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Somatic embryogenesis and plant regeneration in pigeonpea

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Abstract

Somatic embryogenesis in pigeonpea [*Cajanus cajan* (L.) Millsp.] has been achieved using cotyledon segments of mature seeds as explants. A large number of globular somatic embryos were induced directly from cotyledons of genotypes T-15-15, GAUT-82-90 and GAUT-82-99 when cultured on EC6 basal medium supplemented with 2.22, 4.44, 13.32 or 22.2 μM N^6-benzylaminopurine (BAP) and 0.45, 1.36, 2.27, 4.54 and 13.62 μM thidiazuron. Somatic embryos developed into cotyledonary stage when the globular embryos were transferred to Murashige and Skoog's (MS) basal medium containing 2.89 - 14.43 μM gibberellic acid. Maturation of somatic embryos was achieved on half strength MS medium with 0.38 μM abscisic acid. The mature somatic embryos were germinated on MS medium supplemented with 0.44 μM BAP and the plantlets were hardened and transferred to soil.

Additional key words: abscisic acid, *Cajanus cajan*, cotyledon, EC6 medium, gibberellic acid, MS medium, N^6-benzylaminopurine, thidiazuron.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.), a major grain legume of the semi-arid tropics and sub-tropics, is rich in dietary protein. Even though there are reports of somatic embryogenesis in pigeonpea, complete plant regeneration protocols are very few. Though somatic embryos have been induced from immature cotyledons and embryonal axes, well-developed plants could not be derived (George and Eapen 1994). Mallikarjuna *et al.* (1996) observed somatic embryogenesis on various explants of pigeonpea, but transfer of plantlets to soil was not achieved. Haploid somatic embryogenesis and production of embryos upto globular stage has been reported using anther culture technique (Bajaj *et al.* 1980). Somatic embryogenesis was reported in leaf and cotyledon explants cultured on a thidiazuron containing medium (Sreenivasu *et al.* 1998). Patel *et al.* (1994) induced somatic embryogenesis from cotyledons on high cytokinin medium, but transfer of plantlets to the field could not be obtained. Anbazhagan and Ganapathi (1999) obtained somatic embryos from suspension cultures derived from leaf callus. In all these previous reports various developmental (morphological) stages were not reported and no histological studies were made to demonstrate the development of somatic embryos.

This report describes observations on somatic embryogenesis from cotyledon explant of the pigeonpea genotypes and the subsequent development of plants.

Materials and methods

Seeds of pigeonpea [*Cajanus cajan* (L.) Millsp.] genotypes T-15-15, GAUT-82-90 and GAUT-82-99 were obtained from Pulses Scheme, Model Farm, Gujarat Agricultural University, Baroda, Gujarat, India. The seeds

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Abbreviations: ABA - abscisic acid; AdS - adenine sulphate; BAP - N^6-benzylaminopurine; EC6 - Maheswaran and Williams (1984) medium; GA_3 - gibberellic acid; MS - Murashige and Skoog (1962) medium; TDZ - thidiazuron.

