Regeneration of plantlets in *Sapindus trifoliatus* L.

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Continuous production of healthy plantlets of *Sapindus trifoliatus* L. was achieved via somatic embryos from long term cultures of an embryogenic mass (EM). A highly embryogenic culture of *S. trifoliatus* L. was obtained by recurrent embryogenesis from somatic embryos cultured on Murashige and Skoog's (MS) medium supplemented with kinetin (2.3 \( \mu M \)) and benzyladenine (8.8 \( \mu M \)). The cultures could be maintained without reduction of embryogenic competence for more than 20 months by subculture at 4 week intervals. About 90% mature somatic embryos on transfer to basal MS medium germinated to plantlets, of which more than 70% survived when transferred to a sand and soil mixture in greenhouse.

Clonal propagation by enhanced proliferation in in vitro is now possible, often achieving high multiplication rates, using both seedling and mature tree explants in a number of tree species. However, these procedures involve intensive labour and cost. Such systems are also not much suited for most biotechnological applications such as in vitro selection and gene transfer. Somatic embryogenesis offers an alternative technique with several advantages such as cheap, large scale plant production system with possibilities of automation and mechanical planting of synthetic seeds. Although somatic embryogenesis has been obtained in several tree species in recent years, consistent high frequency plant regeneration has been reported in a few cases. In this report we present a procedure adopted for continuous plantlet production from a long term highly embryogenic cultures of an economically important tropical tree species *Sapindus trifoliatus* L. locally known as soapnut. The saponin obtained from the pericarp of the fruits is used in the manufacture of soaps and detergents. Hence the dried fruits have high demand in the soap, shampoo and detergent manufacturing industries. Somatic embryogenesis leading to formation of plantlets in *S. trifoliatus* L. has been reported earlier. However, the said protocol yielded normal somatic embryos at a very low frequency and low level of synchronization. The present procedure demonstrates distinct improvements in terms of continuous production of embryos on a large scale, high frequency of embryos maturation and their germination. Consequently there is pronounced reduction in time taken from callus induction to plantlet regeneration.

**Materials and Methods**

**Plant material**—Young leaves were collected from a mature tree (about 7-8 years old) of *S. trifoliatus* L. growing in the Gujarat Agricultural University Campus, in Anand, India. The leaves were first washed with running tap water for about 30 min and then with mild detergent (Labolin-10% v/v, for 5 min) solution. Surface disinfection was done with 0.1% HgCl\(_2\) solution (w/v) for 2 min followed by repeated washing with sterile distilled water.

**Callus growth and induction of embryogenesis**—Leaf discs without mid-rib measuring 0.5 cm in diameter were explanted on primary medium containing the minerals and vitamins of MS medium supplemented with 2.3 \( \mu M \) kinetin (Kn), 9.0 \( \mu M \) 2,4-dichlorophenoxy acetic acid (2,4-D), 2% sucrose and 0.4% phytagel (sigma). The pH of the medium was adjusted to 5.8 ± 0.1 before the addition of phytagel. After melting phytagel, the medium was dispensed into culture vessels (20 ml medium per 150 x 25 mm tubes with caps and 50 ml medium per 150 ml glass jars with lids). All the media combinations were autoclaved for 20 min at 121 °C (108 kPa). Cultures were maintained at 25°±2°C with a 16 hr photoperiod (30 \( \mu M\) mole m\(^{-2}\) s\(^{-1}\) with a fluorescent
Callus was then transferred to MS medium supplemented with kinetin (2.3 μM) and 2,4-Dichlorophenoxy acetic acid (2.2 μM) for induction of somatic embryos.

**Maintenance of embryogenic cultures**—For further growth of the embryos, the cultures were transferred on an auxin-free MS medium containing both Kn (2.3 μM) and BA (8.8 μM), sucrose (2%) and phytagel (0.4%). Somatic embryos were then isolated at an early developmental stage (globoar, heart-staged) and were subcultured on the same fresh medium for three successive passages, 4 weeks each, to promote an increase in the amount of embryogenic cultures by recurrent embryogenesis. This embryonic mass (EM) was maintained on fresh medium of the same composition by subculturing every 4 weeks.

