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Embryogenesis of *Dipterocarpus retusus* Bl. Syn. D. macrocarpus

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Abstract
Somatic embryogenesis was achieved in tree species *Dipterocarpus retusus* using tender leaf and petiole explants on Murashige and Skoog (MS) medium supplemented with 2, 4-D 2.0+IAA 0.5mg/l and 3% sucrose. MS medium containing 2, 4-D 4.0mg/l was recorded to be the most effective on inducing of embryonic callus. Embryogenesis per culture was varied in MS medium supplemented in different concentration in 2, 4-Dmg/l. Maximum number of somatic embryos was obtained in MS medium containing 2, 4-D 4.0mg/l and proliferation of embryonic callus was enhanced in the cultures having 2, 4-D 2.0mg/l in reducing of 2, 4-D in the media. Somatic embryos in various shapes and sizes after the first subculture on MS medium supplemented with 2,4-D 4.0mg/l. Embryo germination and was observed in the ½ MS medium and optimal results was attained in ½ MS + GA_3 0.1 with leafy cotyledon development with distinct root and shoot pole.

Keywords: Tree species, embryogenesis, 2, 4 dicloro phenoxy acetic acid, germination, Murashige and Skoog media.

Introduction
Dipterocarpus retusus is a timber yielding forestry species belonging to the family Dipterocarpaceae. It is locally known as '*Hollong*'. The height of the tree ranges from 45-48m and girth 3.6-6.7m with clean bole up to 40m (Joshi, 1980) [14]. The species is considered as the 'State tree' of Assam and also endemic to north east India of tropical wet evergreen forest (Champion and Seth, 1968) [9]. The tree is distributed from Brahmaputra valley of upper Assam in the West to South through Jeypore reserve forests of Dibrugargh and Sonari, Doom Dooma and Sibsagar districts, reserve forest in the east towards Hollangapapara, Disoi valley of Jorhat district, foot hills of Tirap, Chanlang, Lohit districts of Arunachal Pradesh and Mokokchung and Tuensang districts of Nagaland. The species has faced an unsustainable exploitation for plywood industries and coupled with poor natural regeneration and included in the red data book of IUCN in 1995. Propagation of the species is difficult due to non-availability of genetically superior seeds and low quality seeds produce due to regular insect and fungal attacks (Kundu, 2001) [16]. Seed storage possesses only 15 days viability along with seed maturity, germination and the chilling sensitivity of the seeds which supports recalcitrant nature (Kundu and Chanda, 2001) [17]. Family also lies in difficult to root category (Adjers and Anti 1996) [1]. Due to low coppicing ability also shows a very limited success in vegetative propagation (Borpuzari and Kachari, 2018) [6]. Hence, the present study was targeted for *in-vitro* study of somatic embryogenesis for regeneration.

Materials and methods
Planting materials were collected from the nursery of Rain Forest research Institute, Jorhat, Assam and the experimental site situated at 26º 47'N latitude and 94º 17'E longitude, altitude 80 msl with annual rainfall is 800mm/annum along with temperature maximum and minimum 33.8 ºC and 21.2 ºC respectively. Collected plant materials were washed thoroughly with running tap water for 1-2 hr to remove the impurities and dust particles then washed with few drops of Tween-20 solution for 10-15 minutes followed by washing with distilled water. Explants were prepared into appropriate sizes leaf (L) 5-6mm, petiole (P) 8-9mm. Sterilizing agent mercuric chloride 0.1% (w/v) were applied for dipping 5-6 min and thoroughly washed six to eight times with sterile distilled water. Both the exposed ends were trimmed and inoculated horizontally singly into the prepared medium. Cultures were kept in culture room maintained the temperature 25±2 ºC and 60-70 percent humidity. Light source was florescent

tubes, Bajaj, 40 Watts emitting at an intensity of 3000 lux approximately at culture levels for 16 hours light and 8 hours dark in a 24 hours cycle. Initially all cultures were exposed under dark condition. Due to high phenol exudation from explants separate experiments were conducted to control of phenol in different auxin, cytokinin with individual treatment with PVP 1.0-2.5(g/l), activated charcoal in different concentration of (0.5-2.0g/l). Sub culturing was done under aseptic condition after 30 days. Frequency of embryonic callus production of each explant was calculated from the percentage of embryonic callus production in each culture tube. Observations were taken after 7 days interval for an incubation period of 30 days. Replicates of 5-10 cultures were set up for every experiment. Each experiment was repeated at least twice. The whole experimental study was recorded for frequency of embryonic callus production, embryo initiation, morphology and texture of embryonic callus, maturation and percent of embryogenesis, etc. Experiments were conducted for selection of optimization basal media with best explants response (Table 1). Individual effect of auxin and cytokinin with IAA 2.0, NAA2.0, 2, 4-D 2.0 and 4.0, Kn 1.0, BAP 2.0 (Table 2).Selection for optimal of growth regulator with selected explants for maximum callus growth type in 2, 4-D 2.0 + Kn 1.0mg/l (Table 3). Influence of different levels of 2, 4-D for embryonic callus production 2.0, 3.0, 4.0 and 6.0mg/l (Table 4). Combine effect of hormonal concentration of 2, 4-D4.0, 2, 4-D4.0+IAA0.5, 2, 4-D2.0+CH100.0, 2,4–D2.0+IAA0.5, 2, 4 D2.0+CH100.0+IAA0.5mg/l (Table 5). Germination of induced embryos was incubated in MS with Kn0.05, Kn0.1, $GA_3$0.05, $GA_3$0.1 and ½ MS.

