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Micropropagation of *Salacia macrosperma* Wight - An Endemic Medicinal Plant of Western Ghats

C. Mahendra[1] and M. S. Sudarshana[1*]

[1]Department of Studies in Botany, Plant Tissue Culture Laboratory, Manasagangotri, University of Mysore, Mysuru - 570 006, Karnataka, India.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Salacia macrosperma Wight. - a potent medicinal plant facing the verge of rare and endemic status in the Western Ghats region of southern India. The effective protocol has been standardized for callus induction and multiple shoot regeneration using leaf and nodal explants. The Murashige and Skoog (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). The leaf explants produced more calli than nodal explants in MS medium supplemented with 2, 4-D and BAP in combination than individual hormones. Likewise, MS medium with 1.5 mg $L^{-1}$of 2, 4-D, 2.5 mg L^{-1} of BAP and 1.5 mg $L^{-1}$of TDZ along with 1% activated charcoal was apt for multiple shoot regeneration (93.33%) from nodal explants with slight embryogenic callus. Further, each developed plantlets were produced maximum rhizogenesis in liquid MS medium supplemented with 1.0 mg L^{-1} of IAA. Furthermore, the cytological study of embryogenic callus revealed variations in callus cells such as multinucleate, multi-nucleolate, cytodifferentiation, chromosomal bridges were noticed, besides normal dividing stages. Further, by scanning electron micrograph (SEM) analysis of embryogenic callus different stages of morphogenic developmental features were recorded.

**Corresponding author: E-mail: mssingh.uom@gmail.com;*

Keywords: Callogenesis; Cytology; Regeneration; Salacia macrosperma; SEM.

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's : Plant Growth Regulators
SDW : Sterile distilled water.

1. INTRODUCTION

Salacia macrosperma Wight. an important medicinal plant belongs to Hippocrateaceae family. It is a woody climber, shrubby, distributed in the peninsular regions of Sri Lanka, India, China, Brazil, Indonesia, Malaysia, Thailand and Philippines [1]. In India, it is found in the South and Western coastal regions of Kerala, Karnataka and Southern parts of Orissa [2,3]. The genus *Salacia* comprises more than 20 species, out of which few species have significant medicinal properties to cure dyspepsia, liver disorders, leprosy, skin diseases, stomachic, urinary disorders, anti-inflammatory properties [4]. It is also used to treat piles and congestion disorders [5]. The root of *S. macrosperma* possessed anti-diabetic properties due to their insulin-like properties [6].

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 [7]. *In vitro* conservation under aseptic conditions for germplasm preservation of potent medicinal plants by the application of plant tissue culture techniques can be an effective strategy [8]. 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 [9,10]. *S. macrosperma* is one such plant that facing the risks for survivability in its 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. reticulate* and Deepak et al., (2015) in *S. oblonga* [11,12]. The *in vitro* micropropagation through tissue culture is very difficult for *Salacia* species due to their recalcitrant nature [13]. 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 over-exploited 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 by micropropagation through tissue culture technique and to standardize suitable protocol using nodes and leaves are the source of explants.

2. MATERIALS AND METHODOLOGY

2.1 Plant Material Collection

S. macrosperma healthy plants (10 saplings) with the same genotypes were collected in June - July 2016 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.

2.2 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 small segments and rinsed in 0.2% Tween-20 (w/v) for 5 min., followed by washing 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 washed with SDW and dried between sterile blotter discs before

transferring on to the culture medium aseptically [14].

2.3 Media Preparation and Culture Condition

The MS medium contains required volume of macronutrients and micronutrients and supplemented with plant growth regulators (PGR's) at various concentrations and combinations [15]. 3% sucrose (Sigma - Aldrich) was used as a carbohydrate source in media compositions and 0.9% bacteriological grade agar (HiMedia) is used as a solidifying agent. The pH is adjusted to 5.8 before autoclaved at 121^{o}C for 20 min. The culture flasks were incubated and maintained at 21±2^{o}C under 12h photoperiod provided by white cool-fluorescent tubes and relative humidity of 85%.

