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Induction, Development and Germination of Somatic Embryos from *in vitro* Grown Seedling Explants in *Desmodium gangeticum* L.: A Medicinal Plant

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ABSTRACT

For high rate multiplication of different medicinal plants, it is necessary to standardize the protocol for high regeneration. The efficiency of any regeneration is primarily depends on factors like type of explants used, composition of the medium and type of genotype. Here we have developed a regeneration protocol using *in vitro* grown seedling explants of *Desmodium gangeticum* (L.) DC (Salparni) a medicinal plant through somatic embryogenesis. Explants from *in vitro* grown seedlings were cultured on Murashige and Skoog's basal medium with BA at different concentrations for callus initiation. Different explants cultured on MS medium containing different concentrations of BA (0.1-1.0 mg L^{-1}) produced friable non-embryogenic callus (brownish white) with many small, smooth, rounded, discreet and greenish unit of embryogenic callus within the first week of inoculation. For induction of somatic embryogenesis, these friable embryonic callus derived from cotyledon, hypocotyl, epicotyl and leaf explants were transferred to MS medium supplemented with various growth regulators like BA, Kn, 2,4-D, NAA, CH (casein hydrolysate) and BA with 3% glucose. Most of the somatic embryos derived from the induction medium germinated like normal embryos producing both tap root and shoot on regeneration medium and also greenish sprouted embryoids with only roots or shoots or secondary somatic embryos i.e., clusters of embryos or poly embryos are found. Only shoots measuring 3 cm or more derived from somatic embryos were excised and planted on semi solid basal medium supplemented with varying concentrations of IBA for induction of rooting. All the explants in the medium containing BA 0.5 mg L^{-1} responded high. In the higher concentration of BA all the explants produced non-regenerative hard callus. However, the cotyledon is best explants over all to produce plants through somatic embryogenesis. Somatic embryogenesis derived plantlets of *D. gangeticum* showed 77% of survival. The *in vitro* propagation protocol standardized can be highly useful in raising quality planting materials of *Desmodium gangeticum* for commercial plantation programmes.

Key words: *Desmodium gangeticum*, axillary bud multiplication, micropropagation, medicinal plant

INTRODUCTION

Desmodium gangeticum (L.) DC (Syn. *Hedysarum gangeticum*) belonging to the family Fabaceae (According to the literature on Indian Materia Medica *Desmodium gangeticum* (L.) DC

belongs to the family Leguminosae) and commonly known as Salparni or Prishniparni (Hindi and Sanskrit) was chosen as the experimental material because of its multiple uses in the Indian system of medicine, particularly in the Ayurveda system. The plant grows mainly in South East Asia and Northern Australia. Prishniparni is used by vaidyas of Travancore and Cochin (Pillai *et al.*, 1981). As a single drug its effect has been described on pregnancy with reference to abortions (Pillai *et al.*, 1981). This plant is widely distributed in India, ascending to 5000 ft in the Himalayas. It contains indole alkaloids in its green parts. 1 kg wet mass of plant material yielded 2.5 g total alkaloids the composition being DMT: 0.41 g, DMT Nb oxide: 0.33 g, N-Methyl-T: 0.77 g, 5 Meo DMT: 0.57 g, 5 MeO DMT Nb oxide: 0.18 g, tetrahydroharman: 0.03 g, 6-methoxy and 2-methyl B carboline: 0.21 g. The dimethyl tryptamine (DMT) and other tryptamine derivatives are used as psychotropic drugs. Three ptero-carpenoids namely gangetin, gangetinin and desmodin were isolated from hexane extract of roots of *D. gangeticum* (Purushothaman *et al.*, 1971). During routine pharmacological screening gangetin exhibited significant anti-inflammatory activity in albino rats (Ghosh and Anandakumar, 1983). Some studies indicate that gangetin exhibited dose dependent anti-fertility properties in albino rats (Pillai *et al.*, 1981; Latha and Govindasamy, 1997; Latha *et al.*, 1997). The different plant parts, as well as the whole plant are used for different ailments. The plant is commonly used as bitter tonic, febrifuge, digestive, anti-catarrhal, anti-emetic, in inflammatory conditions of the chest and in various other inflammatory conditions, which are due to vata-disorders (Nayar *et al.*, 1956; Nadkarni, 1976). The plant shows anthelmintic, aphrodisiac, astringent, diuretic properties. It is used in general anasarca, consumption, cough, diarrhoea, dysentery, chronic fever including enteric fever, piles, respiratory disorder, vomiting, worms, asthma, snake bite and scorpion sting (Kurup and Joshi, 1979; Anonymous, 1952). Kiritikar and Basu (1975) have listed various uses of roots of *D. gangeticum* and have reported that the seeds contain significant amount of β-carboline alkaloids. Roots are chewed daily for the cure of typhoid and pneumonia. Boiled root extract of several plants including *D. gangeticum* is orally administered to overcome weakness. Paste prepared from the root mixed with water and sugar candy when taken orally in empty stomach in the morning daily for about a week prevents spermatorrhoea. The plant parts form part of the compositions of popular Ayurvedic medicines such as Dasamula Kwatha (M/s Zandu Pharmaceuticals), Dasamularishta (Dabur, Himalayan Drugs), Blissful Joy 60 (Maharishi Ayur-Ved), Amlakalp (Maharishi Ayur-Ved), Ere Forte (Dr. J. Vanderstelt, Germany), Mahanarain Taila (Vademecum, Nederland) and JanuTaila (Vademecum, Nederland).

Somatic embryogenesis techniques have been applied in a number of species to obtain propagules at a very rapid rate. Somatic embryos or embryoids in many species can be induced either directly on the explants (direct embryogenesis) or through a callus phase (indirect embryogenesis). However, the rate of clonal propagation is much higher in case of indirect somatic embryogenesis than in direct embryogenesis.

Somatic embryogenesis has following steps: (1) somatic embryo formation (2) somatic embryo maturation (a) culture in high sucrose/agar medium, (b) culture in medium with stress i.e., NaCl, proline and maltose, (c) cold storage at different temperature i.e., 4, 8, 10,15°C, (d) ABA treatments for 4 to 6 weeks, (3) germination of somatic embryo (a) plating mature/dessicated/ treated somatic embryo on MS medium; (b) rooting and shooting should occur simultaneously or one after another and data should be recorded in % of germination and (4) plant establishment.

During the present study attempts were made to obtain somatic embryogenesis indirectly through a callus phase. For this, the effects of different growth regulators supplemented to the culture medium on induction and enhancement of somatic embryogenesis were studied and finally

attempts were made to successfully germinate the somatic embryos into transplantable plants. Results of various experiments carried out during the present investigation on induction, enhancement and germination of somatic embryos in different type of explants of *D. gangeticum* i.e., medicinal plants have been presented in the following sections.

MATERIALS AND METHODS

Explant preparation: Ripe and dried pods of *D. gsngeticum* grown in the green house condition were collected. Seeds were removed and were initially rinsed thoroughly in running tap water for 14 min followed by immersing in a 5% (v/v) solution of detergent (Labolene, Qualigens, India) for 10 min. Then it was surface sterilized with 0.1% (w/v) mercuric chloride for (10-12 min) subjected to repeated washings in sterile distilled water and the sterile seeds were aseptically inoculated on to basal Murashige and Skoog (1962) medium containing Kn 0.1 mg L^{-1} and incubated in an incubator for germination. Segments of cotyledons, hypocotyls and epicotyls (0.5 to 1.0 cm), cotyledonary nodes, roots, leaves and internodal segments were aseptically excised from 15-30 days old aseptically grown seedlings and cultured on different nutrient media. All operations during inoculation including surface sterilization were carried out inside the laminar airflow cabinet (Thermadyne Ltd., India).

