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Induction of Somatic Embryogenesis in Tamarind

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ABSTRACT

Tamarind, being a leguminous tree species, is highly recalcitrant in nature. Very little work has been done on *in vitro* regeneration of tamarind. The present report describes the conditions optimized for regeneration of plants via somatic embryogenesis using immature zygotic embryos (IZEs) as the primary explants. Optimum response (17.5%) was obtained in explants cultured in Murashige and Skoog (MS) medium supplemented with dicamba at 5.0 mg l^{-1}. The cotyledons of IZEs turned brown and necrotic. Somatic embryos (SEs) developed either singly or in multiple from the axes. The response increased to 26.7% in explants cultured in the dark in medium gelled with phytagel in culture tubes. The protocol was tested and found to be effective for IZEs collected from five different trees although the frequency of response varied with the tree. SEs proliferated when subcultured through repetitive embryogenesis but the process was slow. Histological studies confirmed the direct and multicellular origin of the SEs from epidermal and subepidermal tissue of the apical region of the IZE. Development of SEs was restricted to the bipolar and cotyledon stage. Attempts to differentiate the SEs were futile although repetitive proliferation of the SEs and rooting was sporadically noted.

Keywords: dicamba, immature zygotic embryos, repetitive embryogenesis, *Tamarindus indica*

INTRODUCTION

Biotechnological applications of tree tissue culture lags behind those of herbaceous crop species mainly because of the lack of methods for *in vitro* manipulation of tree tissues to yield high frequency plantlets from desired cells or organs. Between the two pathways of morphogenesis for regeneration of plants *in vitro*, somatic embryogenesis has several advantages over organogenesis. Some of these include high frequency regeneration, generation of unlimited number of somatic embryos (SEs) from a single explant, production and handling of thousands of SEs in liquid, etc. Somatic embryogenesis may facilitate the development of new strategies for many biotechnological approaches to plant improvement (Han and Park 1999).

Tamarind (*Tamarindus indica* L.), a multipurpose leguminous tree species, well adapted to semi-arid tropical regions and is a species of choice for sustainable development of wasteland. It is used as food, food preservatives, fodder, drugs, timber and firewood. Tamarind fruit pulp is very rich in ascorbic and tartaric acids and is the most commonly used preservative in pickle industry.

Methods for manipulation of tamarind tissue *in vitro* have been restricted to shoot regeneration from meristematic buds and nodal explants of seedlings and mature trees (Mascarenhas *et al.* 1981; Mascarenhas *et al.* 1987; Jaiwal and Gulati 1991; Jaiwal and Gulati 1992; Sonia *et al.* 1998; Mehta *et al.* 2000) and *de novo* organogenesis from seedlings germinated in thidiazuron (Mehta *et al.* 2004, 2005; Mehta and Hazra 2007). However, there is no report on somatic embryogenesis in this species. This report describes a method for the induction of somatic embryogenesis and demonstrates the origin of these SEs in this recalcitrant and callogenic species. The extensive experimentation carried out to attain the optimum condition for induction of embryogenesis is included in this report.

MATERIALS AND METHODS

Green and tender pods of tamarind (*Tamarindus indica* L.) were collected from locally grown trees. These were treated with 0.1% mercuric chloride ($HgCl_2$) for 15 min. for surface sterilization. The adhering $HgCl_2$ was removed aseptically by washing the pods 4-5 times with sterile water. The pods were cut longitudinally with a sharp blade. The immature seeds were isolated and the white, tender seed coat was removed. Varying concentrations (0.5-10.0 mg l^{-1}) of different growth regulators including 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), di-chloro-o-anisic acid (dicamba) and 4-amino-3,5,6-trichloro picolinic acid (picloram) were tested by supplementing in Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962). Four immature zygotic embryos (IZE) were cultured in each tube. Cultures were incubated in 16 h photoperiod at 25 ± 2°C for 8 weeks.

To determine the appropriate size of IZEs for optimum morphogenic potential IZEs were measured with a scale placed in the laminar airflow. Different sizes of IZE ranging from 1-10 mm were cultured in tubes containing MS basal medium supplemented with dicamba at 5.0 mg l^{-1}, sucrose 60.0 g l^{-1} and phytagel 2.2 g l^{-1}.

