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Histological and scanning electron microscopic observations on plant regeneration in mungbean cotyledon (*Vigna radiata* (L.) Wilczek) cultured *in vitro*

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Abstract

Explanted cotyledons of mungbean *Vigna radiata* (L). Wilczek, variety Pag-asa-1, regenerated shoots directly from the basal adaxial side of the petiolar residue on MS medium supplemented with 8.9 μM 6-benzyladenine. A simplified and rapid procedure for glycol methacrylate sectioning for histological observations was used to observe shoot initiation. At the time of culture, comparatively smaller and differentially stained epidermal cells were present on the basal adaxial region of the petiolar residue. A meristematic cell mass that developed at 48 h after culture appeared to be of epidermal and subepidermal cell origin. Scanning electron microscopy revealed shoot primordia and approximately 2 nodules at the base of the petiole as early as 48 h after culture. All of these structures developed into shoots during incubation.

Abbreviations: FAA – formalin 5% – 70% ethanol, 90% – acetic acid 5%, GMA – glycol methacrylate, BA – 6-benzyladenine

Introduction

Plant regeneration by *in vitro* culture has been obtained from the shoot tip, cotyledonary node and cotyledon explants in *Vigna* (mungbean) (Mathews 1987). The greatest regeneration response reported was from the proximal end of the cotyledon explants (Gulati & Jaiwal 1990). Recently, studies regarding plant regeneration by organogenesis from tissue cultures of other *Vigna* species have been reported (Goel & Gupta 1983; Bhargava & Chandra 1983; Haque et al. 1984; Ozaki 1986; Sato et al. 1989). However, no histological evidence describing shoot origin was available in this genus. Our study reports histological observations on the precise origin of shoots and the distribution of protein and starch bodies with shoot development in explanted mungbean cotyledons based on a simplified and rapid procedure for GMA sectioning of plant tissues. Scanning electron microscopic observations of shoot organogenesis at different culture periods are also presented.

Materials and methods

Mature seeds of green-seeded *Vigna radiata* (L.) Wilczek variety Pag-asa-1, were disinfested as described previously (Mendoza & Futsuhara 1990). Disinfested seeds were imbibed in water for 8 h in the dark at 30°C and then the cotyledons were separated from the plumule. The cotyledon explants were cultured with the

abaxial side in contact with the medium. For regeneration, MS medium (Murashige & Skoog 1962) supplemented with 100 mg l^{-1} yeast extract, 8.9 μM BA and 3% sucrose was used. The pH of the medium was adjusted to 5.8 before 0.6% agar powder (INA BA 30) was added and then autoclaved at 118 kPa for 15 min. Cultures were maintained at 28°C under continuous exposure to a photosynthetic photon flux of 20 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Explants for light microscopy were sampled at 0, 8, 20, 48, 60, 72 and 120 h after culture and for scanning electron microscopy, explants were sampled at 0, 16, 48, 60, 96 and 120 h after culture.

Glycol methacrylate embedding for light microscopy

The sample were trimmed at about 3 mm below the proximal end of the cotyledon, fixed in FAA for 48 h and then dehydrated in a graded acetone series at 30-min intervals. The samples were soaked in propylene oxide for 1 h to facilitate the penetration of resin into the tissue and then processed for GMA sectioning using a simplified and rapid procedure for resin embedding as follows: Resin was gradually infiltrated into the samples by adding 25%, 50%, 75% and 100% volume of GMA solution A on samples containing propylene oxide at 2-h intervals. Finally, the solution was discarded and replaced with 100% GMA solution A. By loosening the caps, infiltration was completed by passing the samples through a vacuum for 2 h at 100 kPA pressure.

GMA solution A was prepared as follows: To a solution of 2-hydroxymethyl methacrylate and n-butylmethacrylate (9:1 v/v), 0.5% activated charcoal (w/v) was added. The mixture was filtered through a Whatman GFC filter. Into the filtrate, 0.5% resin Amberlyst A-21 was added and then passed again through a Whatman GFC filter. Ten percent butoxyethanol and 5% polyethylene glycol 400 (v/v) were added to the filtrate with a neutral to near neutral pH (about pH 6.8). Benzoyl peroxide at 0.7% (w.v) was finally mixed as catalyst to make solution A.