Media preparation and culture condition: MS (Murashige and Skoog (1962) salt composition, sucrose (30 g L^{-1}) and bacteriological grade agar-agar (8 g L^{-1}) were used throughout the study without incorporating any growth regulators and the pH was adjusted to 5.8. All the cultures were incubated in a culture room maintained at 25±2°C under 16/8 h light/dark cycle, 45 µmol m^{-2} sec^{-1} irradiance level provided by cool white fluorescent tubes (Philips, India) and with 55-60% relative humidity. The experiment repeated thrice with each treatment consisted of 20 explants. Duncan's multiple range tests was applied for comparison of the results.

RESULTS

Somatic embryogenesis of *D. gangeticum*: Different explants of *D. gangeticum* gave both callogenesis and adventitious shoots. This callus was embryogenic in nature. So attempts were made to induce somatic embryogenesis in callus culture.

In order to evaluate the possibility of inducing indirect embryogenesis, attempts were made to raise callus cultures from various *in vitro* grown explants of *D. gangeticum* The primary calli were subcultured atleast 2-3 times to obtain homogenous callus for use in the experiments related to induction *in vitro* grown of somatic embryogenesis.

Callus induction: The frequency of callus induction varied with the type of cultured explants and culture medium. The basal medium devoid of growth regulators failed to induce callus in any of the cultured explants. When different explants cultured on MS medium containing different concentrations of BA (0.1-1.0 mg L^{-1}) produced friable non-embryogenic callus (brownish white) with many small, smooth, rounded, discreet and greenish unit of embryogenic callus within the first week of inoculation (Fig. 1a). These calli were subcultured subsequently within 10-15 days interval of culture. Greenish coloured callus contain many meristematic nodular units (which can be compared to dicotyledonous stage of normal zygotic embryos but the number per culture is very less). These embryos produced dwarf shoots having callus formation at the radicle heads (Fig. 1b). The cotyledon explants produced highest percent and degree of callusing irrespective of BA
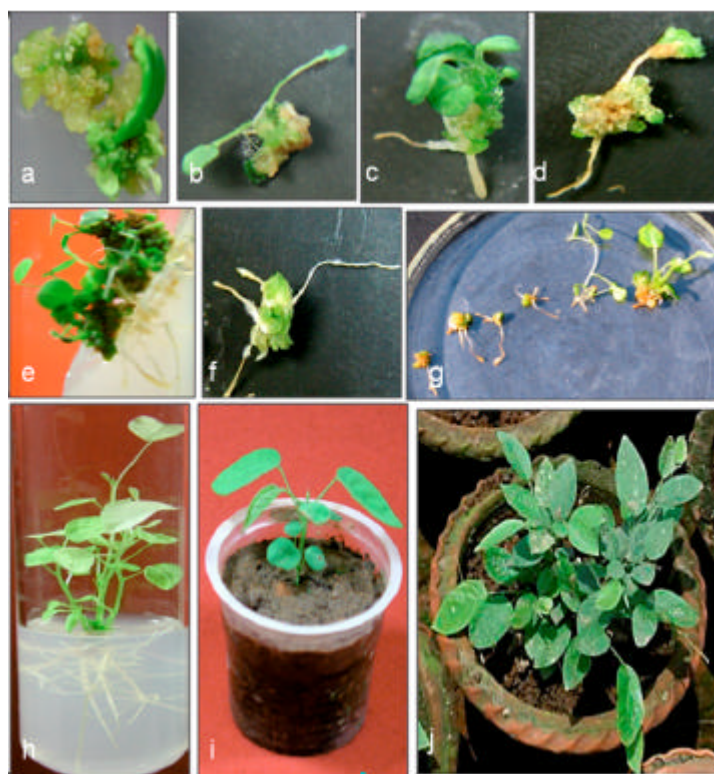
Fig. 1(a-j): Somatic embryogenesis of *D. gangeticum* from different explants. (a) Cotyledon explants on MS medium containing BA (0.5 mg L^{-1}) produced friable non-embryogenic callus (brownish white) with greenish unit of embryogenic callus, (b) Embryos produced dwarf shoots having callus formation at the radicle heads, (c) Some of the embryos were morphologically normal having distinct cotyledons and radicles, (d) Some somatic embryos arose from the base of other embryos to form clusters indicating secondary somatic embryogenesis, (e) Most of the somatic embryos germinated like normal embryos producing both tap root and shoot on regeneration medium, (f) Clusters of embryos or poly embryos, (g) Isolated embryos from hypocotyl explants, (h) In all parameters of rooting medium rooting response was very good, (i and j) Acclimatization of rooted shoots derived from somatic embryos on pot and soil establishment were shown, respectively

concentrations. All the explants in the medium containing BA 0.5 mg L^{-1} responded high (Table 1). In this medium cotyledon explants produced 100+0.57% callusing and 90+0.57% culture with embryonic callus, hypocotyl explants produced 90+2.88% callusing and 30+2.30% culture with embryonic callus, epicotyl explants produced 80+0.57%callusing and 10+0.57% culture with embryonic callus, cotyledonary node explants produced 50+2.88%callusing and 28+1.73% culture with embryonic callus. In the higher concentration of BA all the explants produced non-regenerative hard callus.

Induction of somatic embryogenesis: For proliferation and maintainance of Friable Embryogenic Callus (FEC) it was cultured on somatic embryo induction medium. For induction of

Table 1: Effect of BA on induction of callus in cotyledon, hypocotyl, epicotyl and leaflet segments of *Desmodium gangeticum*

Medium	Cotyledon (%)		Hypocotyl (%)		Epicotyl (%)		Cotyledonary node (%)	
MS with BA (mg L^{-1})	Explants callussing	Culture with embryogenic callus	Explants callussing	Culture with embryogeniccallus	Explants callussing	Culture with embryogenic callus	Explants callussing	Culture with embryogenic callus
	a	b	a	b	a	b	a	b
Control	NR	NR	NR	NR	NR	NR	NR	NR
0.1	30±2.88^{d++}	20±0.57^d	19.6±0.88^{d+0}	25^{d+}	0	20±0.57^{a+}	0	0
0.25	40±0.57^{c++}	38±0.57^c	30±2.88^{c+}	0	38^{c+}	0	25±1.73^{c+}	0
0.5	100±0.57^{a+++}	90±0.57^a	90±2.88^{a+}	30±2.30	80±0.57^{a+}	10±0.57	50±2.88^{a+}	28±1.73
1	80±1.15^{b+++}	75±0.57^b	70±1.15^{b+}	0	72±0.57^{b+}	0	32±1.15^{a+}	0

Data (Mean±SE) of three independent experiments each with 20 replicates. Means followed by the same letter within the column are not significantly different (p<0.05) as tested by the multiple range test (+): Callussing, (++): Middle rate of callussing, (+++): High rate of callussing

somatic embryogenesis, the friable embryonic callus derived from cotyledon, hypocotyl, epicotyl and leaf explants were transferred to MS medium supplemented with various growth regulators like BA, Kn, 2,4-D, NAA, CH (casein hydrolysate) and BA with 3% glucose. All the cultures were incubated at 25+2°C and 16 h photoperiod (cool Phillips white fluorescent light, 45-50 µmol m^{-2} sec $^{-1}$). Each treatment had 20 replicates and the experiment was repeated three times.

After 3 weeks of sub culture data was taken on nature of callus whether it is regenerative or non-regenerative, weight of embryogenic callus, number of somatic embryos per one culture tube and different developmental stages of somatic embryos documented in Table 2. Cotyledon explants on MS medium with 0.5 mg L^{-1} produced 800+5.774 mg embryonic callus with the highest number (88+4.619) of somatic embryos and on 2,4-D 0.5 mg L^{-1}+Kn 0.25 mg L^{-1} produced maximum amount of callus i.e., 900+11.547 mg per culture tube. Epicotyl explants gave minimum number of somatic embryos in comparison to other explants. Somatic embryos obtained from the proliferation or induction media of different explants showed different developmental stages, which remained more or less same against the treatments. On BA 2 mg L^{-1}+2, 4-D 0.5 mg L^{-1} almost less amount of callus and so also less number of somatic embryos produced by all explants. Some of the embryos were morphologically normal having distinct cotyledons and radicles (Fig. 1c). Some produced only shoots or roots or both. Abnormal embryos varied in shape and structure, having one, two or even more unequal cotyledons, which are some times fused into cup shape. Most of the somatic embryos were loosely attached to form aggregate of embryos that could be easily separated. Some somatic embryos arose from the base of other embryos to form clusters (Fig. 1d) indicating secondary somatic embryogenesis.