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were surface sterilized with 70 % (v/v) ethanol for 1 min, 0.1 % $HgCl_2$ (*E. Merck*, Mumbai, India) for 5 min and rinsed with sterile distilled water 4 - 5 times. The seeds were soaked in sterile distilled water for 18 h in dark at 28 ± 2 °C and kept on gyratory shaker at 200 rpm. Cotyledons were removed from the pre-soaked seeds, split into halves and the distal halves (3.5 - 4.0 mm^2 size) of the cotyledons were cultured (2 explants per tube) for a period of 4 weeks on EC6 basal medium with 3 % sucrose and 0.8 % agar-agar (*Qualigens*, Mumbai, India) for induction of somatic embryos. The medium was supplemented with various concentrations of BAP or TDZ. A total of 20 explants were used per treatment and the experiment was replicated thrice. The explants forming globular embryos were then transferred to same medium or to EC6 basal medium supplemented with 0.38 µM ABA or to 2.89 µM GA_3 or to MS basal medium with 0.38 µM ABA or 2.89 µM GA_3. The cotyledonary embryos produced on MS basal medium supplemented with 2.89 µM GA_3 were cultured for one week on MS medium with 0.38 µM ABA for maturation. The mature somatic embryos were then shifted to MS medium containing 0.44 µM BAP for germination and conversion to plantlets. Fully converted embryos with well defined root and shoot apex were transferred to half strength MS medium with 3 % sucrose and 0.2 % *Phytagel* (*Sigma*, St. Louis, USA) for further elongation of roots and shoots. The pH of all the media used during the experiments was adjusted to 5.8. The media were dispensed in 150 mm height culture tubes (*Borosil*, Mumbai, India) and autoclaved at 1.4 kg cm^{-2} for 20 min. All cultures were incubated at 25 ± 2 °C under cool white fluorescent light (38 µmol m^{-2} s^{-1}) under a 16-h photoperiod. The plantlets were hardened in pots containing soil:vermiculite (1:1) mixture in a hardening room at 25 ± 2 °C and relative humidity 60 ± 5 %. The data were analyzed using Fisher's analysis of variance technique for a completely randomized design and the treatment means were compared (Panse and Sukhatme 1967).

For histological confirmation of the origin and structure of somatic embryos, the explants were fixed in formalin:acetic acid:alcohol (5:5:90 v/v) for 72 h at various stages of development. Tissues were dehydrated through a t-butanol series. Paraffin embedding of tissue samples was done as described by Sharma and Sharma (1980). Sections of 10 µM thickness were cut, stained with hematoxylin-eosin and mounted with DPX-4 1889 [2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide] mountant and observed microscopically.

Results and discussion

The cotyledons of genotypes T-15-15, GAUT-82-90 and GAUT-82-99 cultured on EC6 basal media fortified with various concentrations of BAP produced a large number of globular somatic embryos directly on the surface of the cotyledons. The response of cotyledons forming globular embryos varied from 53 - 76 % in the genotype GAUT-82-90, 62 - 80 % in T-15-15 and 40 - 60 % in GAUT-82-99. The average number of globular embryos per explant ranged from 13.9 to 27.6, 10.8 to 16.7 and 9.0 to 18.3 in genotypes GAUT-82-90, T-15-15 and GAUT-82-99, respectively (Table 1).

The maximum number of globular embryos formed as well as the highest percentage response of explants forming globular embryos was observed on 4.44 µM BAP, except for the percentage response in GAUT-82-90 (Table 1). The response of cotyledons to TDZ in forming globular embryos varied from 74 to 97 % in the genotype GAUT-82-90, 75 to 91 % in the genotype T-15-15 and 62 - 92 % in the genotype GAUT-82-99. The genotype GAUT-82-90 produced 19.2 to 38.4 globular embryos per explant while T-15-15 and GAUT-82-99 genotypes recorded 13.0 to 20.9 and 12.0 to 21.6 globular embryos, respectively (Table 2). Cytokinin induced embryogenesis is restricted to a few species such as *Trifolium* (Maheswaran and Williams 1986), *Arachis* (Gill and Saxena 1992), *Phaseolus* (Malik and Saxena 1992), *Cajanus* (Patel *et al.* 1994) and *Cicer* (Murthy *et al.* 1996).

Table 1. Somatic embryo induction [%] and number of globular embryos [explant^{-1}] in cotyledonary segments on EC6 basal medium supplemented with various concentrations of BAP. Means ± SE, $n = 20$, means with different letters differ significantly at $P = 0.05$.

BAP [µM]	GAUT-82-90 induction	glob. embryos	T-15-15 induction	glob. embryos	GAUT-82-99 induction	glob. embryos
2.22	53 ± 3a	14.8 ± 1.1a	62 ± 5a	11.1 ± 0.7a	43 ± 4a	10.5 ± 0.8a
4.44	70 ± 2b	27.6 ± 2.2b	80 ± 1b	16.7 ± 0.6b	60 ± 4b	18.3 ± 1.4b
13.32	76 ± 3b	23.9 ± 1.3b	64 ± 2a	10.8 ± 0.4a	53 ± 6b	10.7 ± 0.6a
22.20	57 ± 2a	13.9 ± 0.5a	69 ± 2a	10.9 ± 0.4a	40 ± 3a	9.0 ± 1.1a

Table 2. Somatic embryo induction [%] and number of globular embryos [explant^{-1}] in cotyledonary segments on EC6 basal medium supplemented with various concentrations of TDZ. Means ± SE, $n = 20$, means with different letters differ significantly at $P = 0.05$.