2.4 Callogenesis and Organogenesis

Prepared leaf and nodal explants were cultured on MS medium supplemented with various concentrations of PGR's 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. The percentage of callus induction was calculated by using the following formula.

$$\%\ \text{Callus induction} = \frac{\text{No.of explants produced callus}}{\text{Total No.of explants cultured}} \text{X}100$$

Multiple shoots were regenerated from the nodal explants on the medium supplemented with 2, 4-D in all the treatments in different concentrations and combinations with BAP, TDZ, IAA (Indole acetic acid), and NAA (Table 2) along with activated charcoal (AC) used as an antioxidant source in some replicates. The regeneration frequency of multiple shoots was calculated by using the following formula [16].

$$\%\ \text{Regeneration frequency} = \frac{\text{NO.of shoots per explants}}{\text{Total No.of explants cultured}} \text{X}100$$

2.5 Cytological Observations

The cytological studies were carried out by the method of Johansen (1940) [17]. The leaf explants with embryogenic callus of 4-week-old 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 min., washed in water and transferred to the hematoxylin stain (2%) for 10 min. The callus is squashed in 45% propionic acid and observed under a light microscope (10X, 40X) to observe the cytological behavior of cultured cells by identifying various stages of cell development.

2.6 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 [18]. This is followed by dehydration through a graded series of ethanol. The samples were dried in desiccator 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 [19].

2.7 Rooting and Acclimatization

For rhizogenesis, the regenerated multiple shoots (6-7cm) were transferred to MS liquid medium supplemented with NAA, IAA or IBA at 0.5 to 2.5 mgL^{-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 [20].

2.8 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.

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Table 1. Effect of plant growth regulators for the induction of callus in nodal explant of *S. macrosperma* on MS medium at different concentrations and combinations

PGR	Concentration (mg L^{-1})	(%) Response	(%) Callus induction	Nature of the callus
		Nodes	Nodes	Nodes
Control	0.0	00.00 ± 0.00	00.00 ± 0.00^{o}	NR
2,4-D	0.5	8.33± 1.45	41.66± 7.26^{l}	W
	1.0	6.66± 1.76	33.33± 8.81^{m}	W
	1.5	10.00± 1.15	50.00± 5.77^{h}	H
	2.0	8.00± 1.52	40.00± 7.63^{l}	H
	2.5	9.66± 1.45	48.33± 7.26^{i}	LG
BAP	0.5	8.33± 0.88	55.00± 13.22^{f}	G
	1.0	9.33± 0.66	46.66± 3.33^{j}	G
	1.5	9.66± 0.88	48.33± 4.40^{i}	G
	2.0	10.66± 1.76	53.33± 8.81^{g}	G
	2.5	11.00± 2.00	55.00± 10.00^{f}	W
Kn	0.5	7.66± 1.20	38.33 ± 6.00^{n}	JC
	1.0	7.33± 0.66	36.66 ± 3.33^{n}	JC
	1.5	0.00± 0.00	0.00 ± 0.00^{o}	NR
	2.0	0.00± 0.00	0.00 ± 0.00^{o}	NR
	2.5	0.00± 0.00	0.00 ± 0.00^{o}	NR
2,4-D + BAP	1.5+0.5	10.66± 1.76	53.33± 8.81^{g}	W
	2.0+1.0	11.00± 0.57	55.00± 2.88^{f}	W
	2.5+1.5	10.66± 1.45	53.33± 7.26^{g}	G
	3.0+2.0	13.33± 0.88	66.66± 4.40^{b}	G
	3.5+2.5	15.66± 0.33	78.33± 1.66^{a}	G
2,4-D + Kn	1.5+0.5	8.00± 1.15	40.00± 5.77^{l}	G
	2.0+1.0	9.00± 1.52	45.00± 7.63^{k}	G
	2.5+1.5	11.66± 0.88	58.33± 4.40^{e}	GH
	3.0+2.0	12.66± 1.76	63.33± 8.81^{c}	GH
	3.5+2.5	11.33± 2.40	56.66± 1.51^{d}	GH
2,4-D +NAA	1.5+0.5	6.66± 0.66	33.33± 3.33^{m}	GJ
	2.0+1.0	10.66± 0.88	53.33± 4.40^{g}	G
	2.5+1.5	8.66± 1.76	43.33± 8.81^{l}	G
	3.0+2.0	0.00± 0.00	0.00± 0.00^{o}	NR
	3.5+2.5	0.00± 0.00	0.00± 0.00^{o}	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, LG: Light green, S: Soft callus and NR: No Response)

