Physiol Mol Biol Plants (July–September 2016) 22(3):381–389
DOI 10.1007/s12298-016-0364-0

RESEARCH ARTICLE

Regeneration of soapnut tree through somatic embryogenesis and assessment of genetic fidelity through ISSR and RAPD markers

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Received: 18 March 2016 / Revised: 11 June 2016 / Accepted: 20 June 2016 / Published online: 1 July 2016

Abstract Somatic embryogenic system was developed in *Sapindus mukorossi* Gaertn. using rachis as explants from a mature tree. Explants showed callus initiation on Murashige and Skoog medium supplemented with TDZ (1-Phenyl-3-(1, 2, 3-thiadiazol-5-yl) urea), zeatin or 6-benzylaminopurine. Induction of somatic embryogenesis was achieved on both MS basal medium and MS medium supplemented with 8.88 µM 6-benzylaminopurine. Hundred percent embryogenesis was observed on MS medium supplemented with 8.88 µM 6-benzylaminopurine with maximum intensity of embryogenesis (51.92 ± 0.40 a). Maximum maturation of somatic embryos (92.86 ± 0.34 a) was observed on induction medium supplemented with 0.0378 µM abscisic and treated for 21 days. Germination of somatic embryos was maximum (77.33 ± 0.58 a) on MS medium supplemented with 8.88 µM 6-benzylaminopurine. In vitro raised plantlets were hardened, acclimatized and transferred to the field. Survival frequency of plantlets was 80 % in field conditions. The genetic fidelity of in vitro regenerated plants was also evaluated and compared with mother plant using random amplified polymorphic DNA and inter simple sequence repeat. Both markers showed similarity in molecular profile of mother plant and in vitro regenerated plants.

Keywords *Sapindus mukorossi* Gaertn. · Rachis · Somatic embryogenesis · Glutamine · Genetic fidelity · Molecular markers

Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
BA	6-Benzylaminopurine
KIN	Kinetin
ZN	Zeatin
TDZ	1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea
ABA	Abscisic acid
RAPD	Random amplified polymorphic DNA
ISSR	Inter simple sequence repeat
FE	Frequency of embryogenesis

Introduction

Sapindus mukorossi Gaertn. (family-Sapindaceae) commonly known as Chinese soapberry, is a medium to large sized deciduous tree found in temperate to tropical and subtropical regions of Asia. The tree is indigenous to northern and central India and is widely distributed in Himalayan region, Haryana, Uttar Pradesh and Chhattisgarh (Anonymous 2005). The tree is well known for its medicinal, industrial and economical importance. Traditionally, soap nut (reetha) is used to treat asthma, piles, snake bite, tooth

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disorder and eye disorders (Balkrishna 2008). Its fruits are rich source of saponins, being used in the preparation of quality shampoos and detergents. The fruits are used for the treatment of several diseases like excessive salivation, epilepsy, hypochromic anemia, migraines, eczema, psoriasis, and freckles (Kirtikar and Basu 1991). Antioxidant activity and polyphenolic compounds were evaluated from leaf and fruit extracts (Singh and Kumari 2015). The different medicinal effects such as antifungal, antiviral, antibacterial, hepatoprotective, anti-inflammatory, anti-trichomonas activity, spermicidal and anticancer activity etc. have been reported from the different parts of the plant (Takagi et al. 1980; Ibrahim et al. 2008; Tsuzuki et al. 2007; Tiwari et al. 2008; Man et al. 2010; Singh et al. 2016).

Seed germination is the major way for conventional propagation of *S. mukorossi*. Poor seed germination and slow growth of seedlings are major obstacles for its large scale plantation. Natural propagation of plant is not fast enough to meet the increasing industrial demand of raw materials. So, there is a need to develop a faster regeneration protocol to fulfill the raw material demand. Various authors have reported somatic embryogenesis from leaf tissue of this plant (Singh et al. 2015; Philomina 2010; Dobhal et al. 2012; Kim et al. 2012). Dobhal et al. (2012) have reported somatic embryogenesis from its petiole explants. Plant regeneration through somatic embryogenesis and organogenesis has been also reported in different species of *Sapindus* such as *S. trifoliatus, S. emarginatus, S. saponaria* etc.