Germination of somatic embryo on regeneration/germination medium: Most of the somatic embryos germinated like normal embryos producing both tap root and shoot on regeneration medium (Fig. 1e). Somatic embryos could develop through globular to cotyledonary-staged embryos. Greenish friable embryonic callus when sub cultured on the germination medium produced germinated somatic embryos with shoot and root, greenish sprouted embryoids with only roots or shoots or secondary somatic embryos i.e., clusters of embryos or poly embryos (Fig. 1f). Hypocotyls explants were responded better in production of somatic embryos at different germination stages (Table 3; Fig. 1g). On ½ MS in comparison to other mediums all the explants responded better. In comparison to induction medium in the regeneration medium more number of somatic embryos at different developmental stages was obtained. However, abnormalities of somatic embryos (i.e., those consisted of rooted globular embryos, additive embryos and callus formation at the root pole) were frequently observed.

Effect of cold storage on somatic embryo maturation and germination: In order to speed up the embryo maturation process and improve the germination frequency somatic embryos were randomly selected from germination medium (Table 3), inoculated on ½ MS medium in petri plates, sealed with parafilm and were stored at low temperature (6-8°C) in dark in a refrigerator for different durations (1,2,3,4,5,6 and 7 days). Thereafter, the cultures were removed and incubated under normal culture room conditions of temperature and light. Following about 2-6 weeks of incubation the percentage of somatic embryos exhibiting germination under each treatment was recorded. The data obtained from this experiment indicated that cold storage had a beneficial effect on the maturation and germination of *D. gangeticum* somatic embryos. The optimal response of

Table 2: Effects of various kinds and concentrations of phytohormones on proliferation of embryonic callus of *Desmodium gangeticum*

MS Medium with Phytohormones	Origin of callus								Development or remark
	Cotyledon		Hypocotyl		Epicotyl		Leaf		
	A	B	A	B	A	B	A	B	
BA 1.5 mg L⁻¹									NRHC
BA 0.5 mg L⁻¹	800±5.774[b,1]	88±4.619[a],*	700 ±11.547[b,3]	80±2.887[a],**	685±8.66[b,3]	78±4.619[a],**	752±4.041[b,2]	83±4.619[a],**	GFEC, D, SH, R, GSE, SSE, GSP.E
BA 0.25 mg L⁻¹	800±2.887[d,1]	75±5.774[b],*	650±11.547[c,2]	70±4.619[b],*	600±11.547[c,3]	68±3.464[b],*	670±5.774[d,2]	71±3.464[b],*	GFEC,D, SH, SSE
BA 0.10 mg L⁻¹	750±2.887[c,1]	80±5.774[a,b,*]	700±8.660[b,2]	75±5.774[a,b,*]	620 ±8.66[c,3]	69±2.887[b],*	710±8.660[c,3]	76±3.464[b],*	GFEC,D, SH, R, GSE, SSEB[a]
0.5 mg/l+ 3%Sucrose	400±5.774[1]	30±2.887[a],*	380±8.660[d,1,2]	28±4.619[a],*	350±8.660[d,3]	25±1.115[c],*	360±5.774[a,2,3]	29±2.887,*	GFEC
2,4 D 0.5+Kn 0.25 mg L⁻¹	900±11.547[a]	114±115[d,1]	870±11.547[a,1]	12±0.577[d],**	800±11.557[b,2]	9±0.577[d],***	880±8.660[a,1]	10±0.577,**,***	FEC, R, GSP.E (VERY LESS)
2,4 D 0.5 mg/l+Kn0.5 mg L⁻¹	300±14.434[b,1]	15±0.577[d],*	220±5.774[f,2]	13±0.577[d],**	200±5.774[f,2]	10±0.577[d],***	225±5.774[f,2]	14±0.577[d,e,f,*],**	GFEC, GSP.E
BA 2 mg/l±2,4-d 0.5 mg L⁻¹	250±14.434[1]	13±0.577[d],*	220±2.887[f,2]	10±0.577[d],**,***	200±2.887[f,2]	9±0.577[d],***	225±2.887[f,2]	11±0.577[e,f],**	GFEC, GSP.E
10 mg/l NAA+Kn 1mg L⁻¹	350±11.547[b,1]	16±0.577[d],*	225±5.774[f,2]	14±0.577[d],**	200±4.041[f,3]	10±0.577[d],***	230±4.619[f,2]	14±0.1[d,e,f],**	GFEC, GSP.E
10 mg/l NAA+Kn 1mg L⁻¹	320±11.547[b,1]	15±0.577[d],*	300±6.928[1]	13±0.577[d],*	5320±8.660[d,2]	10±1.155[d],**	305±2.887[h,1]	14±1.115[d,e,f],*	GFEC,GSP.E (±vitamins)
10 BA 10.5 mg/l±CH 100 gm	450±8.660[1]	22±1.115[c,d,*]	400±14.434[d,2]	16±0.577[d],**,***	350±8.66[d],3	14±0.577[d],***	420±8.660[c,1,2]	18±1.115[d,e,*]	GFEC, GSP.E (SOME)
BA 10.5 mg/l±CH200 gm	400±11.547[1]	20±1.155[d],*	380±8.660[d,1,2]	14±0.577[d],**	320±8.660[e,2]	11±0.577[d],***	390±5.774[1]	20±1.155[d],*	GFEC, GSP.E

A: Fresh weight of embryonic callusin mg /culture tube, B: Number of somatic embryo /one culture tube, NRHC: Non regenerative hard callus (greenish and white in colour), GFEC: Greenish friable embryonic callus, BFEC: Brown friable embryonic callus, SH: Shoot, GSE, GSE: Germinated somatic embryo with root and shoot, SSE: Secondary somatic embryogenesis, FE: Friable embryonic, C: Callus, R: Greenish sprouted embryoids produced roots, GSP.E: Greenish sprouted embryoids without shoot and root, data (Mean±SE) of three independent experiments each with 20 replicates. Means followed by the same letter within the column: Same numerals and star marks within the rows, are not significantly different (p<0.05) as tested by the multiple range test of Duncan (1955)

Table 3: Rsponse of embryonic callus of *Desmodium gangeticum* in the germinating medium

