Research Paper

Overcome the Seasonal Constraint for Somatic Embryogenesis Using *In vitro* Leaf Explants and Genetic Fidelity Testing of *Sapindus mukorossi*

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Sapindus mukorossi is an imperative medicinal and economical tree. The fruit of this tree is as a primary source of saponins. The aim of the current study was to develop a regeneration protocol of *Sapindus* plant from *in vitro* leaf explants to overcome the seasonal constraints. An efficient somatic embryogenesis protocol was developed by using *in vitro* leaf explants. Maximum callusing was found in MS medium supplemented with BAP. All the calli (100%) produced somatic embryos and a number of somatic embryos ($47.10 \pm 1.04a$) were observed on MS medium supplemented with $8.88 \mu\text{M}$ BAP. Maximum maturation percentage of somatic embryos ($95.52 \pm 0.31a$) was observed on MS medium supplemented with 4% w/v sucrose and $0.0378 \mu\text{M}$ abscisic acid (ABA). Highest germination percentage of somatic embryos ($77.79 \pm 0.66a$) was observed on MS basal medium having 4% w/v sucrose and $4.44 \mu\text{M}$ BAP. About 80% plants survived in field condition and grew well. The field transferred plants showed similar morphology as the mother plants. Random amplified polymorphic-DNA and inter-simple sequence repeats analysis also showed analogous banding pattern with mother plants that indicated there are no somaclonal variations.

Keywords: Somatic Embryogenesis; *In vitro* Leaf Explants; Genetic Fidelity; *Sapindus mukorossi*; Molecular Markers

Abbreviations: RAPD-random amplified polymorphic-DNA; ISSR-inter simple sequence repeats; BAP-6-benzylaminopurine; TDZ-Thidiazuron; 2,4-D- 2,4-Dichlorophenoxyacetic acid; KIN-Kinetin; IAA-indole-3 acetic acid; IBA-indole-3 butyric acid; FE-frequency of embryogenesis; IE-Intensity of embryogenesis; ABA-Absciscic Acid

Introduction

Somatic embryogenesis delivers an admirable morphogenetic system for investigating the cellular and molecular process underlying differentiation (Benelli *et al.* 2001) and it is also a model system for studying the genetic basis of embryogenesis and totipotency of somatic cells (Feher *et al.* 2003). It is the most effective method of micropropagation to produce true-to-true type plantlets of valuable and elite genotype for large-scale multiplication in short time period (Lema-Ruminska *et al.* 2013). Somatic embryos (SE) also offer the opportunity to produce artificial seeds and appreciated tools for genetic engineering and germplasm conservation by cryopreservation (Ara *et al.* 2000; Litz and Gray 1995).

In the woody tree system, various explants such as cotyledons and zygotic embryonic axis (*Murraya konigii*), leaf (*Olea europaea* and *Sapindus mukorossi*), rachis (*S. mukorossi*), sepals (*S. trifolius*) etc. have been used for induction of somatic embryogenesis (Paul *et al.* 2011; Mazri *et al.* 2013; Singh *et al.* 2015; Singh *et al.* 2016a; Asthana *et al.* 2017). *In vitro* leaf (IVL) explants have also been used for induction of SE in the rose plant (Kim *et al.* 2004). Generally the auxins have been reported for the induction of somatic embryogenesis but in few reports cytokinins also showed significant role in induction of somatic embryogenesis. Cytokinins play important role in cell division and differentiation. Cytokinins also have the ability to induce caulogenesis and somatic

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embryogenesis (Deo *et al.* 2010). Among all cytokinins, BAP (Singh *et al.* 2015; Singh *et al.* 2016a; Martin 2004; Manjula *et al.* 2000) and thidiazuron (TDZ) (Mithila *et al.* 2003; Lin *et al.* 2004) showed more efficacies for somatic embryogenesis induction. High level of endogenous auxins in explants promotes the somatic embryogenesis in the medium containing cytokinins (Singh *et al.* 2015).