Results

Selection of optimal basal media for callus induction from various explants MS was found best for inducing callus (Table-1).

Table 1: Selection of optimal basal media for callus induction from various explants on 2, 4-D 2.0 + Kn 1.0mg/l

Type of explants	MS			1/2 MS)			WPM		
	Callus induction (days)	% of CR	CI	Callus induction (days)	% of CR	CI	Callus induction (days)	% of CR	CI
SS	30 – 35	10	-	30-50	-	-	30-50	-	-
P	30 – 35	20–25	++	30–50	10-15	+	30–50	10- 20	+
DPL	-	-	-	-	-	-	-	-	-
MPL	30 – 35	20–25	++	30–50	10–15	+	30–50	10–20	+
PPL	30 – 35	5–15	+	30–50	10–15	+	30–50	-	-

= no response, + = poor, ++ = moderate, CR= culture response, CI=callus induction, SS=stem segment, P=petiole, DPL=distal part of leaf, MPL=middle part of leaf, PPL=proximal part of leaf

Among the different explants sources petiole and leaf explants shows best callus initiation as compared with other explants and which was selected for the further experimental process (Table 1).

Table 2: Individual effect of auxin and cytokinin on callus induction and growth

Treatments mg/l	Intensity of callus formation			Observation
	P	MPL	PPL	
MS+IAA2.0	-	-	-	-
MS+NAA2.0	-	-	-	-
MS+2, 4-D2.0	+	+	+	Swelling followed by whitish callus on mid-rib
MS+2, 4-D4.0	+++	+++	++	Good growth of callus dorsal as well as ventral side of the explants on mid rib zone
MS+Kn1.0	+	-	-	Very little callus was observed
MS+ BAP2.0	-	-	-	-

+++ = good

Selection of best explants for optimum callus induction of *D. Retusus* from leaf and petiole in individual effect from leaf (MPL, PPL) and petiole explants of best callus regeneration in 2, 4-D 4.0 incorporated media followed by Kn1.0 with very little callus. Middle part of leaf produced good amount of callus where leaf Calli were off white in colour and formed from mid vein zone (Table 2).

Table 3: Optimization of growth regulator conc. of best selected explants for callus growth

Treatments	Callus growth of 8 months culture	
	Leaf	Petiole
MS+ 2, 4-D 2.0	++	++
MS+ 2, 4-D 4.0	+++	+++
MS+IAA 0.5 + Kn 1.0	+	+

Based on the findings the effect of auxin and cytokinin towards induction of callus for optimization of growth regulator concentrations for maximum callus growth, treatments were tested with 2, 4-D 2.0, 2, 4-D 4.0, IAA 0.5 + Kn 1.0. Callusing took place within 4 weeks of culture. A varied amount of callus growth has been recorded from the explants. All treatments had produced calli in all explants but extent and amount of callus were found to vary with the growth regulator concentrations (Table 3). Best callus regeneration was obtained from 2, 4-D 4.0mg/l incorporated media.

Table 4: Growth of embryonic callus in different concentration of 2, 4-D (2.0-6.0) mg/l

