w/v) Sucrose	No of Somatic Embryos per 200 mg Embryogenic Calli (Mean ± SE)*	
	SG	AI
MS + 0.25 mg/l Kn + 1.0 mg/l NAA	50.4 ± 0.7	0
MS + 0.5 mg/l Kn + 1.0 mg/l NAA	62.6 ± 0.8	8.6 ± 0.9
MS + 0.5 mg/l Kn + 1.5 mg/l NAA	58.2 ± 0.6	22.8 ± 0.7
MS + 0.5 mg/l BA + 1.5 mg/l 2,4-D	14.8 ± 0.4	75.2 ± 0.8
MS + 1.0 mg/l BA + 2.0 mg/l 2,4-D	20.6 ± 0.7	122.2 ± 0.8
MS + 1.5 mg/l Kn + 2.0 mg/l NAA	98.7 ± 0.9	41.6 ± 0.6
MS + 1.5 mg/l BA + 2.0 mg/l 2,4-D	38.7 ± 0.8	80.6 ± 0.9
MS + 1.5 mg/l BA + 2.0 mg/l 2,4-D + 400 mg/l L-proline	42.4 ± 1.1	121.7 ± 1.2
MS + 1.5 mg/l BA + 2.0 mg/l 2,4-D + 600 mg/l L-proline	46.3 ± 0.6	128.4 ± 1.1
MS + 1.0 mg/l Kn + 2.0 mg/l NAA + 600 mg/l L-proline	126.3 ± 0.6	28.4 ± 1.1

(10 replicates per treatment; repeated thrice). *Data collected after four weeks of culture on proliferation medium.

proliferation of somatic embryos., Singh and Chaturvedi [9] reported that 76% of cultures showed somatic embryo development directly from immature zygotic embryos in neem on MS medium having 0.1 µM TDZ and 4 µM ABA. Globular embryos developed into heart and torpedo shaped embryos faster in media containing NAA and BA (**Figures 1(c)** and **2(c)-(d)**). In woody species, explants from immature tissues either seed or seedlings generally exhibited greater ability for somatic embryogenesis as compared to mature tissues [16]. 2,4-D was reported to induce embryogenic callus in a number of plant species including woody perennials [17,18]. In *A.indica*, the highest number of somatic embryos per 200 mg of callus was 152.0 after 8 weeks of culture on the medium containing 0.5 mg/l BA and 1.0 mg/l 2,4-D. Murthy and Saxena [19] included BA in the culture medium for induction of somatic embryogenesis in callus cultures obtained from mature seeds of the neem; in their study, BA was used alone during induction of somatic embryos. Inclusion of L-proline or L-glutamine (600 mg/l) in the induction medium enhanced proliferation of embryogenic calli. Auxin-induced somatic embryogenesis in presence of proline was well documented [20]. Free proline was suggested to act as an osmoticum, a nitrogen storage pool and source of $NADP^+$, necessary for rapidly growing embryos. The mediation of the cellular redox potential that resulted from proline accumulation likely had a significant effect on the flux through redox-sensitive biochemical pathways like the pentose phosphate pathway [21].

3.2. Germination of Somatic Embryos

Both globular and cotyledonary somatic embryos developed on 1/2 strength MS medium supplemented with 0.25 mg/l abscisic acid (**Figure 2(d)**). The medium devoid of growth regulators did not promote germination. Many of the embryos were morphologically normal and showed distinct cotyledons and radicles. But the abnormal embryos varied in shape and structure, having one, two or even more unequal cotyledons which sometimes looked like a cup (**Figure 2(c)**). Most of the somatic embryos were loosely attached to form an aggregate of embryos that could be easily separated; some somatic embryos arose from the base of other embryos to form clusters indicating secondary somatic embryogenesis. High percentage of cotyledonary embryos were recovered when 0.25 mg/l abscisic acid was added to the medium. The efficiency of somatic embryogenesis was strongly dependent on the auxin and cytokinin balance during the initial phase. Though in most of the woody species, it was difficult to stop proliferation of somatic embryos once initiated to shift to maturation phase, it was possible to change over to the maturation phase in most of the cultures using abscisic acid at 1.0 mg/l. For subsequent growth and development of the embryos into plantlets, auxin was not required [22]. Within 20 days of transfer to maturation medium, about 25% of the somatic embryos germinated which developed roots without showing secondary callusing (**Figures 1(c), (d)** and **2(e), (f)**). The somatic embryos so developed due to exogenous application of ABA closely resembled zygotic embryos in both