3. RESULTS AND DISCUSSION

3.1 Establishment of Callus Culture

The effect of different concentrations of individual plant growth regulators such as auxins (2, 4-D and NAA) and cytokinins (BAP and Kn) for callus initiation was investigated on *S. macrosperma*. In PGR's alone, the leaf explants responded 93% (Fig. 1A) at 1.5 $mgL^{-1}$of 2, 4-D produces light green callus, followed by 66. 66% response in BAP (2.0 $mgL^{-1}$of) and KN (1.5 $mgL^{-1}$of) with green callus and friable callus respectively. In the combination of PGR's leaf explants induced a high percentage (98%) of green hard callus in 2, 4-D (2.5 mgL^{-1}) +BAP (1.5 mgL^{-1}), followed by 2, 4-D + Kn (1.5 mgL^{-1} +0.5 mgL^{-1}) produces 86.66% of green hard callus. Among the combination of PGR's tested lowest percentage of callus induction compare to other combination was observed in 2, 4-D +NAA at a concentration of 1.5 mgL^{-1}+0.5 mgL^{-1} induced 78.33% of white hard callus. The best callus initiation from leaf explants of *S. macrosperma* in different concentrations and combinations of PGR's was reported in our previously published research article [21]. In this study, we have reported callus induction from nodal explants and further, we have also carried out the cytological study of cultured callus cells of leaf explants to know the nature of cells.

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

PGR	Concentration (mgL^{-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+2.5 +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)

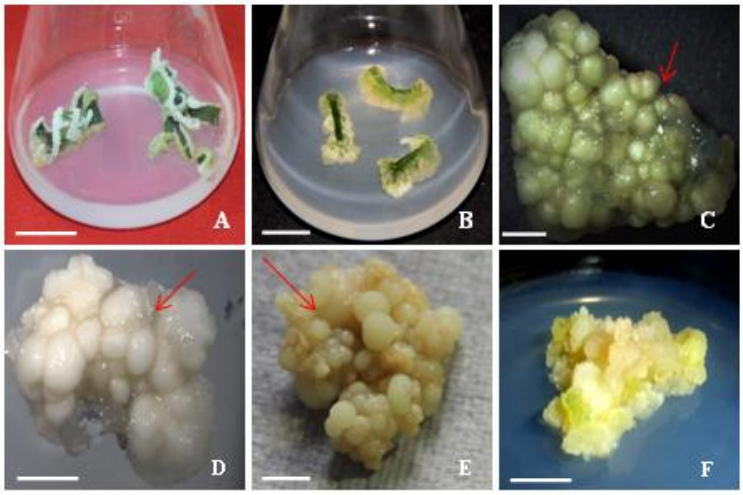

Fig. 1. Induction of callus from leaf and stems explants: A & B Leaf and stem explants after 15 days of inoculation (Scale bars: 2 cm); C to E. Embryogenic callus showing the various shapes of embryos at 40 days old culture (Scale bars: 1 cm); F. Soft friable light green non-embryogenic callus 40 days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