BAP [μM]	GAUT-82-90 induction	glob. embryos	T-15-15 induction	glob. embryos	GAUT-82-99 induction	glob. embryos
0.45	64 ± 9a	27.8 ± 3.4b	84 ± 4b	13.0 ± 2.4ab	92 ± 4c	21.6 ± 2.1b
1.36	88 ± 3b	29.7 ± 2.8b	75 ± 5a	10.4 ± 1.3a	62 ± 9a	12.0 ± 0.5a
2.27	74 ± 2a	28.6 ± 3.3b	80 ± 4ab	20.9 ± 2.3c	90 ± 2c	15.2 ± 0.8a
4.54	97 ± 3b	38.4 ± 4.9c	91 ± 6c	17.8 ± 1.1c	78 ± 4b	13.4 ± 1.0a
13.62	75 ± 5a	19.2 ± 1.7a	90 ± 1c	14.0 ± 1.5b	73 ± 3ab	21.1 ± 2.6b

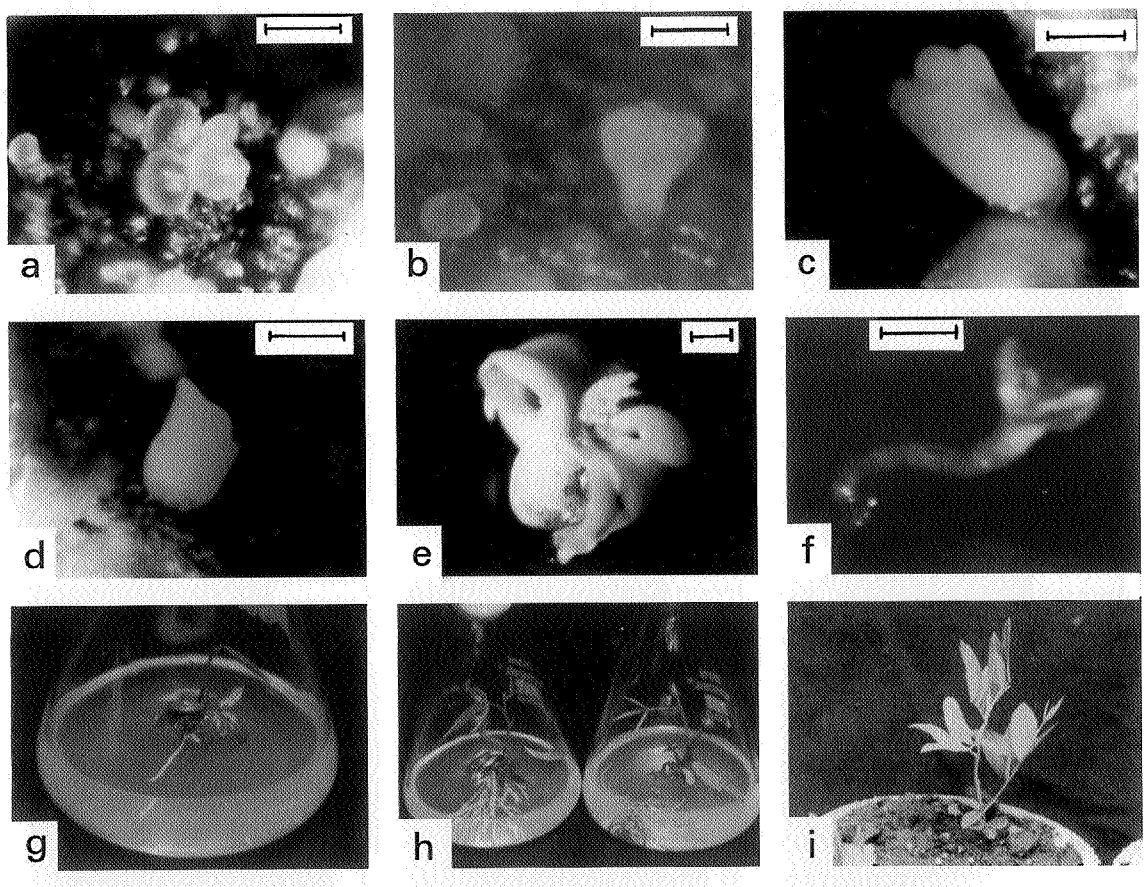

Fig. 1. Plant regeneration via somatic embryogenesis from cotyledon explants of pigeonpea [*Cajanus cajan* (L.) Millsp.]: *a* - globular embryos formed on a mature cotyledon (*bar* = 550 μm), *b* - heart-shaped somatic embryo (*bar* = 500 μm), *c* - cotyledonary stage somatic embryo (*bar* = 650 μm), *d* - cotyledonary stage somatic embryo (*bar* = 825 μm), *e* - mature cotyledonary stage somatic embryo (*bar* = 1000 μm), *f* - germinated somatic embryo (*bar* = 1000 μm), *g* - converted plantlet, *h* - converted plantlets ready to transfer into pots, *i* - plantlets growing in pot.

The explants along with globular embryos were transferred after 6 weeks to various media (see Materials and methods) in order to produce cotyledonary stage embryos. Globular embryos subcultured on the same medium and on EC6 medium supplemented with various growth regulators did not produce any cotyledonary embryos. Cotyledons with globular embryos transferred to MS medium without growth regulators developed into cotyledonary embryos rarely (0.3 % of cotyledonary segments), while 2.25 % of