For embedding, GMA solution B was prepared by mixing N,N-dimethylaniline and polyethylene glycol 400 (1:1 v/v). The embedding GMA solution C was prepared by mixing 50 to 80 parts chilled GMA solution A to 1 part GMA solution B. The resin-infiltrated samples were embedded in plastic caps of 0.7 ml capacity containing GMA solution C. The caps were covered with thin pieces of polyethylene plastic, mounted on a chilled metal block, and then polymerized at 45°C for 30–45 min. After polymerization, the embedded samples were trimmed and mounted on small metal blocks for sectioning at 5 μm on a rotary microtome using steel knives. The sections were serially mounted on oil-free glass slides, stained with iodine: potassium iodide solution according to Johansen (Jensen 1962) for starch and then counterstained with 0.05% toluidine blue for 2 min. For the distribution of protein bodies in the tissue, samples were stained with 0.25% (w/v) Coomassie brilliant blue R-250 in 45% ethanol:10% acetic acid for 2 min and destained in running water for 1 min. The number of starch grains and protein bodies were counted from 20 cells/section/sample obtained from 4 explants sectioned at about the median sagittal longitudinal section of the cotyledonary tissue.

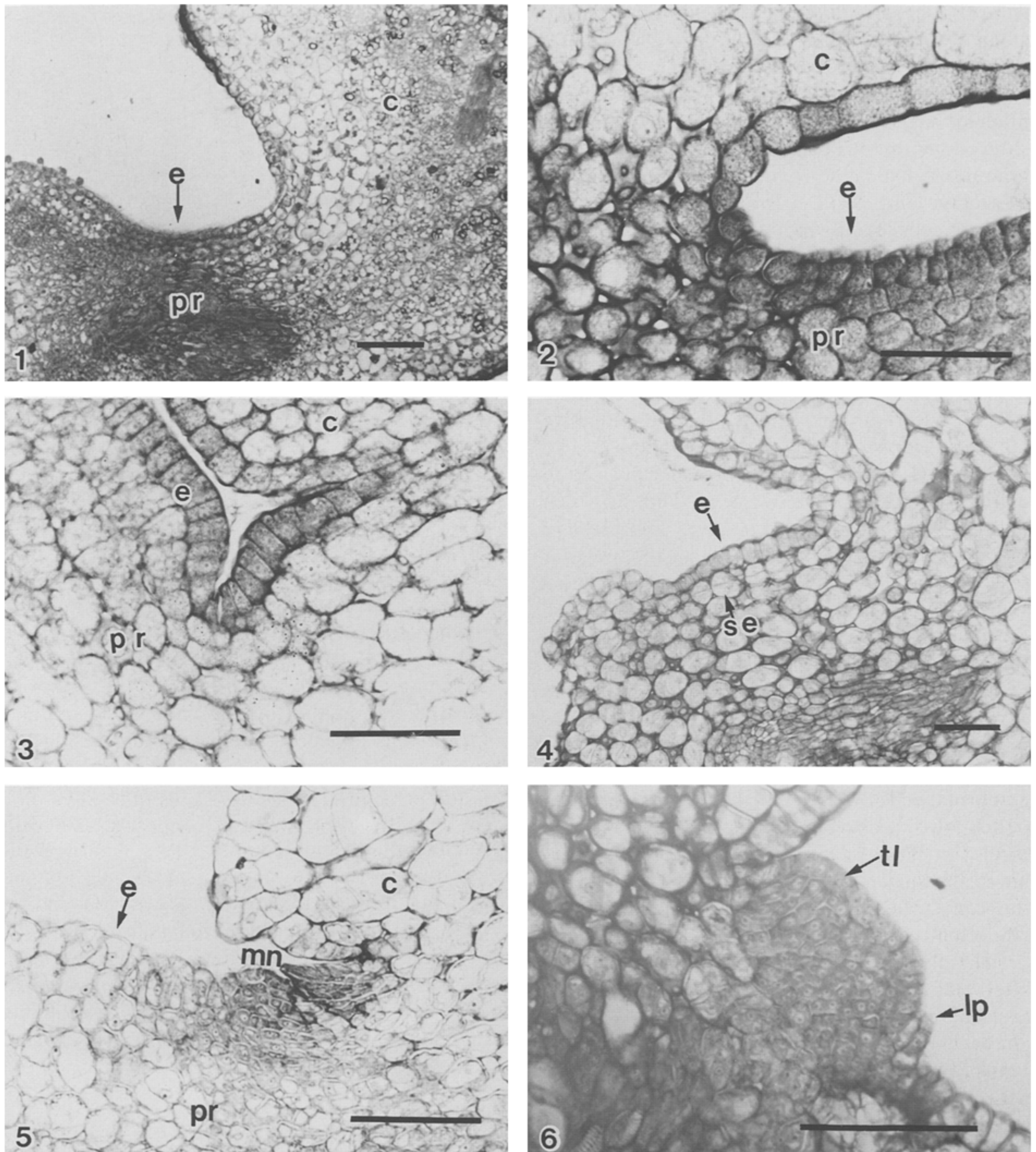
Scanning electron microscopy

Samples fixed in FAA for 48 h were dehydrated in a graded series of acetone at 30-min intervals. The samples were dried to critical point with liquid carbon monoxide for 50 min using an Hitachi Critical Point Dryer HCP-1 and then coated with platinum-paladium ion for 180 s using an Hitachi E-1030 Ion Sputter. Coated samples were mounted on metal blocks and observed under an Hitachi S-2300 scanning electron microscope.

Results

Histological observations on plant regeneration

The proposed procedure for GMA embedding of plant tissues was simple and allowed rapid processing of samples. Histological observations on the proximal end of the explant with the petiolar residue in sagittal longitudinal section and cross section at the time of culture (i.e. 8 h after seeds



Figs 1–6. Anatomical aspects of shoot regeneration in mungbean cotyledon. (*1*) Coomassie brilliant blue stained sagittal longitudinal section of the cotyledon with the petiolar residue at the time of culture. Note numerous darkstained protein and unstained starch bodies in the parenchyma cells of the cotyledon. (*2*) Toluidine blue stained transverse section at the time of culture showing differentially stained epidermal and subepidermal cells on the basal adaxial side of the petiolar residue. (*3*) Transverse section at 8 h after culture showing slight elongation of the epidermal cells on the basal adaxial side of the petiolar residue. (*4*) Sagittal longitudinal section at 20 h after culture showing the first cell division of the subepidermal cells in the basal adaxial side of the petiolar