Somatic embryogenesis is an efficient regeneration system and it is used for propagation and improvement of elite plants (Stasolla and Yeung 2003). Somatic embryogenesis comprises the process of regeneration of whole plants from a single somatic embryo, which in turn, originates from a single cell (Komamine et al. 2005). It is considered better than organogenesis due to bipolar nature of somatic embryos and less chance of genetic aberrations due to single cell origin of somatic embryos (Wang and Bhalla 2004). Trees are mostly heterogeneous population of perennial plants. Propagation of tree species through tissue culture has several bottlenecks such as phenolic exudation, contamination and seasonal dependency with respect to availability of explants. Similarly, development of a regeneration protocol through somatic embryogenesis has several limitations such as low frequency of somatic embryo production, abnormal embryo production, incomplete and low frequency maturation, low germination and less survival in field conditions (Merkle 1995). However, there are several reports of successful regeneration of woody plants via somatic embryogenesis such as *Thymus hyemalis* (Nordine et al. 2014), *Prosopis laevigata* (Buendia-Gonzalez et al. 2012) *Murraya koenigii* (Paul et al. 2011). Initially, somatic embryogenesis was used for large scale propagation and germplasm conservation of plants, but nowadays, the system is also used for the production of secondary metabolites (Shohael et al. 2006).

Genetic fidelity assessment is a prerequisite for the commercial use of regeneration protocols. Mostly isozymes and DNA based molecular markers are used for the evaluation of genetic fidelity. Among various DNA based molecular markers, random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis are used frequently for clonal fidelity assessment. These are technically simple, easy and quick to perform. Both methods require small amount of DNA and do not need genomic information to perform the experiment (Williams et al. 1990; Waugh and Powell 1992).

Rachis is an inter-segment of petiole axis between two successive leaflets in a compound leaf. Rachis is an important part of leaflets in compound leaf. Although, rachis explants have high regeneration potential but it is not used for micropropagation in any plants and no published reports were found. To the best of our knowledge, rachis explants of *S. mukorossi* have not been used for micropropagation till date. To explore the regeneration potential of a new explant (i.e. rachis) this study was undertaken. In the present investigation, an efficient regeneration protocol of *S. mukorossi* was developed through somatic embryogenesis and genetic fidelity of regenerated plants was assessed by RAPD and ISSR.

Materials and methods

Explant and surface disinfection

Fresh flushes of leaves were collected in the month of March from 8 to 10 year old tree of *S. mukorossi* (Fig. 1a, b), growing in Banaras Hindu University, Varanasi, India. The rachis was excised from leaf and washed under running tap water for 15–20 min. After washing, rachis was treated with disinfectant solution containing 2 % (v/v) savlon liquid (Johnsons and Johnsons, India), 4–5 drops of Tween-20 (Hi-Media, India) and 8–10 drops of sodium hypochlorite (Merck, India) in 100 ml of double distilled water for 8–10 min with vigorous shaking. Surface disinfection was also carried out with 0.05 % (w/v) mercuric chloride (Merck, India) for 4–5 min after a brief treatment with 70 % ethanol for 30 s under laminar air flow. After every treatment, explants were rinsed 3–4 times with autoclaved DDW to remove the traces of disinfectants.

Culture media and culture conditions for callus induction

Surface sterilized rachis explants were cut to size of about 1–1.5 cm long and cultured on MS medium supplemented with different concentrations of growth regulators i.e. 2,4-D

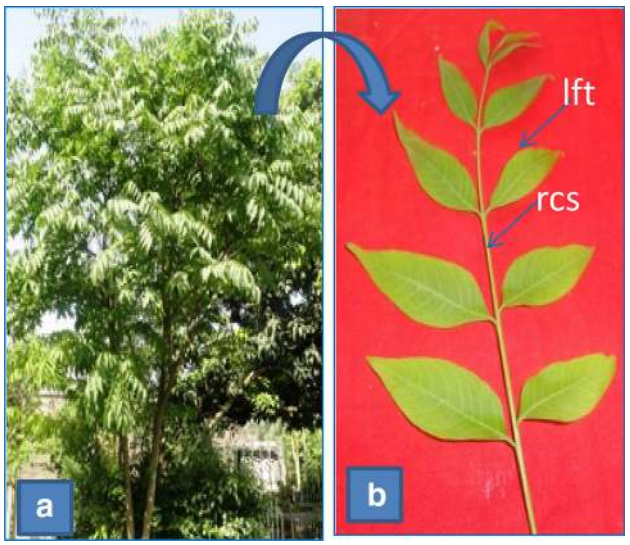

Fig. 1 A tree of *S. mukorossi* Gaertn. growing in the campus of Banaras Hindu University, Varanasi, **a** mature tree, **b** compound leaf (*rcs* rachis, *lft* leaflet)