Studies on optimization of various parameters were conducted with IZEs 1-6 mm in size. Subsequently influence of culture vessels, gelling agent and light conditions were tested. Media were gelled using either agar (Qualigens Fine Chemicals, India) or Phytagel™. Media (20 ml) with agar or phytagel were distributed in Borosil make, cotton plugged glass tubes (25 × 150 mm) or in disposable plastic Petri dishes (10 ml) (55 mm × 15 mm). Four IZEs were cultured in each tube and six IZEs were cultured in each Petri dish. The cultures were divided into two groups. One group was incubated in dark whereas the other group was incubated in 16 h light at 25 ± 2°C for 60 days.

The protocol for induction of somatic embryogenesis was extended to IZEs of size 1-6 mm, which were collected from five different trees to test their viability. The trees were identified as I, II, III, IV and V.

Cultures with SEs were shifted to fresh medium along with the original explant for further proliferation in MS basal medium supplemented with dicamba at 5.0 mg l^{-1}, sucrose 60.0 g l^{-1} and

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phytagel 2.2 g l^{-1} and subcultured on the same fresh medium every 60 days.

Histological studies were carried out to determine the origin and morphological status of the SEs. Tissues were fixed in formalin: acetic acid: alcohol (FAA; 5: 5: 90) for 72 h and processed following the method described by Sharma and Sharma (1980). The 10 µm sections were double stained using haematoxylene and eosin.

SEs were transferred on to MS basal medium with or without charcoal, with 10% sucrose, lower concentration of dicamba and ABA to achieve maturation, germination and conversion. Media supplemented with different cytokinins including KN, BAP, zeatin, TDZ alone or in combinations were also tested for conversion of the SEs.

Data on the embryogenic response in IZEs were transformed and subjected to analysis of variance (ANOVA) and the treatment means were compared using Duncan's multiple range test (DMRT) wherever required.

RESULTS AND DISCUSSION

Flowering in tamarind starts in May. Until the end of August the IZE was jelly-like and the seed coat was too tender to be removed without damaging the IZE. Therefore the pods were collected in the period from the first week of September to the first week of October. Thus the estimated ages of the IZEs used were of 12-16 weeks.

The results of the experiments conducted to identify the suitable auxin for SE in tamarind is included in **Table 1**. Varied responses were noted in the explants. In MS medium the cotyledons and radicle turned brown and necrotic. In some explants the plumule was elongated, whereas in some,

Table 1 Effect of different plant growth regulators on induction of somatic embryogenesis in immature zygotic embryos of tamarind.

Medium (PGR conc. in mg l^{-1})	Frequency of response (%)	Frequency of response in form of multiple embryos (%)
2,4-D		
0.5 (80)	1.3 ± 1.8 de	1.3 ± 1.8 ab
1.0 (80)	2.5 ± 3.5 cde	1.3 ± 1.8 ab
3.0 (80)	1.3 ± 1.8 de	0.0 ± 0.0 b
5.0 (80)	1.3 ± 1.8 de	0.0 ± 0.0 b
10.0 (80)	0.0 ± 0.0 e	0.0 ± 0.0 b
NAA		
0.5 (80)	7.5 ± 10.6 bcd	1.3 ± 1.8 ab
1.0 (80)	8.8 ± 12.4 abcd	1.3 ± 1.8 ab
3.0 (80)	6.3 ± 8.8 bcde	1.3 ± 1.8 ab
5.0 (80)	1.3 ± 1.8 de	0.0 ± 0.0 b
10.0 (80)	1.3 ± 1.8 de	0.0 ± 0.0 b
2,4,5-T		
0.5 (80)	11.3 ± 1.8 ab	1.3 ± 1.8 ab
1.0 (80)	12.5 ± 3.5 ab	1.3 ± 1.8 ab
3.0 (80)	3.8 ± 1.8 bcde	0.0 ± 0.0 b
5.0 (80)	1.3 ± 1.8 de	0.0 ± 0.0 b
10.0 (80)	0.0 ± 0.0 e	0.0 ± 0.0 b
Dicamba		
0.5 (99)	6.6 ± 3.0 abc	0.0 ± 0.0 b
1.0 (95)	11.1 ± 6.0 ab	0.0 ± 0.0 b
3.0 (151)	7.4 ± 3.0 abc	1.2 ± 1.3 ab
5.0 (234)	17.5 ± 10.4 a	3.1 ± 2.8 a
10.0 (95)	6.8 ± 5.1 abc	0.8 ± 1.4 b
Picloram		
0.5 (80)	12.5 ± 0.0 ab	0.0 ± 0.0 b
1.0 (80)	14.1 ± 11.1 ab	0.0 ± 0.0 b
3.0 (80)	4.7 ± 2.2 abcd	0.0 ± 0.0 b
5.0 (80)	0.0 ± 0.0 e	0.0 ± 0.0 b
10.0 (80)	0.0 ± 0.0 e	0.0 ± 0.0 b
MS		
(80)	0.0 ± 0.0 e	0.0 ± 0.0 b