residue. (*5*) Two meristematic nodules on the basal adaxial side of the petiolar residue at 48 h after culture. (*6*) Early stage of shoot organogenesis at 60 h after culture illustrating the tunica layer of cells and leaf primordia from a meristematic dome. (Abbreviations: c, cotyledon; e, epidermis; lp, leaf primordia; mn, meristematic nodule; pr, petiolar residue; tl, tunica cell layer; se, subepidermal cells). Scale bar = 50 μm

were imbibed in water) showed differentially stained epidermal and cortical cells on the basal adaxial side of the petiolar residue (Figs 1–2). A one-dimensional observation of tissue slices in median sagittal longitudinal sections at 5 μm showed numerous starch grains, from 7–20 granules/cell in the parenchyma cells of the cotyledon and about 0–5 granules/cell in the cortical cells of the petiole based on 20 cells scored per explant sample. In the cotyledon parenchyma, protein bodies appeared more numerous when smaller in size and fewer in number when larger. Relatively fewer protein bodies in the cortex of the petiolar residue were present. The onset of morphogenesis became evident after 8 h of culture on the inductive medium. Most of the cells of the explant had enlarged (Fig. 3). The cytoplasmically dense epidermal cells had elongated. The first cell division, which was transverse, was completed in the adaxial subepidermal cells of the petiolar residue at 20 h after culture (Fig. 4). At this stage, cell division was not observed in the cotyledon, but it occurred sometimes in the cortical cells of the petiole and mostly near the wound site. At 48 h after culture, and sometimes after 60 h, one to three meristematic nodules were formed at the basal adaxial side of the petiolar residue of the cotyledon (Fig. 5). The epidermal cells of the cotyledon divided either in periclinal or transverse (i.e. parallel to the surface), anticlinal or longitudinal (i.e. perpendicular to the surface), or tangential (i.e. tangent to the cell surface) division while, the less dense epidermal cells of the petiole generally divided periclinally or tangentially. Most nodules at 60 h after culture showed a distinct tunica layer and the leaf primordia became prominent (Fig. 6). Shoot organogenesis became evident at 72 h after culture on the basal adaxial side of the petiolar residue. After 120 h of culture on the medium, the leaves of the regenerated shoots had already developed epidermal hairs (Fig. 7).

Shoot initiation was associated with rapid depletion of starch and protein in the parenchyma cells of the cotyledon. From 48 to 60 h after culture, marked differences in the density of protein bodies at 10–13 cell layers away from the meristematic nodules were observed. It appeared that proteins were depleted first before starch because no protein body was detected in the same cell layer at 72 h after culture while starch bodies remained present.