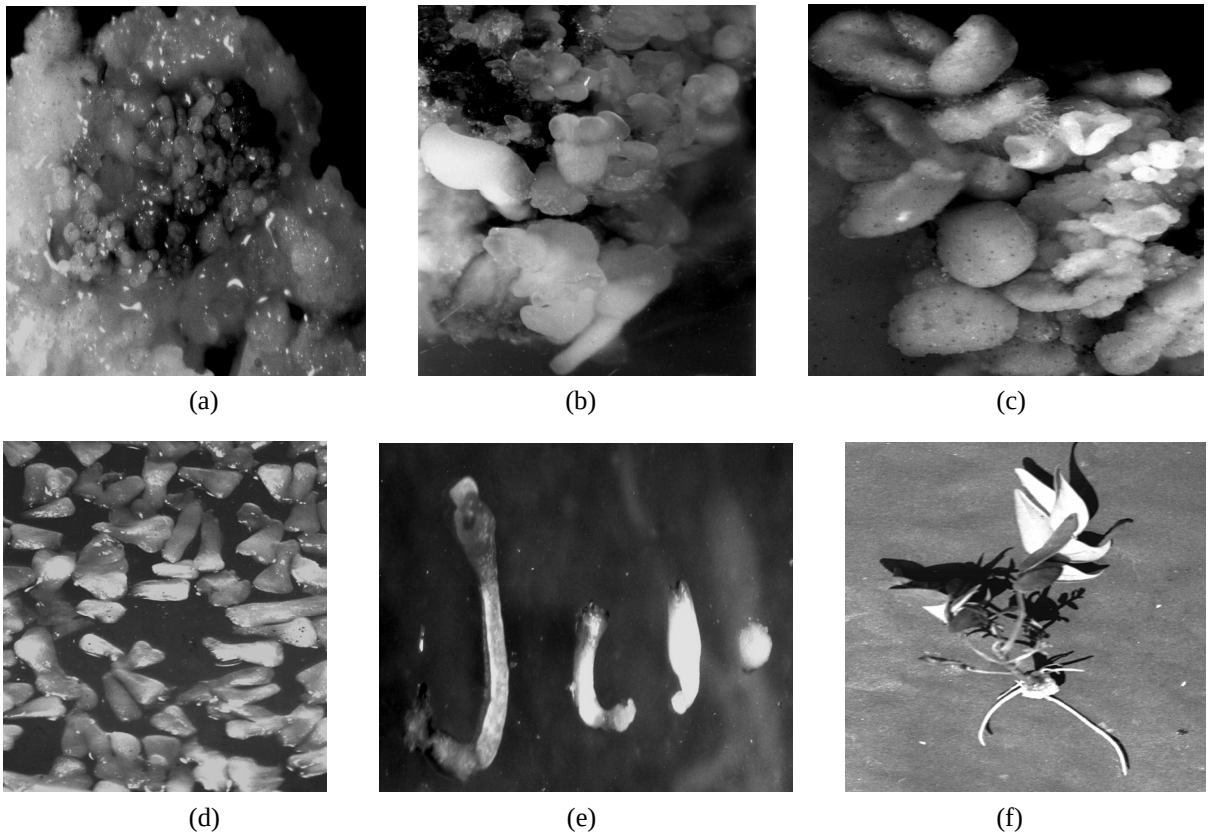


Figure 2. *In Vitro* somatic embryogenesis in *Simarouba glauca*. (a) Development of embryogenic callus from immature zygotic embryos after 4 weeks of culture. (b) and (c) Primary and secondary somatic embryogenesis after 4 weeks of subculture. (d) Somatic embryos in different shapes & sizes. (e) Germination of somatic embryos on 1/2 strength MS medium supplemented with 1 mg/l abscisic acid and 2% sucrose after 2 weeks of culture. (f) Somatic embryo derived plantlet.

structure and behaviour. About 50% of the plantlets survived under greenhouse conditions.

4. Conclusions

The somatic embryogenesis reported here in two oil yielding tropical tree species *i.e. Simarouba glauca* & *Azadirachta indica* can be employed for mass cloning of superior and elite candidates lines without resorting to gametes fusion. This protocol can be utilized for genetic improvement and development of stress tolerant lines through *in vitro* selections for adoption to stressful environments.

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