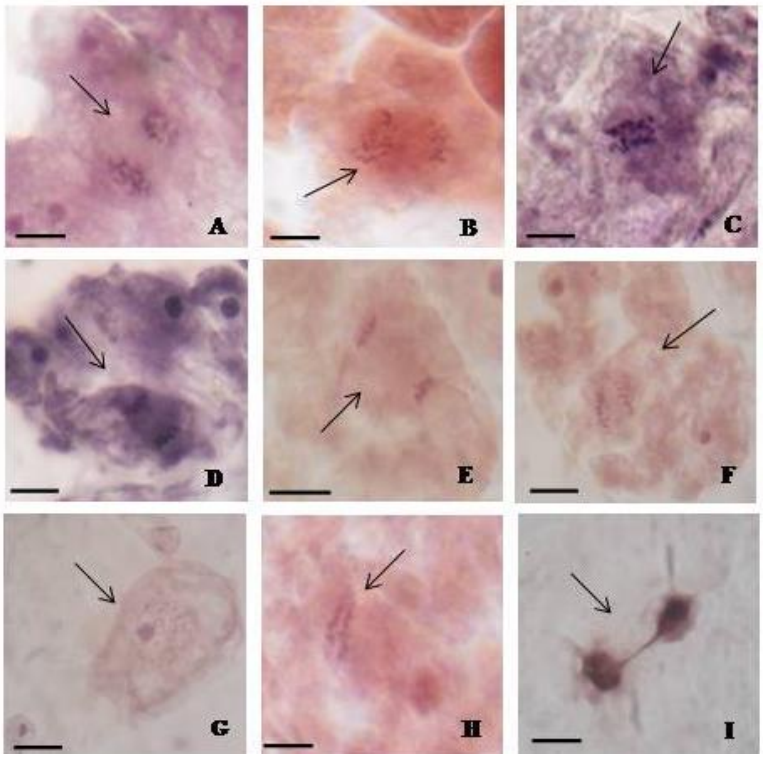

Fig. 2. Cytological studies of *in vitro* cultured cells showing variations: A. Uneven cell divisions with chromosomes at early metaphase and late metaphase stage; B. Unequal distribution of chromosomes at two opposite poles in a single cell at anaphase stages; C. Chromosomal Bridge; D & E. Anaphase stage with laggard chromosomes, sticky chromosomes and uneven cell wall morphology; F. Early anaphase stage with fragmentation of chromosome; G. Uni-nucleate cell and chromosome breakages at prophase stage; H. Normal metaphase stage; I. Cell with nuclear connections in binucleate conditions (Scale bars: 30µm)

The leaf explants induced callus became embryogenic in the subsequent subcultures (Fig. 1C & 1D) but failed to induce shoots. Nodal explants responded 50% (Fig. 1B), particularly at 1.5 $mgL^{-1}$of 2, 4-D. In BAP 55% green callus was observed from nodal explants at 2.5 mgL^{-1} concentration followed by KN 38% juicy callus at 0.5 mgL^{-1} concentration and poor callogenesis was observed in higher as well as lower concentrations of PGR's. As callus gets six-week older it became embryogenic in callus of nodal explants (Fig. 1E & 1F). In the combination of 2, 4-D and Kn (3.0 + 2.0 mgL^{-1}) at higher concentration, 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 of 2, 4-D and BAP at 2.0+2.0 mgL^{-1} [22]. Likewise, Kumar Meena, (2017) was also achieved the highest percentage of callus induction in *Nigella sativa* at 3.0 $mgL^{-1}$of 2, 4-D supplementation in MS medium [23]. Dhanasri *et al.,* (2013) successfully micropropagated *S. reticulata* on MS medium fortified with different PGR's such as benzylaminopurine and Indole-3-acetic acid (3.5 + 0.5 mg/L) by using nodal segments [11].

3.2 Cytological Variations

The cytological examinations in 40-days old callus cells exhibited variations such as asynchronous division within a single cell (Fig. 2A), unequal distribution of chromosomes at the 2 pole ends of the anaphase stage (Fig. 2B), early anaphase with fragmented chromosomes (Fig. 2C), chromosome breakages at prophase (Fig. 2D). 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 [24]. Presence of chromosomal bridge (Fig. 2E), anaphase with laggard, sticky chromosomes (Fig. 2F& 2G) were observed at the anaphase stage agrees with the report of Anju and 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 [25]. The normal metaphase stage (Fig. 2H) and nuclear connection in binucleated cells with cytodifferentiation (Fig. 2I) 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 *Roeynemia cilliaris* and *Psathyrostachys luashanica* [26]. Callus cells with a high ploidy level were also observed in tobacco, sugarcane and other plants [27,28,29]. According to Demoise and Partanen, (1969) chromosome number in callus cells increased as the culture period became longer in a large number of plants [30]. These observations may be due to the somaclonal variations in PGR's or the composition of nutrients in the medium [31].

3.3 SEM Analysis

The ontology of the embryogenic callus (Fig.3H) and its morphology was revealed by SEM studies. The MS medium supplemented with 2.5 mgL^{-1} of 2,4-D, the leaf explant cells undergo differentiation 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 (Fig. 3A). 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 [32]. In 35 days old nodal callus non-embryogenic cells in clusters on the medium supplemented with 2, 4-D (Fig. 3B). In the sixth week, the leaf callus cultures showed the presence of embryo-like cells which vary in their shape and morphology (Fig.3C). Similar observations were also reported by Jainol and Gansau, (2017) in *Dimorphorchis lowii* [33]. In the subsequent sub-cultures, the callus developed globular, torpedo, cordite and elongated shaped embryoids (Fig.3D, E, F & G). Likewise, our findings are in line with the previous reports of Andi Brisibe et al., (1992) in *Oryza glabarima* and by Popielarska et al., (2010) in *Actinidea deliciosa* wherein, the developmental and morphological features of *in vitro* cultured cells were observed in SEM monographs [18,34].