All values means ± S.D. Figures in parenthesis indicate numbers of replicates. Each treatment was repeated at least twice. Data was subjected to transformation for statistical analysis. Means in each column followed by different letters are significantly different according to DMRT at $P < 0.05$.

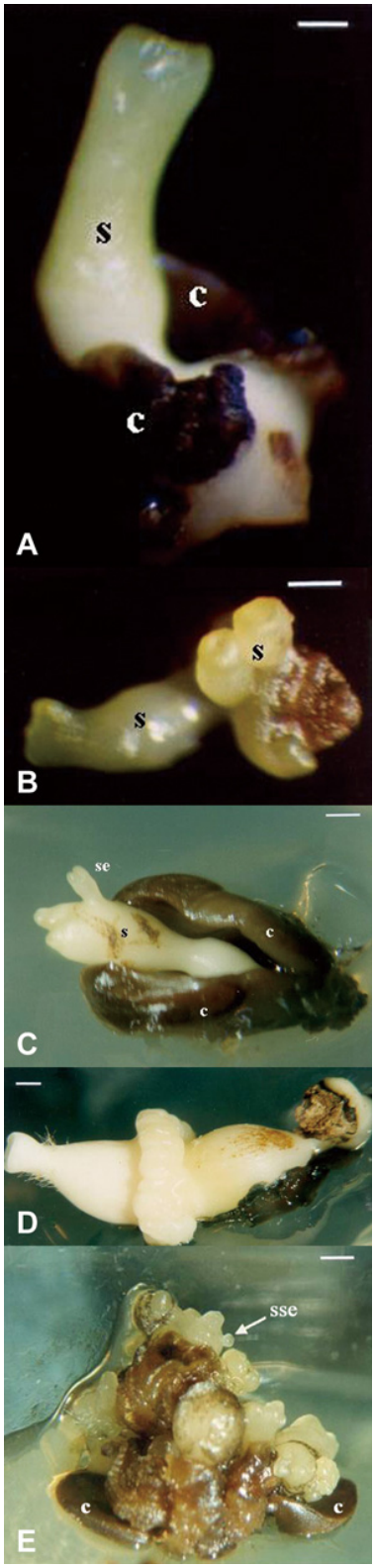

Fig. 1 Immature zygotic embryo (IZE) cultured in 5.0 mg l^{-1} dicamba. (**A**) Cotyledons (c) of the IZEs turned brown and necrotic, plumule developed to form structures (s) similar to bipolar somatic embryo (SE) with fused cotyledons. The base of the structures were constricted. Hypocotyl of IZE was swollen and radicle did not differentiate (bar = 500 µm). (**B**) SE-like multiple structures developed from the IZE in 5.0 mg l^{-1} dicamba. The structures initiated from the plumular region of the IZE and from the cotyledonary nodal region (bar = 660 µm). (**C**) Primary SEs (se) developing from the outer surface of the plumular region of the embryogenic structure (s) in 5.0 mg l^{-1} (bar = 588 µm). (**D**) Primary SEs developing from the epicotyl region of the embryogenic structure in 5.0 mg l^{-1} dicamba (bar = 500 µm). (**E**) Secondary/repetitive somatic embryogenesis in the cultures maintained by repeated subcultures, due to slow proliferation of the secondary SEs the cotyledons, which turned brown in the initial stage of culture, could still be seen with the cluster of SEs after one year in culture (bar = 1000 µm).

the hypocotyl region elongated. However, these axes neither differentiated nor produced SEs.

