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HISTOLOGICAL ASPECTS OF IN VITRO ROOT AND SHOOT DIFFERENTIATION FROM COTYLEDON EXPLANTS OF *BRASSICA JUNCEA* (L.) CZERN.

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Under fairly simple culture conditions, excised cotyledons of *Brassica juncea* undergo high frequencies of either root formation on growth regulator-free Murashige and Skoog (MS) medium or shoot formation on MS + 5.0 μM N^6-benzyladenine (BA). Temporal histological events of organ differentiation processes revealed that while root formation was endogenous in origin and did not involve callusing, shoot bud differentiation occurred after the formation of meristematic nodules at the cut end. While root primordia differentiated from the petiolar cut end after 4 days in culture, the formation of a meristematic nodule at the same end occurred by 4–6 days which further lead to the differentiation of shoot meristem by 8–10 days. Although the initial accumulation of starch in organogenic cotyledons was the same during both root and shoot formation, its depletion was faster in shoot forming cultures suggesting that this process is more energy requiring than rhizogenesis.

Key words: *Brassica juncea*; cotyledon culture; histology; organogenesis; regeneration; tissue culture

Introduction

Although it is known that phytohormone balance is a factor that controls in vitro-organogenesis, how this control is exerted is still largely unknown. According to Christianson and Warnick [1], a series of events must occur between the placement of an explant on an inductive medium and the appearance of a recognizable organ. These events have been divided into two sets: (1) the events that lead to 'determination' of competent cells for a particular developmental pathway, and (2) the events concerned with the process of morphological differentiation and development.

Our knowledge of the physiology and biochemistry of the organ formation process is still limited due to the lack of a truely suitable experimental system. According to Thorpe [2], a suitable experimental system should fulfill the following requirements: (1) the precise location of cells, which are synchronously undergoing dedifferentiation followed by differentiation de novo, can be observed routinely and manipulated in situ, and (2) these cells should be easily removable and available in large quantities for detailed biochemical studies. Following these suggestions, some attempts have been made to develop such a system, which include *Torenia* stem segments [3], zygotic embryos of *Picea abies* [4] and cotyledons of *Pinus radiata* [5,6]. Excised cotyledons of *Brassica juncea* seem ideal for such studies [7]. They form both adventitious roots or shoots with high frequencies (85–90%) under fairly simple culture conditions, and the time and site of differentiation is predictable. Under specified culture conditions, roots or shoots are formed at the same morphological site (cut end of the cotyledonary petiole) within 6 days (for root formation) and 8–10 days (for shoot formation). In seeking to develop the *B. juncea* cotyledon system as an

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ideal experimental system, we undertook detailed histological studies to elucidate the developmental events leading to in vitro root and shoot bud differentiation from cotyledon explants.

Materials and Methods

Aseptic seedlings of *B. juncea* cultured on Murashige and Skoog [8] basal medium (MS) consisting of MS salts, vitamins, 3% sucrose and 0.8% Difcobacto agar, served as the source of cotyledon explants. Cotyledons from 5-day-old seedlings were cultured either on root inducing medium (MS; RIM) or on shoot inducing medium (MS + 5.0 μM BA; SIM). The details of culture conditions were the same as previously described [7].

Histological studies

To study the ontogeny of adventitious root and shoot differentiation in cultures, cotyledons at day 0 and those cultured on RIM and SIM for 1—13 days were fixed at 24-h intervals and processed for glycol methacrylate (GMA) sectioning according to the procedure described by O'Brien and McCully [9]. After fixing the material in a freshly prepared, chilled 5% (v/v) solution of glutaraldehyde in Sorensen's phosphate buffer (pH 7.2) for 24 h at 4 °C, it was dehydrated by passing through 2-methoxyethanol, ethanol, *n*-propanol and *n*-butanol series and infiltrated with the monomer mixture composed of 92.2% (v/v) glycol methacrylate (Merck), 0.3% (w/v) 2-2′-azobis (2-methyl propionitrile; Merck) and 7.5% (v/v) polyethylene glycol-400 (BDH) with three changes at 24-h intervals. The infiltrated material was embedded in gelatin capsules containing the monomer mixture and after capping the capsules tightly, the mixture was polymerized at 60 °C for 48 h. The trimmed blocks were mounted on a clean wooden support and sectioned on a Spencer A.O. 820 rotary microtome, using dry glass knives. The sections were cut at a thickness of 2 μm and mounted serially on glass slides precleaned with 90% ethanol. All sections were stained with periodic acid Schiff's reaction (PAS) for total insoluble polysaccharides [10] and counterstained with mercuric bromophenol blue [11] for proteins.