3.4 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 PGR's 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 (Fig.4A to 4D) on the MS medium supplemented with 2, 4-D (1.5 mgL^{-1}) + BAP (2.5 mgL^{-1}) + TDZ (1.5 mgL^{-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 Mahendra et al., (2020) in *S. chinensis* wherein higher percentage of shoot inductions was achieved in a combination of BAP and NAA (2.0+0.8 mgL^{-1}) [35,21]. In some replicates of 60 days old culture exhibited 88% of multiple shoots on MS medium+1.5 mgL^{-1} 2, 4-D + 1.5 mgL^{-1}BAP with 3% AC (Fig.4E to 4H) and this is followed by 86% shoots inductions on MS medium+1.5 mgL^{-1}2,4-D +1.5 mgL^{-1} TDZ with 1% AC. The outcome of these results is corroborated with the findings of Faisal et al., (2014) where they have achieved direct shoot multiplication in *Mentha arvensis* [36]. The use of TDZ growth regulator in all the explants played a vital role in inducing more number of multiple shoots in *S. macrosperma.*

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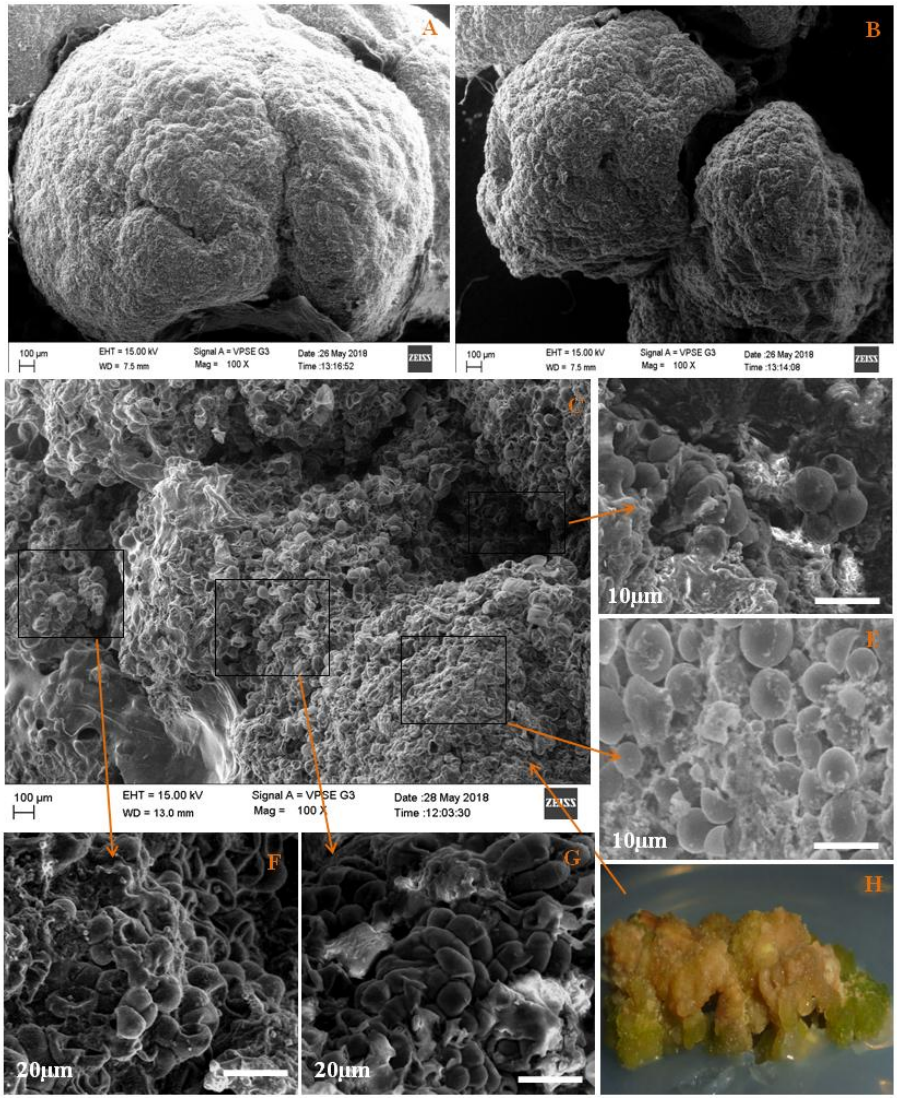

