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RESEARCH PAPER

Multiple shoot regeneration, establishment of callus and cell suspension cultures in *Salacia macrosperma* Wight

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KEYWORDS
Callogenesis; Cytology; Histology; Regeneration; *Salacia macrosperma*; SEM; Suspension culture.

Abstract: *Salacia macrosperma* Wight. - a potent medicinal plant facing the verge of rare and endemic status to the Western Ghat of southern India. Effective protocol has been established for induction of callus and multiple shoot regeneration using leaf and nodal explants on MS medium fortified with various plant growth regulators like 2,4-dichlorophenoxyacetic acid (2,4-D), benzyl amino purine (BAP), thidiazuron (TDZ), Indole acetic acid (IAA), Kinetin (Kn), Naphthalene acetic acid (NAA) and Indole butyric acid (IBA). Leaf explants produced more calli (98.33%) than nodal explants at 2.5 + 1.5 mg L^{-1} of 2, 4-D and BAP in combination. MS medium with 1.5 mg L^{-1} of 2, 4-D, 2.0 mg L^{-1} of BAP and 1.5 mg L^{-1} of TDZ along with 1% activated charcoal was apt for multiple shoot regenerations (93.33%) from nodal explants with embryogenic callus. Rhizogenesis was achieved in liquid MS medium supplemented with 1.0 mg L^{-1} of IAA. Friable callus when agitated in liquid MS medium at 100 rpm embryogenic callus followed by morphological changes were observed. Cytogenetic variations such as multinucleate, multinucleolate, cytodifferentiation, chromosomal bridges were noticed, besides normal dividing stages. Anatomical features were observed by microtome sections of the embryogenic callus revealed the presence of active centers, embryoids, and shoot bud formation. Further, by scanning electron micrograph (SEM) analysis of embryogenic callus different stages of morphogenic developmental features were recorded.

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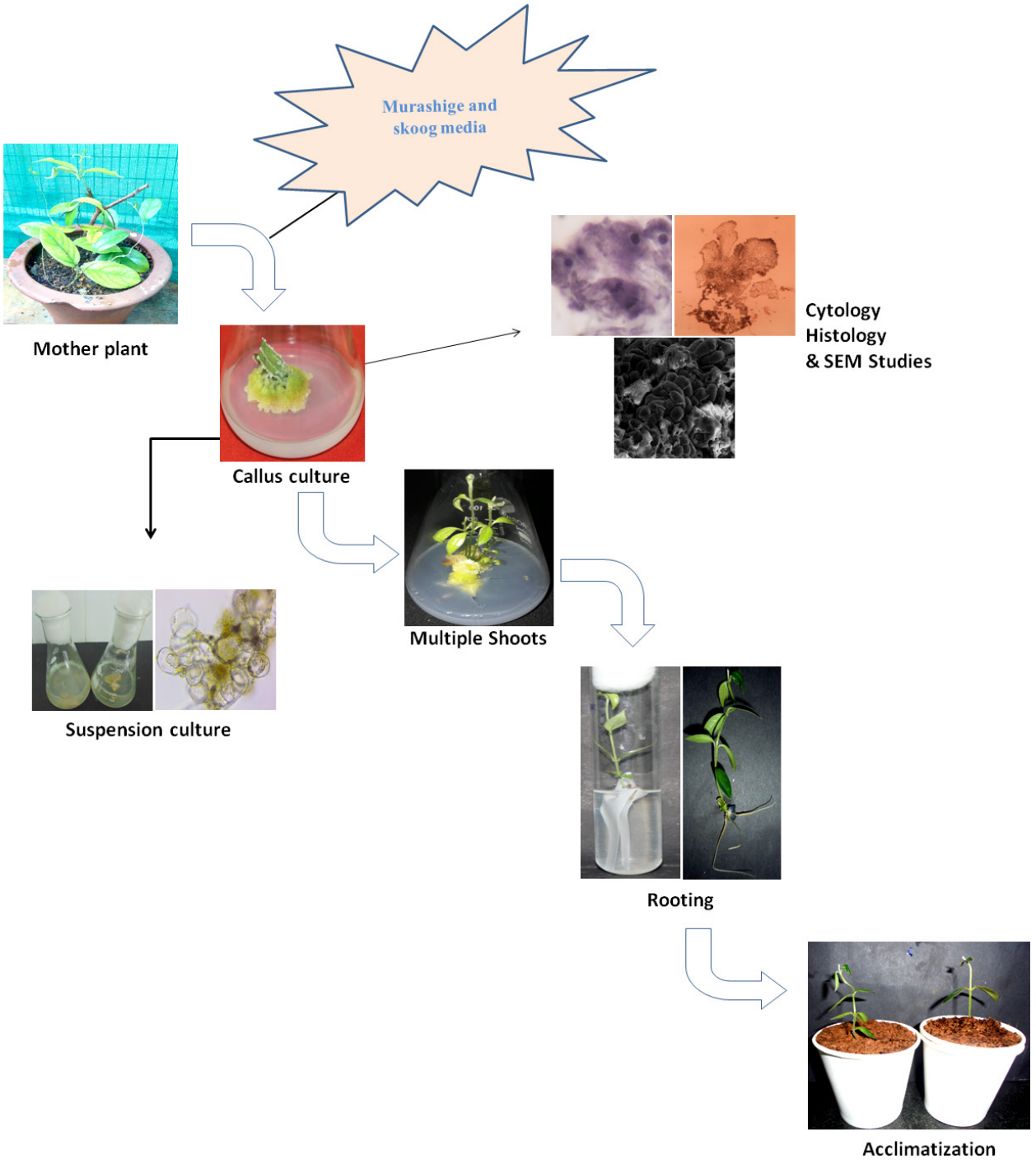
Graphical Abstract



HIGHLIGHTS

For the first time the protocol has been standardized for *in vitro* micro propagation of *Salacia macrosperma*.

The histological and cytological variations were studied and suspension culture was established for *in vitro* leaf derived callus.

SEM (Scanning Electron Microscopy) analysis was also done on the subsequent development of embryogenic callus.

Introduction

Salacia macrosperma Wight. an important medicinal plant belongs to the family Hippocrateaceae. It is a woody climber, shrubby, distributed in the peninsular regions of Sri Lanka, India, China, Brazil, Indonesia, Malaysia, Thailand and the Philippines (Saldanha, 1998). In India, it is found in the South and Western coastal regions of Kerala, Karnataka and Southern parts of Orissa (Hooker & Hooker, 1875; Rahangdale

& Rahangdale, 2017). The genus *Salacia* comprises more than 20 species, out of which few species have highly medicinal properties to cure dyspepsia, liver disorders, leprosy, skin diseases, stomachic, urinary disorders, anti-inflammatory properties (Nadakarni, 1964). It is also used to treat piles and congestion disorders (Chopra & Nayar, 1956). The root of *S. macrosperma* possessed anti-diabetic properties due to their insulin-like properties (Venkateswarlu et al., 1993).