Some cotyledons bearing young shoot buds (8 days after culture on SIM) were also processed for obtaining thick (10 μm) paraffin sections. The material was fixed in formaldehyde/acetic acid/70% ethanol (1:1:18, by vol., FAA) and dehydrated through an ethanol-xylene series. The sections were stained with safranin-fast green [9]. The stained sections were air dried and mounted in DePeX (BDH) and photographed with a Nikon Optiphot photomicroscope.

Results

In transverse section, the petiole at the time of culture appeared crescent-shaped (Fig. 1A). It had 4—5 layers of cortex made up of isodiametric parenchymatous cells (Figs. 1A and 1B). Besides the main vascular supply, 2—4 lateral traces traverse the petiole (Fig. 1A). Vascular bundles were collateral, with the phloem facing the abaxial side and xylem towards the adaxial side. The xylem consisted of 3—6 vessels with spiral thickenings and a large number of richly cytoplasmic parenchymatous cells each with a prominent nucleus (Fig. 1B). The phloem is largely composed of highly cytoplasmic, parenchymatous cells (Fig. 1B).

In the petiole, starch grains occurred in the cells of the cortex but were absent from the vascular parenchyma (Fig. 1B). The grains found in the innermost layer of the cortex were larger than those in the outer cortex. Laminar cells were highly vacuolated and contained 10—15 chloroplasts each with a small starch grain (plastid starch; Fig. 1C). Both lamina and petiole contained some randomly distributed cells containing myrosin protein (Figs. 1B and 1C). Myrosin cells are characteristic of the zygotic embryos of *Brassicaceae*; they are a special type of idioblast assumed to contain the enzyme myrosinase [12].