the cotyledons cultured on MS medium supplemented with 0.38 μM ABA and 15.48 % of the cotyledonary segments cultured on MS medium with 2.89 μM GA_3 produced cotyledonary embryos. When globular embryos were transferred to various concentrations of GA_3 (2.89 - 14.43 μM), the maximum number of cotyledonary embryos produced was 2.5 per explant on the medium containing 2.89 μM GA_3 (Table 3). The effect of GA_3 on development of somatic

embryos has been very well demonstrated in papaya (Chen *et al.* 1987), tepary bean (Kumar *et al.* 1988), black mustard (Vibha *et al.* 1990) and spinach (Komai *et al.* 1996). The media with increased sucrose concentration (6 %), casein acid hydrolysate (200 to 600 mg dm^{-3}), 3 % polyethylene glycol, 3 % mannitol, or 3 % sorbitol did not result in the formation of cotyledonary embryos (data not shown).

Table 3. Cotyledonary embryo formation [%] and number of cotyledonary embryos [$explant^{-1}$] in cotyledonary segments on MS basal medium supplemented with various concentrations of GA_3. Means ± SE, n = 20, means with different letters differ significantly at P = 0.05.

GA_3 [μM]	GAUT-82-90 induction	coty. embryos	T-15-15 induction	coty. embryos	GAUT-82-99 induction	coty. embryos
2.89	15 ± 4d	2.50 ± 0.58c	10 ± 3c	2.57 ± 0.53c	10 ± 6b	1.00 ± 0.19b
5.77	5 ± 2b	1.00 ± 0.27b	5 ± 1b	1.67 ± 0.33b	0 ± 0a	0.00 ± 0.00a
8.66	0 ± 0a	0.00 ± 0.00a	5 ± 1b	1.00 ± 0.57b	0 ± 0a	0.00 ± 0.00a
11.54	10 ± 5c`	1.00 ± 0.43b	0 ± 0a	0.00 ± 0.00a	0 ± 0a	0.00 ± 0.00a
14.43	5 ± 1b	1.25 ± 0.31b	0 ± 0a	0.00 ± 0.00a	0 ± 0a	0.00 ± 0.00a