	Cotyledon				Hypocotyl				Epicotyl				Leaf			
Medium	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
MS	$6\pm0.58^{b*}$	$10\pm0.58^{a,b,1}$	$5\pm.58^{a,2}$	$8\pm0.58^{a,b,3}$	$5\pm0.58^{a**}$	$9\pm0.58^{a,1}$	$4\pm0.58^{a,2}$	$7\pm0.58^{a,2}$	$4\pm1.15^{c*}$	$8\pm1.15^{a,1}$	$3\pm0.58^{a,1}$	$6\pm0.58^{a,b,3}$	$5\pm0.58^{**}$	$8\pm1.73^{b,b,1}$	$5\pm1.15^{a,2}$	$6\pm0.58^{a,b,3}$
1/2MS	$7\pm0.58^{a,b***}$	$11\pm1.73^{a,1}$	$6\pm0.58^{a,2}$	$9\pm0.58^{a,3}$	$11\pm0.58^{a**}$	$7\pm1.15^{a,b,1}$	5 ± 0.73^{a}	$28\pm0.58^{a,3},$	$10\pm0.58^{a**}$	$10\pm1.15^{a,1}$	$4\pm0.58^{a,1}$	$7\pm1.15^{a,3}$	$6\pm0.58^{***}$	$10\pm0.58^{a,b,1}$	$6\pm0.58^{a,2}$	$8\pm0.58^{a,3}$
1/4MS	$8\pm0.58^{a,b***}$	$7.6\pm2.19^{a,b,1,1}$	5 ± 0.58^{a}	$28\pm0.58^{a,b,3}$	$10\pm0.58^{a,b**}$	$6\pm1.15^{a,b,1*}$	4 ± 0.58^{a}	$27\pm58^{a,b,3,3}$	$9\pm0.58^{a,b**} ***$	9 ± 1.15^{a}	$1,1^{a}3\pm0.58^{a}$	$26^{a,b,3,3}$	$6\pm0.58^{a****}$	$11\pm0.58^{a,1}$	$4\pm1.15^{a,2}$	$5\pm0.58^{b,3}$
MS+0.1 mg L^{-1}	$9\pm1.73^{a,b*}$	$7\pm0.58^{a,b,1}$	4 ± 1.15^{a}	$27\pm0.58^{b,c,3}$	$8\pm0.58^{c,d*}$	$5\pm1.73^{b},1$	3 ± 1.15^{a}	$26\pm0.58^{b,c,3}$	$7\pm0.58^{b*}$	$7\pm1.15^{a,1}$	$4\pm1.15^{a,2}$	$5\pm0.58^{a,b,3}$	$8\pm0.58^{a**}$	$4\pm0.58^{a,1}$	$5\pm1.15^{a,2}$	$5\pm0.58^{b,3}$
1/2MS+0.1 mg L^{-1}	$10\pm0.58^{a**}$	$7\pm1.15^{a,b,1}$	$5\pm0.58^{a,2}$	$7\pm0.58^{b,3}$	$9\pm0.58^{b,c**}$	$6\pm0.58^{a,b,1}$	$4\pm1.15^{a,2}$	$6\pm0.58^{b,c,3}$	$8\pm0.58^{a,b**}$	$8\pm0.58^{a,1}$	$5\pm2.31^{a,2}$	$5\pm1.15^{a,b,3}$	$8\pm1.73^{**}$	$6\pm0.58^{c,d,1}$	$6\pm1.15^{a,2}$	$4\pm1.15^{b,3}$
1/4MS+0.1 mg L^{-1}	$8\pm1.15^{a,b*}$	$6\pm1.15^{b,1}$	$5\pm1.73^{a,2}$	$6\pm0.58^{b,3}$	$7\pm0.58^{b**}$	$5\pm0.58^{b,1}$	$4\pm1.73^{a,2}$	$5\pm0.58^{b,3,3}$	$7\pm1.15^{b*}$	$7\pm0.58^{a,1}$	$4\pm1.51^{a,2}$	$3\pm1.15^{b,3}$	$7\pm0.58^{a**}$	$5\pm0.58^{a,1}$	$4\pm1.73^{a,2}$	$4\pm0.58^{b,3,3}$

A: Shoot per culture, B: No. of shoots having roots, C: Somatic embryo with only roots, D-secondary somatic embryo, data (Mean±SE) of three independent experiments each with 20 replicates. Means followed by the same letter in groups within the column; same numerals between groups within rows are not significantly different ($p<0.05$) as tested by the multiple range test of Duncan (1955).
(A: Shoot per culture, B: Number of shoots having roots, C: Somatic embryo with only roots, D: Secondary somatic embryo)

about 30% germination was recorded when somatic embryos received a cold treatment at 6-8°C of about 3 days. The detrimental effect of cold storage was evident as the germination percentage sharply declined after a storage period of 3 days. Thus the percentage of embryo germination was recorded following 7 days of cold storage was as low as 28% that was even lower than the control set of embryos i.e., 30% (Fig. 2).

Root induction: Only shoots developed from somatic embryos were given roots within 7-10 days when grown on MS medium containing different concentrations of IBA. Percentage of root induction was 56-69%. In all parameters of rooting medium rooting response was very good (Table 4; Fig. 1h). At 2.0/2.5/3.0 mg L^{-1} IBA the rooting response was varied from 96 to 100%. In the Table 4, response data up to 2.0 mg L^{-1} concentration was given.

Hardening and soil establishment of plantlets derived from somatic embryos of *D. gangeticum*: The present study demonstrated that production of somatic embryos, their germination, rooting and their subsequent development into whole plants. Shoots, which are derived from embryogenic callus, gave roots and subsequently they survived on vermiculite medium. The percent of survival of germinated somatic embryos derived from germination medium were presented in Fig. 3. Germination of cold treated somatic embryos with root and shoot after cold

Table 4: Effect of various medium on rooting of shoots of *D. gangeticum* obtained from different explants grown on root induction medium

MS with IBA mg L^{-1} control	Respond (%)	Days to rooting	No. of roots	Mean root length (4 weeks)	Mean root length (8 weeks)	Survival *in vivo* (%)
control	NR	NR	NR	NR	NR	NR
0.5	90.6+0.33[b]*	12.6+1.20[a]	3+0.57[a]	2.9+0.26[c]	3.1+0.37[b]	57+0.57[c]
1.0	95.0+0.57[a]	12.6+0.88[a]	4.0+0.57[a]	3.5+0.28[b,c]	3.8+0.43[b]	65+0.57[b]
1.5	95.0+1.00[a]	11.0+0.57[a]	4.0+1.15[a]	4.0+0.10[b]	4.0+0.23[a,b]	65.3+0.33[b]
2.0	96.3+1.20[a]*	10.0+0.57[a]	5.0+0.57[a]	4.8+0.18[a]	4.9+0.18[a]	68.6+0.33[a]

Data (Mean+SE) of three independent experiments each with 20 replicates. Means followed by the same letter within the column are not significantly different (p<0.05) as tested by the multiple range test of Duncan (1955). *Callus formation
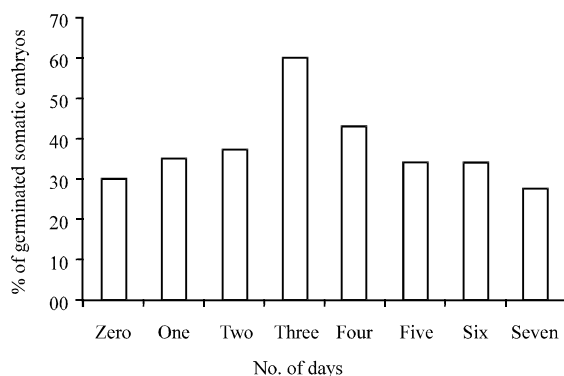

Fig. 2: Effect of cold storage on % of somatic embryos germination (derived from callus of various *in vitro* grown seedling explants) of *D. gangeticum*. Results are Mean±SE of independent experiments
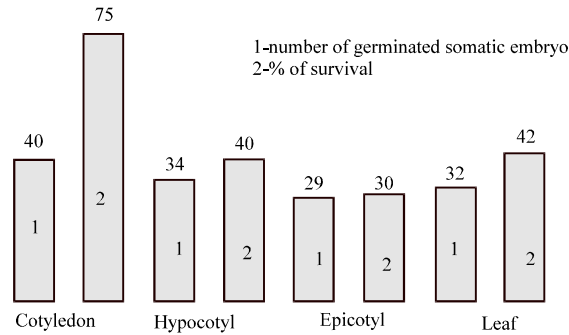
Fig. 3: *In vivo* survival of somatic embryos derived from different explants of *D. gangeticum*. Results are Mean±SE of 3 independent experiments
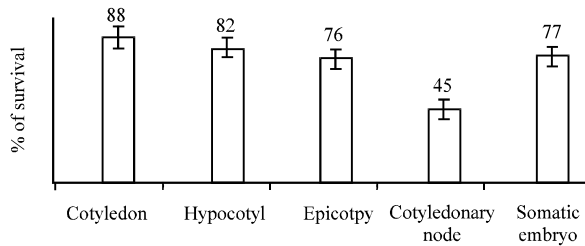

Fig. 4: Percent survival (*in vivo*) of different plants obtained from different plants of *D. gangeticum*. Results are Mean±SE of 3 independent experiments each with 10 replicates
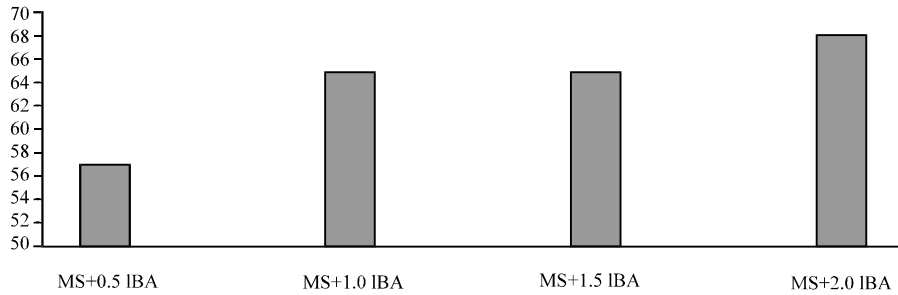

Fig. 5: Survival of plantlets (in %) derived from *D. gangeticum* somatic embryos rooted on different rooting medium. Results are Mean±SE of 3 independent experiments

treatment and subsequent survival of a single shoot let on vermiculite medium and pot were shown in Fig. 1i and acclimatization of rooted shoots derived from somatic embryos on pot and soil establishment were shown in Fig. 1j.