**Synchronization and maturation**—To achieve synchronization in embryo formation and their maturation, the embryogenic cultures were transferred into half strength MS liquid medium supplemented with meso inositol (5 g/l) and 4% sucrose (30 ml per 150 ml Erlenmeyer flask with cotton plug). After agitating the cultures on a gyratory shaker (120 rpm) with 12 hr photoperiod for 2 weeks, the somatic embryos were aseptically filtered through nylon mesh (2.0 mm) to get globular to heart stage embryos and through 4.0 mm nylon mesh to get late heart-stage and early cotyledonal stage embryos. The isolated embryos were again transferred to fresh liquid medium. Within 2 to 4 weeks, they all developed into cotyledonal stage embryos.

**Germination and plantlet development**—When mature embryos were transferred to 0.4% phytagel based MS half strength medium with 4% sucrose, germination started within 1 week and small plantlets were produced within 4 weeks. For further growth, these plantlets were then incubated on half strength MS medium supplemented with 4% sucrose, 250 mg/l (NH₄)₂SO₄, and 0.4% phytagel in tubes.

**Transplantation**—Plantlets 4 to 6 cm in height bearing 6 or more leaves and a well-developed tap root system (10 to 12 weeks old) were transferred to polythene bags containing a sterile sand:soil (1:1) mixture. They were watered daily with tap water and twice per week with one fourth strength MS mineral salts.

**Results and Discussion**

A slow growing semi-hard, yellow callus was obtained from young leaves of an elite mature tree after 4 weeks on primary MS medium. This primary callus rapidly proliferated into friable, whitish to pale yellow callus upon three successive subcultures on the same medium. Induction of embryogenesis was achieved by lowering the level of 2,4-D to 2.2 μM. Early stages of regeneration (globoar and heart stage) were obtained after 4 weeks on MS medium supplemented with Kn (2.3 μM) and BA (8.8 μM) that is developmental medium. Maturation of these embryos and germination of the same was obtained on MS medium with 4% sucrose. However, the number of embryos was low and the germination rate was only 2% (ref. 15).

Isolated primary embryos subcultured on developmental medium underwent recurrent embryogenesis to form a granular mass. Since it has been termed as an embryogenic mass, it has been termed as embryogenic mass (EM) in this report. The origin of EM from somatic embryos and its proliferation by recurrent embryogenesis distinguishes it from an embryogenic callus. Somatic embryos at an early developmental globular stage proved to be an excellent source for the production of EM and the developmental medium is effective for its maintenance (Fig. 1a). These cultures have been maintained without reduction of embryogenic competence for more than 20 months. This EM is similar to the friable EM described in coffee and eucalyptus.

<table>
<thead>
<tr>
<th>Meso inositol (g/l)</th>
<th>Number of embryos per embryogenic mass</th>
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<tr>
<td></td>
<td>Globular stage</td>
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<tr>
<td>0.0</td>
<td>31 ± 4</td>
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<tr>
<td>0.5</td>
<td>33 ± 7</td>
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<tr>
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<td>45 ± 5</td>
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<td>29 ± 3</td>
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<td>25 ± 4</td>
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<td>10.0</td>
<td>17 ± 4</td>
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Somatic embryos counted under stereoscopic microscope.