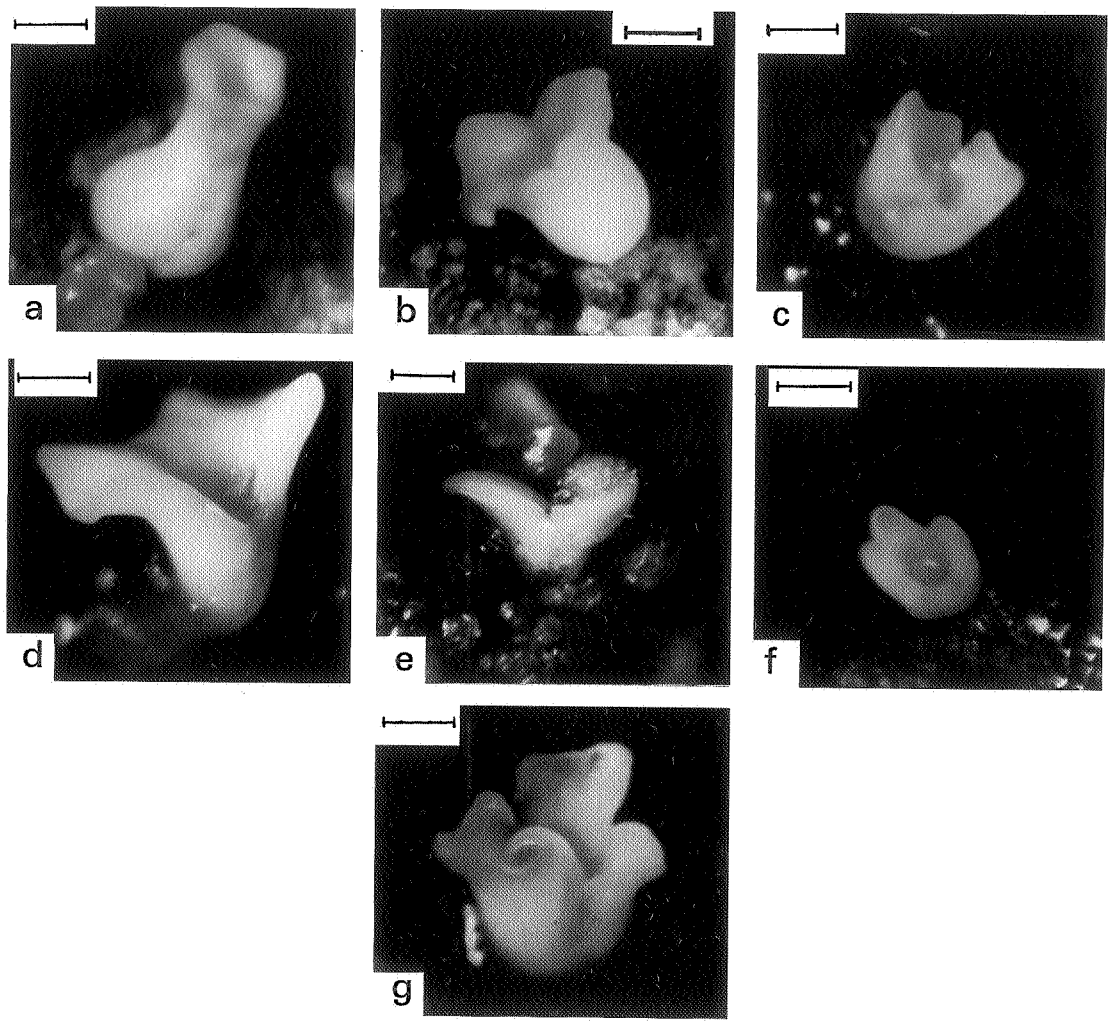

Fig. 2. Morphological variations of cotyledonary stage embryos:
a - horn-shaped embryo (*bar* = 600 μm), *b* - bell-shaped embryo (*bar* = 550 μm), *c* - cup-shaped embryo (*bar* = 550 μm), *d* - somatic embryo with single cotyledon (*bar* = 750 μm), *e* - dicotyledonary somatic embryo (*bar* = 1000 μm), *f* - tricotyledonary somatic embryo (*bar* = 750 μm), *g* - multicotyledonary somatic embryo (*bar* = 625 μm).