(2.26 –22.65 μM), BA (2.22–22.2 μM), KN (2.32–23.25 μM), IAA (2.85–28.55 μM), IBA (2.45–24.5 μM), ZN (0.0045–2.28 μM) or TDZ (0.0045–2.27 μM) (Sigma-Aldrich, USA) for callus induction. Explants were also inoculated on woody plant media (WPM) with different concentrations of BA (2.22–22.2 μM). Growth regulator free MS medium was used as control. Medium was supplemented with 3 % (w/v) sucrose (SRL, India) and solidified with 0.8 % (w/v) agar (Hi-Media, India) and pH of the medium was adjusted to 5.8 ± 0.02 with 0.1 N NaOH and 0.1 N HCl prior to autoclaving. 15–20 ml media was poured in 25 × 150 mm test tube and autoclaved at 121 °C temperature and 1.06 kg cm^{-2} pressure for 15 min. Cultures were maintained at 25 ± 1 °C with 16/8 h (light/dark) photoperiod at a photon flux of 50–70 μmol m^{-2} s^{-1} by cool white fluorescent tubes (Philips, Mumbai, India).

Induction and development of somatic embryo

For induction of somatic embryos, callus was sub-cultured either in the induction medium of same composition or in MS basal medium after 1, 2, 3 or 4 weeks of callus formation. Glutamine (100–400 mg l^{-1}) was also added to MS basal medium to observe its impact on the induction of somatic embryogenesis. Similarly to optimize the concentration of sucrose, different concentrations of sucrose were tried in induction medium.

Maturation and germination of somatic embryos

For the maturation, early stage cotyledonary somatic embryos were transferred to MS medium + 8.88 μM BA with different concentrations of sucrose. Both MS medium supplemented with different concentrations of ABA alone or combinations of different conc. of ABA with 8.88 μMBA were also tried for maturation. MS medium supplemented with different concentrations of sucrose and BA were tried to optimize the effects of both factors on germination of embryos.

Ex vitro plantation of plantlets

Well-developed plantlets were transferred to paper cups containing a mixture of sterilized red sand and garden soil (3:2), red sand and soilrite (3:2), garden soil and soilrite in the ratio of 1:1 or garden soil alone. Plantlets were covered with polyethylene bags to maintain high humidity and irrigated with tap water or DDW. Potted plantlets were kept in culture room for 4–5 weeks and after 5 weeks polyethylene bags were punched with needle for gradual decrease of humidity. Plantlets were maintained in culture room condition up to 8 weeks during hardening. After that the pots were transferred to normal room conditions and then plantlets were gradually exposed to sunlight for acclimatization. After, 10 weeks of hardening and acclimatization, plants were transferred to the field.

Genetic stability assessment

Clonal fidelity between mother plant and 9 randomly selected field transferred micropropagated plants (about 18 months old) was assessed using PCR based RAPD and ISSR analysis. About 200 mg young leaves of mother plant and in vitro raised plants were used for DNA extraction. Genomic DNA was extracted by CTAB method (Doyle and Doyle 1990) with slight modification. The quantity and quality of isolated DNA was checked by using 0.8 % agarose gel stained with ethidium bromide and Dyna Quant 200 Fluorometer. Initial screening was done by a set of 10 ISSR and 6 RAPD primers (Operon Technologies Inc., CA, USA). Amplification of DNA was done using 25 μL PCR mixture consisting of 2.5 μL 10× buffer (GeNei™, Banglore, India), 1.0 μL primer (5 pM), 1.0 μL DNA (about 25 ng μL^{-1}), 0.2 μL Taq polymerase (GeNei™), 1.0 μL dNTP (10 μM: 2.5 μM each of the dNTPs viz. dATP, dTTP, dCTP and dGTP), GeNei™ and 19.2 μL Milli-Q water. Amplifications were carried out by a chain of processes: an initial denaturation of DNA at 94 °C for 5 min followed by denaturation at 94 °C for 1 min, 1 min annealing (35 °C), 2 min extension (72 °C) and final extention for 3 min at 72 °C in an iCycler™Thermal Cycler (Model 4.006, Bio-Rad, Hercules, CA, USA). This process was continued for 45 cycles.