Plant tissue culture is one of the essential components in plant biotechnology, which immensely contributing to crop improvement and has given great buddings for future generations (Kasagana & Karumuri, 2011). *In vitro* conservation under aseptic conditions for germplasm preservation of potent medicinal plants can be an effective strategy (Hill & Schaller, 2013). In general, micropropagation of medicinal plants plays a vital role in pharmaceuticals as well as in increasing their populations of individual species which are encountered with reproductive problems (Sharma et al., 2010; Hussain et al., 2012). *S. macrosperma* is one such plant that is facing the risks for survivability in their natural habitat. However, so many micropropagation works have been carried out in the same genus of other species by Dhanasri et al. (2013) in *S. reticulata* and Deepak et al. (2015) in *S. oblonga*. The *in vitro* micropropagation through tissue culture is very difficult for *Salacia* species due to their recalcitrant nature (Deepa & Narmada, 2010). Several attempts have been made earlier by many researchers and failed to achieve indirect regeneration for this genus. Generally, *Salacia* species are propagated naturally through seeds and the percentage of seed germination and their percentage of viability is less hence because of this reason the population undergoes decreasing in their original habitat. Owing to the ever-increasing demand in the pharmaceutical industries, this plant being overexploited due to its high medicinal properties. Propagation of *S. macrosperma* through sexual means is less due to poor seed germination thereby threatening nature in the wild. Since there are no reports available on conventional propagation by seed germination and cuttings on this species, hence the present work is undertaken to conserve this species for micropropagation through tissue culture technique and to standardize suitable protocol using node and leaf explants.

Materials and methodology

Plant material collection

S. macrosperma healthy plants (10 saplings) with same genotypes were collected in the month of June and July from Virajpete Taluk, Makkuta; a place of Western Ghats of Karnataka, India latitude (12° 25' 37 N) and longitude (75° 44' 51 E). A herbarium specimen (Voucher Number: MC.BOT/05-2018) is submitted to the Department of Botany, University of Mysore, Mysuru and few plants were also maintained in the medicinal garden for further research work.

Explant preparation and surface sterilization

The young and healthy leaf and nodal explants were excised from the mother plant (6 months old) maintained in the medicinal garden. The explants were washed thoroughly in running tap water, made into segments and rinsed in 0.2% Tween-20(w/v) for 5 min, followed by washed with sterile distilled water (SDW). The explants again treated with 1% Bavistin (w/v) (Biostadt. Com. Ltd. India) for 10 min and then washed with SDW. Further, retreated with 0.1% (w/v) $HgCl_2$ (Sigma - Aldrich) for 3 to 4 min and followed by 3-4 times wash with SDW and dried between sterile blotter discs before transferring on to the culture medium aseptically (Fernando et al., 2016).

Media preparation and culture condition

Macronutrients and micronutrients stock solutions (Murashige & Skoog, 1962) supplemented with growth regulators at various concentrations and combinations and 3% sucrose (Sigma - Aldrich) was used as a carbohydrate source in media compositions. 0.9% bacteriological grade agar (HiMedia) is used as a solidifying agent and the pH is adjusted to 5.8 before autoclaved at 121°C for 20 min. The cultures were incubated and maintained at 21 ± 2°C under 12 h photoperiod provided by white cool-fluorescent tubes and relative humidity of 85%.

Callogenesis and organogenesis

Prepared leaf and nodal explants were cultured on MS medium supplemented with various concentrations of plant growth regulators (PGR) such as 2, 4-D (2, 4-dichlorophenoxyacetic acid), Kn (Kinetin), NAA (Naphthalene acetic acid), BAP (Benzylaminopurine), TDZ (Thidiazuron) (Table 1). After 4 weeks, proliferated callus was further subcultured on MS medium fortified with various PGR`s at different concentrations and combinations (Table 2). The percentage of callus induction was calculated by using the following formula.

$$\% \text{ Callus induction} = \frac{\text{No.of explants produced callus}}{\text{Total No.of explants cultured}} \text{X100} \quad (1)$$

Multiple shoots were regenerated from the nodal explants on the medium supplemented with 2, 4-D in all the treatments in combination with BAP, TDZ, IAA (Indole acetic acid), and NAA along with activated charcoal used as an antioxidant source in some replicates. The regeneration frequency of multiple shoots was calculated by using the following formula (Alonso-Herrada et al., 2016).

$$\% \text{ Regeneration frequency} = \frac{\% \text{ of shoots per explants}}{\text{Total No.of explants cultured}} \text{X100} \quad (2)$$

Establishment of cell suspension culture

The MS liquid medium supplemented with BAP, Kn, and TDZ in combination at different concentrations in Erlenmeyer flasks (250 mL capacity). 4 weeks - old friable callus was

Table 1. Effect of plant growth regulators (PGR) on MS medium at different concentrations for the induction of callus in *S. macrosperma*.

PGR in mg L^{-1}		(%) Response		(%) callus induction		Nature of the callus	
		Leaf	Nodes	Leaf	Nodes	Leaf	Nodes
Control	0.0	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00^{k}	00.00 ± 0.00^{f}	NR	NR
	0.5	16.66± 0.88	8.33± 1.45	83.33± 4.40^{abcd}	41.66± 7.26^{cde}	G	WC
	1.0	16.66 ± 0.88	6.66± 1.76	83.33± 4.40^{abcd}	33.33± 8.81^{e}	G	WC
2,4-D	1.5	18.66± 0.33	10.00± 1.15	93.33± 1.66^{ab}	50.00± 5.77^{bcde}	LG	HC
	2.0	18.00± 0.57	8.00± 1.52	90.00± 2.88^{abc}	40.00± 7.63^{de}	HC	HC
	2.5	18.33± 1.20	9.66± 1.45	91.66± 6.00^{abc}	48.33± 7.26^{bcde}	HC	LG
	0.5	10.00± 1.15	8.33± 0.88	50.00± 5.77^{ij}	55.00± 13.22^{bcde}	LG	GC
	1.0	10.33± 1.85	9.33± 0.66	51.66± 9.27^{ij}	46.66± 3.33^{bcde}	LG	GC
BAP	1.5	11.33± 0.88	9.66± 0.88	56.66± 4.40^{hij}	48.33± 4.40^{bcde}	G	GC
	2.0	13.33± 0.33	10.66± 1.76	66.66± 1.66^{defghi}	53.33± 8.81^{bcde}	G	GC
	2.5	8.33± 0.88	11.00± 2.00	41.66± 4.40^{j}	55.00± 10.00^{bcde}	G	WC
	0.5	10.00± 1.15	7.66± 1.20	50.00± 5.77^{ij}	38.33 ± 6.00^{de}	FG	JC
	1.0	11.66± 0.88	7.33± 0.66	58.33± 4.40^{ghij}	36.66 ± 3.33^{de}	FC	JC
Kn	1.5	13.33± 0.66	0.00± 0.00	66.66± 3.33^{defghi}	0.00 ± 0.00^{f}	FC	NR
	2.0	11.66± 0.88	0.00± 0.00	58.33± 4.40^{ghij}	0.00 ± 0.00^{f}	FC	NR
	2.5	11.33± 0.88	0.00± 0.00	56.66± 4.40^{hij}	0.00 ± 0.00^{f}	FC	NR
	1.5+0.5	17.66± 0.33	10.66± 1.76	88.33± 1.66^{abc}	53.33± 8.81^{bcde}	JC	WC
	2.0+1.0	18.33± 1.20	11.00± 0.57	91.66± 6.00^{abc}	55.00± 2.88^{bcde}	GH	WC
2,4-D + BAP	2.5+1.5	19.66± 0.33	10.66± 1.45	98.33± 1.66^{a}	53.33± 7.26^{bcde}	GH	GC
	3.0+2.0	18.66± 0.33	13.33± 0.88	93.33± 1.66^{ab}	66.66± 4.40^{ab}	EC	GC
	3.5+2.5	15.66± 1.20	15.66± 0.33	78.33± 6.00^{bcdef}	78.33± 1.66^{a}	EC	GC
	1.5+0.5	17.33± 0.88	8.00± 1.15	86.66± 4.40^{abc}	40.00± 5.77^{de}	GH	G
	2.0+1.0	16.00± 2.30	9.00± 1.52	80.00± 1.54^{abcde}	45.00± 7.63^{bcde}	GH	G
2,4-D + Kn	2.5+1.5	15.33± 0.88	11.66± 0.88	76.66± 4.40^{bcdefg}	58.33± 4.40^{abcd}	GH	GH
	3.0+2.0	12.00± 1.52	12.66± 1.76	60.00± 7.63^{fghij}	63.33± 8.81^{abc}	GC	GH
	3.5+2.5	10.33± 1.45	11.33± 2.40	51.66± 7.26^{ij}	56.66± 12.01^{bcd}	GC	GH
	1.5+0.5	15.66± 0.33	6.66± 0.66	78.33± 1.66^{bcdef}	33.33± 3.33^{e}	WH	GJC
	2.0+1.0	14.66± 1.85	10.66± 0.88	73.33± 9.27^{cdefgh}	53.33± 4.40^{bcde}	S&J	GC
2,4-D +NAA	2.5+1.5	15.33± 1.85	8.66± 1.76	76.66± 9.27^{bcdefgh}	43.33± 8.81^{cde}	NC	GC
	3.0+2.0	12.33± 1.20	0.00± 0.00	61.66± 6.00^{efghi}	0.00± 0.00^{f}	WC	NR
	3.5+2.5	11.00± 1.52	0.00± 0.00	55.00± 7.63^{hij}	0.00± 0.00^{f}	WC	NR