Root differentiation

On MS medium, which favours root differen-

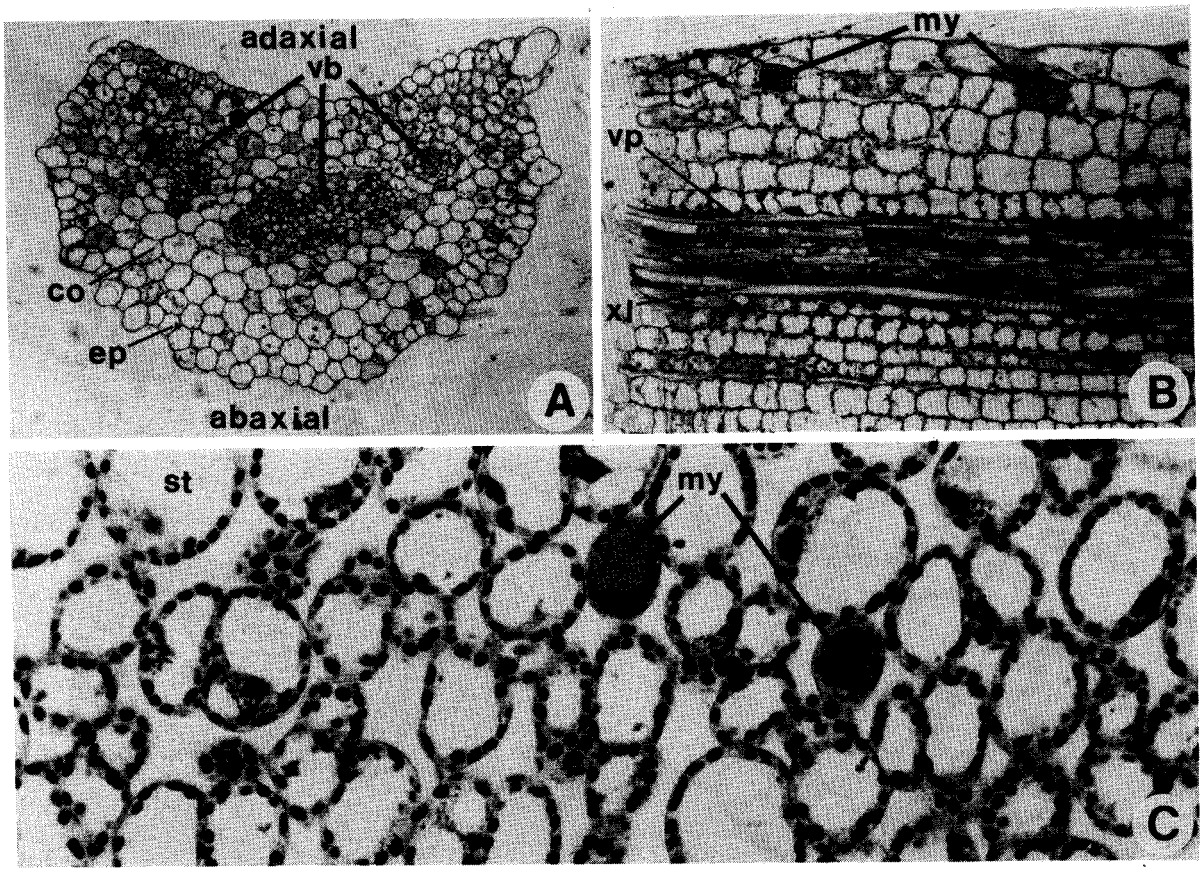

Fig. 1. Transverse (A) and longitudinal (B) sections of the petiole and vertical section of the lamina (C) at the time of *B. juncea* cotyledon culture. co, cortex; ep, epidermis; my, myrosin cell; st, starch; vb, vascular bundle; vp, vascular parenchyma; xl, xylem. (A) Transection of petiole showing one main vascular bundle and two lateral traces (×68). (B) Longisection passing through the main vascular bundle showing the presence of comparatively large starch grains in the innermost layer and small grains in the rest of the cortex. Also note richly cytoplasmic cells of vascular parenchyma. Two myrosin cells can be seen in the cortex (×120). (C) Section showing the presence of plastid starch in the peripheral layer of the cytoplasm and myrosin cells randomly distributed in the mesophyll tissue (×442).

tiation, the most notable change within 12 h after culture was the appearance of starch grains in the vascular parenchyma of the petiole (Fig. 2A). At the end of 24 h, the cells of the cortex had enlarged and become more vacuolated. Two-day-old cultures exhibited further increase in size and vacuolation of the cortical cells. The cells next to the cut surface of the petiole had collapsed giving a pointed appearance to the proximal end of the petiole. The collapsed cells completely withered after 3 days and the vascular parenchyma and cortical cells around the vascular supply near the cut end, had become meristematic (Fig. 2B). At this stage, the starch content of the cortical cells was considerably reduced but the laminar cells contained large grains which appeared to be lying free in the cells after being released by the plastids (Fig. 2C).

Root primordia had differentiated from the meristematic cells after 4 days of culture (Fig. 2D), and a well organized root growing through