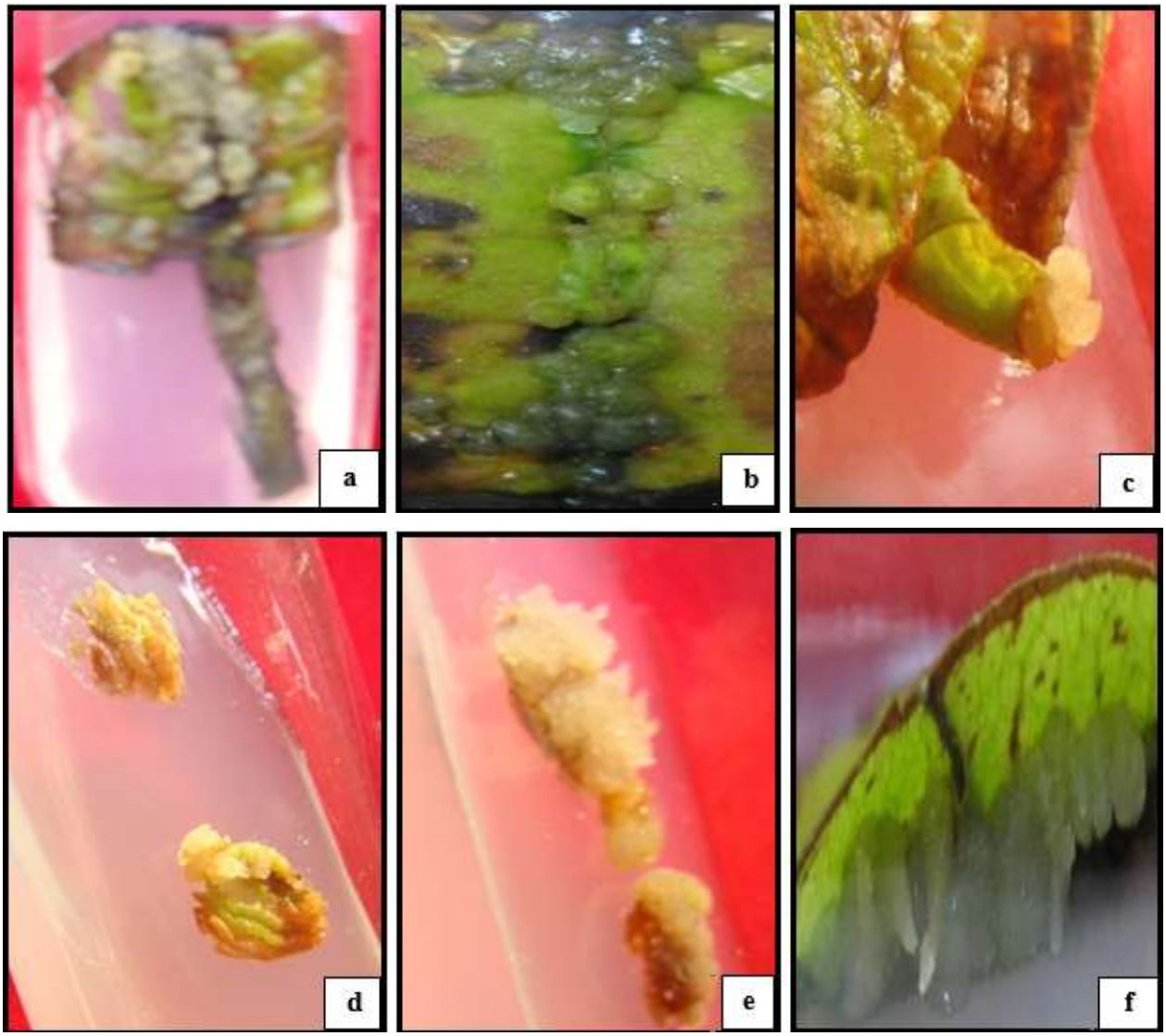
Tr. no.	Efficiency of EC	Morphology & texture
MS+2, 4D 2.0	+	White, loose
MS+2, 4D 3.0	+	White, loose
MS+2,4D 4.0	+++	Organized, compact, slow growing proliferative & soft, shiny, nodular
MS+2, 4D 6.0	++	Unorganized, very compact, hard

Supplementation of 2, 4-D with different concentration in the media it was observed that calli induced was mostly embryonic type. In respect to their explants types the result of these embryonic calli was not much varied amongst themselves of embryonic callus morphology and efficiency of calli (Table-4). It was observed that the production of embryonic callus morphology, efficiency and their growth in respect to their different 2, 4-D concentrations, 2, 4-D 2.0 and 2, 4-D 3.0 not responded for embryonic callus even after 3rd subculture. While from (2, 4-D 4.0) best embryonic callus was initiated as best. They were compact, organized and slow growing. Beyond this optimal conc. other treatment such as 2, 4-D (6.0) produced callus which was hard and non-embryonic type. The nature of leaf callus induced in the medium was soft, and compact. However, calli regenerated from petiole were cream colour, granular and embryonic type.

Table 5: Effect of media on the percentage of embryonic callus formation and culture response on leaf and petiole explants

Tr. no.	Petiole		Leaf	
	% CR	%EC	% CR	%EC
MS+2, 4-D4.0	40.0±2.6	20.7±2.8	42.5±1.6	21.4±6.6
MS+2, 4-D4.0+IAA0.5	42.3±6.0	22.8±6.2	48.7±2.5	25.9±7.4
MS+2, 4-D2.0+CH100.0	48.1±3.2	24.8±6.1	45.4±3.8	30.5±2.3
MS+2, 4–D2.0+IAA0.5	45.0±0.0	28.6±6.6	50.7±2.0	61.1±7.3
MS+2,4D2.0+CH100.0+IAA0.5	10.1±1.2	12.3±4.8	15.7±4.4	10.6±1.8

Among the all treated media proliferation of leaf calli was highest 61.1±7.3 in the media 2, 4-D 2.0+IAA 0.5 and percentage of culture response was 50.7±2.0. The lowest 10.6±1.8 was recorded from 2, 4-D 2.0+CH100.0+IAA0.5 (Table. 5).