Experimental design and data analysis

Twenty replicates were used for each treatment and each treatment was repeated thrice. The cultures were examined at regular intervals of 1 week. The frequency of embryogenesis was calculated as the percentage of cultures showing at least one somatic embryo and intensity of embryogenesis was calculated as the mean number of somatic embryos per culture. Total number of somatic embryos formed from responsive cultures was counted using simple magnifying glass. The results were expressed as a mean ± SE of three independent experiments. The data were analyzed statistically using one-way analysis of variance (ANOVA) and significant differences between means were assessed by Tukey's multiple range tests at a 1 % probability level by SPSS (version 16).

Results

Effect of different factors on callusing

1.2–1.5 cm long explants were found responsive for callus induction (Fig. 3a). Explants less than 1 cm in length either failed to respond or produced negligible callus. Callusing was significantly affected by orientation and exogenously applied plant growth regulators. In both horizontal and vertical orientations of explants, callusing frequency was 100 %. But, horizontally placed explants formed callus on whole surface whereas, callus development was restricted to only some portions of vertically orientated explants.

Effect of different growth regulators on callusing

Media supplemented with different types and concentrations of growth regulators showed different response for callus initiation. The explants showed callus initiation only on medium containing 2, 4-D, TDZ, ZN or BA after about 15–20 days of inoculation. Percent callusing was higher in MS medium having TDZ or ZN (Table 1) in comparison to BA and percent callusing was higher in MS medium with BA when compared to WPM having BA (Fig. 2). There was no callus initiation on medium containing IAA or IBA and callusing of explants was very less in MS medium supplemented with 2, 4-D (data not shown). Callus initiation and proliferation was maximum on MS medium supplemented with 8.88 µM BA (Fig. 3b). Maximum proliferation of callus was observed after 21 days of sub-culturing.

Induction of somatic embryogenesis

Induction of somatic embryogenesis was observed on both MS basal medium and MS medium with various concentrations of BA (Table 2). Initiation of somatic embryos occurred after 3 weeks of transfer of proliferated callus (Fig. 3c). Frequency of embryogenesis (FE) in MS basal medium was 91.33 ± 0.79 b, while 100 % FE was observed on MS medium with 8.88 µM BA. However, number of somatic embryos was very less (8.94 ± 0.28 f) on MS basal medium. Maximum number of somatic embryos (51.92 ± 0.40 a) was observed on MS medium + 8.88 µM BA. Somatic embryos was developed asynchronously (Fig. 3d).

Table 1 Callusing percentage in MS media with different concentration of TDZ and ZN

Conc. of TDZ (µM)	Conc. of ZN (µM)	Percent callusing (Mean ± SE)
0.0	0.0	0.00 ± 0.00 f
0.0045		0.00 ± 0.00 f
0.045		38.00 ± 0.28 e
0.45		88.00 ± 0.28 b
1.36		75.50 ± 0.28 c
2.27		100.00 ± 0.00 a
	0.0045	75.83 ± 0.44 c
	0.045	87.58 ± 0.22 b
	0.45	100.00 ± 0.00 a
	1.37	100.00 ± 0.00 a
	2.28	50.50 ± 0.28 d

Medium: MS medium with 3 % (w/v) sucrose + 0.8 % (w/v) agar with different concentration of TDZ and ZN. All values are significant at $p \leq 0.001$

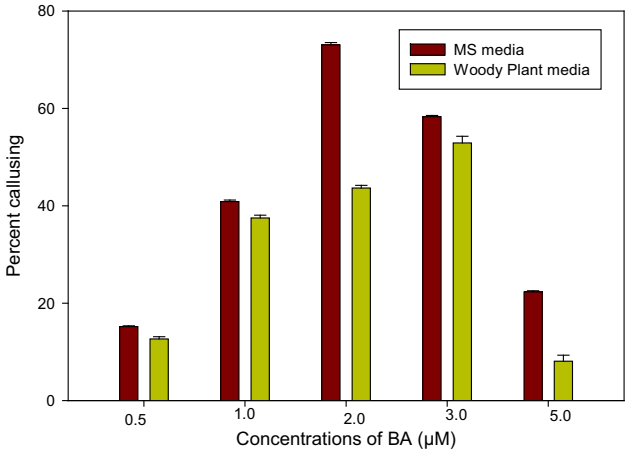

Fig. 2 Effect of BA on percent callusing of rachis explants in MS media and woody plant media

Maturation and germination of somatic embryos

After formation of somatic embryos, embryos were transferred to different maturation medium. Maximum