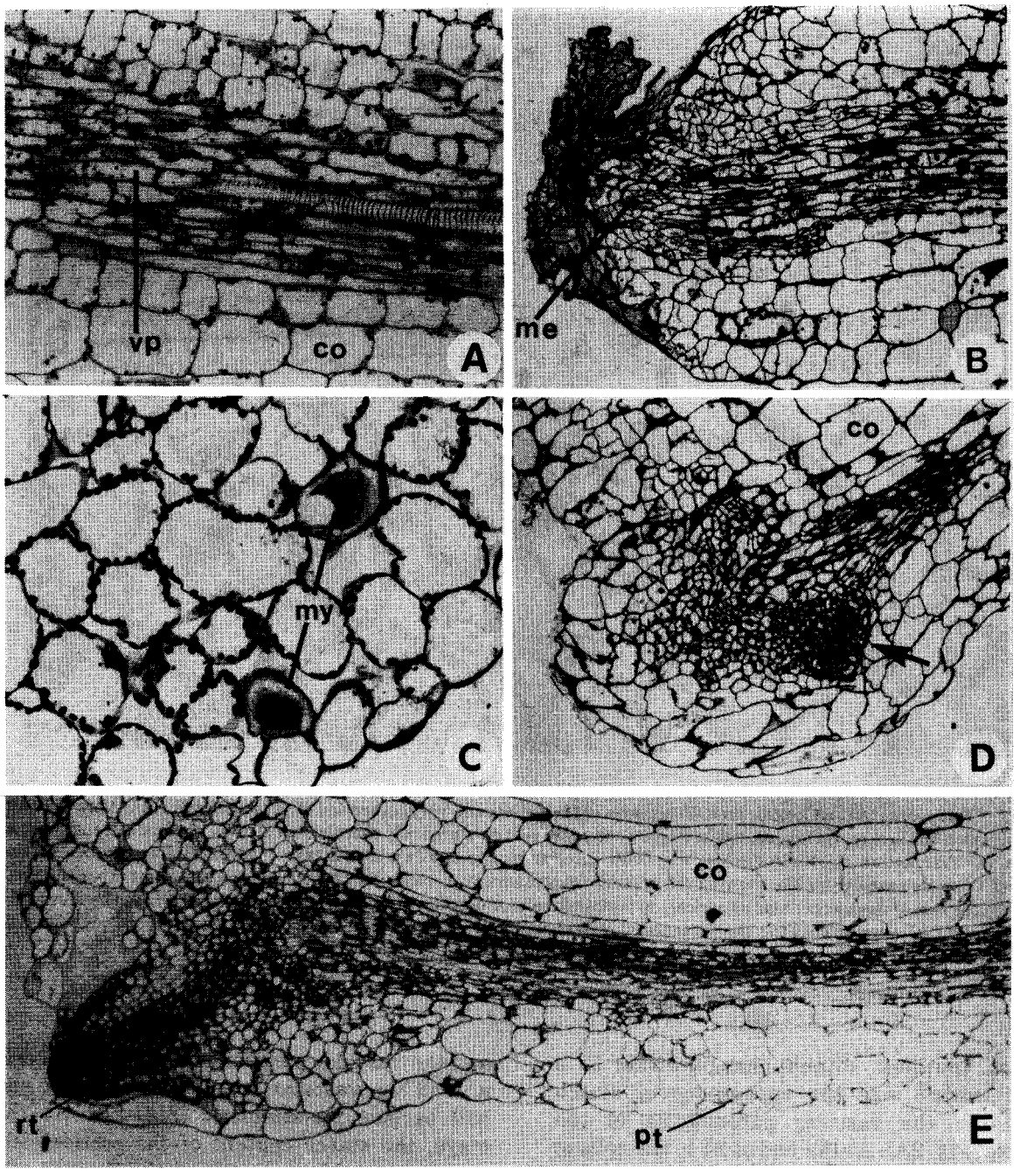

Fig. 2. Longitudinal sections of the proximal end of the petiole (A,B,D,E) and vertical section of the lamina (C) from *B. juncea* cotyledon cultures on MS medium (RIM). co, cortex; me, meristematic cells; my, myrosin cell; pt, petiole; rt, root; vp, vascular parenchyma. (A) 12-h-old culture showing the accumulation of small starch grains in the cells of vascular parenchyma (× 146). (B) 3-day-old culture. The collapsed cells have completely withered. Note meristematic activity next to the degenerating cells (× 63). (C) Section of lamina from 5-day-old culture. Note the increase in the size of the cells and starch grains. The cells show hardly any cytoplasm and the myrosin cells are degenerating (X268). (D) 4-day-old culture showing the initiation of a root primordium (arrow) close to the vascular supply. Starch grains have completely disappeared from cortex and vascular parenchyma (× 76). (E) 5-day-old culture showing a well developed root just emerging from the cortex (× 66).

the cortex was discernible after 5 days (Fig. 2E). In these cultures, while the laminar cells were loaded with large starch grains, most of the cells of the petiole were devoid of starch. In cultures older than 4 days, the myrosin cells were absent from the petiole, and those present in the lamina were undergoing degradation. The roots became macroscopic after 6 days of culture. Thus, root differentiation was endogenous in origin and it was not preceded by any callus formation.

Shoot differentiation

As in the case of RIM the only apparent change noticed in cotyledons 12 h after culture on SIM was the appearance of starch grains in vascular parenchyma (Fig. 3A). In 1-day-old cultures the cells of the cortex had enlarged and become more vacuolated, and the amount of starch grains in the petiole, especially towards the cut end had declined. Occasionally, cell divisions were observed in the peripheral cells of the cortex. At this stage, the plastid starch grains in the lamina had increased in size. Another important change noticed after 24 h was the shrinkage and degradation of some of the mesophyll cells. After 2 days on shoot-forming medium, the meristematic activity in the proximity of the vascular supply had considerably increased. While the cells in the meristematic areas contained some starch grains, the remaining cells of the petiole, which had enlarged appreciably, were almost empty (Fig. 3B). Although some collapsed cells at the cut end were also seen on SIM, their number was considerably less than on RIM (see Figs. 2B and 3B). After 3 days even the mesophyll cells lacked starch, although plastids could be identified in the peripheral layer of the cytoplasm (Fig. 3C). The myrosin cells persisted in the lamina but not in the petiole (Figs. 3C and 3D). At this stage, some anticlinal and periclinal divisions were apparent in the epidermal and hypodermal layers at the cut end of the petiole (Fig. 3D).