The cotyledonary embryos derived from MS basal medium containing 2.89 μM GA_3 were matured for one week on half strength MS medium supplemented with 0.38 μM ABA and then transferred to MS medium containing 0.44 μM BAP for germination and conversion. Precocious germination is a major problem during somatic embryo development and this is controlled either by increasing the osmolarity of the maturation medium with additional sucrose (Carman 1989) or by incorporating ABA into the medium (Ammirato 1974). The use of 0.38 μM ABA for maturation was based on earlier report in chickpea (Suhasini *et al.* 1994). Maturation of somatic embryos was 54 %. 39 % of the embryos germinated and converted to plantlets. The survival of plantlets in the pots was 42 % (Figs. 1,2).

A large number of cotyledonary embryos formed were morphologically abnormal and such abnormal embryos failed to develop further. A total of 158 embryos were obtained out of which 100 embryos were found to be abnormal. Out of 58 normal embryos 31 matured and 12 converted into plantlets. Only 5 plants survived in the pots after hardening. This observation was similar to the earlier reports in peanut (Hazra *et al.* 1989, Ozias-Akins 1989), soybean (Hartweck *et al.* 1988, Lazzeri *et al.* 1987, Buchheim *et al.* 1989) and chickpea (Suhasini *et al.* 1996).

At the time of culture, the cotyledonary segments showed a single layered epidermis and the parenchyma was filled with food reserves (Fig. 3*a*). Sections of cotyledonary segments showed nodular outgrowths after 30 d of culture on induction medium (Fig. 3*b*). A section passing through the cotyledonary segment revealed the development of globular embryos directly from epidermal and subepidermal layers along the periphery of the explant (Fig. 3*c*). Single cell initiation of embryos was not observed. A section of early cotyledonary stage somatic