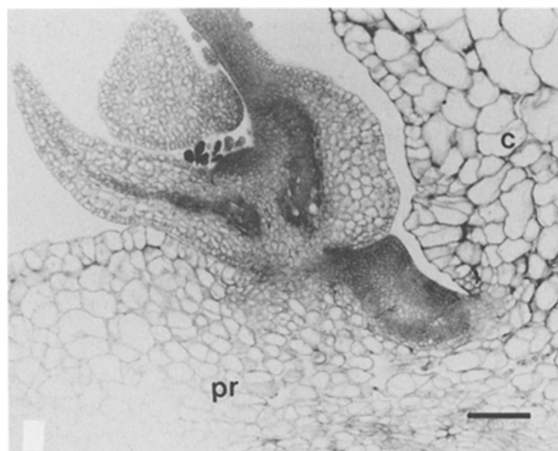


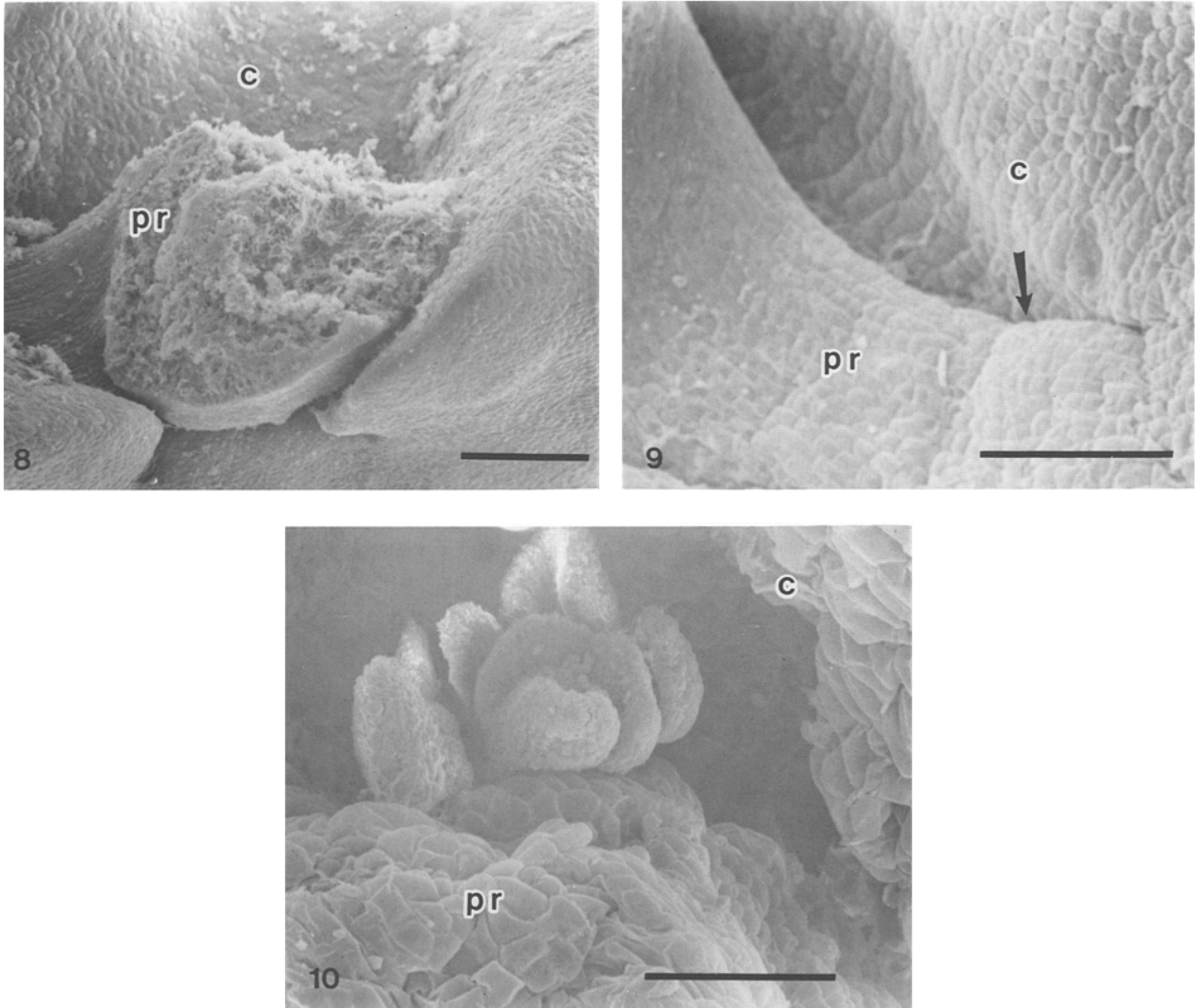
Fig. 7. A regenerated shoot and a shoot bud on the basal adaxial side of the petiolar residue at 120 h after culture. Scale bar = 50 μm.