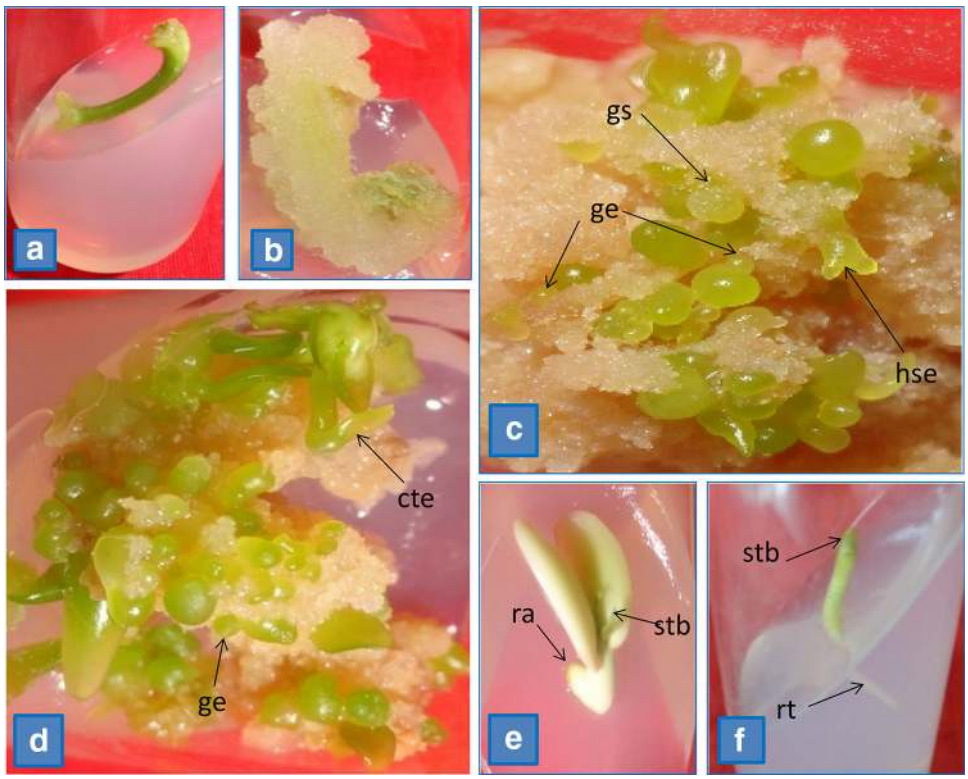

Fig. 3 Somatic embryogenesis in *S. mukorossi* using rachis explant of (**a**–**j**). **a** rachis explant after 7 days of inoculation, **b** Callus proliferation, **c**, **d** Different stages of embryos, **e** Germination of cotyledonary stage embryo, **f** Germination from torpedo stage somatic embryo (*ge* globular stage embryo, *gs* globular structure, *cte* cotyledonary stage embryo, *hse* heart shape embryo, *ra* radicle end, *stb* shoot buds, *rt* root)

Table 2 Effect of different concentrations of BA on frequency and intensity of somatic embryogenesis in *Sapindus mukorossi*

BA (μM)	Frequency of embryogenesis (FE)	Intensity of embryogenesis (IE)
00	91.33 ± 0.79 b	8.94 ± 0.28 f
2.22	50.53 ± 0.37 e	13.17 ± 0.19 e
4.44	75.11 ± 0.25 d	39.56 ± 0.27 c
8.88	100.00 ± 0.00 a	51.92 ± 0.40 a
13.32	86.97 ± 0.27 c	46.22 ± 0.32 b
22.2	49.87 ± 0.20 e	28.24 ± 0.56 d

Medium: MS with 3 % sucrose + 0.8 % agar supplemented with different concentration of BA. All values are significant at $p \leq 0.001$

Table 3 Effect of sucrose percentage and ABA on maturation of somatic embryos

Sucrose percentage (w/v)	ABA (μM)	Maturation frequency (Mean ± SE)
1	0	31.40 ± 0.65 i
2	0	48.11 ± 0.82 h
3	0	81.50 ± 0.51 d
4	0	59.04 ± 0.50 f
5	0	51.62 ± 0.48 g
3	0.000378	82.52 ± 0.18 cd
3	0.00378	85.18 ± 0.24 bc
3	0.0378	92.86 ± 0.34 a
3	0.378	87.93 ± 0.37 b
3	0.756	71.86 ± 0.46 e

Medium: Full strength MS medium solidified with 0.8 % (w/v) agar + 8.88 μM BA + different concentration of sucrose or ABA. Values are significant at $p \leq 0.001$, mean was compared with Tukey multiple range test

maturation frequency (81.50 ± 0.51 a) was found on MS medium with 3 % sucrose while minimum maturation frequency (31.40 ± 0.65 e) of somatic embryos was found in 1 % sucrose (Table 3; Fig. 3e). Among different concentration of ABA, the MS medium containing 3 % sucrose along with 0.0378 μM ABA was found best (92.86 ± 0.34 a) for somatic embryos maturation.