Note: Experiment repeated thrice (n=10 & 20 bits of explants used). Values represented mean ± SE followed by the same letters within the column are not significantly different at (p<0.05) according to Duncan's Multiple Range Test (DMRT). (G: Green, F: Friable, E: Embryogenic, H: Hard, N: Nodular, W: White, J: Juicy Callus and NR: No Response).

transferred to the liquid medium and agitated at 150 rpm on a gyratory shaker at 27°C. The process of subculturing was repeated once in 4 days using fresh liquid medium and the developmental changes of cultured cells were examined under a light microscope by using a drop of the medium on a glass slide. Various modifications of the growth medium were used to study their effect on the growth and friability of the suspension cultures. Cell volume and viability were observed by using a 2% hematoxylin stain (Vasil & Vasil, 1981; Chee & Tricoli, 1988).

Table 2. Effect of plant growth regulators (PGR) on MS medium for multiple shoots regeneration from nodal explants of *S.macrosperma*.

PGR	Concentration (mg L^{-1})	Avg. No. of explants responded	Regeneration frequency (%)	Avg. No. of shoots/explant	Length of shoots (cm^{-1})	Remarks
Control	Nil	00.00 ± 0.00	00.00 ± 0.00^{h}	0.00 ± 0.00	0.00 ± 0.00^{e}	-
2,4-D + BAP + Charcoal	0.5+1.0 + (1%)	12.33 ± 0.88	61.66 ± 4.40^{defg}	3.33 ± 0.33	1.86 ± 0.40^{abcd}	S+C
	1.0+2.0 + (1%)	10.66 ± 0.66	53.33 ± 3.33^{g}	3.66 ± 0.33	1.90 ± 0.25^{abcd}	S+C
	1.5+3.0 + (1%)	17.66 ± 0.33	88.33 ± 1.66^{ab}	4.66 ± 1.76	2.06 ± 0.03^{abcd}	S+C
	2.0+4.0 + (1%)	11.33 ± 0.66	56.66 ± 3.33^{efg}	4.66 ± 0.33	1.90 ± 0.65^{abcd}	S+C
	2.5+5.0 + (1%)	11.00 ± 1.00	55.00 ± 5.00^{fg}	4.33 ± 1.20	1.70 ± 0.32^{bcd}	S+C
2,4-D + TDZ + Charcoal	0.5+ 0.5 (1%)	14.66 ± 0.66	73.33 ± 3.33^{abcdefg}	3.00 ± 0.57	2.13 ± 0.49^{abcd}	S
	1.0+1.0 (1%)	17.00 ± 0.57	85.00 ± 2.88^{abc}	10.33± 0.88	2.13 ± 0.28^{abcd}	S
	1.5+1.5 (1%)	17.33 ± 0.66	86.66 ± 3.33^{abc}	10.00± 0.57	2.26 ± 0.46^{abc}	S+C
	2.0+2.0 (1%)	13.00 ± 0.57	65.00 ± 2.88^{defg}	6.00 ± 0.57	2.26 ± 0.57^{abc}	S+C
	2.5+2.5 (1%)	14.33 ± 0.66	71.66 ± 3.33^{bcdefg}	4.66 ± 1.76	2.86 ± 0.29^{abc}	S+C
BAP + IAA	1.0+0.5	13.33 ± 0.66	66.66 ± 3.33^{cdefg}	4.00± 0.57	1.53 ± 0.31^{cd}	S
	2.0+1.0	14.33 ± 0.88	71.66 ± 4.40^{bcdefg}	4.33± 0.88	1.10 ± 0.15^{d}	S
	3.0+1.5	11.33 ± 1.33	56.66 ± 6.66^{efg}	7.66± 0.88	2.63 ± 0.36^{abc}	S
	4.0+2.0	11.66 ± 1.85	58.33 ± 9.27^{defg}	3.66± 0.33	2.43 ± 0.18^{abcd}	S+C
	5.0+2.5	11.33 ± 0.66	56.66 ± 3.33^{efg}	6.33± 0.33	2.06 ± 0.32^{abcd}	S+C
BAP+NAA+TDZ	1.0+0.5+0.5	17.66 ± 0.33	88.33 ± 1.66^{ab}	13.00± 0.57	2.33 ± 0.29^{abcd}	S
	2.0+1.0+1.0	17.33 ± 0.33	86.66 ± 1.66^{abc}	7.33 ± 0.66	2.73 ± 0.66^{abc}	S
	3.0+1.5+1.5	15.00 ± 0.57	75.00 ± 2.88^{abcdef}	7.00 ± 0.57	3.20 ± 0.50^{a}	S+C
	4.0+2.0+2.0	15.66 ± 0.66	78.33 ± 3.33^{abcd}	4.66 ±1.20	2.66 ± 0.40^{abc}	S+C
	5.0+2.5+2.5	15.33 ±0.33	76.66 ± 1.66^{abcde}	5.66 ± 0.66	2.90 ± 0.05^{abc}	S+C
2,4-D+ BAP+TDZ+Charcoal	0.5+1.0 +0.5 (1%)	18.00 ± 0.57	90.00 ± 2.88^{ab}	10.33± 0.88	2.60 ± 0.40^{abc}	NEC+S
	1.0+1.5 + 1.0(1%)	18.33 ± 0.33	91.66 ± 1.66^{ab}	8.00 ± 1.15	3.03 ± 0.57^{ab}	NEC+S
	1.5+2.0 +1.5 (1%)	18.66 ± 0.66	93.33 ± 3.33^{a}	7.33 ± 0.33	2.53 ± 0.43^{abc}	NEC+S
	2.0+2.5+2.0 (1%)	17.00 ± 1.00	85.00 ± 5.00^{abc}	10.66± 0.33	1.76 ± 0.28^{abcd}	S+C
	2.5+5.0 + 2.5(1%)	12.33 ± 0.88	61.66 ± 4.40^{defg}	10.00 ± 0.57	1.50 ± 0.68^{cd}	S+C

Note: Observations were made at weekly intervals. Each set of conc. contains 10 culture flasks and 20 bits of explants used and repeated thrice. Values represented means ± SE followed by the same letters within the column are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test (DMRT). (S+C: Shoots + Callus, NEC+S: Non-Embryogenic Callus + Shoots).