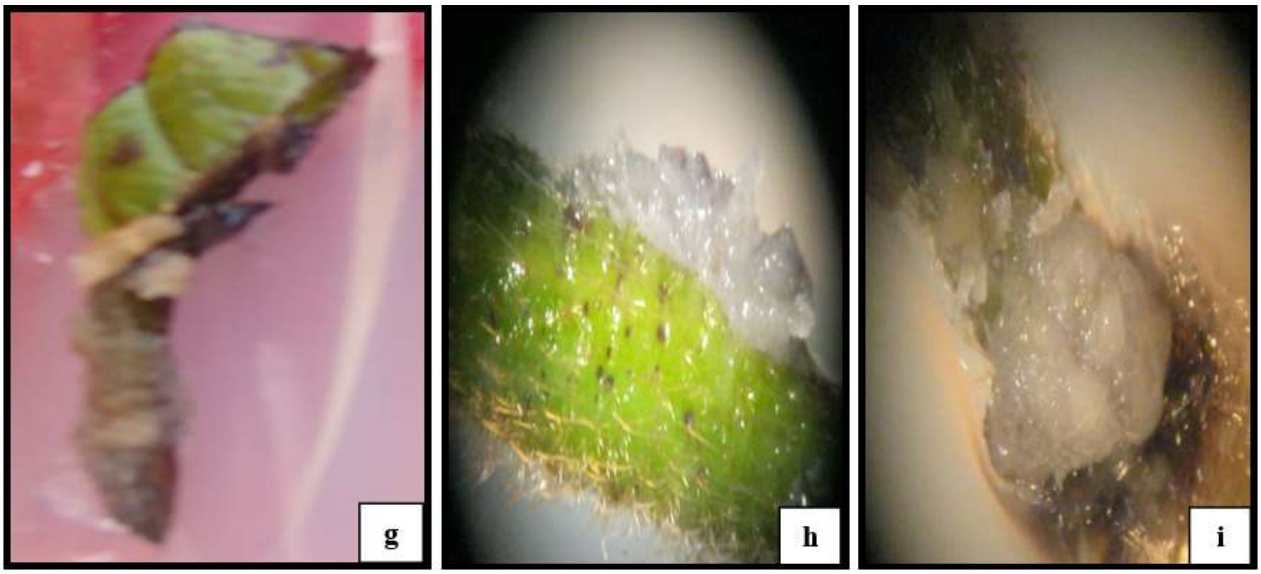
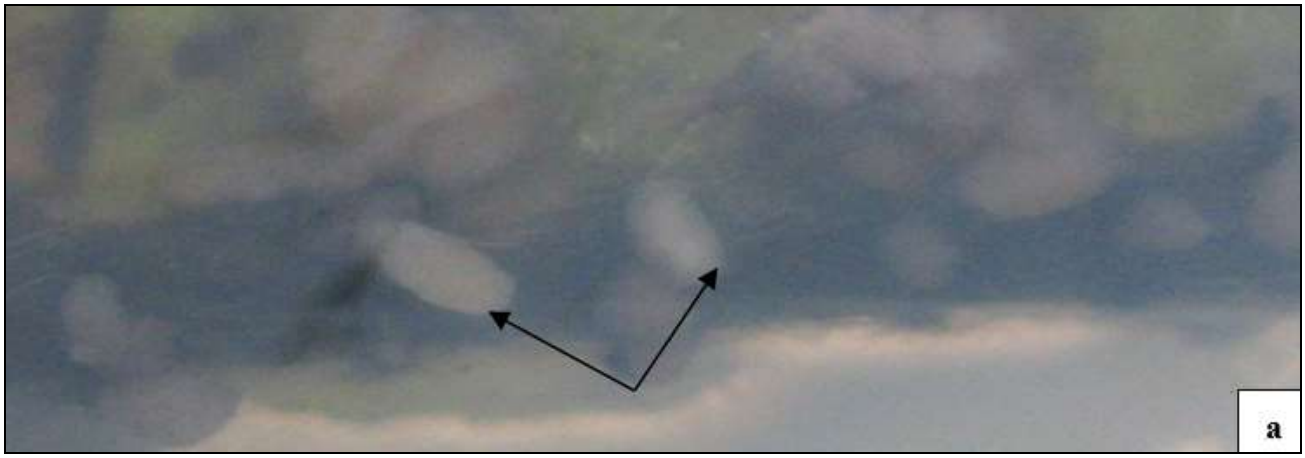


Fig 1: Embryonic callus regeneration of different parts of leaf and petiole with different morphology of *D. Retusus* a) white embryonic callus distributed on the ABXIAL side of leaf, b) grayish white globular embryonic callus restricted on leaf mid vein area, (c-e) soft cream colour proliferated embryonic leaf callus, f) deep Gray colour soft embryonic callus on adxial side of the leaf, (g-i) proliferated embryonic callus at the end of the petiole