After 4 days, localized meristematic activity at the proximal end resulted in the formation of nodular structures (Fig. 3E) which later became vascularized. This was followed by proliferation of the nodules, and after 10 days, shoot buds differentiated from the peripheral cells of the nodules (Fig. 3F).

Discussion

In *B. juncea* the presence or absence of a cytokinin in the medium determines the nature of organogenic differentiation from cotyledon explants. Whereas on MS alone (RIM) only roots are formed, in the presence of 5.0 μM BA (SIM) only shoot differentiation occurs [7]. It was also shown earlier that if cotyledons were cultured on RIM for a period of 6 days the competent cells at the cut petiolar end become determined to form roots, and their subsequent transfer to SIM does not alter the density of these cells. Similarly, the minimum time required on SIM for any shoot differentiation is 3 days, and within 7 days, cells become determined for shoot formation.

In order to pursue any physiological and biochemical studies on organogenesis, it is necessary to understand the developmental sequence leading to organ formation. On the basis of histological examinations Aitken-Christie et al. [5] concluded that the high productivity of shoots from radiata pine cotyledons depends on the selection of young cotyledons from 3-day-old seedlings, when they are still in a meristematic state. In such explants the epidermal and sub-epidermal cells are not fully determined. Responsive cotyledons have been characterized by the presence of immature stomatal complexes. Bornman [13] reported that, in cotyledonary needles of *P. abies* cultured on media containing BA, cell divisions appeared to be initiated mainly in the subsidiary stomatal cells and epidermal cells proper. In thin cell layers of *Begonia rex*, bud formation occurred mainly in the epidermis, in cells surrounding the basal glandular hair cells [14]. Such discrete markers were not apparent in the cotyledons of *B. juncea* at the time of culture. However, most of the cells of vascular tissues in the cotyledonary petioles of 5-day-old seedlings were richly cytoplasmic with a prominent nucleus. The cortical cells were isodiametric with a large vacuole and small starch

