A reliable *in vitro* protocol for rapid mass propagation of *Sapindus mukorossi* Gaertn.

Narender Singh*, Avneet Kaur and Kuldeep Yadav

Department of Botany, Kurukshetra University Kurukshetra -136119 (India)
*Email: nsheorankuk@yahoo.com

Abstract: An efficient regeneration protocol under *in vitro* conditions has been developed for *Sapindus mukorossi* Gaertn.- an important medicinal plant using nodal segments. Nodal explants of this species were cultured on Murashige and Skoog medium supplemented with various concentrations of auxins and cytokinins individually and in various combinations. BAP was found to be more effective than Kinetin for shoot multiplication. The highest number of shoots (2.0 ± 0.29) was achieved on MS medium augmented with 2.0 mg/l BAP. The medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA responded better than all other media combinations. MS half strength medium supplemented with 2.0 mg/l IBA proved better with forty percent rooting after 22 days of implantation. Most of the roots were long and healthy. The micropropagated plantlets were hardened and acclimatized. They were successfully transferred in pots containing sterilized soil and sand mixture (1:1) with 60% survival rate under field conditions. [Nature and Science 2010;8(10):41-47]. (ISSN: 1545-0740).

Key words: Multiple shoots, Nodal segments, *Sapindus mukorossi* ,auxins, cytokinins.
Abbreviations : BAP-6-benzylamino purine, Kn-Kinetin, IAA-indole-3-acetic acid, 2,4-D- 2,4-dichlorophenoxy acetic acid, NAA-á-naphthalene acetic acid, IBA-indole butyric acid.

1. Introduction

Sapindus mukorossi (family: Sapindaceae) popularly known as 'Ritha' and 'Soapnut', is a most important deciduous tree of tropical and sub- tropical regions of Asia. The fruit of this tree contains saponins, the most active secondary metabolites extracted from this plant. It is a good substitute for washing soap and is as such used in preparation of quality shampoos, detergents etc .The fruit is of considerable importance for its medicinal value for treating a number of diseases like common cold, pimples, epilepsy, constipation, nausea etc. It is also used as expectorant and anthelmintic in small doses. It was utilized by Indian jewelers for restoring the brightness of tarnished ornaments made of gold, silver and other precious metals. It was also used for washing and bleaching cardamoms (Anonymous, 1992). Central Drug Research Institute, Lucknow has developed a contraceptive cream from this fruit which has anti-*Trichomonas* activity (Tiwari *et al.*, 2008).

The vegetative propagation of this tree does not yield satisfactory results and propagation through seeds is also unreliable because the per cent survival of the seedlings proved to be meager due to heavy incidence of mortality at seedlings stage in the natural habitat. Moreover, the seeds have hard seed coat due to which these became physically dormant. Due to these limitations, the conventional methods of vegetative propagation of this species have not proved easy. Therefore, an alternate *in vitro* method of propagation has been developed.

2. Materials and Methods

Nodal segments were excised from the plants growing in Herbal Garden of Botany Department, Kurukshetra University, Kurukshetra. All the explants were washed with liquid detergent under running tap water to remove dust particles. The explants were then treated with 0.1% (w/v) mercuric chloride for 3-5 minutes under aseptic conditions. After this these explants were then thoroughly washed 4-5 times with sterilized double distilled water to remove the traces of mercuric chloride. The nodal segments (1.0-1.5cm), after trimming the ends, were finally inoculated on MS medium supplemented with various concentrations (0.5-2.0 mg/l) of auxins (IAA, NAA, 2, 4-D and IBA) and cytokinins(BAP and Kn) alone and in various combinations for shoot regeneration and callus induction.

The cultures were incubated at a temperature of 25±2ºC and a photoperiod of

16hrs light (intensity of 2000 lux) and 8hrs of dark.

Visual observations like callus induction, growth of callus, number of days taken for bud break, percentage of bud break and number of shoots regenerated per explants were recorded regularly. A mean of 20 replicates was taken per treatments.

The *in vitro* raised single/multiple shoots (2.0 – 3.0 cm long) were excised and transferred to cultural tubes containing full and half strength MS medium fortified with IBA, IAA and NAA under aseptic conditions for rooting.