Histological and cytological observations

The histological studies were carried out by the method of S'lesak et al. (2014) and Navarro-garcía et al. (2016). The leaf explants with embryogenic callus (4-week-old) proliferated on MS medium was fixed in Carnoy's fixative for 24 h. The material is dehydrated in the alcohol-xylene series followed by infiltration using paraffin. Microtome sections (11µm) were taken and stained with hematoxylin and thus made into permanent. For the cytological study, a 4-week old callus was fixed in Carnoy's fixative for 24 h and washed thoroughly in SDW and stored in 70% alcohol. The callus is dropped in 0.5% mordant for 5 minutes, washed in water and transferred to the hematoxylin stain (2%) for 10 minutes. The callus is squashed in 45% propionic acid and observed under a light microscope (10X and 40X) for their cytological behavior of cultured cells by identifying various stages of cell development (Johansen, 1940).

SEM analysis

Embryogenic leaf derived callus was prefixed for SEM analysis in 5% glutaraldehyde (0.1 M phosphate buffer solutions (PBS) (pH 7.2) for 24 h at room temperature and then washed thrice with PBS (Andi Brisibe et al., 1992). This is followed by dehydration through a graded ethanol series. The samples were dried in desiccators by using silica beads. After critical drying, materials were sputter-coated with gold and then examined under a scanning electron microscope (HITACHI, S-3400N made in Japan) operating with 10 kV acceleration to

observe the morphology and development of various shapes and sizes of embryoids (Haque et al., 1998).

Rooting and acclimatization

For rhizogenesis, the regenerated shoots (6-7cm) were transferred to MS liquid medium supplemented with NAA, IAA or IBA at 0.5 to 2.5 mg L^{-1} concentrations. The well-developed shoots with rooted plantlets were removed from the culture vessels and transferred to the plastic pots containing vermiculite and perlite mixture (1:1) which is covered with a polythene bag for maintaining humidity. After 8 weeks, which is kept under shade in greenhouse conditions and then transferred to the field conditions for evaluating the percentage of survivability (Karthikeyan et al., 2009).

Statistical analysis

The experiments were performed in triplicates and each treatment with at least 10 culture flasks and consisted of 20 explants. Statistical analysis was done by using IBM SPSS 21 ver. (IBM. Corp.US) software Duncan`s Multiple Range Test at $p<0.05$ and the data were represented means of SE among the three replicates.

Results and discussion

Establishment of Callus culture

The effect of different auxins (2, 4.D and NAA) and cytokinins (BAP and Kn) and their combinations on callus initiation was investigated in *S. macrosperma*. In PGR alone, the leaf explants responded 93% (Figure 1A) particularly at 1.5 mg L^{-1} of 2, 4-D and nodal explants responded 50% (Figure 1B). In BAP and Kn, 66% callus induction was observed from leaf explants at 2.0 mg L^{-1} each. Similarly, 55% callus was observed from nodal explants at 2.5 mg L^{-1} BAP and it was also observed that in higher as well as lower concentrations of PGRs the callogenesis was poor. In the combination of 2, 4-D (2.5 mg L^{-1}) and BAP (1.5 mg L^{-1}) 98% of the leaf explants induced callus and became embryogenic in the subsequent subcultures (Figure 1C & 1D) but failed to induce shoots. As callus gets older it became embryogenic in callus of nodal explants (Figure 1E & 1F). In the combination of 2, 4-D and Kn, moderate callus was induced from nodal explants and there was no response on the same medium with higher concentrations of 2, 4-D and NAA. Similar results were also reported by Chavan et al. (2015) in *S.chinensis* wherein, the highest percentage of callus was achieved in a combination

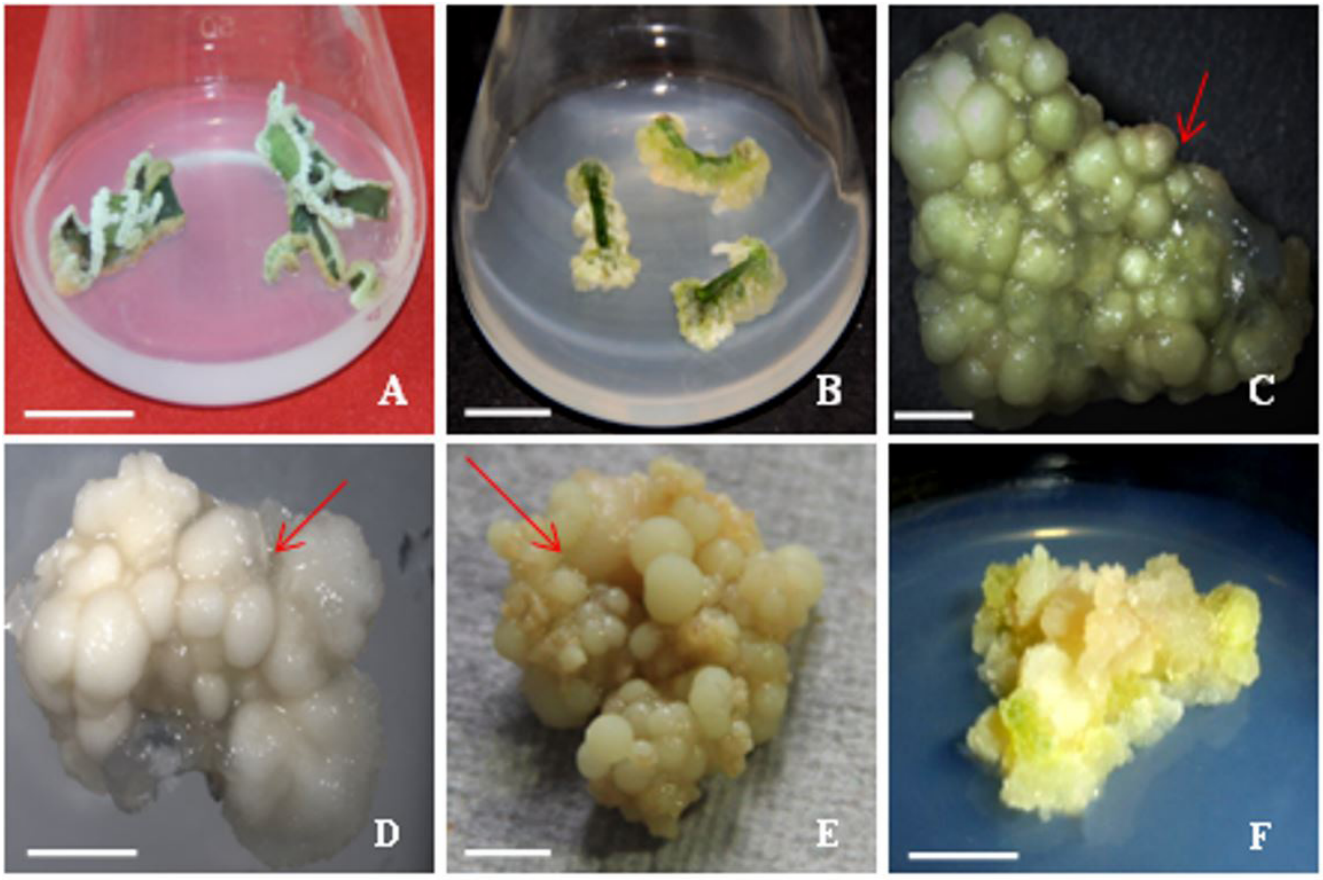


Figure 1. Induction of callus from leaf and stems explants: (A, B) Leaf and stem explants after 15 days of inoculation (Scale bars: 2 cm); (C to E) Embryogenic callus showing the various shapes of embryos at 40 days old culture (Scale bars: 1 cm); (F) Soft friable light green non embryogenic callus 40 days old culture (Scale bars: 1 cm).

of 2, 4-D and BAP at 2.0 + 2.0 mg L^{-1}. Likewise, Kumar Meena (2017) was also achieved the highest percentage of callus induction in *Nigella sativa* at 3.0 mg L^{-1} of 2, 4-D supplementation in MS medium.

