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Somatic embryogenesis in pigeonpea, *Cajanus cajan* L.

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Somatic embryogenesis was obtained from immature leaflet segments, root discs, epicotyl and hypocotyl segments, as well as from mature cotyledons or MS medium supplemented with different concentrations of NAA and BAP. Conversion of somatic embryos to plants was obtained only from the cotyledons.

Pigeonpea (*Cajanus cajan* L.) is one of the major grain legume crops of the semi-arid tropics and sub tropics. Though plant regeneration has been reported for many legumes, there are only a few reports on somatic embryogenesis in legumes[2,3]. Somatic embryogenesis is useful in studies on somaclonal variation, cell selection for desirable traits, genetic transformation, clonal propagation and production of artificial seeds. The present paper reports somatic embryogenesis from leaflet segments, cotyledons, root discs and epicotyl and hypocotyl segmenets of pigeonpea.

Mature seeds of *C. cajan* cv. ICPL 87 were surface sterilized for 20 min in 50% clorox and 2 drops of tween 20. They were washed four times in sterilized distilled water and kept for germination on moist filter paper in sterile petriplates. Leaflet segments, root discs, epicotyl and hypocotyl segments excised from 7 days old seedlings and cotyledons from mature seeds were used as explants. Murashige and Skoogs (MS) medium was supplemented with napthalene acetic acid (NAA) at 1, 5, 10, 25 and 50 mg L^{-1} and benzylaminopurine (BAP) at 0.1, 1 and 10 mg L^{-1}. Pieces of callus tissue (3 mm^2 in surface area) containing putative embryos were fixed in absolute alcohol and acetic acid (3:1, v/v) and squashed in 2% acetocarmine stain. An average of five observations was taken to determine the number of embryos produced from different explants of pigeonpea. Tissues for microtomy were fixed in formalin solution for 48 hr, dehydrated in ethyl alcohol-xylene series and embedded in paraplast (Monojet Scientific, St. Louis) at 56°C. Embedded tissues were cut into 8 μm thick sections and stained with saffranin and fast green.

For scanning electron microscopy studies, embyros were fixed in 3% glutaraldehyde in phosphate buffer *p*H 2.5. The samples were then post fixed in 2% osmium tetroxide for 4 hr and washed with double distilled water. The samples were dehydrated in graded series of acetone 30-100%, critical point dried in CPD-750 using liquid carbon dioxide, mounted on aluminium stubs, coated with a 20 nm layer of gold in an EM scope FD 500 sputter coating unit and observed under JEOL JSM 35 CF scanning electron microscope.

Cotyledons (75%) enlarged and turned green on MS medium containing NAA (50 mgL^{-1}). Tiny globular outgrowths (0.5-0.75 mm) were observed by 15 days of culture (Figs 1A, B), but no well formed structures were observed. Inclusion of BAP (1.0 mgL^{-1}) in combination with NAA (50, 25, 10 and 5 mgL^{-1}) in the culture medium gave rise to well deveoped embryo like structures. Acetocarmine squash of the callus from cotyledons showed embryogenic callus intermixed with proembryos at different stages embryogeny (Fig. 1C). Basically, two types of proembryos were observed, filamentous and spherical (Figs 1D, F). The filamentous proembryo at times had transverse divisions in some of the apical and subapical cells (Fig. 1F). These embryos did not develop further. The globuar proembryo formed as a result of first transverse division in the embryogenic cell. The basal cell gave rise to the suspensor and the distal cell to the proembryo (Figs 1D, E). The suspensor was distinct as a single enlarged cell or a group of cells and persisted even at later stages of embryo development. Well developed somatic embryos with cotyledons and shoot and root regions (Fig. 1H) were observed on the cotyledons by 60 days of culture.

Cotyledons cultured on the medium with 1 mgL^{-1} of NAA and 1 mgL^{-1} BAP gave rise to shoot buds at the nodal end of cotyledon, with tiny globular outgrowths on all over the surface.