The shoots with well developed roots were gradually pulled out from the medium and immersed in water to remove the remains of agar-agar particles sticking to the root system by using a fine brush. These plantlets were transferred to pots containing sterilized soil and sand mixture (1:1). The potted plantlets were covered with a transparent polythene bag to ensure high humidity around the plants. The pots were supplied with MS (half strength) salt solution on alternate days. After about two weeks the polythene bags were removed for 3-4 hours daily to expose the plants to the conditions of natural humidity for acclimatization. These plants were shifted to bigger pots after one month of its transfer and were maintained under green house conditions.

3. Results and Discussion

3.1 Direct organogenesis

MS basal medium devoid of growth regulators served as control. No bud break was achieved on MS medium devoid of growth regulators. Similarly no shoot buds developed on MS basal medium in *Peganum harmala* (Saini & Jaiswal, 2000),*Celastrus paniculatus* (Lal and Singh, 2010).

Nodal explants responded to all the concentrations of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) tried. MS medium fortified with 2.0 mg/l BAP supported better results as compared to other concentrations of BAP in terms of period required for bud break, per cent bud break, number of shoots regenerated and shoot length (Table-1, Figure-1-a). The effectiveness of BAP on the induction of bud break and shoot proliferation has been reported in *Rotula aquatica* (Sebastian *et al.,* 2002), *Vigna Radiata* (Sonia *et al.,* 2007). Regeneration of shoots in response to Kn has been observed in *Kaempferia galangal* (Chirangini *et al.,* 2005) and *Phyllanthus niruri* (Karthikeyan *et al.,* 2007). Shoot regeneration was not noticed in nodal segments inoculated on the medium supplemented with various concentrations of IAA and NAA.

A combined effect of BAP + IAA and BAP + NAA was also studied on the nodal explants. The per cent bud break increased with increase in the concentration of BAP from 0.5 mg/l to 2.0 mg/l. Among the various media fortified with different concentrations of BAP and IAA, 2.0 mg/l BAP+1.0 mg/l IAA was proved better in terms of per cent bud break and number of shoots differentiated per explant (Figure-1-b). The explants cultured on MS medium fortified with BAP + NAA gave better results as compared to BAP + IAA. Among the three concentrations of BAP (0.5, 1.0 & 2.0 mg/l) + NAA (1.0 mg/l) applied, most favorable results were obtained on the MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA. Similar observations have been made in *Albizzia lebbeck* (Vargeese & Kaur, 1988), *Tagectes erecta* (Vanegas *et al*., 2002), *Spilanthes acmella* (Saritha *et al*., 2003). High cytokinins to auxins ratio has been shown to promote shoot formation in *Rhodiola rosea* (Kirichanko *et al.,* 1993). The development of axillary shoots from nodal explants was accompanied by basal callusing of the explants. However this remained undifferentiated. Same type of observation have been made by Singh and Lal (2007) and Nandwani and Ramawat (1991) working with *Prosopis juliflora and Leucaena leucocephala* respectively.

3.2 Indirect organogenesis

Callus formation was noticed from the nodal explants in the media supplemented with auxins (NAA and 2, 4-D) and cytokinins (BAP and Kn). Among auxins, 2,4-D exhibited better response in terms of callus induction as compared to NAA supplemented in the MS medium. The callus so produced was whitish green in colour and soft in texture. Presence of 2, 4-D has been shown to be essential for callus formation in *Capsicum annum* (Gupta *et al.,* 1990). NAA played an important role in callus formation in *Withania Somnifera* (Kannan *et al.,* 2005). Similarly among cytokinins, BAP was more effective than Kn for callus formation. MS medium fortified with 2.0mg/l BAP resulted in hundred per cent callus formation after 12 days of inoculation (Table-2). Shoot regeneration from the callus was also noticed at higher concentration of BAP (1.0, 1.5 and 2.0 mg/l) (Figure-1-d). The callus obtained on 1.5 and 2.0 mg/l of BAP was whitish green and soft.