Maintenance of genetic integrity is a key for large-scale multiplication of *in vitro* plants. The genetic fidelity can be evaluated by isozyme electrophoresis. The modern day PCR based molecular markers such as random amplified polymorphic-DNA (RAPD), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), or Restriction fragment length polymorphism (RFLP) etc. are some of the clonal fidelity methods that are rapid and easy for detection of somaclonal variations. ISSR proved to be more effective and reproducible for detecting genetic uniformity (Martins *et al.* 2004), whereas RAPD marker is easy in handling and cost effective also. The limited number of informative makers and its proneness to developmental and environmental variations are the major restraints of this technique (Rani *et al.* 1995).

Sapindus mukorossi Gaertn. (family-Sapindaceae) is a well-known economical and medicinal tree. Fruits contain large amount of saponins, but conventional propagation is not enough to accomplish the extensive demand of soap industry. This plant possesses various pharmacological properties such as antioxidant activity (Singh and Kumari 2015; Singh and Kumari 2020), antibacterial and antifungal activity (Singh *et al.* 2016b; Singh *et al.* 2016c; Tsuzuki *et al.* 2007), hepatoprotective (Ibrahim *et al.* 2008), and anti-cancer activity (Man *et al.* 2010) etc. The present research paper deals with the regeneration of *S. mukorossi* through somatic embryogenesis by using *in vitro* leaf explants and assessment of genetic fidelity through RAPD and ISSR markers.

Material and Methods

Source of Explants, Culture Media and Culture Conditions

Leaves were harvested from the *in vitro* plants, raised

by following the protocol described by Singh *et al.* (2015). Leaf explants were inoculated after removal of the apical and basal portion. Culture media and culture conditions were similar to the earlier published reports of Singh *et al.* (2015).

Callus Induction and Development of Somatic Embryos

For callus induction, leaf explants were cultured on full strength MS medium having 3% (w/v) sucrose and 0.8% (w/v) agar supplemented with different concentrations of PGRs such as 2,4-Dichlorophenoxyacetic acid (2,4-D) (2.26, 4.52, 9.04, 13.56 or 22.65 μ M), 6-benzylaminopurine (BAP) (2.22, 4.44, 8.88, 13.32 or 22.2 μ M), kinetin (KIN) (2.32, 4.64, 9.28, 13.92 or 23.25 μ M), indole-3 acetic acid (IAA) (2.85, 5.7, 11.4, 17.1 or 28.55 μ M) or indole-3 butyric acid (IBA) (2.45, 4.9, 9.8, 14.7 or 24.5 μ M). After callus initiation, cultures were sub-cultured either on a medium of the same composition or on MS basal medium for callus proliferation and initiation of SE.

Role of Media Supplements on Induction of Somatic Embryos

To evaluate the effect of sucrose concentration on SE induction, embryogenic calli were transferred to MS basal medium with different concentrations of sucrose (1, 2, 3, 4, 5 or 6% w/v). Embryogenic calli were also transferred to MS medium fortified with different concentrations of casein hydrolysate (100, 200, 300 or 400 mg l^{-1}).

Maturation and Germination of Somatic Embryos

For maturation of somatic embryos, early cotyledon stage somatic embryos were transferred to MS medium supplemented with different concentrations of sucrose (1, 2, 3, 4, 5 or 6% w/v). Different concentrations of ABA (0.000378, 0.00378, 0.0378, 0.378 or 0.756 μ M) were also tried in the medium containing 4% w/v sucrose for maturation of embryos.

Mature embryos were transferred to either MS basal medium with different concentrations of sucrose (1, 2, 3, 4, 5 or 6% w/v) or MS medium + 4% w/v sucrose and supplemented with different concentrations of BAP (4.44, 8.88 or 13.32 μ M).

Field Transfer of Plantlets and Genetic Fidelity Testing

Hardening, acclimatization and field transfer processes followed were similar to as reported by Singh *et al.* (2015).