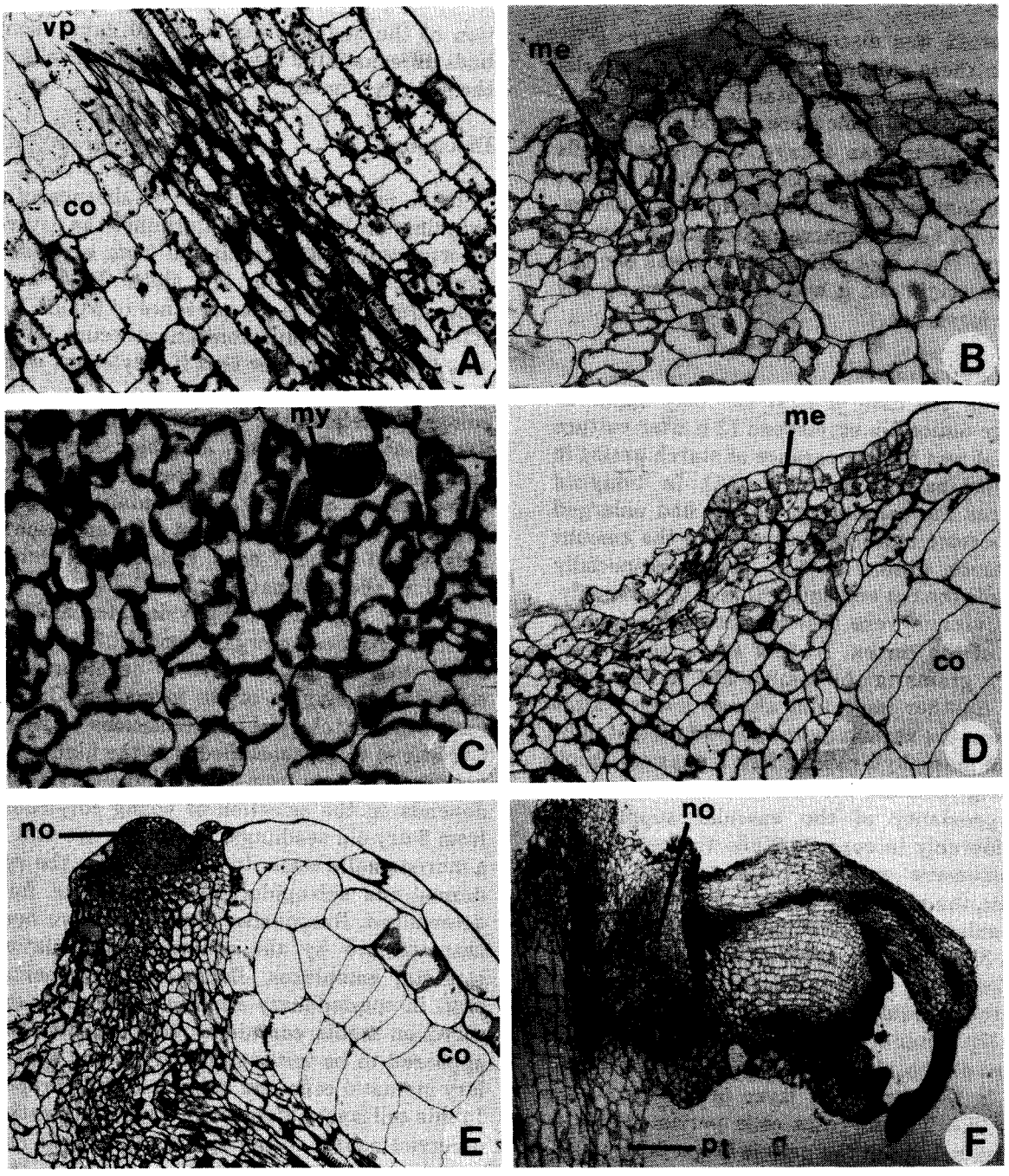

Fig. 3. Longitudinal sections of the proximal end of petioles (A,B,D,E,F) and vertical section of the lamina (C) from *B. juncea* cotyledon cultures on MS + BA medium (SIM) [co, cortex; me, meristematic cells; my, myrosin cell; no, meristematic nodule; pt, petiole; vp, vascular parenchyma]. (A) 12-h-old culture showing the appearance of starch grains in the cells of vascular parenchyma and slight enlargement of cortical cells (× 134). (B) 2-day-old culture showing meristematic cells next to the degenerating cells at the cut end. Cells behind the meristematic zone are devoid of starch (× 143). (C) Section of lamina from 3-day-old culture. The cells contain plastids, but are totally devoid of starch. Myrosin cells persist (× 207). (D) 3-day-old culture showing anticlinal and periclinal divisions in the epidermal and sub-epidermal cells at the cut end of the petiole (× 127). (E) 4-day-old culture showing a young meristematic nodule formed at the cut end (× 64). (F) 11-day-old cultures showing shoot buds with leaf primordia arising from vascularized nodules (× 31).

grains. During root and shoot formation the starch was rapidly mobilized first from the petiole and then from the lamina. While in root-forming cotyledons starch persisted in the cells of the lamina, in shoot-forming cotyledons all the laminary cells were completely depleted of starch. Thus shoot bud differentiation is probably a more energy requiring process than root formation. The mobilization and utilization of starch as a prime source of energy in shoot bud differentiation has been emphasized earlier in tobacco [15] and *P. abies* [16]. In *P. abies*, the organogenic embryos started to accumulate starch during the first day in culture mainly in the cortex and vascular system in the part of the embryo in contact with the medium [16]. Patel and Berlyn [17] correlated the occurrence of strong amylase activity with the degradation of starch in the organogenic centres of cultured explants of *Pinus coulteri*.

In the cotyledons of *B. juncea*, roots originate from the vascular parenchyma and cortical cells around the vascular supply of the petiole, close to the cut end which turns meristematic after 3 days of culture on RIM. Root primordia differentiate from these cells after 4 days of culture and well organized roots growing through the cortex were discernible after 5 days. Unlike root differentiation, shoot differentiation in this species was preceded by the formation of a small nodular callus. The nodules originated from the meristematic activity of the cortical cells around the vascular supply within 1.0 mm from the cut end of the petiole. The temporal histological changes during shoot formation were as follows: (1) initiation of mersitematic activity (12–24 h), (2) formation of meristematic nodules (4–7 days) and (3) differentiation of shoots from the periphery of the nodules (8–10 days). The limit of the second stage is marked by an increased requirement for nutrients and less or no dependence on BA [7].

In conclusion, the excised cotyledons of *B. juncea* may be a good system in which to carry out detailed studies of the physiology and biochemistry of organogenesis. Under specified conditions, roots and shoots are formed with high frequencies at the same morphological site within 6 and 10 days, respectively. This system is therefore potentially useful for basic studies on organogenesis, as well as for more applied work, such as selection of somaclonal variation (Arora and Bhojwani, unpublished results) and *Agrobacterium*-mediated gene transfer [18].

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