The internodal explants favoured callus induction on MS medium supplemented in combinations of 2, 4-D with NAA and BAP. The

Table 1. Effect of cytokinins and auxins supplemented individually and in various combinations on nodal segments of *Sapindus mukorossi*

Auxins/cytokinins (mg/l)	Concentration Of growth regulators (mg/l)	%age of bud break	Number of days required for bud break	Number of shoots (Mean±SE)	Shoot Length (cm) (Mean ± SE)
Control	_	_	_	_	_
BAP	0.5	60	12	1.3 ± 0.17	1.1 ± 0.26
	1.0	70	11	1.4 ± 0.34	1.5 ± 0.34
	1.5	70	12	1.5 ± 0.31	1.7 ± 0.35
	2.0	90	10	2.0 ± 0.29	1.6 ± 0.31
Kn	0.5	50	13	1.0 ± 0.00	1.2 ± 0.24
	1.0	50	12	1.1 ± 0.37	1.4 ± 0.38
	1.5	60	11	1.2 ± 0.24	1.4 ± 0.33
	2.0	80	11	1.5 ± 0.36	1.5 ± 0.27
MS+BAP+ IAA	0.5 +1.0	50	12	1.4 ± 0.18	1.5 ± 0.26
	1.0 +1.0	80	10	1.7 ± 0.27	1.7 ± 0.14
	2.0 +1.0	90	09	2.0 ± 0.24	2.2 ± 0.38
MS+BAP+ NAA	0.5 +1.0	70	11	1.8 ± 0.38	1.7 ± 0.29
	1.0 +1.0	70	10	2.2 ± 0.34	1.8 ± 0.32
	2.0 +1.0	100	08	2.6 ± 0.25	2.2 ± 0.25

(–) No Response

*Data based on 20 explants per treatment and taken after 28 days of culture

combination of 2, 4-D + NAA induced callus on all the concentrations applied. The per cent response of callus induction was maximum on MS medium supplemented with 1.0mg/l 2, 4-D + 2.0 mg/l NAA. The callus so obtained was whitish brown in colour and soft in texture on all the concentrations (Table-3). The combination of 2,4-D + BAP was less effective than the combination of 2,4-D + NAA for callus induction(Figure-1-c). A good callus was obtained on higher concentrations of 2, 4-D + BAP.

The leaf segments responded to callus formation on the MS medium fortified with cytokinins (BAP and Kn). BAP was better in inducing callus as compared to Kn. Induction of callus in response to BAP has also been reported in *Lagerstroemia* indica (Niranjan & Sandarshana, 2005). Young leaf explants of Soapnut were more responsive than older ones. This could be attributed to high plasticity cells at younger age. The younger tissues are physiologically and biochemically more active as well as they has less rigid cell walls.

Shoot bud regeneration was also observed from the leaf segments derived calli on MS medium supplemented with 1.5 and 2.0 mg/l BAP (Table-4) (Figure-1-e). Lower concentration of BAP (0.5 mg/l) did not respond to callus induction. Callus formation was not observed on the leaf segments in MS medium supplemented with various concentrations of NAA and IAA.

3.3 Rooting of *in vitro* regenerated shoots

In vitro developed shoots were excised and implanted on MS medium without growth regulators as well as on half strength MS medium with IBA and NAA for rooting. The medium without growth regulators failed to initiate roots. Better rooting was supported by the MS medium fortified with 2.0mg/l IBA. The promotive effect of IBA has been reported for *in vitro* rooting on woody plants *Eclipta alba* (Bhaskaran and Jayabalan, 2005), *Chlorophytum borivilianum* (Sharma & Mohan, 2006), *Prosopis cineraria* (Kumar and Singh, 2009). Callus formation was observed at the base of the shoots implanted on the media supplemented with NAA (Table-5).

Table 2. Effect of cytokinins and auxins supplemented individually on callus formation on nodal explants of *Sapindus mukorossi.*

Media Composition	Concentration of growth regulators (mg/l)	No. of days required for callus induction	%age of callus induction	Nature of callus	Callus growth
MS control	–	–	–	–	–
MS + Kn	0.5 1.0 1.5 2.0	19 – 18 16	60 – 60 80	Whitish green, soft - Whitish green, soft Whitish brown, soft	C+ – C++ C++
MS+BAP	0.5 1.0 1.5 2.0	15 14 14 12	70 60 90 100	Whitish green, soft Whitish brown, soft Whitish green, soft Whitish green, soft	C+ C++ C+++ C+++
MS+ NAA	0.5 1.0 1.5 2.0	24 22 – 18	50 70 – 90	Whitish green, soft Whitish green, soft – Whitish green, soft	C+ C++ – C+++
MS+ 2,4-D	0.5 1.0 1.5 2.0	22 20 19 17	60 60 80 100	Whitish green, soft Whitish green, soft Whitish green, soft Whitish brown, soft	C++ C+++ C+++ C+++

(–) No Response, (C+) Poor growth, (C++) Moderate growth, (C+++) Good growth.
*Data based on 20 explants per treatment and taken after 28 days of culture