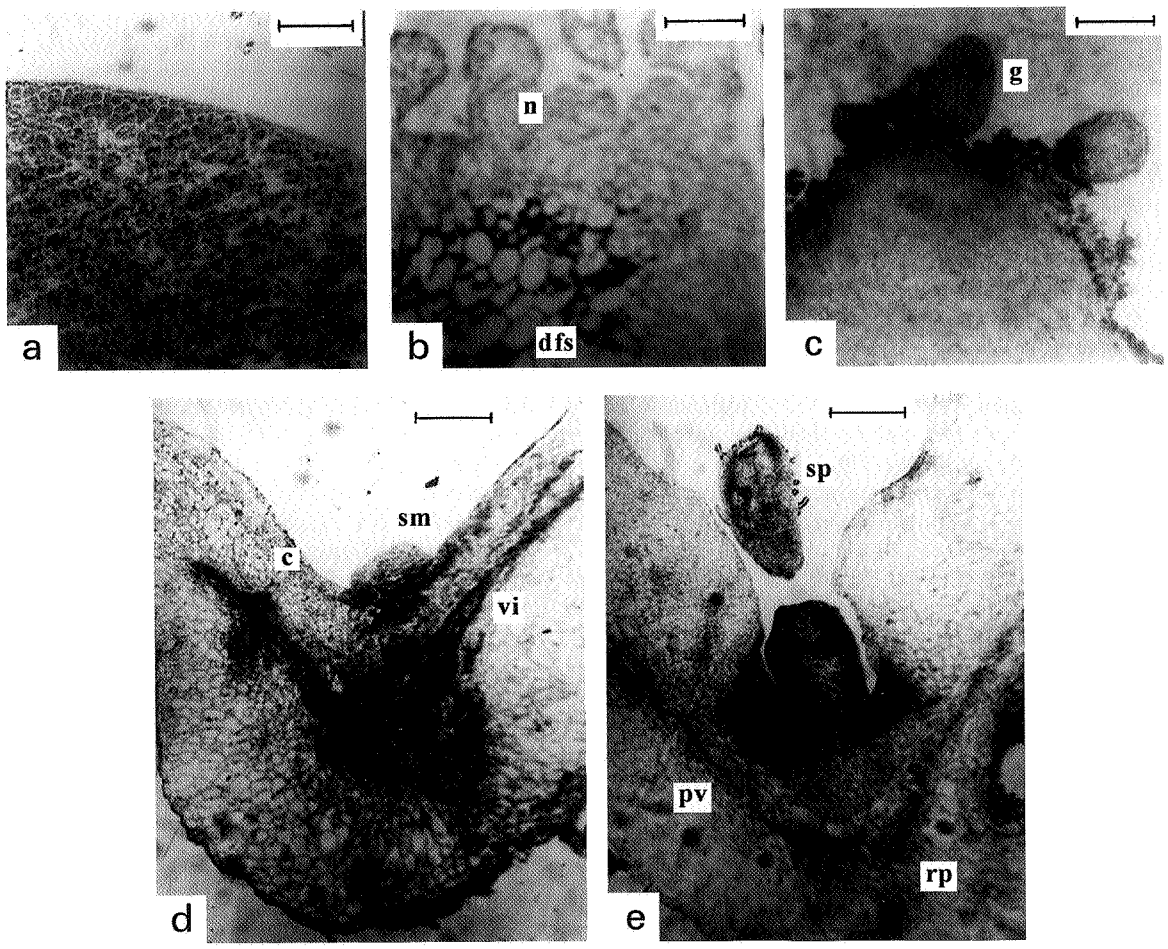

Fig. 3. Histology of developmental stages of somatic embryos of pigeonpea:
a - section of explant at the time of culture, showing parenchymatous cells filled with food reserves (*bar* = 500 μm), *b* - nodular outgrowths (n) arising on the surface of cotyledonary segments after 30 d of culture, 'dfs' indicates cells with depleted food reserves (*bar* = 650 μm), *c* - section of cotyledonary segment showing formation of globular (g) embryos (*bar* = 500 μm), *d* - section of early cotyledonary stage embryo with cotyledon (c) developing shoot meristem (sm) and vascular initials (vi) (*bar* = 835 μm), *e* - longitudinal section of mature cotyledonary stage embryo showing root pole (rp), shoot pole (sp) and provascular strand (pv) (*bar* = 500 μm).

embryo showed vascular initials, cotyledons and a developing shoot meristem region (Fig. 3*d*). A mature somatic embryo exhibits well developed cotyledons with provascular strands, a shoot pole and a root pole. The section passing through the centre of the mature somatic embryo revealed leaf primordia at the shoot pole and a prominent root pole (Fig. 3*e*).

Patel *et al.* (1994) reported induction of somatic embryogenesis in cotyledons of pigeonpea when cultured on media supplemented with 22.2 μM BAP, 2.3 μM kinetin and 271.0 μM AdS. In our experiments, however, somatic embryogenesis was observed when the medium was supplemented with BAP alone. Somatic embryo formation was observed on calli derived from cotyledon and leaf tissue of pigeonpea by Sreenivasu *et al.* (1998). However, we observed direct appearance of globular embryos on cotyledonary segments without callus formation. TDZ did not support conversion of globular structures into cotyledonary structures as observed by Visser-Tenyenhuis *et al.* (1994).

George and Eapen (1994) could produce normal embryos using immature cotyledons and embryonal axes as explants on auxin supplemented medium, but failed to get plantlets. We observed somatic embryogenesis and formation of normal plantlets using mature cotyledonary segments which are available throughout the year for experiments. It was observed that the cytokinin supplementation instead of auxin was necessary for induction of somatic embryos. Mallikarjuna *et al.* (1996) also reported somatic embryogenesis on a medium containing a combination of NAA and BAP. In contrast, only BAP or TDZ supplementation was sufficient to induce somatic embryos in our experiments. Previous reports of somatic embryogenesis in pigeonpea (George and Eapen 1994, Mallikarjuna *et al.* 1996, Patel *et al.* 1994, Sreenivasu *et al.* 1998) did not show different morphological and histological developmental stages of somatic embryogenesis.

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