Some common responses were noted in IZEs. In all the auxins, at all the concentrations, radicle differentiation was restricted and the cotyledons turned brown. Only the IZE axis responded differently in various media. Initially in the responding IZEs, a white, glistening mass was formed at the plumule end. These masses were of various shapes and sizes. Often these were tubular in shape with swelling around the middle region. Subsequently SEs developed from these masses. Therefore any growth or swelling in the IZEs was scored as response (**Table 1**). In 2,4-D the response of the axis was very poor (1.3-2.5%) and the cotyledons did not open. However, in 1.3% of the responding SEs multiple SEs were also seen. Compared to 2,4-D, in NAA more IZE axes (1.3-8.8%) partially elongated and in some of the cultures a pair of fleshy leaves differentiated from the plumule of the IZE axis. Similar to 2,4-D, multiple SEs were seen in some of the responding IZEs. In 2,4,5-T and dicamba treatments the response was similar but the frequency of response varied greatly. In these two auxins, the plumule of IZE swelled in 30-40 days to develop into structures similar to torpedo/cotyledonary stage SEs (**Fig. 1A**). The base of these structures was constricted near the cotyledonary node and swollen around the middle region. Tips of these structures were either tapering or funnel-shaped. On extended incubation of these structures in the same media for 30-40 days SEs appeared from the cotyledonary nodes of the IZE and the swollen plumular end. The swollen IZE, together with these newly developed SEs, were identified as multiple SEs (**Fig. 1B**). The frequency of response decreased as the 2,4,5-T concentration increased. In dicamba, the frequency of response varied from 6.6 to 17.5%. Both the frequency of response and the frequency of multiple SE formation were highest for dicamba at 5.0 mg l^{-1}. For picloram, only lower concentrations were more effective in inducing a response in the IZE, and were totally ineffective at higher concentrations. Since, the response was highest (17.5%) in 5.0 mg l^{-1} dicamba and more IZEs gave rise to multiple SEs, this concentration was used in the subsequent experiments to study the effect of various other factors.

The embryogenic response was in two forms: single and multiple structures. For dicamba at 5.0 mg l^{-1}, out of 17.5% total response, 14.3% of the cultures were single structures of various shapes (**Fig. 1A**) whereas 3.1% of the cultures showed multiple SEs (**Fig. 1B**). For 2,4-D (0.5 and 1.0 mg l^{-1}), NAA (0.5-3.0 mg l^{-1}) and 2,4,5-T (0.5 and 1.0 mg l^{-1}), multiple SE formation was noted at frequencies lower than that obtained in dicamba at 5.0 mg l^{-1}. Picloram inhibited the formation of multiple SEs at all concentrations tested.

Though dicamba has mostly been used in monocots to obtain embryogenic callus, it was used by Finer (1987) to induce SEs from immature embryos of hybrid sunflower (*Helianthus annus* L.). The effect of dicamba on the frequency of SE was also noted in a grain legume, *Cicer arietinum* L. (Eapen and George 1994). From the five growth regulators tested in the present experiment, dicamba was found to be most effective in inducing embryogenic response in the IZEs of tamarind without involving a callus phase. To the best of our knowledge, dicamba was used for the first time for induction of direct embryogenesis in a woody legume.

Table 2 Influence of the stage of development of IZE on induction of somatic embryogenesis in tamarind.