Initially all the leafy explants underwent an extent of shrinkage of tissues while, explants like petiole not swelled and followed by formation of callus growth. In the leafy explants callusing took place at the mid vein touching the media and on the abaxial side of the mid rib portion. Callusing were restricted at the mid vein of the explants and within 12-20 days of incubation it is gradually proliferated on the abaxial surface along the mid vein region and gradually distributed over the leaf blade. Among the auxin conc. 2, 4-D (4.0) mg/l was considered as optimal doze for induction of embryonic callus and shows a high embryonic potentiality became evident which was confirmed by microscopically. With the increased concentration of 2, 4-D 6.0 callus development also increased but compact, hard and non-embryonic type. Likewise two cytokinins, BAP (2.0) and Kn (2.0) induced poor amount of callus. Presence of 2, 4-D followed by its gradual withdrawal from the culture medium favours for maturation of somatic embryos. Leaf callus produced good amount of callus off white in colour. Explants supplied with individual complex substances like CH (100.0) and YE (100.0) moderate callus initiation was observed from petiole explants.YE incorporated media leaf induced soft, compact embryonic callus and calli regenerated from petiole were embryonic type. While petiole calli were initially white, compact and later converted to granular type. Embryonic callus shiny and during gradual subculture became soft and slimy. Petiole calli proliferation was only at the proximal region and gradually extends to the mid vain region (Fig. 1, g-i). The results confirmed that part of leaf blade can influence the initiation of visible globular embryos and percentage of embryogenesis. Moreover, depending upon the incubation period percentage of embryonic callus also increased. Optimal callus induction of leaf blade and petiole explants was taken around 240 days from inoculation.

Physiological age of the explants source also played an important role for obtaining of successful results. Explants collected from 1 to 2 years old plants from green house condition produced almost similar results whereas the explants collected 5-6 years old was failed to induce callus even incorporation of selected optimal media. Monsoon season (May- August) was selected as the best period for collection of explants. In exudation of phenol in the media, different anti-oxidants were tested and absorbents at the same time could diminish sometimes. As pretreatment, explants soaking the in water for few hours and washing of explants in running tap water also controls phenols. After 2-3 days of culture, explants can sometimes be rescued by removing and re culturing on fresh medium to save calli from deep black exudation in the media.



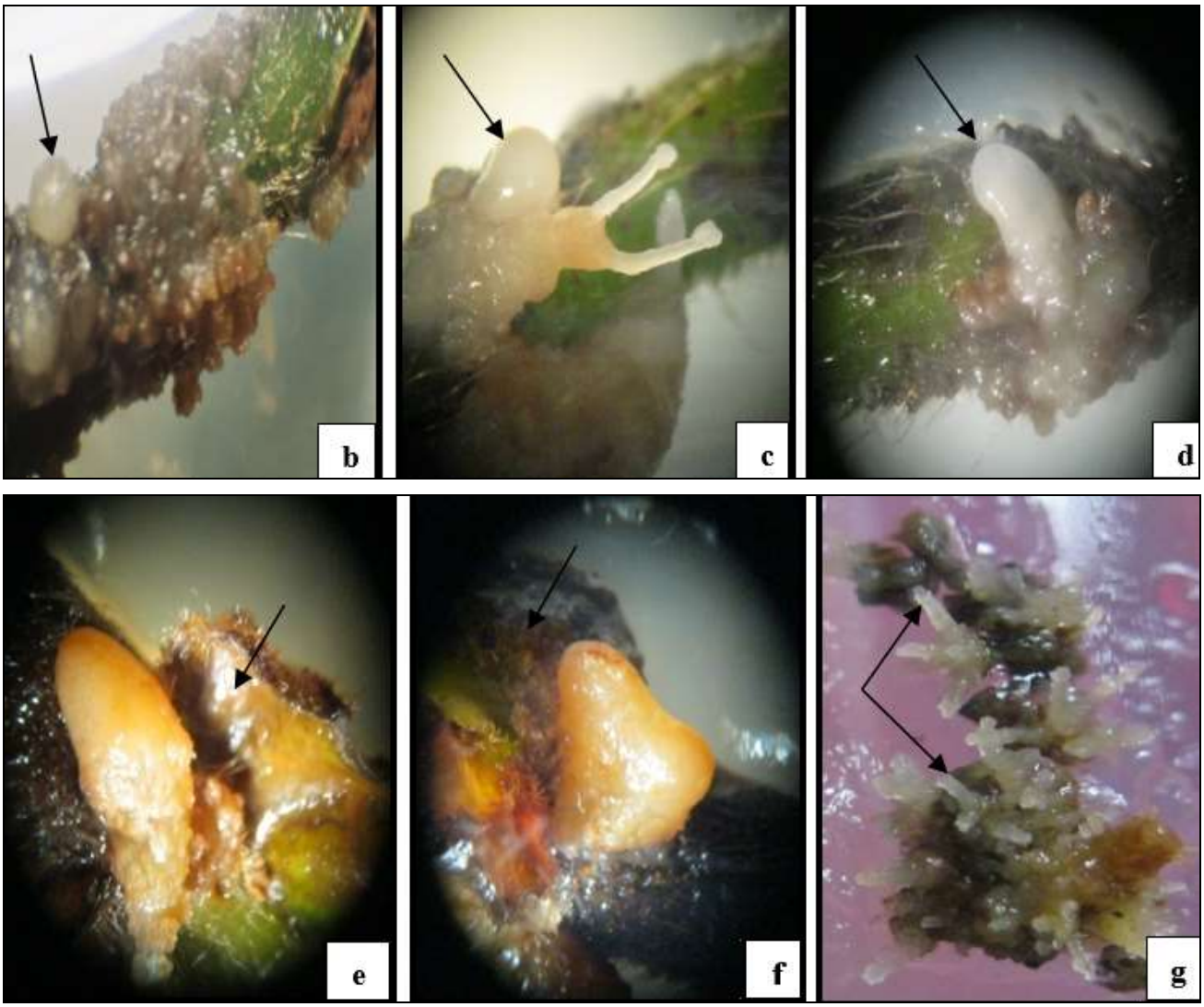
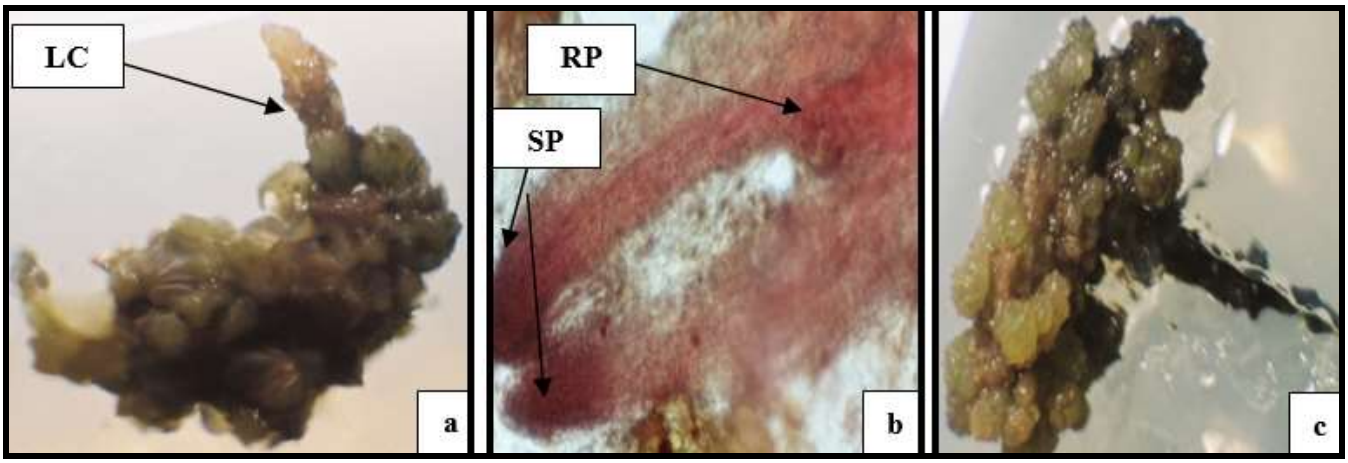