Table 3. Effect of various combinations of 2,4-D with NAA and BAP on callus formation on internodal explants of *Sapindus mukorossi.*

Media Composition	Concentration of growth regulators (mg/l)	No. of days required for callus induction	%age of callus induction	Nature of callus	Callus growth
MS+ 2,4-D +NAA	1.0+0.5 1.0+1.0 1.0+1.5 1.0+2.0	17 16 16 15	70 80 70 90	Whitish brown, soft Whitish brown, soft Whitish brown, soft Whitish brown, soft	C++ C+++ C+++ C+++
MS+ 2,4-D +BAP	1.0+0.5 1.0+1.0 1.0+1.5 1.0+2.0	– 20 18 17	– 60 60 80	– Whitish green, soft Whitish green, soft White, soft	– C+ C+++ C+++

(–) No Response, (C+) Poor growth, (C++) Moderate growth, (C+++) Good growth.
*Data based on 20 explants per treatment and taken after 28 days of culture

Table 4. Effect of cytokinins and auxins supplemented individually and in various combinations on callus formation on leaf explants of *Sapindus mukorossi.*

Media Composition	Concentration of growth regulators (mg/l)	No. of days required for callus induction	%age of callus induction	Nature of callus	Callus growth
MS control	–	–	–	–	–
MS + Kn	0.5 1.0 1.5 2.0	20 17 19 –	60 70 80 –	Whitish green, soft Whitish brown, soft Whitish green, soft –	C+ C++ C++ –
MS+BAP	0.5 1.0 1.5 2.0	– 16 15 14	– 80 70 80	– Creamish brown, soft Whitish brown, soft Whitish brown, soft	– C++ C++ C++

(–) No Response, (C+) Poor growth, (C++) Moderate growth, (C+++) Good growth.
*Data based on 20 explants per treatment and taken after 28 days of culture

Table 5. Root formation on different media composition in *Sapindus mukorossi.*

Media composition (mg/l)	Days required for root induction	% age of Root formation	Remarks
MS full strength without growth regulators	–	–	–
MS half strength without growth regulators	–	–	–
MS half strength +0.5 mg/l IBA	–	–	–
MS half strength +1.0 mg/l IBA	20-23	30	Short, Stout
MS half strength +2.0 mg/l IBA	22-25	40	Long, Thin
MS half strength +0.5 mg/l NAA	–	–	+
MS half strength +1.0 mg/l NAA	–	–	+
MS half strength +2.0 mg/l NAA	–	–	+

(-) No Response, (+) Callusing at basal end
*Data based on 20 explants per treatment and taken after 28 days of culture

3.4 Acclimatization and Transfer of plantlets to the soil

Complete regenerated plantlets with sufficient roots were taken out from the culture tubes and washed several times with sterile distilled water to remove the traces of MS medium by putting the roots in water with the help of fine brush. The *in vitro* regenerated plantlets were then transferred to small earthen pots containing sterilized soil and sand mixture (1:1) (Figure-1-f). Similar soil composition i.e. Soil and Sand was used to acclimatize *Dalbergia latifolia* (Raghwaswamy *et al.,* 1992), *Dendrocalamus asper* (Arya, 1997).Each pot was covered with polythene bags with small holes to maintain high humidity and kept them in the culture room (Figure-1-f). The plantlets were initially irrigated with half strength (salts only) MS medium without sucrose on alternate days. The

plantlets were exposed to 3-4 hours daily to the conditions for natural humidity after 10 days of transfer. After about 30 days the plants were transferred to bigger pots in greenhouse and were maintained under natural conditions of day length, temperature and humidity. Finally the plants were transferred to the field conditions. Sixty per cent of the regenerants survived well. Further hardening and acclimatization procedures for establishment of micropropagated plantlets were also developed for many species such as *Melia azadirach* (Shahzad & Siddiqui, 2001), *Salvadora persica* (Mathur *et al.*, 2008), *Peganum harmala* (Goel *et al.*, 2009).