Total ten samples were processed for genetic fidelity assessment (mother plant M1 and M2 + eight *in vitro* raised plants R1-R8). Genetic fidelity was assessed using PCR based RAPD and ISSR analysis. About 200 mg young leaves of the mother plant and *in vitro* raised plants were used for DNA extraction by following the standard method of Doyle and Doyle (1990). A similar procedure was used as earlier reported by Singh *et al.* (2016).

Experimental Design and Data Analysis

For each treatment, twenty replicates were used and each experiment was repeated thrice. The cultures were examined at regular intervals of one week each. The results were expressed as a mean $\pm$ SE of three independent experiments. The data were analysed statistically using one-way analysis of variance (ANOVA) and significant differences between means were assessed by Tukey's multiple range tests at $p \leq 0.001$ using SPSS (version 16). Frequency of somatic embryogenesis was calculated in terms of % cultures showing somatic embryo formation, whereas, intensity of embryos was calculated by calculating average number of somatic embryos per culture.

Results and Discussion

IVL explants have shown robust embryogenic potential similar to that of leaf explants of this plant. *In vitro* raised explants not only overcome the limitations of seasonal availability of explants but also seasonal variations in embryogenic responses. *In vitro* explants are more juvenile and have high and fast regeneration potential as compared to *in vivo* explants.

Effect of Different Growth Regulators on Callusing

Callusing induction was observed on MS media supplemented with different concentrations of 2,4-D, BAP or KIN. Maximum percentage callusing (100%) was observed on MS media supplemented with 4.44, 8.88 or 13.32 μ M BAP (Fig. 1a). But callus formed

on 8.88 μ M BAP was more friable and embryogenic in nature, so, further experiments were carried out using this callus. However, maximum percentage callusing of leaf explants was found only on 8.88 μ M BAP supplemented medium (Singh *et al.*, 2015). Hundred percent callusing by *in vitro* leaf explants on suitable media may be due to less development of vasculature and poor differentiation of tissue.

MS media supplemented with different concentrations of 2,4-D or KIN were not effective for callus induction as compared to BAP containing media. Callus induction was also observed on media containing different concentrations of KIN but the callusing percentage was very low. Among various concentrations of KIN, maximum percent callusing ($44.10 \pm 0.66a$) was observed on 13.92 μ M KIN supplemented medium. Similarly in many plants, somatic embryogenesis induced by cytokinins have been observed (Lakshmanan and Taji 2000; Sagare *et al.*, 2000; Kaparakis and Alderson 2002). There are several parameters which affect somatic embryo formation, such as endogenous and/or exogenously supplied plant growth regulators (Claudia *et al.*, 2016). In present study, presence of endogenous hormones especially auxins might have played a crucial role.

Induction of SE and Development of Somatic Embryos

Callus formed on all concentrations of BAP showed embryogenic responses when transferred to MS basal medium (Fig. 1b) but the frequency of embryogenesis (FE) and intensity of embryos (number of somatic embryos) differed. Maximum FE (100%) was observed from the calli, whose induction media was MS medium supplemented with 4.44 or 8.88 μ M BAP (Table 1). But maximum IE ($47.10 \pm 1.04 a$) was observed from the callus transferred from MS medium with 8.88 μ M BAP to MS basal medium. In case of *in vivo* leaf explants, initiation and development of somatic embryos took place on MS medium supplemented with 8.88 μ M BAP. But, in case of IVL explants somatic embryos developed on MS basal media. The maximum number of somatic embryos was observed on MS medium containing 3% w/v sucrose. Effect of sucrose concentrations on the development of somatic embryos on MS basal medium has been reported in other plants such as *Sapindus trifoliatus* (Asthana *et al.*, 2017), *Quercus rubra*

Fig. 1: Somatic embryogenesis from *in vitro* leaf explant of in *S. mukorossi* (a-f). a-Initiation of callusing, b- Asynchronous development of somatic embryos, c-Germination of somatic embryos, d-hardening of plantlets, e-acclimatization, f-Field transferred plantlet in pot, (gse-globular stage embryo, ege-elongated globular embryo, ctse-cotyledonary stage embryo, Bar scale = 2 mm)

(Vengadesan and Pijut 2009), and *Eucalyptus camaldulensis* (Prakash and Gurumurthi 2010).