Somatic embryogenesis derived plantlets of *D. gangeticum* showed 77% of survival in comparison to plantlet regeneration from other explants (Fig. 4).

When the embryonic callus grown on the germination medium i.e., full / half or fourth strength of MS without or with BA gave different developmental stage of somatic embryos. Only shoots obtained from this medium after being transferred into rooting medium produced roots (Fig. 5).

DISCUSSION

In a wide variety of plant species it has been noted that explants of young tissues still undergoing cell division generally form callus more readily than those in older parts of the plant and the chance of organogenesis from such callus is much greater. Even in case of direct organogenesis, the young parts respond much better than the parts at adult phase of growth. In many kinds of woody trees or shrubs, cultures of all kinds are most easily established from plant parts at juvenile phase (George and Sherrington, 1984). Swain and Pattanaik (1987) have achieved greater success in callus induction and shoot bud regeneration in case of seedling explants of *Dalbergia sissoo*. In various plants organogenic regeneration and shoot multiplication is best achieved in MS and 1/2 MS with combination of BA, Kn, NAA, GA3 and IAA (Chaichana *et al.*, 2011; Dash *et al.*, 2011; Elavazhagan and Arunachalam, 2010; Htwe *et al.*, 2011) which also happened in the present study

The size of the explants determines the survival of the culture. Generally, the larger the explant, the better the chance of survival (Dale, 1975). Basing on the reports on various species, the size of the explants cultured was correlated and the larger explants have shown higher survival rates. Hence the large explants instead of minute meristems have been selected for *in vitro* culture. Instead of taking a number of small cuttings of various *in vitro* grown plant parts like cotyledons, hypocotyls and epicotyls etc, the total part of cotyledon, hypocotyls and epicotyls etc were taken for culture which gave very good results. However, if eradication of diseases becomes one of the objectives, meristems of smaller size will be more preferable.

The juvenile explants (hypocotyl segments, cotyledonary leaves and primary roots) have shown better results in terms of callus induction and shoot bud regeneration. The seedling explants directly and indirectly through a callus phase regenerated into whole plants. Induction of callus from both mature and juvenile plant parts would be a good starting point for mass production of the plantlets. In tissue culture of the medicinal plant species, generally, the pattern of development of juvenile explants differs from that of mature explants. The juvenile explants grow and proliferate faster than mature ones, may be due to the accumulation of inhibitors in the cut surface of the mature plant parts which are released in to the medium and cause poor response. Although some workers have achieved success in culturing explants from mature plant parts, in regenerating plantlets, in some plant species as: *Acacia nilotica* (Mathur and Chandra, 1983), *Dalbergia sissoo* (Dawara *et al.*, 1984), *Ficus religiosa* (Jaiswal and Narayan, 1985), *Populus alba* (Park and Son, 1988) and *Robinia pseudoacacia* (Davis and Keathley, 1987), in most of the species, poor performance of mature plant parts has been reported. Rather the embryonal, seedling or juvenile tissues yield vigorously growing cultures that are potentially more regenerative. In addition to this, the time period for regeneration in these juvenile plant parts is quite striking as it occurred in the following examples: *Acacia albida* (Duhoux and Davies, 1985), *Albizzia lebbeck* (Verghese and Kaur, 1988), *Leucaena leucocephala* (Dhawan and Bhojwani, 1985), *Pterocarpus santalinus* (Patri *et al.*, 1988) and *Sesbania grandiflora* (Shankar and Ram, 1990a).

Of the various explants used, the cotyledonary leaf explants showed the maximum callus induction although initially both cotyledon and hypocotyl segments showed a better response compared to the epicotyl and root explants. Callus induction and shoot multiplication or somatic embryo formation response occurred in a progressive parameter that was root ≤cotyledonary node ≤epicotyl ≤leaf ≤hypocotyl ≤cotyledon. In case of some parameters hypocotyl segments gave better response than cotyledons. This can be observed in somatic embryogenesis of *D. gangeticum*

in the germinating medium. This may possibly be due to the presence of large number of cells undergoing division in cotyl region having greater chance for callogenesis and organogenesis. This may be dependent upon endogenous auxin levels, which vary according to the age of the tissues. Some times the explants sequence towards the response may vary or they may respond equally to callus induction.

Nature of calli: Variations in induction of callus could only be possible through nutritional manipulations. Hence the media and cultural environment need to be varied from one species or variety of a plant to another. Sometimes, even very closely related varieties may differ in their cultural requirements and there are several instances where callus from closely related varieties of plants may differ in texture, colour as well as in morphogenetic capacity (George and Sherrington, 1984). It has also been established that the capacity for direct morphogenesis in any medium can also found to vary between closely related plant varieties. During the present investigation, in *D. gangeticum* the rate of callus formation was quite high and the calli were soft, compact, friable, granular and light green in colour. For callusing, *in vitro* grown seedling explants were subjected to culture and out of these explants, cotyledonary leaves and hypocotyls responded better. Possibly, a different callus response in decreasing order of magnitude for cotyledonary leaf, hypocotyl, epicotyl, cotyledonary node, internodal segment and primary root derived from *in vitro* grown seedling explants of these species may be due to different morphogenetic potentialities. While cotyledonary leaf is a determinate organ and root is indeterminate, hypocotyl is intermediate between the two and considered an organ of transition. On the other hand, the time period taken for seed germination being quite long, the attainment of suitability by the cotyledonary leaf for use as explants is considerably delayed as compared to that of the primary root. By the time the cotyledonary leaf becomes suitable for use as an explant, the primary root has grown to a greater extent, giving rise to lateral roots in *D. gangeticum*. Since the meristematic zone is restricted in matured rootless, the callusing might have become restricted. Callus response of hypocotyl explants have also been reported in a number of tree species like *Santalum* (Bapat *et al.*, 1985), *Sesbania* (Shankar and Ram, 1990b), *Eucalyptus* (Subbaiah and Minocha, 1990) and *Dalbergia* (Swain, 1993). This may possibly be due to the presence of a number of cells undergoing division in hypocotyl region having a greater chance to form callus and organogenesis. Nagori and Purohit (2004) cultured *Annona squamosa* on MS with NAA, IAA, IBA, 2, 4-D, BA and TDZ and observed hypocotyl towards root was more responsive. This may also be dependent upon the endogenous auxin levels, which vary according to the age of the tissues.

Since the explant response in the medicinal plants was found to be different, it is quite evident that variation in explants might be influencing the callus formation and morphogenesis. George and Sherrington (1984) stated that, there might be a group of genes capable of shifting plant development in different directions and influencing the process of cell proliferation and morphogenesis. Such genes have been thought to exert their influence by regulating the effective levels of growth substances which may vary in different genotypes and explants.