Size of explants (mm)	Frequency of response (%)
1 (19)	10.4 ± 15.3 ab
2 (143)	15.6 ± 15.3 a
3 (333)	18.7 ± 18.2 a
4 (368)	12.6 ± 9.2 a
5 (312)	6.3 ± 10.4 ab
6 (242)	0.6 ± 2.1 b
7 (143)	0.0 ± 0.0 b
8 (86)	0.0 ± 0.0 b
9 (15)	0.0 ± 0.0 b
10 (4)	0.0 ± 0.0 b

All values means ± S.D. Figures in parenthesis indicate numbers of replicates. Each treatment was repeated at least thrice. Data was subjected to transformation for statistical analysis. Means in each column followed by different letters are significantly different according to DMRT at $P < 0.05$.

The days of post-anthesis were not available to identify the developmental stage of the IZE used for culture. Therefore size was considered for identification of the IZE with highest morphological potential. Explants ranging from 1 mm to 6 mm were responsive while explants from 7 mm size upwards produced phenolics and turned brown. Among the different sizes of explants, embryogenic response was highest in explants of 3 mm size (**Table 2**). The IZEs above 6 mm in size did not respond in culture even after extended incubation of 8-9 weeks. There was no callusing either. The IZEs turned brown and necrotic. These results emphasize the importance of the developmental status of the IZE on induction of somatic embryogenesis in tamarind.

For this experiment, tamarind pods were collected from the first week of September to the first week of October. This limits the period for availability of desired explant material to practically 4-5 weeks only, for optimal response.

It is apparent (**Tables 1, 2**) that the embryogenic response in the IZE of tamarind was poor and inconsistent. Therefore an experiment was conducted to optimize the culture conditions to enhance embryogenesis in tamarind. Keeping in view the limited availability of IZEs at the appropriate size in the particular season several parameters, including type of culture vessel, gelling agent and light conditions were tested in a single experiment.

The response induced in culture is the result of an integrated effect of the nature of the explant, chemical factors in form of nutrients and growth regulators and physical factors such as microenvironment of the culture, temperature, light, gelling agent, etc. Data were subjected to a 3-way ANOVA at P=0.05. Irrespective of the culture vessel the embryogenic response in IZEs is higher in cultures incubated in the dark. Between tubes and Petri dishes, the response was consistently higher in the tubes compared to Petri dishes; between phytagel and agar, the response was higher in the former. However, none of the explants cultured in Petri dishes formed multiple SEs. Thus the optimum embryogenic res-

Table 3 Influence of culture conditions (culture vessel, gelling agent, light and dark) on induction of somatic embryogenesis in tamarind.

Treatments				Frequency of response (%)	Frequency of response in form of multiple embryos (%)
Tube	Agar	Light 16 h	(60)	6.7 ± 11.6 b	0.0 ± 0.0 b
	Phytagel		(340)	13.4 ± 6.8 a	2.7 ± 1.9 a
Petri dish	Agar		(54)	1.9 ± 3.2 b	0.0 ± 0.0 b
	Phytagel		(66)	4.4 ± 7.7 b	0.0 ± 0.0 b
	Agar	Dark	(48)	17.6 ± 13.7 a	0.0 ± 0.0 b
	Phytagel		(54)	19.0 ± 7.9 a	0.0 ± 0.0 b
Tube	Agar		(60)	25.0 ± 13.2 a	1.7 ± 2.9 ab
	Phytagel		(60)	26.7 ± 18.9 a	5.0 ± 8.7 a

All values means ± S.D. Figures in parenthesis indicate numbers of replicates. Each treatment was repeated at least thrice. Data was subjected to transformation for statistical analysis. Treatments were compared using 3-way ANOVA at 5% level. For embryogenic response, treatment effect of culture vessel was nonsignificant. Gelling agent and Light factor found to be significant with CD ($P < 0.05$) = 0.31.

Table 4 Effect of genotype on embryogenic response in immature zygotic embryos of tamarind.

Genotype	Frequency of response (%)
I (171)	21.6 ± 7.0 ab
II (280)	28.0 ± 13.3 a
III (1083)	4.3 ± 3.3 c
IV (232)	14.2 ± 6.5 ab
V (248)	6.3 ± 1.1 bc

All values means ± S.D. Figures in parenthesis indicate numbers of replicates. Each treatment was repeated at least thrice. Data was subjected to transformation for statistical analysis. Means in each column followed by different letters are significantly different according to DMRT at $P < 0.05$.

ponse of 26.7% was induced in IZEs of tamarind when they were cultured in the dark on medium in cotton plugged tubes, in medium gelled with phytagel. Out of this, 21.7% were single SEs while 5% were multiple SEs (**Table 3**).