Maturation and Germination of Somatic Embryos

About 88.81% somatic embryos showed maturation on MS basal medium with 4% w/v sucrose. Maturation percentage of somatic embryos increases by the addition of ABA in media. Maximum maturation ($95.52 \pm 0.31a$) was observed on MS medium supplemented with 4% w/v sucrose and $0.0378 \mu\text{M}$ ABA. Presence of ABA in maturation medium enhances the physiological maturity and produces

higher quality of somatic embryos (Deo *et al.* 2010). Promoter effects of ABA on the maturation of somatic embryos were also reported in *Psidium guajava* (Rai *et al.* 2007), and *Corydalis yanhusuo* (Kuo *et al.* 2002). ABA-induced an enhancement in protein, polyamines, free proline and starch levels as a response to desiccation tolerance in somatic embryos of sugarcane (Nieves *et al.* 2001). ABA also plays an important role in inhibition of precocious germination of somatic embryos and promotes the accumulation of reserve food materials (Singh *et al.* 2016a; Rai *et al.* 2011).

Table 1. Development of somatic embryos on MS basal medium from calli formed from different concentration of BAP

BAP (μ M)	FE (Mean $\pm$ SE)	IE (Mean $\pm$ SE)
2.22	35.70 $\pm$ 1.06 c	16.74 $\pm$ 0.80 d
4.44	100.0 $\pm$ 0.00 a	28.80 $\pm$ 0.64 b
8.88	100.0 $\pm$ 0.00 a	47.10 $\pm$ 1.04 a
13.32	49.58 $\pm$ 0.68 b	24.78 $\pm$ 1.34 bc
17.76	40.77 $\pm$ 0.88 c	19.34 $\pm$ 0.44 cd
22.2	24.75 $\pm$ 0.68 d	14.98 d

Medium: MS + 3 % w/v sucrose + 0.8 % w/v agar. All data are highly significant at $p \leq 0.001$.

Mean values followed by the different letter under different treatments within a column are significantly different from each other at $p \leq 0.001$ (Tukey's multiple range test). Letter (a-d) is from higher to lower values of the observation

Among different concentrations of sucrose tried, maximum germination percentage (67.58 $\pm$ 0.58b) was observed on MS basal medium with 4% w/v sucrose (Table 2). Combined role of sucrose and BAP was observed on the germination of somatic embryos (Table 2). Optimum medium for germination was MS medium with 4% w/v sucrose and 4.44 μ M BAP and an enhanced germination (77.79 $\pm$ 0.66 a) was found on this media (Fig. 1c). Somatic embryos developed

Table 2: Effect of sucrose and BAP on germination of somatic embryos

Sucrose (w/v %)	BAP (μ M)	Germination (%) (Mean $\pm$ SE)
1	0	20.97 $\pm$ 0.72 f
2	0	41.00 $\pm$ 1.47 e
3	0	58.61 $\pm$ 1.12 d
4	0	67.58 $\pm$ 0.58 b
5	0	62.15 $\pm$ 0.81 cd
6	0	40.03 $\pm$ 0.94 e
4	2.22	73.56 $\pm$ 0.93 ab
4	4.44	77.79 $\pm$ 0.66 a
4	8.88	65.11 $\pm$ 0.67 cd

Medium: MS + 0.8 % w/v agar with different concentrations of sucrose or BAP. All data are significant at $p \leq 0.001$. Mean values followed by the different letter under different treatments within a column are significantly different from each other at $p \leq 0.001$ (Tukey's multiple range test). Letter (a-d) is from higher to lower values of the observation

from *in vivo* leaf explants of this plant have shown maximum germination on MS medium supplemented with 8.88 μ M BAP (Singh *et al.* 2015). Germination of embryos developed from IVL explants required a lower concentration of BAP for germination; it may be due to the presence of high concentration of endogenous cytokinin in IVL explants. The efficiency of germination is a major factor for the success of plant regeneration (Singh *et al.* 2016; Martinelli and Mandolino 2009). Germination of somatic embryos at higher concentration of sucrose was also reported in *Psidium guajava* (Rai *et al.* 2007) and germination of somatic embryos in BAP supplemented media was reported in *Quercus rubra* (Vengadesan and Pijut 2009).