Micropropagation via somatic embryogenesis is reported to be more advantageous than organogenesis, particularly for plant breeding programmes (Tomar and Gupta, 1988). This is because somatic embryos are believed to originate from single cells whereas organ regenerates through the collective organization of many cells. Therefore, plants derived from somatic embryos tend to be similar while plant reared through organogenesis may show wide genetic variations. Somatic embryogenesis has been reported in many different medicinal plant species (Rout *et al.*,

1991; Purohit *et al.*, 1994) in safed musli by 2,4-D; Te-Chato and Rungnoi (2000) in *Azadirachta excelsa* by 2,4-D BA, NAA; Das *et al.* (2001) in *Santalum album* by sucrose, nitrate, ammonium and abscisic acid; Nakagawa *et al.* (2001) in *Cucumis melo* L.; Das *et al.* (1999) in *Typhonium trilobatum*; Rout *et al.*, 2000, 2001 in *Cephaelis ipecacuanha*) and also including a number of tree species (Ammirato, 1983; Tulecke, 1987; Cardoza and D'Souza, 2002; Jones *et al.*, 1990; Rout *et al.*, 1996 in *Muntingia calabura* by Kn and 2,4-D; Das *et al.*, 1997 in *Dalbergia sissoo* by Kn +2,4-D+3% Sucrose; Rout *et al.*, 1995) in *Acacia catechu* a multipurpose leguminous tree on WPM medium supplemented with Kn, NAA and proline; Rout and Das (1994) produced direct somatic embryogenesis on cotyledon explants of *Simarouba glauca* by BA, NAA and ascorbic acid; Rout *et al.* (1991) in *Rosa hybrida* producing dicotyledonous stage of somatic embryo on MS supplemented with BA ,NAA, 2,4-D, proline and adenine Sulphate; Das *et al.* (1993) in *Rosa hybrida* reported effect of carbohydrates on somatic embryogenesis; Martin *et al.*, 2001; Gallego *et al.*, 2001. Through the phenomenon of somatic embryogenesis has been reported in tissue culture of more than thirty families, it is not common in trees as it is in herbaceous species. Trees are still considered to be recalcitrant and mass propagation by somatic embryogenesis is the mot sought after method for rapid propagation.

In the present study, somatic embryogenesis was responded very well. In case of *D. gangeticum* both by direct and indirect somatic embryogenesis was found. The steps adopted for somatic embryogenesis are; 1) induction, 2) proliferation, 3) histo-differentiation and maturation, 4) desiccation, 6) germination and conversion.

Induction of callusing was influenced by the presence of growth regulators in the medium. Medium devoid of growth regulators failed to induce callus. Though callusing was quicker in the medium supplemented with BA, NAA and 2,4-D at higher concentration, the calli were yellowish and soft having smaller number of embryogeneic response. Such calli subsequently turned deep brown or black after first passage. This observation is in accordance with the results reported by Sita and Swamy (1992) in *Dalbergia latifolia*. They reported that maintenance of callus in the 2,4-D enriched medium mostly resulted in the browning of callus. A slow growing semi-hard greenish white calli was observed in the medium enriched with 0.5 mg L^{-1} BA +0.1 mg L^{-1} NAA and 0.1 mg L^{-1} 2,4-D agrees with the result of Arumugam and Gopinath (2011). Although, BA alone at 0.1 to 0.5 mg L^{-1} only in terms of formation of embryogeneic tissues, addition of auxins like NAA, 2,4-D improved the growth and proliferation of somatic embryo and produced embryogenic callus at a slower rate which did not show any further differentiation. This result is in corroboration with the direct somatic embryogenesis and plant regeneration of *Dianthus caryophyllus* (Yantcheva *et al.*, 1998) by 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BA. Thus a combination of BA 0.5 mg L^{-1} + NAA 0.1 mg L^{-1}+2,4-D 0.1 mg L^{-1} was found to be optimal for inducing the formation of S.E. while 1 mg L^{-1} BA + 0.5 mg L^{-1} IAA+0.5 mg L^{-1} 2,4, D helped in the proliferation of embryogeneic callus in the first passage only. More or less similar response was reported in *Santalum album* by Rout and Das (1994) in *Simarouba glauca*. The results in the present study indicated that incorporation of BA at 0.5 mg L^{-1} along with NAA and 2, 4 -D at 0.1 mg L^{-1} showed better response in inducing somatic embryo formation. In such cultures most of the embryos were morphologically normal and showed distinct cotyledons and radicles. Rout *et al.* (1991) produced cotyledonary stages of somatic embryos from *Rosa hybrida*. Many of these embryos were loosely attached to form an aggregation of embryos. Some germinate and form bud like trifoliate structure failed to develop complete plant. Similar result was obtained in case of *Rosa hybrida* (Rout *et al.*, 1991). Also it was observed that some embryos arose from the base of these embryos to form cluster

indicating secondary somatic embryogenesis. This pattern of secondary somatic embryogenesis was somewhat similar to the results obtained in *Trifolium repens* (Maheswaran and Williams, 1986) and *Simarouba glauca* (Rout and Das, 1994), *Myrtus communis* L. (Parra and Amo-Marco, 1998).

The result suggested that the induction of callusing and somatic embryogenesis from cotyledon segments is greatly influenced by BA NAA and 2, 4-D concentrations in the medium, which is in agreement with the findings that BA induce somatic embryogenesis in a number of species (Murthy and Saxena, 1998); 2,4-D has been used to induce somatic embryogenesis in a number of other species (Siwach *et al.*, 2011; Jha *et al.*, 2011; Sita, 1981; Parra and Amo-Marco, 1998; Craig *et al.*, 1997) and so also BA and 2,4-D in *A. marmelos* (Islam *et al.*, 1996).

In case *D. gangeticum* embryogenic callus were introduced by the supplementation of BA (0.1-1.0 mg L^{-1}) in the induction medium. Cotyledon gave highest percentage of embryonic callus in respect of other explants. Cotyledon is the best explants in response to organogenesis as explained in the study of Otroshy *et al.* (2011). The production of somatic embryo depends on explants origin (Hu and Wang, 1983).

It was observed that addition of auxin was found to be essential for inducing embryogenic callus formation and subsequent induction of development of somatic embryos. In the present study induction and proliferation of somatic embryogenesis was achieved in full strength medium with or without BA. But the germination of somatic embryos derived from induction and proliferation medium of somatic embryogenesis was achieved in full, $^1/_2$ and $^1/_4$ strength medium with or without BA. The mode of action of auxin in somatic embryogenesis may be somewhat similar to that in the zygotic embryo development. Auxin transport in immature seed embryos is strictly basipetal and during embryogenesis physiologic polarity precedes morphologic polarity (Fry and Wangermann, 1976). From these facts of zygotic embryogenesis, it may be extrapolated that a polarized distribution of endogenous auxin in tissue culture may be a pre-requisite for induction of somatic embryogenesis (Rangaswamy, 1986). At the molecular level, the mechanism of auxin action in somatic embryogenesis is less understood. But it was observed that tissues when initially grow in auxin-rich medium and subsequently on auxin free medium showed higher DNA content (Wochok, 1973; Masuda *et al.*, 1984).

When the embryonic callus grown on the germination medium i.e., full/half or fourth strength of MS without or with BA gave different developmental stage of somatic embryos. Only shoots obtained from this medium after being transferred into rooting medium produced roots. This result is quite similar to the result in Te-Chato and Rungnoi (2000) in *Azadirachta excelsa* (somatic embryos developed only shoots were rooted on ½ MS supplemented with 15 μM NAA) and *Gloriosa superba* L. by Jadhav and Hegde (2001); where friable embryonic callus was initiated from shoot apices on MS+2,4-D (4 mg L^{-1})+Kn (5 mg L^{-1})+CH (10 mg L^{-1}); Ghatge *et al.* (2011) found rooting in ½ MS with NAA. Subsequent development was occurred on the same medium with subsequent shoot and root development by supplementation of BA, Kn and IBA.