Scanning electron microscopic observations of plant regeneration

Scanning electron microscopic observations showed that at the time of culture, no nodular protrusions appeared on the petiolar residue of the cotyledon (Fig. 8). The epidermal cells of the petiole appeared smaller than the epidermal cells of the cotyledon. After 8 h of culture on MS medium supplemented with BA a slightly swollen region on the basal adaxial side of the petiolar residue adjacent to the cotyledon appeared (Fig. 9). By 48 h after culture, nodules became evident. Sometimes a distinct bud primordium with prominent leaf buttresses was observed. At 96 h after culture most explants had already regenerated two shoots from the same site where the meristematic nodules were observed (Fig. 10).

Discussion

In the application of genetic manipulation techniques in mungbean through tissue culture, knowledge on the mechanism of cytokinin action leading to organ formation and location of the precise origin of competent cells is important.



Figs 8–10. Scanning electron microscopic observations of shoot regeneration in mungbean cotyledon. (*8*) The morphology of the explant at the time of culture. (*9*) Slight protrusion of the epidermal cells (arrow) on the basal adaxial side of the petiolar residue at 8 h after culture. (*10*) A shoot primordium and another shoot with leaves still devoid of epidermal hairs 72 h after culture. (Abbreviations: c, cotyledon; pr, petiolar residue). Scale bar = 200 μm

Such studies that are important to developmental research are still limited. However, *in vitro* culture studies in a few plant species have provided information on the precise location of cells that dedifferentiate and undergo shoot differentiation *de novo*. In *Brassica juncea* (Sharma & Bhojwani 1990) and *B. campestris* (Hachey et al. 1991) cotyledons, shoots were observed to develop from meristematic nodules resulting from the meristematic activity of the vascular parenchyma cells below the cut end of the petiole. Reynolds (1989) reported that adventive organogenesis from stem segments of *Solanum carolinense* arose from mitotic activity of the external phloem, which was later extended to the inner cortex. Whereas the vascular cells were involved in direct shoot regeneration in these species, in *Begonia rex* the epidermal cells at the base of glandular hairs developed into bud meristems (Chlyah & Tran Thanh Van 1984). In young cotyledons of *Picea abies* the hypodermal cells as well as the mesophyll cells below them were recognized to give rise to meristemoids and finally to adventitious buds (Bornman 1983).

The exogenous origin of regenerated plants was also observed in green bean, *Phaseolus vulgaris* L. (Franklin et al. 1991). In that study, it was reported that a ring of meristematic tissue was produced at the base of the axillary bud in the cotyledonary node from which shoots and buds regenerated. In mungbean, the epidermal cells at the basal adaxial side of the cotyledonary petiole devoid of epidermal hairs and the subepidermal cell layer below these competent cells were involved in meristematic nodule formation from which shoots regenerated. Since shoot regeneration was rapid, with the shoot primordia visible as early as 48 h after culture on the inductive medium, it may imply that BA activates cell division of the competent cells. Similar to other tissues cultured *in vitro* (Sharma & Bhojwani 1990; Hachey et al. 1991; Redway 1991), shoot regeneration in mungbean utilizes starch and protein rapidly from cotyledonary cells close to the developing shoot.

In soybeans, Cheng et al. (1980) claimed the existence of totipotent cells at the cotyledonary node region that can be activated with an appropriate concentration of BA. These cells, found to be differentially stained in seeds germinated on BA-containing medium subsequently developed into shoots when cultured *in vitro* (Wright et al. 1986a). Moreover, Wright et al. (1986b) observed that the meristematic zone in the cotyledonary petiole is another site for *de novo* shoot organogenesis that produced cell divisions in the epidermis and in approximately 4-5 subtending subepidermal cell layers. That observation appeared similar to mungbean. However, in mungbean, the participation of subtending cortical cells below the subepidermal layer occurred at about 72 h after culture, during the formation of vascular connection between the explant and the regenerated shoot. This slight discrepancy possibly could be due to species variation or differences in cultural protocol. The presence of existing meristems in the cotyledonary node also accounted for plant regeneration in *Vigna mungo* (Gill et al. 1987). Since the location of organogenic cells in mungbean is exogenous in origin and the regeneration site predictable, plasmid DNA microinjection techniques or *Agrobacterium*-mediated transformation may be possible in obtaining genetically engineered plants.

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