Field plantation and Genetic Fidelity Analysis

Micropropagated plants were successfully hardened and acclimatized in a mixture of red sand and soilrites (3:2) (Fig. 1d-e). About 80% plants survived under field condition (Fig. 1f).

RAPD and ISSR photographs showed clear, scorable and monomorphic bands. In RAPD, total 27 bands were observed with 6 primers and the average number of bands was 4.5 (Fig. 2). In ISSR, the total number of bands was 30 with 10 primers and the average number of bands was 3 (Table 3 and Fig. 2). Amplification bands showed the genetic similarity between mother and *in vitro* raised plants. Presence of monomorphic bands in both RAPD and ISSR assays shows that use of IVL for regeneration purpose is safe, and there is no adverse effect of *in-vitro* conditions and culture media, as both mother plants and cultures have been maintained in *in-vitro* conditions for the longer duration. Combinations of both markers have been used by several researchers to assess the genetic stability in many micropropagated plants such as *Spilanth esacmella* (Yadav *et al.* 2014), *Sapindus mukorossi* (Singh *et al.* 2019) and *Simmond siachinensis* (Kumar *et al.* 2011).

Conclusions

In vitro plants of *S. mukorossi* successfully regenerated using IVL explants through somatic embryogenesis pathways. The use of IVL explants overcomes the seasonal variations that affect the explants' responses. Genetic fidelity testing proved the genetic similarity between the mother plants and

Fig. 2: RAPD amplified molecular profile with primers OPA-06 and OPP-10 and ISSR amplified molecular profile with primers UBC-807 and UBC-810 for mother plant and randomly selected regenerated plants (L-molecular ladder-1 Kb, M1 & M2-mother plant, R1-R8 the micropropagated plants)

Table 3: DNA-based primers amplified for *in vitro* raised plants from *in vitro* leaf explants

RAPD primer	Primer sequences	Total no. of bands amplified	No. of scorable bands	Range of amplification (bp)
OPE-03	CCAGATGCAC	80	8	350-1250
OPA-06	GGTCCCTGAC	30	3	350-950
OPE-12	TTATCGCCCC	50	5	350-1200
OPC-07	GTCCCGACGA	60	6	400-1200
OPP-10	TCCCGCCTAC	20	2	500-1000
OPH-04	GGAAGTCGCC	30	3	350-650
Total		270	27	
ISSR Primer				
UBC-824	TCTCTCTCTCTCTCTCG	20	2	400-800
UBC-834	AGAGAGAGAGAGAGACYT	40	4	250-900
UBC-856	ACACACACACACACCTA	20	2	400-1000
UBC-825	ACACACACACACACACT	40	4	500-1150
UBC-814	CTCTCTCTCTCTCTCTA	30	3	450-950
UBC-807	AGAGAGAGAGAGAGAGT	40	4	400-900
UBC-810	GAGAGAGAGAGAGAGAT	20	2	400-1100
UBC-840	GAGAGAGAGAGAGAGAY	30	3	400-1250
UBC-842	GAGAGAGAGAGAGAGAYG	30	3	450-950
UBC-854	TCTCTCTCTCTCTCTCAGG	30	3	700-1250
Total		300	30	

Note: 10 samples (plants) were tested

regenerated plants. This regeneration protocol will be beneficial for production of a large number of *in vitro* plants without genetic variations in the limited time period. The *in vitro* raised plants, callus and *in vitro* raised leaf can also be used as raw materials for the production of natural antioxidants, and other important phytochemicals and pharmacophores. Production and isolation of saponins is also possible by using this reliable and reproducible protocol.

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