Table 1—Effect of meso inositol concentration on somatic embryo differentiation from embryogenic mass (1 g) upon transfer from maintenance medium onto synchronization and maturation medium. (MS/½4% sucrose+meso inositol concentration as specified in addition to 100 mg/l)

[Values are Mean ± SE of 3 replications]
Fig. 1—(a) An embryogenic mass (EM) developed from primary embryos on MS medium supplemented with Kn (2.3 μM) and BA (8.8 μM) after 12 weeks showing numerous somatic embryos at various stages of development in half strength MS medium with 4% sucrose (Liquid); (b) Isolated somatic embryos, globular (g), early heart stage (eh) and heart stage (h) obtained by aseptically filtering the culture grown in half strength MS medium supplemented with meso inositol (5.0 g/l) and sucrose (4%) after 2 weeks through 2.0 mm nylon mesh; (c) Isolated somatic embryos, late heart staged (lh) and early cotyledonary staged (ec) obtained by filtering through 4.0 mm nylon mesh; (d) An isolated germinated embryo (e) after 1 week and plantlets regenerated after 2 weeks in half strength MS medium with 4% sucrose (note the cotyledons persist at the base of the shoot); (e) Plantlet development after 8 weeks in half strength MS medium supplemented with 250 mg/l of (NH₄)₂SO₄ and 4% sucrose; and (f) Regenerated soapnut plants under greenhouse conditions six months after transplantation.

When EM was transferred to half strength MS liquid medium supplemented with 4% sucrose, development and maturation of embryos occurred. However, the development of embryos in this medium was asynchronous and all stages as shown in Fig. 1a occurred simultaneously. Embryo differentiation was better achieved after transferring EM to a high osmoticum medium by incorporating higher levels of meso inositol 5.0 g/l (Table 1). This medium had the ability to block further EM proliferation, facilitating the onset of somatic embryo development. Synchronization in the developmental stages was further achieved by filtering the somatic embryos grown in liquid medium (Fig. 1b) and (Fig. 1c). The isolated embryos obtained after filtration, when transferred to the fresh liquid medium, developed into cotyledonary stage embryos within 2 to 4 weeks. On an average of 100 ± 20 normal fully developed embryos could thus be obtained from 1 g fresh weight of EM.

In the experiment conducted, around 500 synchronized, white cotyledonary stage embryos were kept for germination on the standardized germination medium (10 embryos per vessel). Out of these, 411 embryos germinated and developed into healthy plantlets. A higher frequency of germination (>80%) was achieved by culturing the mature white embryos on germination medium. After 1 week, chlorophyll development, cotyledon growth and hypocotyl elongation had occurred and a strong tap-root system had also developed. Small plantlets were thus produced within 2 to 3 weeks (Fig. 1d). Mathews et.
al. have reported improvement of efficiency of plant regeneration from somatic embryos by optimizing the maturation regime and incorporating a desiccation stage prior to inducing germination in cassava.

When the mature embryos were transferred to germination medium, shoot and root development proceeded simultaneously, while the cotyledons persisted at the base of the shoot (Fig. 1d). After 2 weeks however, the growth of the shoot was suppressed since the leaves turned yellow and dropped. This premature leaf drop was controlled by transferring the plantlets to half strength MS medium supplemented with 250 mg/l of (NH4)2SO4, which was found to be more beneficial than glutamine. This resulted in normal development and healthy growth of the plantlets with appropriate root and shoot ratio. Healthy plantlets were produced within 80 to 90 days depending upon the developmental stage of the embryos when transferred and subsequent growth of the embryos (Fig. 1e).

More than 100 plantlets have been transferred to the sand : soil mixture, of which over 70% have survived. S. trifoliatus L. plants once established in soil, showed vigorous and uniform growth (Fig. 1f).

The protocol described here defines a somatic embryogenic system that has allowed continuous isolation of normal mature embryos from an EM. The regeneration system presented here utilizing an EM is thus a very efficient and reproducible method for large-scale production by permitting recurrent embryogenesis. To proceed from primary embryos, a subculturable system is feasible without resorting to the callus phase. The latter cuts short the total period of plantlet production from callus induction by more than half (from 180-200 days to 80-90 days). Somatic embryos obtained by this method have been encapsulated in calcium alginate and their germination is obtained under in vitro conditions (unpublished result of this laboratory).

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References