Histology and cytology

Microtome sections of 1-week old callus showed that the mother tissue undergoes dedifferentiation to form a mass of cells and in the subsequent week which developed into pro embryos (Figure 2A). In the third week, the cells transferred into vessels here and there, i.e. cytodifferentiation, which gives the way for organogenesis (Figure 2B & 2C). Similar results have been reported by Arumugam & Rao (2000) in *Aegle marmelos* and by Figueiredo et al. (1989) in *Datura insignis*. In the section of embryogenic callus showed differentiated shoot buds formation and the development of embryoids (Figure 2D & 2E). These observations are in line with Bespalhok-Filho & Hattori (1997) in *Stevia rebaudiana*. This histological study is evidence for the regeneration of plantlets under *in vitro* conditions.

The primary callus culture was observed under a microscope squashing with 2% hematoxylin which revealed the presence of normal diploid dividing cells with binucleated conditions (Figure 2F). The cytological examinations in 40-days old callus cells exhibited variations such as asynchronous division within a single cell (Figure 2G), unequal distribution of chromosomes at the 2 pole ends of the anaphase stage (Figure 2H), early anaphase with fragmented chromosomes (Figure 2L), chromosome breakages at prophase (Figure 2M). The results are in line with earlier reports of D'Amato (1977) where the number of cytological variations like cytodifferentiation and other abnormalities has been reported in the *in vitro* cultured cells which supports our present findings. Presence of chromosomal bridge (Figure 2I), anaphase with laggard, sticky chromosomes (Figure 2J & 2K) were observed at the anaphase stage is in agreement with the report of Anju & Sarbhoy (1990) in *Pisum*, where they have reported cultured cells showing cytological variations like compactly arranged cells with sticky chromosomes and more number of enucleated cells. The normal metaphase stage (Figure 2N) and nuclear connection in binucleated cells with cytodifferentiation (Figure 2O) may be a source of evidence in inducing polyploidy which is quite similar to the earlier findings of Yen et al. (1993) in inter-generic hybrids of *Roeyneria cilliaris* and *Psathyrostachys luashanica*. These observations may be due to the somaclonal variations in PGR or the composition of nutrients in the medium (Chen et al., 2015).

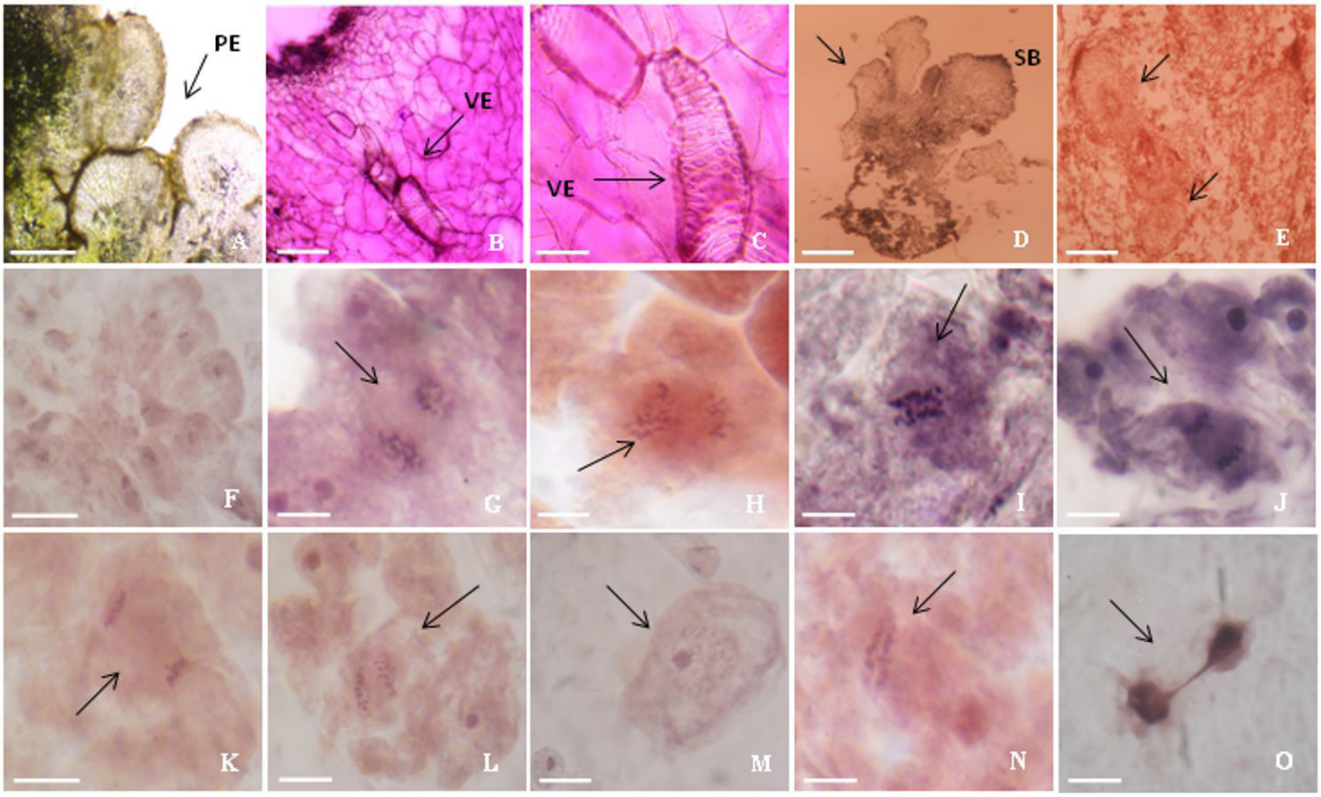


Figure 2. Histological and Cytological studies of *in vitro* cultured cells showing variations. **(A)** Freehand sections were taken (40x) in 35 days old callus showing the capacity of development of pro embryoid; (B, C) Vessel elements in 35 days old callus culture; (D, E) Microtome sections showing the development of shoot buds; (F) Cells with mono and binucleated conditions; (G) Uneven cell divisions with chromosomes at early metaphase and late metaphase stage; (H) Unequal distribution of chromosomes at two opposite poles in a single cell at anaphase stages; (I) Chromosomal Bridge; (J, K) Anaphase stage with laggard chromosomes, sticky chromosomes and uneven cell wall morphology; (L) Early anaphase stage with fragmentation of chromosome; (M) Uni nucleate cell and chromosome breakages at prophase stage; (N) Normal metaphase stage; (O) Cell with nuclear connections in binucleate conditions (Scale bars: 30µm).