Enhancement of maturation and germination of somatic embryos: When the embryonic callus grown on the proliferation medium (which contains BA, Kn, 2, 4-D, NAA, CH, vitamins 10 and 3% Sucrose) produced somatic embryo with different developmental stage. In *Gloriosa superba* somatic embryogenesis was obtained on MS with BA, 2,4-D, Kn, CH and AdSO$_4$ (Jadhav and Hegde, 2001); Gill *et al.* (1995) in *Citrus reticulata* Blanco founded somatic embryogenesis from epicotyl explants by using NAA (10 mg L^{-1})+Kn (1 mg L^{-1}) +vitamins (10 times of normal MS levels). Das *et al.* (1997) in *D. sissoo* also got somatic embryogenesis by Kn, vitamins, ABA and 3%

glucose and these somatic embryos matured on 1/2 MS with ABA, vitamins and with 2% w/v sucrose. Some of the embryos were morphologically normal and distinct cotyledons and radicles. Some produced only shoots, roots or both. Abnormal embryos varied in shape and structure, having one, two or even more unequal cotyledons, which are some times fused into cup shape. Most of the somatic embryos were loosely attached to form an aggregate of embryos that could be easily separated some somatic embryos arose from the base of other embryos to form clusters, indicating secondary somatic embryogenesis (Parra and Amo-Marco, 1998) in *Myrtus communis* by 2,4-D and 1/2 MS without any growth regulators; Rout *et al.* (1995) in *Muntingia calabura* by Kn and 2,4-D and on 1/2 MS with 2% w/v sucrose without any growth regulators somatic embryo matured) and so also on the germination medium i.e., full/half or fourth strength of MS without or with BA, about 15-20% of the somatic embryos germinated like normal embryos, producing both tap root and shoot. As reports in many earlier works on somatic embryogenesis, the induction medium yields a mixture of embryos in different stages of development, but these embryos are required to be sub-cultured on a different medium in order to germinate (Parra and Amo-Marco, 1998). This has also been reported for the *Myrciaria* (Litz, 1984), *Feijoa* (Cruz *et al.*, 1990) and *Eucalyptus* (Muralidharan *et al.*, 1989), Borad *et al.* (2001) in *Sapindus trifoliatus* with BA and Kn. In most of the cultures by supplementation of BA in the medium produced more number of shoots produced which agrees with the result obtained by Singh and Tiwari (2010) in *Clitoria ternatea*; Remarkers *et al.* (1993) in *Cassava* where cyclic somatic embryogenesis produced at four stage.

The shoots obtained from the proliferation and germination medium were rooted on full strength of MS with supplementation of IBA also reported by Dvin *et al.* (2011).

A pre-culturing of the somatic embryo of *D. gangeticum* for 3-4 weeks in a medium supplemented with 0.5 mg L^{-1} BA under the cold treatment osmotica for 1-7 days at 6-8°C stress condition in the medium so as to stimulate and also improve the conversion rate of embryos to enter into the maturation phase just similar type of effect of heat stress and irradiation on embryogenesis (Tuncer and Yanmaz, 2011). Incubation of the embryos at low temperature also initiates a slow desiccation process. Development of normal zygotic seeds culminates in the loss of water that is either coincident with or subsequent to the acquisition of desiccation tolerance by the embryo. Although the occurrence and extent of this drying varies, in excess of 70% of cellular water is lost and as a direct result the seed becomes quiescent and metabolism stops. Under favourable conditions of moisture, temperature and in some cases light, metabolism is reactivated and germination begins. At this stage, the seed can be brought back to its original moisture level without adversely affecting viability (Simon, 1974). In fact dehydration or partial desiccation seems to change the physiology of an embryo leading it to a maturation phase just prior to germination and development of seedlings (Kennode *et al.*, 1986). Poor germination of somatic embryos may result from incomplete internal or external conditions for germination. Therefore, the induction of desiccation tolerance and dehydration may be necessary to induce germination in somatic embryos. ABA has long been proposed to play an important role in seed and embryo maturation (Das *et al.*, 1997) and the suppression of precocious germination. In developing seeds, it stimulates accumulation of reserve substances and prepares embryos for dormancy. It also increases cold and desiccation tolerance of embryos (Attree *et al.*,1991). Dormancy has been documented in somatic *Vitis* embryos (Gray, 1987). It may be more common in somatic embryos usually treated with ABA noticed. Thus, germination quality may also result from the dormancy of somatic embryos. If poorly germinating somatic embryos are well developed, the dormancy might be detected with dormancy-

breaking treatments such as cold treatment or exogenous growth regulators (Hepher and Roberts, 1985). In fact, in the present study, both supplementations of growth regulators to both induction and the germination media as well as cold pretreatment of somatic embryos could significantly boost the ultimate germination frequency. Methods have been developed in plant cell culture to induce somatic embryo formation from vegetative cells and then to develop and mature those embryos into a dry quiescent state that may be used as an artificial or synthetic seed (McKersie and Bowley, 1993). Drying the somatic embryo allows long-term (years) storage of a clonal propagule. Whether encapsulated or not, the artificial seed then becomes a true analogue of conventional seed to be used for germplasm conservation in seed storage banks, or simply for bulking up propagules (Gray and Purohit, 1991). The objective in the development of artificial seed technology is to produce a propagule that is genetically, developmentally and morphologically as close as possible to the seed of the species from which it has been derived. Zygotic embryo formation begins with the double fertilization of the egg and polar nuclei, whereas somatic embryos are formed by the differentiation of somatic cells (Gray and Purohit, 1991). Despite this difference in initiation, both somatic and zygotic embryos originate and pass through similar morphological stages of development, including globular, heart, torpedo and cotyledonary stages (Gray and Purohit, 1991). In late maturation, somatic embryos may acquire desiccation tolerance. Numerous methods have been used to induce desiccation tolerance in these somatic embryos including exposure to exogenous ABA and treatment with sub-lethal stress (Senaratna *et al.*, 1989). Torres *et al.* (2001) observed abscisic acid and osmotic induction of synchronous somatic embryo development of sweet potato. The first is to prevent precocious germination. The second is to induce the genetic program that initiates biochemical and physical changes associated with the acquisition of desiccation tolerance.

Alternative means of inducing tolerance involve sub-lethal stress. For example, thermal stress and cold treatment have induced tolerance to dehydration in microspore-derived embryos of *Brassica napus* (Anandarajah *et al.*, 1991). In alfalfa, sub-lethal stresses such as nutrient deprivation, cold stress, thermal treatment and water stress all induce tolerance to desiccation (Senaratna *et al.*, 1989). Partial water stress can be applied in two ways. Senaratna *et al.* (1989) simply removed the seal from the petri plate containing medium and embryos, letting the moisture stress occur over 23 days. A more controlled method was that employed by Attree *et al.* (1991) who used polyethylene glycol (PEG-4000) to induce water stress. Sucrose, other sugars or sugar alcohols may play a role in desiccation tolerance by acting as osmotic agents. A desiccation state was achieved in *Medicago* somatic embryos simply by the inclusion of 6% sucrose in the maturation medium (Anandarajah and McKersie, 1990). Maturation of somatic embryos of some species including soybean (*Glycine max* L. Merr.), orchardgrass (*Dactylis glome rata* L.) and grape (*Vitis longii*) allowed survival of water loss without any planned inductive treatment (Gray, 1987).

Overall propagation efficiency: *In vivo* vs. *in vitro*: Through micropropagation, it was possible to multiply these two medicinal plants through out the year irrespective of the seasons of the year. Once the explants are successfully established in *in vitro* culture, no matter whether they are nodal explants, calli derived from mature or seedling explants or plants derived from somatic embryo, it was only routine affair of subculturing them at regular intervals year after year for production of propagules. Roughly four to seven culture cycles can be possible in a single year. Depending on the species the propagation rate in field per single culture varies from 29-75% in case of *D. gangeticum* and through manipulation of some culture parameters and proper pre-treatment conditions considerably high number of somatic embryos can be pushed towards maturation and germination. These ultimately boost the embryo to plantlet conversion rate.