SEs of tamarind formed at the tip of the plumule (**Fig. 1C**) or from the epicotyl region (**Fig. 1D**) of the embryogenic IZE. The growth and differentiation of the embryogenic IZE with or without SEs is restricted. Instead, the SEs also turned into glistening masses. Subsequently new SEs developed from these structures.

The establishment and maintenance of morphogenic cultures for leguminous species is often genotype dependent (Oelck and Scheider 1983). In this study, we observed that explants from all plants responded in culture at different frequencies. Plant number II was most responsive while plant number III was least reactive (**Table 4**). This confirms earlier observations in other leguminous tree species e.g. *Cercis canadensis* L. (Trigiano *et al.* 1988) and *Robinia pseudoacacia* L. (Arrillaga *et al.* 1994) that the embryogenic response is genotype dependent.

Secondary/repetitive somatic embryogenesis

Cultures formed multiple structures when these, together with the original explant, were shifted and subcultured on dicamba at 5.0 mg l^{-1} with sucrose 60.0 g l^{-1} and phytagel 2.2 g l^{-1}. On this medium secondary SEs (**Fig. 1E**) could be proliferated for more than two years although the rate of proliferation was very slow.

Histological studies of somatic embryos

In the histological preparation of IZE explants (**Fig. 2A**), an IZE axis with two attached cotyledons could be seen. The plumule and the radicle of the IZE axis were distinguished by the presence of meristematic cells. On exposing these explants to medium containing dicamba at 5.0 mg l^{-1}, the differentiation of plumule and radicle were restricted. The apical meristem of the IZE, which was determined to form the shoot primordia, changes the course of development to form an elongated, fleshy, glistening, white, cellular structure. These structures often appeared like torpedo stage or cotyledonary stage (funnel-shaped) SEs. This gave the impression of the formation of a SE from the plumule of the IZE. On fixing the IZE-derived structures in fixative (FAA) the cotyledons, which were brown and brittle, broke off. In the section of the torpedo-shaped IZE axis it is apparent that the cells of the apical meristem proliferated to form a fleshy embryogenic structure with small, compact, meristematic cells with dense cytoplasm and prominently stained nuclei. However, the integrity and differentiation of vasculature was maintained. Therefore the strand of vasculature could be seen passing through this embryogenic structure. On examination of the funnel-shaped structures it appeared that the tip developed a cup-like structure. It was observed that the vascular connection was maintained from the tip of the structure to the radicle end. The cotyledonary nodal region or the portion of epicotyl immediately above the node was swollen due to the proliferation of the cells in the subepidermal layers and in the central part of the IZE.