Embryogenic cell suspension culture and its observations

Cell suspension cultures were initiated by transferring 3 weeks-old friable calli to liquid MS medium (Figure 3A). Within 4 days the friable callus disintegrates into single cells and small cell aggregates. The results are in accordance with the previously reported by Fujimura & Komamine (1979) in *Daucus carota* in the first week after observation. It was necessary to transfer the cells to fresh medium to avoid browning of the callus. In the second week after subculturing the cell suspensions appeared to be homogeneous and contain spherical or elongated cells and small aggregates. In the third week, two types of calli were observed one is small, fast-growing calli with cells rich in the cytoplasm (Figure 3B) and other non-dispensable, slow-growing calli with highly vacuolated cells. In subsequent subcultures, cells differentiated to form various morphological features like elongated cells (Figure 3C & 3D) sometimes 2 celled, one with spherical vacuolated and the other normal cell (Figure 3E & 3F) with dense cytoplasm and deeply stained nucleolus. Similar observations was also reported by Iantcheva et al. (2006) in *Medicago truncatula*. Besides, embryogenic spherical cells (Figure 3G) are also observed. In some replicates transversely dividing cells forms into 2- 4- 8 celled pro-embryos (Figure 3H, 3I & 3J). These cells are nearly 60% of them are viable, observed under a light microscope using hematoxylin stain with well developed cellular contents. As an evidence, these observations supports with the previous observations made by Vasil & Vasil (1982) wherein nearly 60% of the cells are viable in *Pennisetum americanum* cell suspension culture.

SEM analysis

The ontology of the embryogenic callus (Figure 4H) and its morphology was revealed by SEM studies. The MS medium supplemented with 2.5 mg L^{-1} of 2,4-D, the leaf explant cells

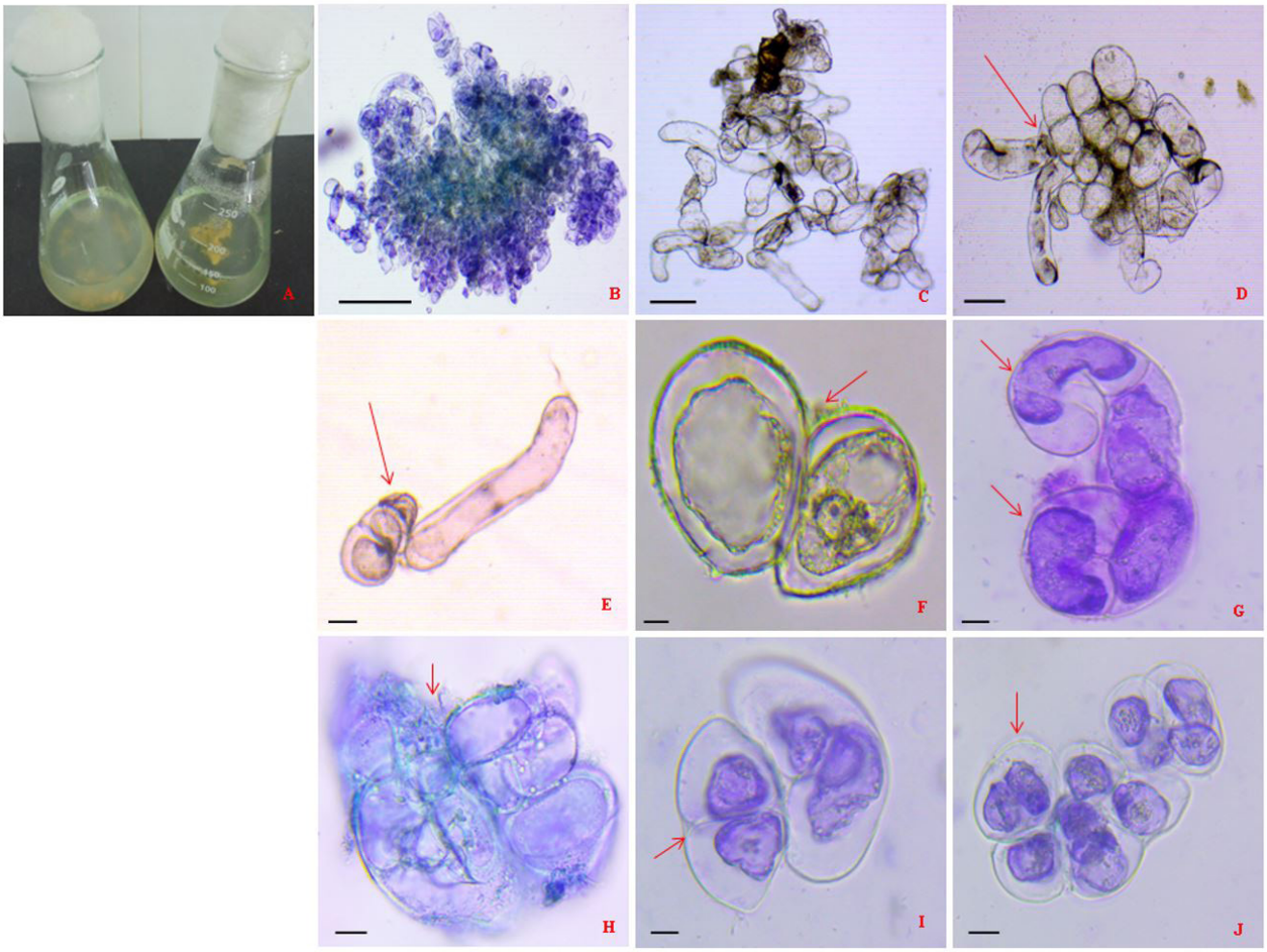


Figure 3. Development of suspension culture from 4 weeks old callus culture showing various stages of development and morphological changes: (A) Liquid MS media suspension culture (1.5 mg L^{-1} 2, 4-D and 1.0 mg L^{-1} TDZ); (B) Colony of uniform cells (1week old culture) (4X); (C, D) Elongated cells developed from single cells observed after 2 weeks of incubation (10X); (E) Two celled stage with elongated embryogenic vacuolated cell (10X); (F) Two cells stage one with vacuolated and other with normal cell (40X); (G) Embryogenic cells with movement of cytoplasmic division (10X); (H) 4 cell stages of cell division after 3 weeks of incubation (40X); (I) Division of nuclear contents at 4 celled stages conditions (40X); (J) Colony of two-celled dividing stages of cells (10X); (K, L) Viable cultured cells observed under (40X); (Scale bars: 30µm and arrow mark indicate the events occurred in cells).