The results of the present study indicated that *in vitro* propagation protocols could be far more superior to the conventional vegetative propagation techniques for the mass multiplication of the medicinal plant *D. gangeticum*. However, there still remains enough scope to refine the techniques further. Further research and developmental inputs, particularly for development of the maturation and germination frequencies of the somatic embryos achievable in *D. gangeticum* can open up the possibilities for artificial seed technology as well as exploring the automated propagule production system through bioreactor technology (Redenbaugh *et al.*, 1986). The genetic variation if present in tissue culture derived plants that should be established. The *in vitro* propagation results and protocols developed in the present study, thus can be effectively utilized in establishment of medicinal plant gardens for the utilization by pharmaceutical industries. But, nonetheless, depending upon the available infrastructure situations, the vegetative propagation approach can also be a useful alternative or can be adopted to serve as an adjunct to the *in vitro* approaches (Murashige, 1974).

Effect of growth regulators on induction of rooting: Most of the commercial laboratories are now turning to extra Vitrum methods, although *in vitro* rooting was historically the most widely used system for obtaining plantlets from micropropagated shoots. However, *in vitro* rooting is still being commonly utilised in research and may be the only practical method in some kinds of plants. In the present study, plantlets regenerated in cultures of both the species, failed to root despite their transfer to phytohormone free MS media. The same media were tried with cytokinins but were of no avail. Possibly cytokinin is instrumental in inhibiting rooting. Hence, regenerated shoots were taken in media containing auxins like NAA, IAA and IBA. Under these conditions, *D. gangeticum* induced well-developed roots with laterals. In all cultures of *D. gangeticum* with higher levels of IAA (1.0 and 1.5 mg L^{-1}) or IBA (0.5, 1.0 and 2.0 mg L^{-1}) treatments, root primordia emerged from the shoot base starting from day 6 to day 16 after shoot inoculation and soon after that the root growth was rapid. IBA was more effective than IAA in induction of rooting as days required to rooting. On further trial it was found that half strength media with IBA, although rooting was initiated further growth was not observed. However, another problem crept into the induction of roots that was the development of callus at the cut ends at the development site of the roots. Possibly, higher concentration of auxin was responsible for inducing the callus.

Concentration of auxin in the medium was found to be the critical factor in producing healthy roots. A range of concentration of IAA and IBA was tested (0.25 -5.0 mg L^{-1}) for rooting in the present studies. Both these auxins were effective in the concentration range between 1.0-5.0 mg L^{-1}. IAA at 0.25-0.5 mg L^{-1} concentrations was not effective as the shootlets did not produce any roots. It was also observed that low concentration of IBA (0.5 mg L^{-1}) on the shootlets of nodal explants origin also from *in vitro* grown seedling explants completely failed to induce rooting. In comparison to IAA, IBA produced better rooting response. But the overall rooting response with IBA at higher concentration (>0.5) was better, while IAA responded better between 1.0-1.5 mg L^{-1}. Higher concentration of these auxins also resulted in basal callusing. Pradhan *et al.* (1998) reported that the rooting of *in vitro* shootlets of *D. latifolia* was better at 2.0 mg L^{-1} of IBA. Ndoye *et al.* (2003) observed the highest rooting response at 20 mg L^{-1} IBA while working in *Balnites aegyptiaca*. This is a similar type of findings that at 1.0-5.0 mg L^{-1} concentrations these auxins showed effective respond in all the experiments. Similarly it may be also be worthwhile to mention that rooting of *in vitro* propagated shoots was achieved on the half strength MS medium supplemented with IAA or IBA. Initially full strength MS medium along with

the auxins IAA and IBA was tried which proved to be ineffective. So the strength was reduced to half strength. Al-Wasel (2000) reported that *in vitro* shootlets of *Acacia senegal* produced longer and normal roots on the half strength MS medium whereas, cultures on full strength MS medium produced shorter and abnormal roots. Though to some extent this is in accordance with the results obtained by Al-Wasel (2000) in *A. senegal* but it did not give any satisfactory results i.e., <0.5 cm root length was obtained.

In most of the reports on rooting, reduced strength of macronutrients was favoured. This favourable effect of reduced macronutrient concentration on rooting is probably due to total ionic concentration than to the need for only a small amount of total nitrogen. IBA has been widely used as root inducing hormones both under *in vitro* and *in vivo* conditions (Minocha, 1987). The beneficial effects of reduced salt and sucrose concentrations during the rooting phase has been described in several reports (Purohit *et al.*, 1994) in *Wrightia tomentosa*; Purohit and Singhvi (1998). Previous investigators have also obtained comparable results like, on the effect of temperature and light on rooting cv. Bridal pink, Khosh-Khui and Sink (1982a, b); lower illumination of light is required for rooting in *Rosa hybrida* by Rout *et al.* (1991); Singh *et al.* (2002) in *Psidium guajava* reported rooting of micro shoots was achieved best on ½ strength MMS with 4.90 µM IBA along with 100 mg L^{-1} activated charcoal; lower concentration of MS salts in rooting by Rout *et al.* (1990). AC supplementation is based on its capacity to adsorb toxins, excess phytohormones and other phenolics. Hence optimum levels of AC for rooting vary with plant species, as higher levels make auxin unavailable (Narayanaswamy, 1994). These shoots rooted normally and bore fresh leaves when transferred to pots after rooting.

Concentration of agar was also found to affect the root formation and elongation. Medium with low concentration of agar or liquid medium was found to be more suitable for rooting possibly due to better aeration. Anderson (1980) considered that the agar concentration should be as low as possible. Since lowering of agar content may facilitate better availability of nutrients and hormones for rooting. Agar generally serves to support the propagules (Skirvin, 1980; Nemeth, 1978). Agar and activated charcoal influence on uptake of gibberellin and Plant morphogenesis (Yassen, 2001).

Roots developing from explants in *D. gangeticum* quite thin, well branched and apparently healthy while the latter were weak and slender and appeared as the primary root structure. Such a condition had also been reported by Lee and Rao (1980) in *P. indicus*. Sita *et al.* (1992) obtained a single thick root in *P. santalinus* on MS medium with high concentration of IAA. But they got the best results with formation of adventitious roots on using lower concentrations of auxins. Similar success with diluted MS medium supplemented with IBA was reported by Swain, 1993) in *D. latifolia* and *D. lanceolaria*, whereas, he found NAA in combination with low concentration of cytokinin (BAP) to be more suitable for root induction in *D. sissoo*, Nayak *et al.* (2002) found root production in orchids on MS medium with 2.0 mg L^{-1} IBA. In earlier studies, Mukhopadhyay and Ram (1981) induced roots in MS medium with IAA alone or in combination with NAA.

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). The auxins IAA and IBA were used singly to induce rooting from *in vitro* raised shootlets. There were distinct differences in rooting behaviour of the shootlets originated from different explants source. *In vitro* shootlets failed to respond to rooting in the auxin free medium irrespective of its origin. Addition of either IAA or IBA was essential for rooting of *in vitro* shootlets. Between the two auxins used, IBA proved to be better than IAA in terms of roots per rooted shootlets and total root length. IBA has been widely used as a root-

inducing hormone in difficult-to-root plants both under *in vitro* and *in vivo* conditions (Minocha, 1987). Pradhan *et al.* (1998) observed a positive rooting response when both IAA and IBA were added together. But, in the present study IBA IAA alone was found to be sufficient for rooting of *in vitro* shoots though IBA when used singly stimulated better rooting response in all the three tree species.

Another noteworthy observation in the present investigation was that rooting was significantly affected by the explant origin as well as length of the shoot which is a similar observation by Pati *et al.* (2006) in roses where they found activated charcoal, growth regulators, carbohydrate content and size of the explants affects the rooting. Shootlets less than 1.75 cm long showed comparatively poor rooting and took longer time to form roots. Shootlets obtained from *D. gangeticum* showed root initiation after about one week of culture. Negussie (1997) observed response to rooting sometimes is very difficult while working on *Juniperus excelsa*. He reported that rooting of *in vitro* shoots from cotyledon explant was difficult.

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