Fig. 3. SEM showing embryogenic leaf callus of *S. macrosperma*. A. Abaxial surface view of 25 days old callus from leaf explants; B. An overview of (nodal callus 35 days old) of non-embryogenic cells; C. Loosely arranged mass of embryogenic callus cells (40 days old); D. Heart, round and ovule shaped 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 mgL^{-1} concentration of 2, 4.D, BAP

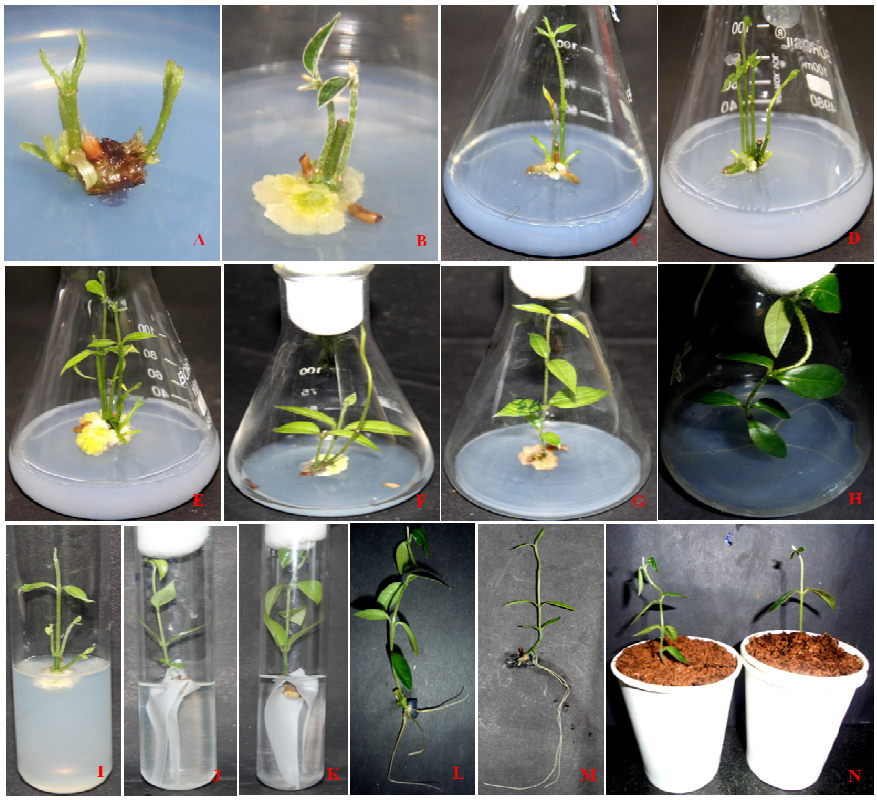

Fig. 4. A. Multiple shoots development from nodal explants: B to D. Induction of multiple shoots with callus from nodal explants; E to I. Sub cultured multiple shoots with callus development transferred after 40 days; J & K: Induction of roots from developed plantlets in MS liquid medium; L & M. Well-developed 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*

PGR	Concentrations (mg L^{-1})	No. of shoots cultured	Roots response (%)	No. of roots /plantlets	Length of roots (cm)
Control	0.0	10.54 ± 0.54	00.00 ± 0.00c	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
NAA	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}
	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}
IBA	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}
	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}
IAA	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}
	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 tubes. 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)

3.5 Rooting and Acclimatization

For induction of roots, the excised shoots from the culture vessels are aseptically transferred to the MS medium containing NAA or IAA or IBA in the range 0.5 - 2.5 mgL^{-1}. A maximum of 85.36 % of rooting was induced in IAA (1.0 mgL^{-1}) in MS medium with 4.33 average number of roots per shoots and the average length of roots is 2.3 cm (Fig.4I, 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 concentrations [37]. 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 polycups (Fig.4N) 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 mgL^{-1} of IBA [20]. Similarly, Mastiholi *et al.,* (2018) got the best result of micropropagation with maximum survival percentage of *in vitro* regenerated plantlets of *S. chinensis* from nodal explants on MS medium supplemented with BAP (2 mgL^{-1}) and NAA (0.8 mgL^{-1}) [38].

4. CONCLUSION

An effective direct regeneration protocol has been developed for *S. macrosperma* economically and medicinally important plant species from the Western Ghat region, India. 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 into their natural habitat. The studies of cytology and SEM observations revealed various aspects such as cellular 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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