Germination of somatic embryos (Fig. 3f) was clearly affected by sucrose and BA concentrations. Highest germination frequency (77.33 ± 0.58 a) was observed on MS medium with 3 % (w/v) sucrose and supplemented with 8.88 μM BA. Germination frequency (74.71 ± 0.26 b) was less on MS basal medium (Table 4) than on MS medium with BA. The present study showed that BA plays a significant role in germination of somatic embryos. However, supplementing MS with 4.44 and 13.32 μM BA was not as effective as that with 8.88 μM BA for germination.

Establishment of plants in soil

In vitro regenerated plantlets (Fig. 4a) through somatic embryogenesis were successfully hardened and established

Table 4 Effect of sucrose and BA on germination of somatic embryos

Sucrose concentration in percent (w/v)	BA (μM)	Germination frequency (mean ± SE)
1	0	12.75 ± 0.04 d
2	0	22.18 ± 0.14 c
3	0	74.71 ± 0.26 a
4	0	68.77 ± 0.22 b
5	0	63.55 ± 0.20 d
3	4.44	67.24 ± 0.26 b
3	8.88	77.33 ± 0.58 a
3	13.32	70.01 ± 0.95 b

Medium: full-strength MS medium + 0.8 % (w/v) agar. All values are significant at ($p \leq 0.001$) according to Tukey's multiple range test

in the mixture of red sand and soilrite (Fig. 4b). Finally plantlets were transferred to the field conditions (Fig. 4c) and survival frequency was observed 80 %. The acclimatized plants were phenotypically similar to the mother plants.

Genetic fidelity analysis

A total of 16 primers (10 ISSR and 6 RAPD) were used for production of scorable and reproducible bands. Figure 5 (RAPD) and Fig. 6 (ISSR) showing monomorphic amplified bands of genomic DNA of parent and micropropagated plants. All primers generated a clear, scorable and reproducible banding pattern ranging from 350 to 1250 bp for RAPD (Table 5) and 250–1250 bp for ISSR markers (Table 6). All these RAPD primers produced a total of 25 bands and number of scorable bands for each primer varied from two (OPP-10, OPH-04) to eight (OPE-03) with an average of 4.16 bands per primer (Table 5). In ISSR primers total number of scorable bands was 28 and varied for each primer from one (UBC-824) to four (UBC-834, UBC-825, UBC-807) with an average 2.8 bands per primer (Table 6).

Discussion

The present study clearly shows the potential of rachis tissue isolated from an adult tree of *S. mukorossi* for somatic embryogenesis through callus culture. Somatic embryogenesis from rachis explants follows secondary pattern of embryogenesis. Similar pattern of somatic embryogenesis was also reported from leaf explants of *S. mukorossi* (Singh et al. 2015) and other plants (Nordine et al. 2014). Formation of callus on the whole horizontly placed explants, may be due to more contact area of explants with the medium than in the case of vertically placed explants. Types of calli were observed different in different orientations of explants such as green, compact and non embryogenic from vertically placed explants and yellowish whitish, friable, glossy and embryogenic callus in horizontally placed explants. Similar reports are there in *Phaseolus coccineus* (Genga and Allavena 1991). In contrast to our result, somatic embryo formation was observed on green compact hard callus in *Desmodium motorium* (Devi and Narmathabai 2011).