Fig 2: Different types of embryo regeneration on leaf and petiole callus of *D. Retusus* (a) Distinct globular embryo on the adaxial side of leaf callus, (b) distinct globular embryo of petiole, (c) callus late globular embryo, (d) torpedo shaped embryo, (e) heart shaped embryo, (f)late torpedo shaped embryo, (g) secondary embryogenesis of leaf callus

Calli of cut leaf and petiole explants became embryonic and proliferation of the cells occurred from 20-30 days onwards from incubation and started to form visible white embryos on 2,4-D media which were attached to the surface of the callus and induce distinct visible globular embryos after 8 weeks of serial subculture (Fig 2, a-c). These embryonic calli continued to proliferate even after 3 months of subculture. Leaf calli produced torpedo (Fig 2, d-e) to heart shaped embryos (Fig 2, f). Moreover, this frequency of visible embryo formation and percentage of culture response varied with the variant calli and according to the different media treated. Initially white petiole callus became compact and nodular (Fig. 2, b) and later formed prominent globular to torpedo shaped embryos region after gradual subculture. However, continuous exposure of these calli to 2, 4-D caused secondary embryogenesis (Fig 2, g). The initiation of secondary embryogenesis last up to 6-8 weeks. MS media supplemented with singular supplement of 2, 4-D was obtaining a secondary embryogenesis of embryos. Medium fortified with 2, 4-D2.0 proved to be most suitable for somatic embryogenesis.