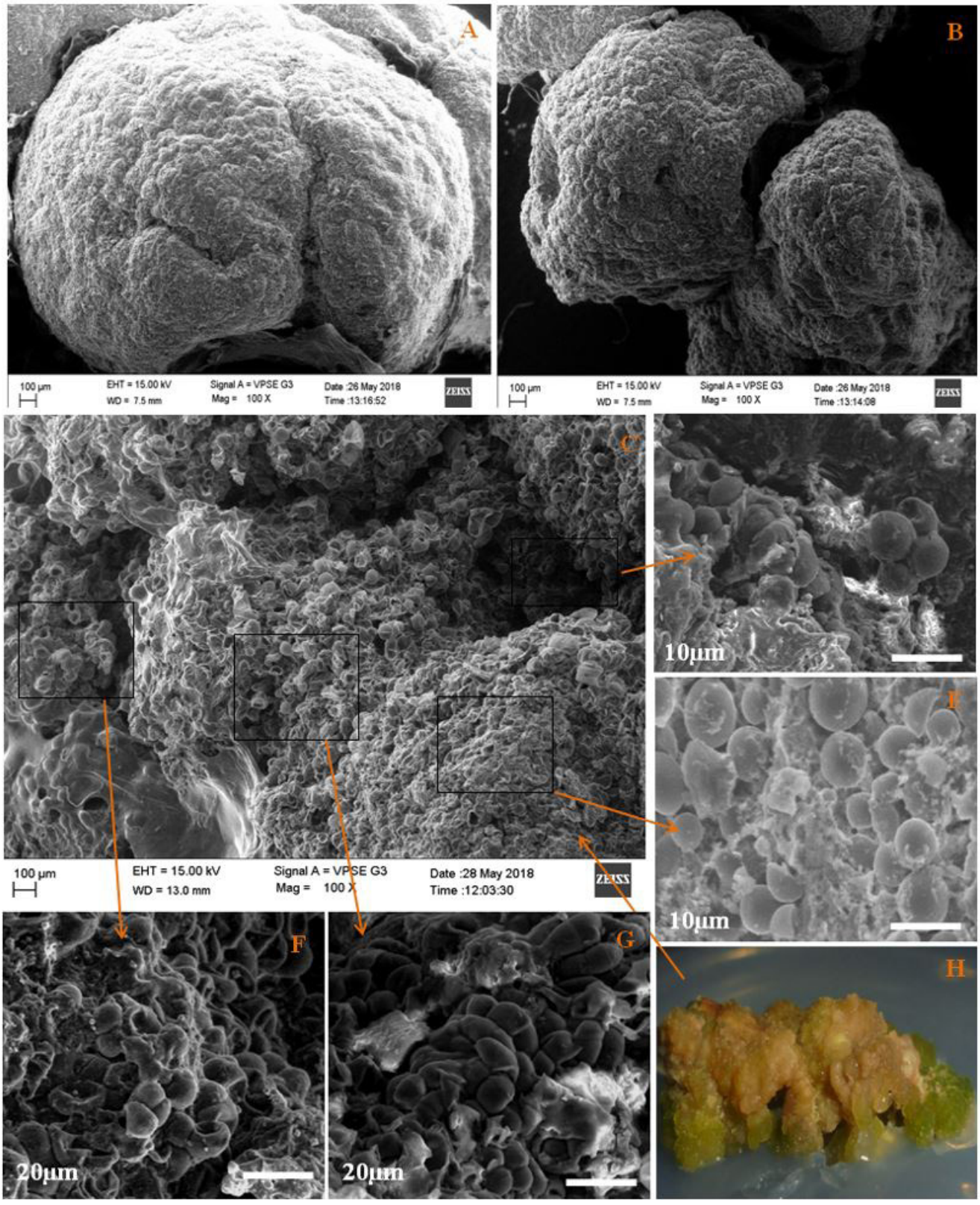


Figure 4. SEM showing embryogenic leaf callus of *S.macrosperma*. (A) Abaxial surface view of 25 days old callus from leaf explants; (B) An overview of (nodal callus 35 days old) of non embryogenic cells; (C) Loosely arranged mass of embryogenic callus cells (40 days old); (D) Heart, round and ovule shaped compactly arranged cells; (E-G) Compactly arranged elongated pro-embryogenic nodular callus cells; (H) Four weeks old Embryogenic nodular green friable callus at 2.5 + 1.5 mg L^{-1} concentrations of 2, 4.D & BA.

undergo differentiated at the cut end portion that triggered cells at wounding regions and forming a pro-embryos type of cells due to dedifferentiation after 25 days of incubation on the abaxial surface of the leaf explant (Figure 4A). These findings are in accordance with the work of Ban et al. (2016) who cultured *Panax assamicus* on the medium supplemented with 2,4.D and BAP wherein the bunch of embryonic cells protruded from the explants. In 35 days old nodal callus non-embryogenic cells in clusters on the medium supplemented with 2,4-D (Figure 4B). In the sixth week, the leaf callus cultures showed the presence of embryo-like cells which vary in their shape and morphology (Figure 4C). Similar observations were also reported by Jainol & Gansau (2017) in *Dimorphorchis lowii*. In the subsequent sub-cultures, the callus developed globular, torpedo, cordite and elongated shaped embryoids (Figure 4D, E, F, and G). Likewise, our findings are in accordance with the previous reports of Andi Brisibe et al. (1992) in *Oryza glabarima* and by Popielarska-Konieczna et al. (2010) in *Actinidea deliciosa* wherein, the developmental and morphological features of *in vitro* cultured cells were observed in SEM monographs.

Multiple shoots induction

The direct regeneration was achieved from nodal explants on MS medium but failed to succeed indirect regeneration from leaf induced callus of *S. macrosperma*. The inductions of multiple shoots from nodal explants on nutrient medium supplemented with various PGRs were tried and the results were exhibited in Table 2. The Nodal explants in some replicates induced slight callus and about 93% of multiple shoots were induced simultaneously (Figure 5A to 5D) on