Percent callusing was different in different media supplemented with various growth regulators. Generally, exogenously applied plant growth regulators are considered as major factor for callus induction. But, endogenous level of different phytohormones in explants tissues might be a factor that influenced the required concentration of exogenously applied plant growth regulators (Deo et al. 2010). Generally, auxins specially 2,4-D is reported for induction of embryogenic reponses but role of cytokinins was also reported. Endogenous level of phytohormones is

Fig. 4 Hardening, acclimatization and field transfer of plantlets. **a** In vitro raised complete plantlet, **b** Hardened and acclimatized plantlet, **c** Field transferred plantlet in pot

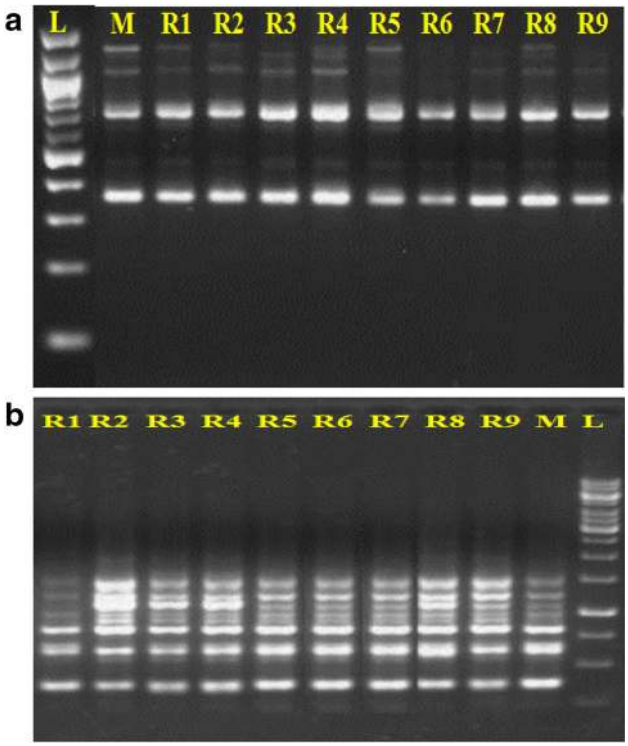

Fig. 5 RAPD amplified molecular profile with primers OPE-12 (**a**) and OPE-03 (**b**) for mother plant and randomly selected regenerated plants (L- molecular ladder, M- mother plant, R1-R9 the micropropagated plants)

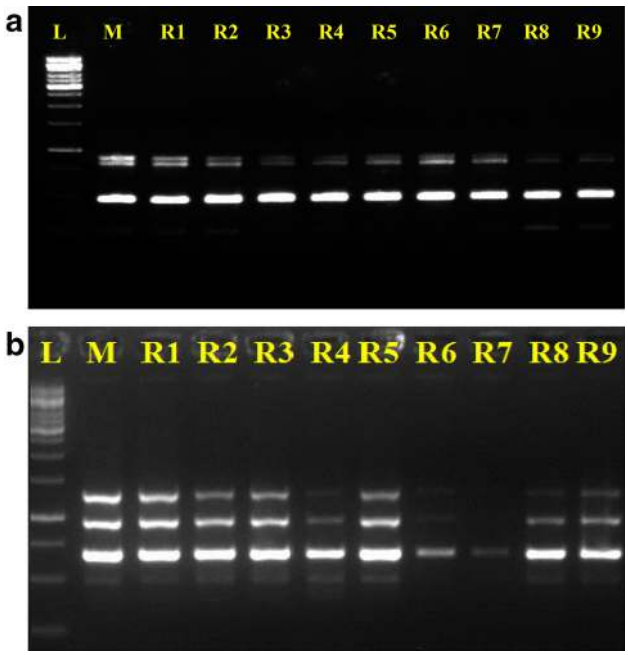

Fig. 6 ISSR amplified DNA profile observed with primers UBC-834 (**a**) and UBC-825 (**b**) for mother plant and randomly selected regenerated plants (*L* molecular ladder, *M* mother plant, *R1–R9* the micropropagated plants)

also considered to be one of the crucial factors for determining the embryogenic potential of explants (Feher et al. 2003; Gaj 2004).

We observed induction and development of somatic embryos on MS medium supplemented with BA. Mostly, somatic embryogenesis (induction and development of somatic embryos) was observed on MS basal medium. But, in *Ocimum basillicum* maximum induction of somatic embryogenesis was reported in BA, NAA and KN containing medium (Gopi and Ponmurugan 2006). Development of somatic embryos in BA containing medium is also reported in *Coffea arabica* (Yasuda et al. 1985), *Leptadenia reticulate* (Martin 2004), *Curculigo orchioides* (Patel et al. 2011). Somatic embryos was also present on MS medium or MS medium with 8.88 μM BA, supplemented with different concentrations of glutamine and maximum number of somatic embryos was found on medium with 200 mg l^{-1} glutamine (data not shown). But, there was no significant effect of glutamine on induction of somatic embryogenesis as compared to control (MS medium with 8.88 μM BA). Effect of amino acids on somatic embryogenesis was also studied in other plants (Rai et al. 2009).