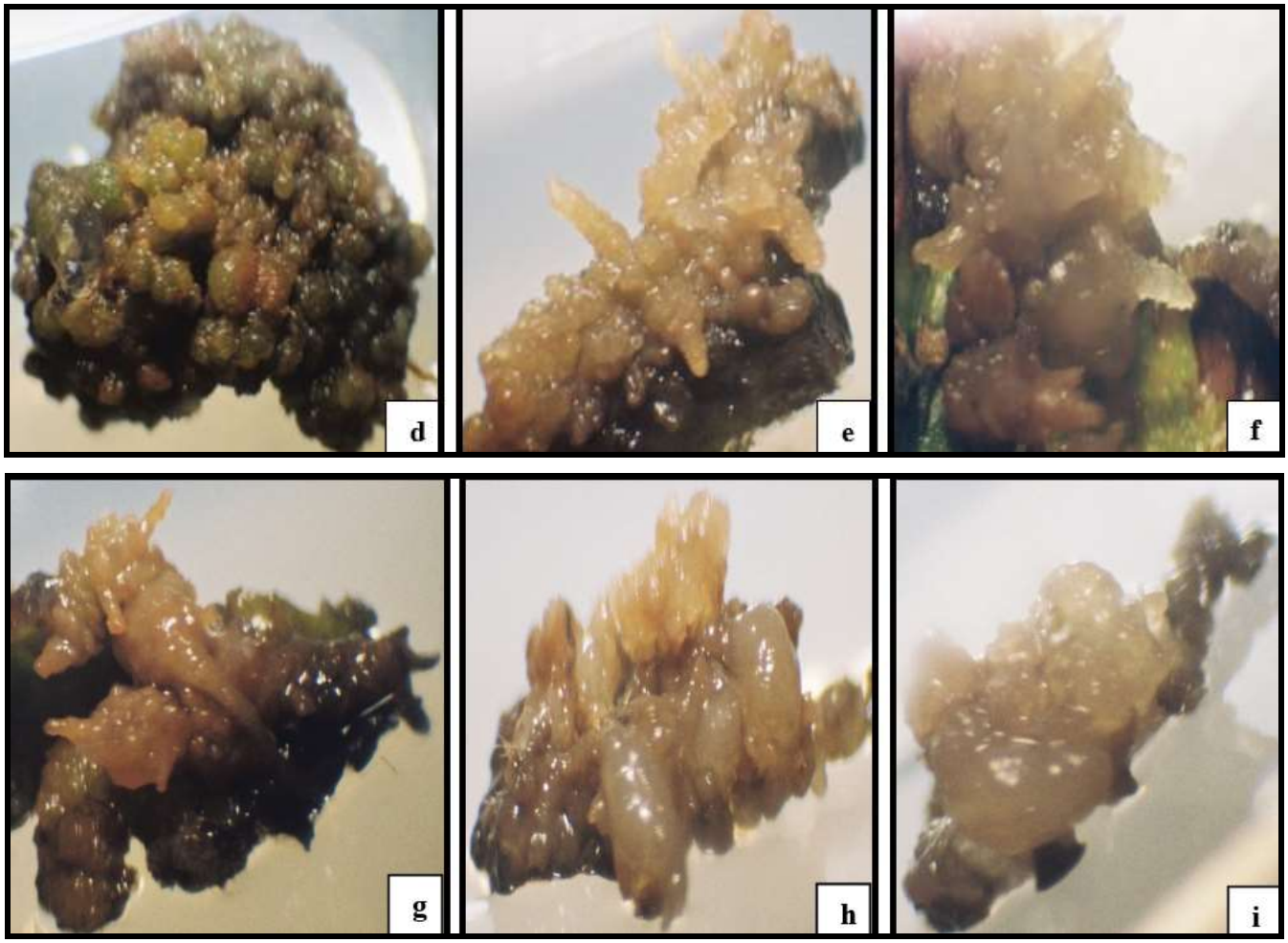


Fig 3: Shoot regeneration and late embryonic leaf callus of *D. Retusus* (a)Shoot regeneration of germinated embryo, (b) distinct root and shoot pole after anatomical study,(c-d) green callus of embryos of shoot regeneration, (e-g) embryo germination,(h-i) late mucilaginous embryonic callus. LC- leafy cotyledon, RP-root pole, SP- shoot pole

Somatic embryos gradually enlarged slightly before a gradual greening of the entire embryo. These embryos developed into small, greenish protuberances or shoot buds regenerated from the callus (Fig. 3a). Histology of embryos shows and globular proembryos were embedded in mucilaginous matrix. Distinct root and shoot pole were visible (Fig. 3, b) and these calli were composed of many embryonic cells. Embryogenesis was marked by the appearance of green globular structures (Fig 3 c-d). Germination of these embryos occurred within 4 months after the maturation of the globular embryo (Fig. 3 e-g). Most of the regenerated embryonic callus remained mucilaginous and watery embedded in a matrix when kept without sub culturing for another 2 to 3 weeks as late embryonic callus (Fig 3 h-i) and translucent even after 3 months of incubation. These calli later failed to regenerate plantlets. Both the explants germination in ½ MS medium treated as best. After 4 weeks induced somatic embryos were transferred to half-strength hormone free MS medium. In this leaf calli average 20 per cent of embryo germination was observed in the GA_3 0.1 medium up to leafy cotyledon development. On the other hand in the nutrient media supplemented with Kn, embryos produced only rhizogenesis instead of shoot formation.