Histological sections revealed a broad origin of the SEs

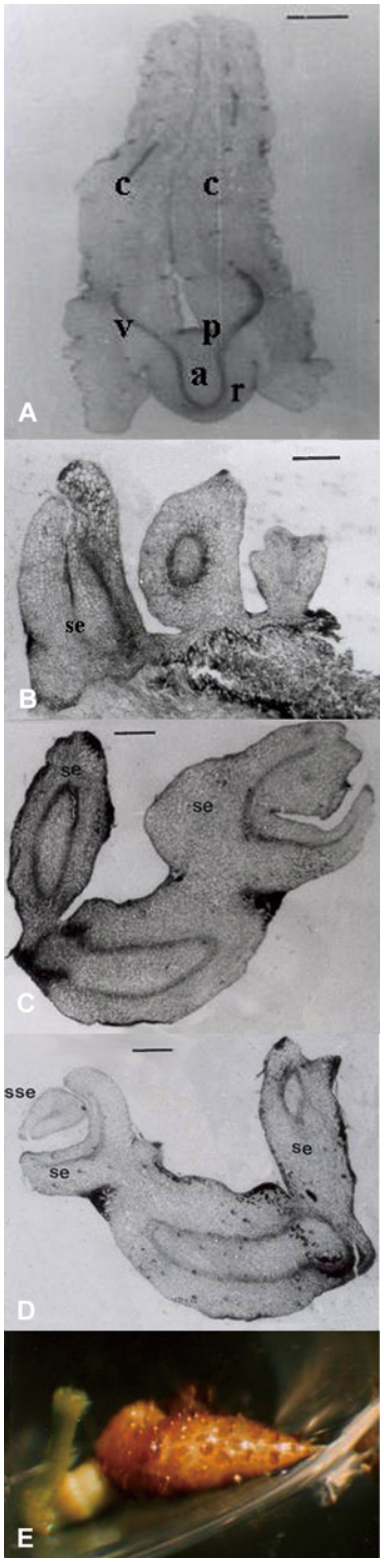

Fig. 2 (**A**) Explant IZE on histological examination show two cotyledons (c) attached to the embryo axis (a). The embryo axis shows a vascular strand (v) diverting into the two cotyledons. The plumule (p) and the radice (r) of the embryo axis shows highly concentrated meristematic cells (bar = 500 μm). (**B**) Asynchronous development of SEs which are multicellular in origin. No vascular connection between the SEs and the IZE (bar = 227 μm). (**C**) SE developed from both apical meristem and cotyledonary nodal junction. In this cluster, the SE developed from the nodal region was torpedo-shaped whereas that developed from the tip was at the cotyledonary stage. These SEs appear to originate directly from the epidermal and subepidermal layers of the embryogenic structure. The SEs are multicellular in origin (bar = 250 μm). (**D**) Formation of secondary SE (sse) from the tip of the primary SEs (bar = 250 μm). (**E**) Germinating SE in half-strength MS basal media supplemented with 0.25% activated charcoal. Structures in (**B**), (**C**), (**D**) and (**E**) developed on IZEs in the presence of 5.0 mg l^{-1} dicamba.

suggesting their multicellular origin. Embryogenesis was asynchronous. SEs at various stages of development were noted in the same section (**Fig. 2B**). No vascular connection was seen between the IZE and the SEs that developed from them.

In some of the IZE-derived embryogenic structures primary SEs developed from the apical region. Histological studies on structures with multiple SEs confirmed that these developed from both the apical region and the cotyledonary node region (**Fig. 2C**). The SEs appeared to originate directly from epidermal and subepidermal layers of the explant with no visible callus, suggesting that the cells of the meristematic region may be preembryonically determined cells (PEDCs) (Sharp *et al.* 1982). It also confirms that the SEs are multicellular in origin. The formation of secondary SEs from the primary SE was also confirmed by histological studies (**Fig. 2D**).

Maturation/germination/conversion of somatic embryos

SEs, developed from the IZE explants, were subjected to various treatments for further morphogenetic development to achieve maturation and conversion of SEs to plantlets. The treatments included basal medium with or without activated charcoal (AC), basal medium with 10% sucrose, medium with reduced dicamba concentrations, media supplemented with different cytokinins like KN, BAP, Zeatin, TDZ singly or in combinations or ABA treatment. Rooting was observed in the embryogenic structures sporadically in the following media: (i) Half strength MS basal medium → Half strength MS basal medium supplemented with 0.25% AC; (ii) MS basal medium supplemented with 10% sucrose; (iii) MS basal medium supplemented with 0.1 mg l^{-1} ABA; (iv) MS basal medium supplemented with 0.3% phytagel; (v) MS basal medium supplemented with NAA 10.0 mg l^{-1} → half-strength MS basal medium.

However, in most cases the shoot did not differentiate and the root also was thick and stunted (**Fig. 2E**), which deteriorated.

There are several reports describing apparently well formed SEs that failed to produce plantlets (Ammirato 1987). In several legumes species, SE development and conversion are not always successful. Precocious germination and/or aberrant SE development is a common problem with legume cultures (Venkatachalam *et al.* 2003). More effort should be given to develop repetitive embryogenic systems with high frequency of germination and regeneration, since such systems will find immediate application in mass propagation and other crop improvement programmes (Lakshmanan and Taji 2000).

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