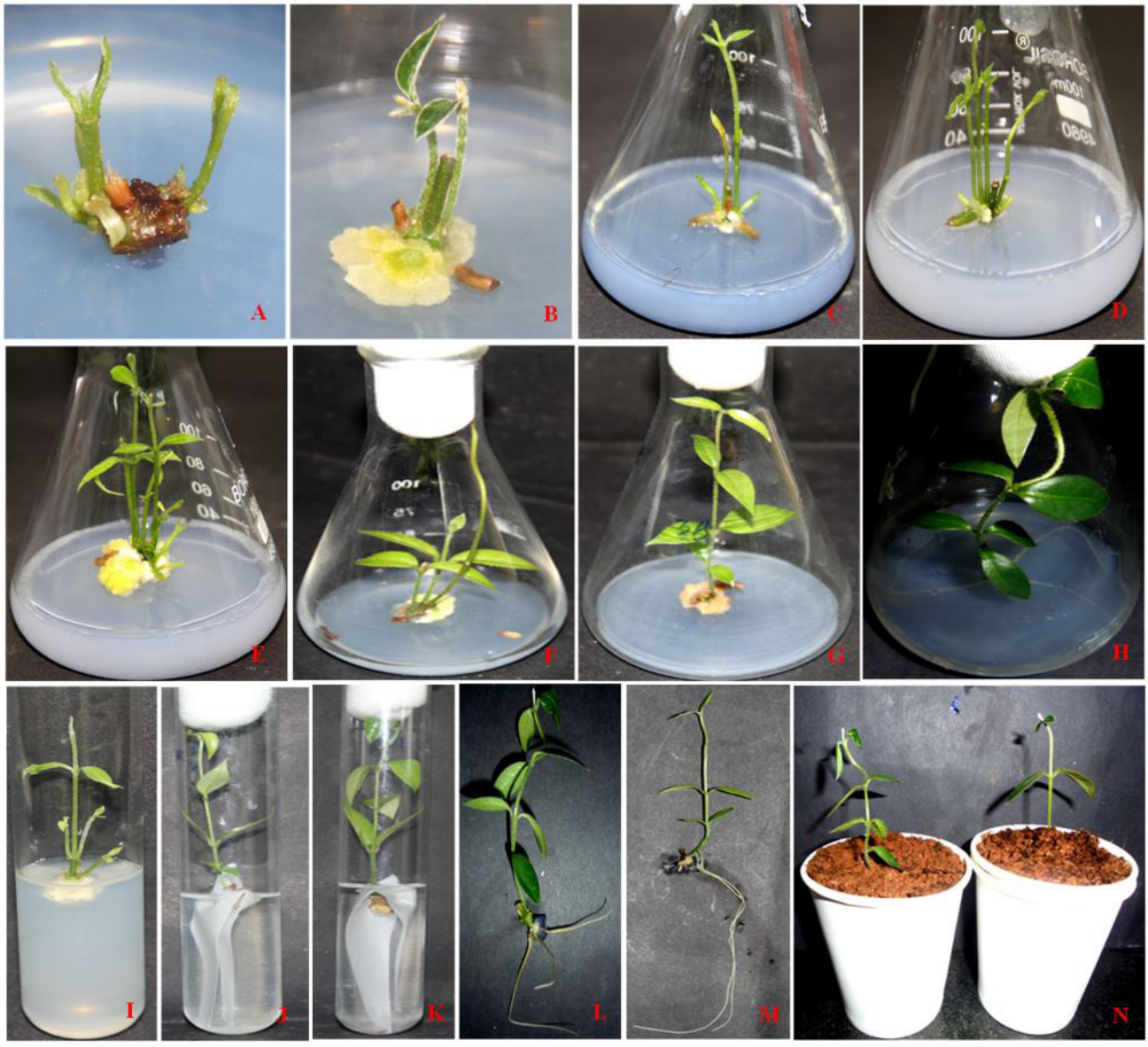


Figure 5. (A) Multiple shoots development from nodal explants; (B to D) Induction of multiple shoots with callus from nodal explants; (E to H) Sub cultured multiple shoots with callus development transferred after 40 days; (I to K) Induction of roots from developed plantlets in MS liquid medium; (L, M) Well developed roots from plantlets; (N) Acclimatized plantlets.

Table 3. Effect of different concentrations of PGR on MS medium for root induction from *in vitro* developed shoots of *S. macrosperma*.

Medium	PGR	Concentrations (mg L^{-1})	No of shoots cultured	Roots response (%)	No of roots/ plantlets	Length of Roots (cm)
Control	Nil	0.0	10.54 ± 0.54	00.00 ± 0.00c	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
		0.5	11.33 ± 0.66	67.22 ± 4.33^{ab}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
		1.0	12.66 ± 0.88	54.99 ± 5.23^{ab}	1.00 ± 0.57^{cd}	1.16 ± 0.61^{b}
	NAA	1.5	11.66 ± 1.20	53.89 ± 2.08^{ab}	1.33 ± 0.33^{bcd}	2.36 ± 0.34^{a}
		2.0	11.33 ± 0.33	67.67 ± 7.88^{ab}	3.33 ± 0.88^{ab}	2.30 ± 0.35^{a}
		2.5	13.66 ± 1.45	55.03 ± 5.03^{ab}	2.00 ± 0.57^{bcd}	1.66 ± 0.33^{ab}
		0.5	14.00 ± 1.15	46.92 ± 4.67^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
		1.0	12.66 ± 0.88	61.70 ± 10.54^{ab}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
MS	IBA	1.5	13.66 ± 0.88	64.75 ± 10.58^{ab}	2.66 ± 1.20^{abc}	2.16 ± 0.24^{a}
		2.0	14.66 ± 0.88	77.64 ± 3.51^{ab}	3.00 ± 1.15^{abc}	1.96 ± 0.03^{ab}
		2.5	12.33 ± 0.88	66.30 ± 12.11^{ab}	3.00 ± 0.57^{abc}	2.50 ± 0.26^{a}
		0.5	12.33 ± 1.20	58.02 ± 10.66	2.33 ± 0.33^{abc}	2.66 ± 0.37^{a}
		1.0	14.33 ± 1.76	85.56 ± 1.93^{a}	4.33 ± 0.88^{a}	2.30 ± 0.36^{a}
	IAA	1.5	13.66 ± 1.45	77.21 ± 5.15^{ab}	3.33 ± 0.33^{ab}	2.16 ± 0.27^{a}
		2.0	13.33 ± 1.33	79.86 ± 2.50^{ab}	2.33 ± 0.88^{abc}	2.03 ± 0.13^{ab}
		2.5	14.33 ± 2.02	52.71 ± 3.66^{ab}	2.00 ± 0.57^{bcd}	2.30 ± 0.30^{a}

Note: Experiment repeated thrice and each set of conc. contains 10 culture flasks. Values represented Mean ± SE: followed by the same letter within columns are not significantly different ($p < 0.05$) according to Duncan's Multiple Range Test (DMRT).

the MS medium supplemented with 2, 4-D (1.5 mg L^{-1}) + BAP (2.5 mg L^{-1}) + TDZ (1.5 mg L^{-1}) besides 1% activated charcoal as an antioxidant. These results are in concurrence with earlier work carried out by Zhai et al. (2011) in *Caragana fruiticosa* and Chavan et al. (2015) in *S. chinensis* wherein higher percentage of shoot inductions was achieved in combination of BAP and NAA (2.0+0.8 mg L^{-1}). In some replicates 88% of multiple shoots were observed on MS medium + 1.5 mg L^{-1} 2, 4-D + 1.5 mg L^{-1} BAP with 3% AC (Figure 5E to 5H) and this is followed by 86% shoots inductions on MS + 1.5 mg L^{-1} 2,4-D + 1.5 mg L^{-1} TDZ with 1% AC. The outcome of this results is corroborated with the findings of Faisal et al. (2014) where they have achieved direct shoot multiplication in *Mentha arvensis*. The use of TDZ growth regulator in all the explants played a vital role in inducing more number of multiple shoots in *S. macrosperma*.

Rooting and acclimatization

For induction of roots, the excised shoots from the culture vessels are aseptically transferred to the liquid MS medium containing NAA or IAA or IBA in the range 0.5 - 2.5 mg L^{-1}. A maximum of 85.36% of rooting was induced in IAA (1.0 mg L^{-1}) in MS medium with 4.33 average number of roots per shoots and the average length of roots is 2.3 cm (Figure 5I, J, K, L, and M) the results were mentioned in the Table 3. These results are also similar to the work carried out by Kumar et al. (2016) in *Hibiscus sabdariffa* wherein the IAA induced the maximum number of roots at higher concentration. No induction of roots on hormone-free medium. All the plantlets with well-developed roots were removed from the culture vessels washed thoroughly in distilled water to remove the traces of medium. Subsequently, plantlets were transferred to poly cups (Figure 5N) having autoclaved coir pit with sterile soil. Each pot was covered with a polythene bag and after a few days, the acclimatized plantlets were transferred to the field with 80% survivability. Similar results were also reported by Karthikeyan et al. (2009) in *Centella asiatica* at 1.5 mg L^{-1} of IBA.

Conclusion

An effective direct regeneration protocol has been developed for *S. macrosperma* - economically and medicinally important plant species from the Western Ghat region. This study investigated the consistent effect of plant growth regulators in inducing callus and regeneration of plantlets by using leaf and nodal explants. This standardized protocol can be useful for mass multiplications of disease-free plants which could help to conserve the genetic diversity of this valuable medicinal plant by reintroducing their natural habitat. The studies of cytology, histology, suspension culture, and SEM observations revealed various aspects such as cellular, anatomical and morphological developmental features. This will lead to obtaining true- to- type genotypes by genetic engineering methods for commercialization of active phytoconstituents of this plant.

Conflict of interests

The authors declare that there are no conflicts of interest.

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Abbreviations

2, 4-D: 2, 4 dichlorophenoxyacetic acid, **TDZ**: Thidiazuron, **KN**: Kinetin, **BAP**: Benzylaminopurine, **NAA**: Naphthalene acetic acid, **IAA**: Indole acetic acid, **IBA**: Indole butyric acid, **SEM**: Scanning Electron Microscope, **AC**: Activated charcoal, **PGR**: Plant Growth Regulators.

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