Discussion

Selection of plant material is the most crucial factor in determining the success of embryogenesis and as well as the culture media. It reported like our findings that the size, source and physiological age of the explants influence the morphogenetic response (Okazawa *et al.* 1967, Raju and Mann 1970, Narayanaswamy 1977) [29, 34, 26]. Similarly, the type of the explants is also considered one of the main factors and regeneration ability is influenced to a great extent by the media composition that attracted the concern of many investigators (Amstrong and Green 1985; Bohorova *et al*., 1995 and Carvalho *et al*. 1997) [3, 8]. 2, 4-D is the most commonly used auxin for embryogenesis and other auxin may be required for certain species (Ammirato 1983) [2]. The shorter period of time that cultures are exposed to 2, 4-D may help the decrease the incidence of abnormalities that could arise from 2, 4-D treatment (Larkin and Scowcroft 1981) [18]. In case of age of the explants, Murthy and Saxena (1994) [25] recorded a similar type of effect on age of the explants in *Arachis hypogeal* that the younger plantlets were higher in their embryogenic competence while the explants of older age failed to produce somatic embryos, which indicated that the physiological age of the plant is most important in determining the embryogenic capability which is also indicated the effect of embryogenesis in our studies.

As reported earlier work on Dipterocarpaceae, most of the explants of *D. Grandiflorus* for tissue culture work were contaminated (Smits and Struycken 1983) [38]. As similar, during our experimental time we have also recorded high contamination to establish aseptic culture. Scott *et al.* (1995) [36] obtained development of axillary shoots on cotyledonary nodes and stem nodes of embryos of *Hopea odorata* and reported that excised axillary shoots formed few buds in BAP containing medium. Linington (1991) [20] also has reported *in vitro* propagation of *D. Alatus* and *D. Intricatus* where

aseptically grown seedlings in Woody Plant Medium (WPM) supplemented with the cytokinins BAP or 2-isopentyl adenine axillary shoots developed from excised cotyledonary nodes. Rapid multiplication was obtained in *D. Intricatus* and rooting obtained on an IBA containing medium. In *Shorea contorta*, callus formation was the only response in explants taken from mature dipterocarp trees although only 2-3 shoots were obtained from axillary buds (Pollisco, 1996) [32]. In *Anisoptera Thurifera*, dome like structures were produced in callus cultures and single shoots from axillary buds (Pollisco, 1994a & b) [30, 31]. *Vateria macrocarpa* nodes and shoot tip explants taken from young trees failed to respond in all the media tested. Considering growth regulator it is reported that, the specificity on the effect of various growth regulators which varies from plant to plant. This is in conformity with Monotoro *et al*. (1993) [23] who working with *Hevea brasiliensis* reported that the basic mineral media used could also modify callus texture and types in which media components and plant growth regulators are quite often responsible. As in our study level of auxin induced different type of callus same as Carrier *et al*. (1990) [7] also recorded on excess level of synthetic auxin induced friable callus on *Gingo biloba* L. Likewise, Lazzeri *et al.* (1987b) [19] reported the requirement of benzyl aminopurine enriched medium to obtain friable calli in *Glycine max* Merrill. Similar to our result, in *Prosopis Juliflora* 2, 4-diclorophenoxy acetic acids were found to be the most effective among other auxins for embryonic callus induction (Naudiwani and Ramavat 1991) [27]. Results confirmed that size and cutting of leaf blade can influence the initiation of visible globular embryos. Moreover, depending upon the incubation period percentage of embryogenic callus also increased. Both the finding supports and reported by Baker and Wetzstein, 1998 [4] that the influence of leaf size on per cent of embryogenesis. The potentiality of 2, 4-D in the production of cultured embryos was firmly established (Raghavan 2003, Choi *et al*. 2002, Kumar *et al*. 2002, Martin, 2003) [33, 11, 15, 21] that the frequency of compact callus formation was increased with the increase of 2, 4-D conc. was also reported by Vikrant and Rashid (2003) [40]. From the visual observation in our study calli shows a high embryonic potentiality became evident which was later on confirmed by microscopically. This similar result on the effect of 2, 4-D was also reported by El-Itriby *et al.* (2003) [12] in normal type E*gyptian maize*. Superior to other auxins for induction in 2, 4-D callus in many cases was reported by (Hu and Wang 1983) [13]. Moreover, in most of the plant species, presence of 2, 4- D followed by its gradual withdrawal from the culture medium favoured maturation of somatic embryos (Rangaswamy 1886, Beena and Martin 2003) [35, 5, 21]. So, during experimental time a reduced concentration of 2, 4-D 2.0mg/l was added. This finding is in agreed with those of Charleen and Hazal (1992) [10] and Sellars *et al.* (1990) [37] on leaflets of peanut and immature zygotic cotyledons of pea respectively. Stamp and Henshaw (1987) [39] reported in *Mannihot esculenta* that primary somatic embryos produced and secondary embryos are produced with additional period exposed to 2, 4-D medium. With *Medicago truncatula* secondary embryos were observed by sub-culturing single axis and bicotyledonary primary embryos in a basal medium (Nolan *et al.*1989) [28]. Prolong incubation in 2, 4-D incorporated media shows secondary embryogenesis. Embryo germination of leaf calli possesses average 20 per cent of embryo germination and was observed in the ½ MS medium. But best result was attained in GA_3 0.1 medium leafy cotyledon development and distinct root and shoot pole. This finding supports the results of Mathur *et al.* (2002) [22] in three different *Panax spp.* Gibberllins medium have showed faster maturation of somatic embryos and enhanced their planting efficiency study reported by (Rangaswamy, 1986) [35].

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