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Corresponding Author:
Dr. Narender Singh
Associate Professor,
Department of Botany,
Kurukshetra University,
Kurukshetra,Haryana 136119,India.
E-mail: nsheorankuk@yahoo.com

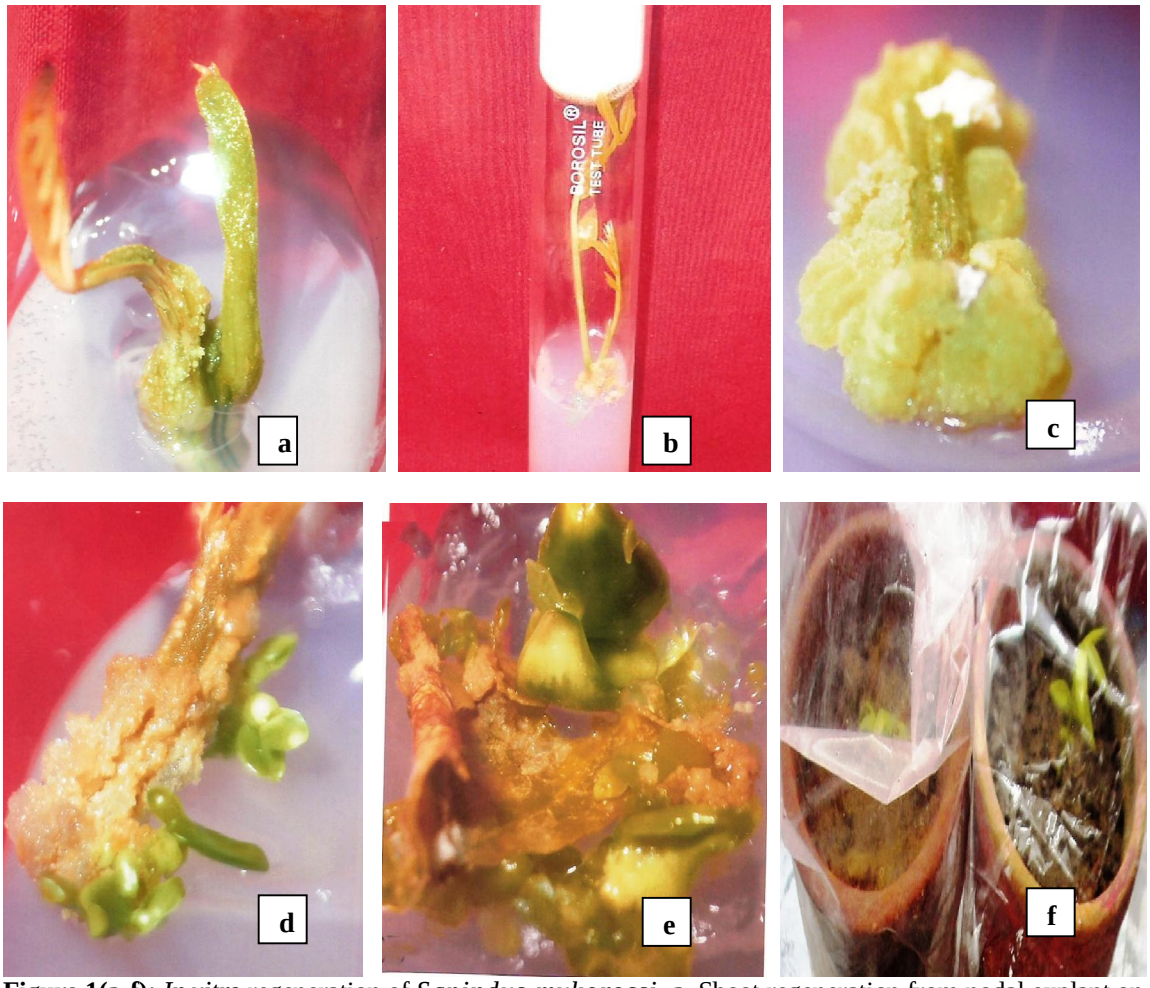

Figure 1(a-f): *In vitro* regeneration of *Sapindus mukorossi*. **a,** Shoot regeneration from nodal explant on MS medium + BAP (2.0mg/l) ; **b,** Regeneration of multiple shoots from nodal explant on MS medium supplemented with BAP(2.0mg/l + IAA (1.0mg/l); **c,** Callus growth from internodal segment on MS medium supplemented with NAA(1.0mg/l + 2,4-D (1.0mg/l); **d,** Indirect regeneration through callus formation from nodal segment on MS medium + BAP (1.0mg/l); **e,** Indirect regeneration through callus formation from leaf segment on MS medium + BAP (1.5mg/l); **f,** Establishment of plantlets under natural conditions.

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