Since further development of embryos was not observed on the medium with different concentration of NAA and BAP or on the basal medium, they were transferred to the medium with 10 mgL^{-1} of BAP for 15 days and then to 1 mgL^{-1} of BAP. Three per cent of the transferred cotyledons showed direct germination of the embryos into plans. Most of the embryos showed a tendency for proliferation of shoots (Fig. 1G) with a disorganised radicle region. To determine the structural malformations of the embryos, they were examined by scanning electron microscope

(SEM). SEM observations showed that embryoids had a distinct shoot region with trichomes and stomata whereas the root region was stunted, lacking trichomes and stomata (Figs 2 A, B).

In order to increase the conversion of cotyledon-derived somatic embryos to plants embryos were desiccated by transferring them to empty sterile petriplates and incubating them for 7, 14 and 21 days at room temperature (25°C) in the dark. On transfer to either the basal medium or to the medium with BAP (10 mgL^{-1} and later to 1 mgL^{-1}) none of the embryos germinated instead callus formation was observed.

Leaflet segments and root discs enlarged with a tendency to callus at their cut ends when cultured on the medium with both NAA (50 $gl.^{-1}$) and BAP (1 mgL^{-1}). By 30 days of culture, globular outgrowths were observed on the callus. Further growth of these structures was never observed. Similar results were observed when epicotyl and hypocotyl segments were cultured on the medium with 5 $mgl.^{-1}$ NAA and 1 $mgl.^{-1}$ BAP (Table 1).

Although different explants of pigeonpea exhibited the capacity to produce somatic embryos, conversion of somatic embryos to plants was obtained only

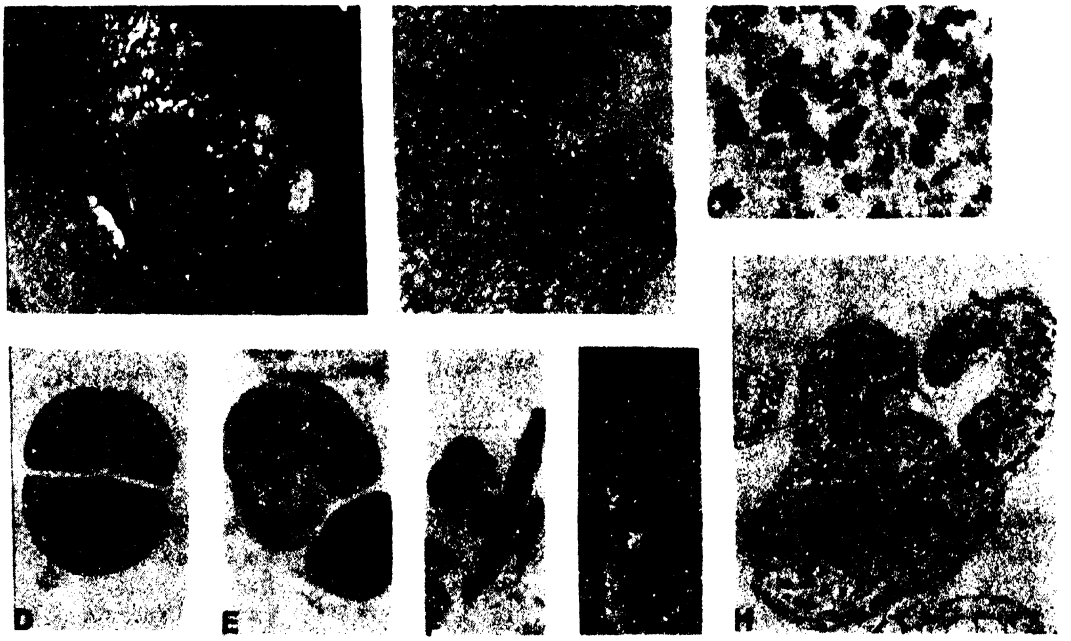

Fig. 1—(A) Cotyledon, enlarged with outgrowths, × 5; (B) Microtome section through the outgrowths, × 49; (C) Embryogenic callus, × 200, (D) Three celled embryo, × 495; (E) Multicellular globular embryo, × 495; (F) Filamentous embryo, × 495; (G) Multiple shoots, × 0.44; and (H) Microtome section through an embryo, × 49.