In our study, treatment of ABA for 21 days showed positive effect on maturation of somatic embryos. Role of ABA in maturation of somatic embryos was also studied in other plants such as sugarcane (Nieves et al. 2001) *Quercus suber* (Garcia-Martin et al. 2005), etc. Exogenously supplied ABA showed positive effect in maturation phase of *Hybrid larch* somatic embryos (Gutmann et al. 1996). In maturation phase of somatic embryos, reserve food materials were accumulated and respiration was reduced (Trigiano and Gray 1996). Maturation stage is a very crucial and important phase of somatic embryogenesis because germination frequency of somatic embryos depends on maturation phase up to some extent. Somatic embryogenesis response, number of somatic embryos, maturation and germination of somatic embryos and field survival of plantlets was less in rachis explants than leaf explants (Singh et al. 2015) but better than the other previous reports (Philomina 2010; Dobhal et al. 2012).

RAPD and ISSR analysis showed similar banding pattern in mother plant and in vitro regenerated plants. RAPD alone has been successfully applied for evaluation of clonal fidelity in many micropropagated woody plants such as *Terminalia arjuna* (Gupta et al. 2014), *S. mangifera* (Tripathi et al. 2012) and *S. trifoliatus* (Asthana et al. 2011). Similarly, ISSR was also applied for evaluation of genetic homogeneity in *Tylophora indica* (Sharma et al. 2014) and *Ochreinauclea missionis* (Chandrika and Rai 2009). RAPD and ISSR both were used for assessment of genetic fidelity by some previous researchers (Yadav et al. 2013; Kumar et al. 2011).

Table 5 RAPD primers amplified in *Sapindus mukorossi*

Primer	Primer sequences	Total no. of bands amplified	No. of scorable bands per primer	No. of polymorphic bands	Range of amplification (bp)
OPE-03	CCAGATGCAC	80	8	0	350–1250
OPA-06	GGTCCCTGAC	30	3	0	350–950
OPE-12	TTATCGCCCC	40	4	0	350–1200
OPC-07	GTCCCGACGA	60	6	0	400–1200
OPP-10	TCCCGCCTAC	20	2	0	500–1000
OPH-04	GGAAGTCGCC	20	2	0	350–650
Total		250	25	0	

Table 6 ISSR primers amplified in *Sapindus mukorossi*

ISSR Primer	Nucleotide sequence (5′–3′ motif)	Annealing Temp (°C)	Total no. of bands amplified	No. of scorable bands per primer	No. of polymorphic bands	Range of amplification (bp)
UBC-824	TCTCTCTCTCTCTCTCG	55	10	1	0	800
UBC-834	AGAGAGAGAGAGAGACYT	55	40	4	0	250–900
UBC-856	ACACACACACACACACCTA	56	20	2	0	400–1000
UBC-825	ACACACACACACACACT	50	40	4	0	500–1150
UBC-814	CTCTCTCTCTCTCTCTA	55	20	2	0	450–950
UBC-807	AGAGAGAGAGAGAGAGT	55	40	4	0	400–900
UBC-810	GAGAGAGAGAGAGAGAT	55	20	2	0	400–1100
UBC-840	GAGAGAGAGAGAGAGAY	58	30	3	0	400–1250
UBC-842	GAGAGAGAGAGAGAGAYG	59.1	30	3	0	450–950
UBC-854	TCTCTCTCTCTCTCTCAGG	58	30	3	0	700–1250
Total			280	28		

Conclusions

The present reports described regeneration protocol for *S. mukorossi* through somatic embryogenesis by using rachis explants. This protocol provides a successful and rapid technique of regeneration that could be useful for in vitro propagation, secondary metabolite production, germplasm conservation and genetic transformation. Callus extracts can be used for production of phytochemicals either through suspension culture or normal reculturing methods and in vitro leaf can also be used for phytochemical production. RAPD and ISSR molecular markers were used to assess the genetic integrity of in vitro regenerated plants. Combined studies of both markers showed that there is no somaclonal variation in the present protocol.

Acknowledgments Author (RS) acknowledged to Council of Scientific and Industrial Research (CSIR) New Delhi, India is for providing financial support as Junior Research Fellowship. Financial assistance by University Grants Commission (UGC), India is also acknowledged by the corresponding author.

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