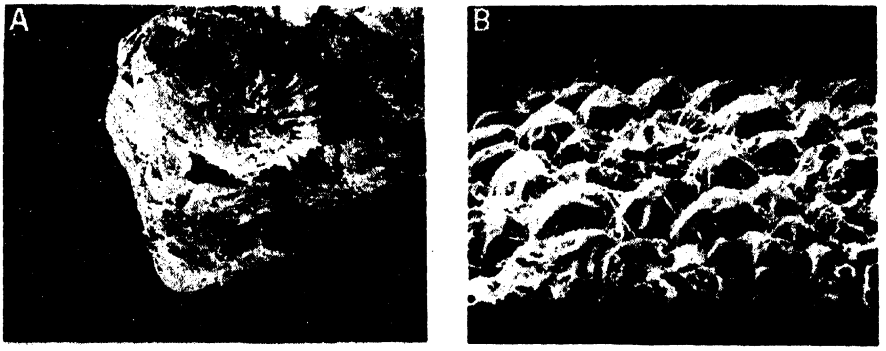

Fig. 2—(A) Somatic embryo with trichomes (t) on shoot region. Root region not regular, × 35; (B) Shoot region with the stomata,

Table 1—Callus growth on pigeonpea explants

Explant	Hormone (mg/L)	Response (%)	Type of callus produced	
			Embryogenic[1]	Nonembryogenic[2]
Cotyledons	NAA 50 BAP 0	76	Green, semicompact	
	NAA 50 BAP 1	84	Green, compact	
	NAA 25 BAP 0	68	Green, semicompact	
	NAA 25 BAP 1	80	Green, compact	
	NAA 10 BAP 0	64	Green, semicompact	
	NAA 10 BAP 1	76	Green, compact	
	NAA 5 BAP 0	72	Green, semicompact	
	NAA 5 BAP 1	84	Green, compact	
Roots	NAA 50 BAP 0	48		White, friable
	NAA 50 BAP 1	36	White, compact	
	NAA 5 BAP 0	52		White, friable
	NAA 5 BAP 1	56	White, compact	
Leaflet	NAA 50 BAP 0	44		Green, friable
segments	NAA 50 BAP 1	56	Green, semicompact	
	NAA 5 BAP 0	48	Green, friable	
	NAA 5 BAP 1	52	Green, compact	
Epicotyl	NAA 5 BAP 0	64		White, friable
segments	NAA 5 BAP 1	72	White, friable	
Hypocotyl	NAA 5 BAP 0	64		Green, friable
segments	NAA 5 BAP 1	72	Green, friable	

1 Embryogenic callus contained densely stained cells (Fig. 1C). 2 Nonembryogenic callus contained elongated and vacuolated cells.

from the cotyledons. SEM observations showed that somatic embryos were abnormal in their development. The shoot region of the embryo was normal with trichomes and stomata. The shoot region of the pigeonpea plant is generously endowed with trichomes. Trichomes are present on the embryonic leaves too. The root region of the somatic embryo had stunted growth, at times accompanied by callus. This is the main cause of low conversion frequency of somatic embryos into plantlets in pigeonpea.

Suspensors were observed in the somatic embryos of pigeonpea, but it is not known if the suspensor had any specific role to play in viability or conversion of somatic embryos. However, the presence of a suspensor in the somatic embryo shows resemblane to zygotic embryogenesis in pigeonpea. Multiple embryoids were observed with a common suspensor. Similar variations in morphology in somatic embryos have also been observed in *Vigna* sp.[2].

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