

HISTOLOGY OF ORGANOGENIC AND EMBRYOGENIC RESPONSES IN COTYLEDONS OF SOMATIC EMBRYOS OF *QUERCUS SUBER* L.

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In cork oak (*Quercus suber* L.), recurrent embryogenesis is produced *in vitro* through autoembryony without exogenous plant growth regulators (PGRs); secondary embryos appear on the embryo axis but seldom on cotyledons. Focusing mainly on the histological origin of neoformations, we investigated the influence of the embryo axis and exogenous PGRs on the embryogenic potential of somatic embryo cotyledons. Isolated cotyledons of somatic embryos became necrotic when cultured on PGR-free medium but gave secondary embryos when cultured on media containing benzyladenine and naphthaleneacetic acid. Cotyledons of cork oak somatic embryos are competent to give embryogenic responses. Isolated cotyledons without a petiole showed a lower percentage of embryogenic response than did those with a petiole. In petioles, somatic embryos arose from inner parenchyma tissues following a multicellular budding pattern. Joined to the embryo axis, cotyledons did not show morphogenic responses when cultured on PGR-free medium but revealed budlike and phylloid formations when cultured on medium with PGRs. The different morphogenic behavior displayed by somatic cotyledons indicates an influence of the embryo axis and indicates a relationship between organogenic and embryogenic regeneration pathways.

Keywords: autoembryony, cork oak, cotyledon, micropropagation, somatic embryogenesis.

Introduction

Somatic embryos of cork oak (*Quercus suber* L.) produce secondary embryogenesis on medium that lacks plant growth regulators (PGRs) (El Maâtaoui et al. 1990; Fernández-Guijarro et al. 1995). This phenomenon, known as autoembryony, has been described for several species and is the basis of embryo cloning, giving this technique the potential to produce high multiplication rates (Merkle 1995). However, secondary embryogenesis is also a problem when somatic embryos need to be converted into viable plants.

In a previous paper on cork oak recurrent somatic embryogenesis (Puigderrajols et al. 1996), we reported that on PGR-free medium, secondary embryos arose from a slight initial proliferation at the external layers of the root cap and developed mainly following a multicellular budding pattern. Under these culture conditions, we seldom observed secondary embryos originating from cotyledons. Thus, in cork oak, secondary embryogenesis appeared to be associated with the embryo axis. The absence of secondary embryogenesis in the cotyledons of somatic embryos has been shown in several species, such as *Aesculus hippocastanum* (Profumo et al. 1986), *Quercus robur*, and *Tilia cordata* (Chalupa 1990). In assays with zygotic embryos of *Pisum sativum*, the formation of embryogenic callus was associated exclusively with the embryonic axis and was independent of the maturation of embryos and the combinations and/or concentrations of PGRs used (Kysely and Jacobsen 1990). However, some species in which cotyle-

dons produce secondary embryogenesis are known; the best-known of these species is *Juglans regia* (Tulecke and McGranahan 1985).

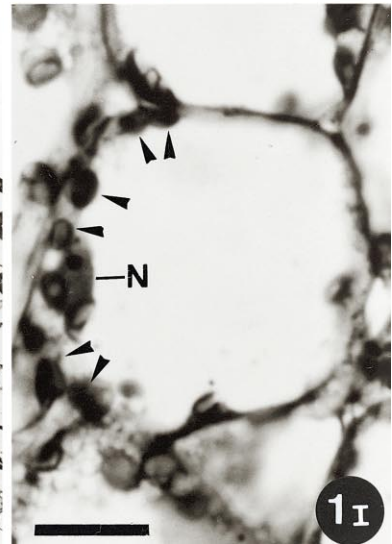
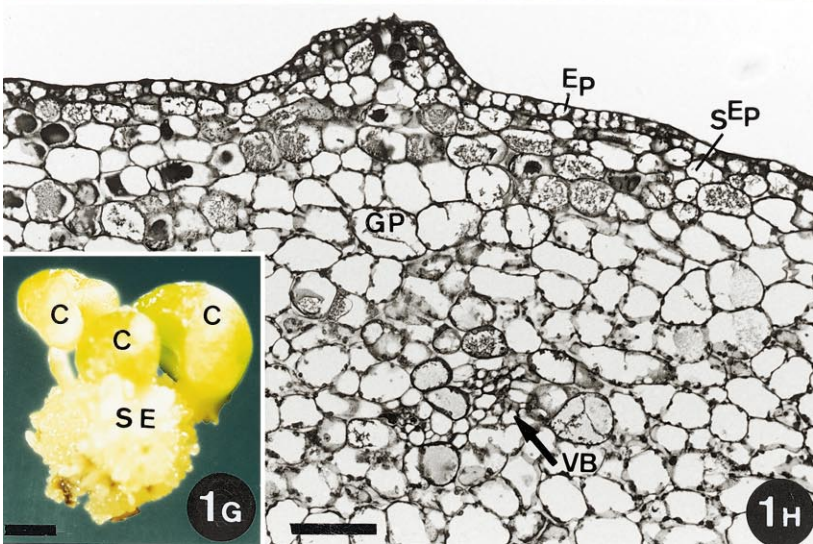
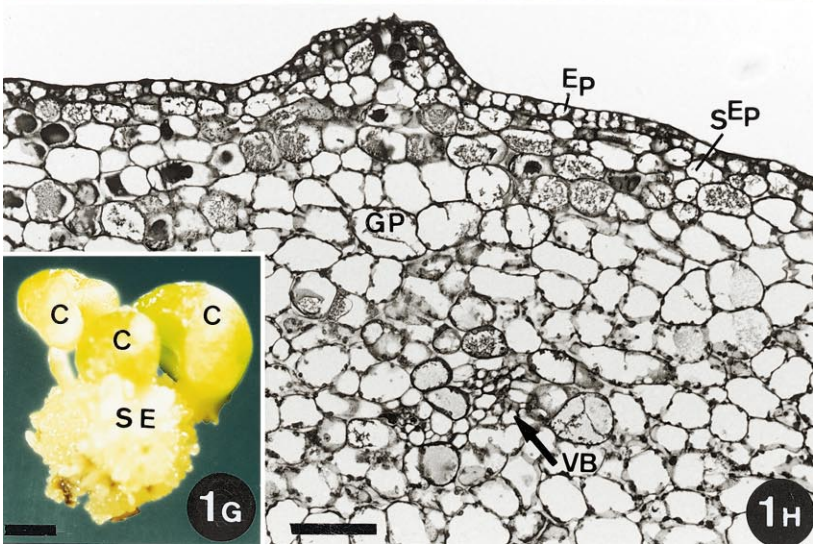
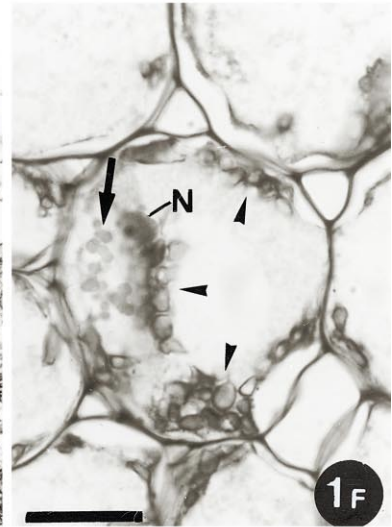
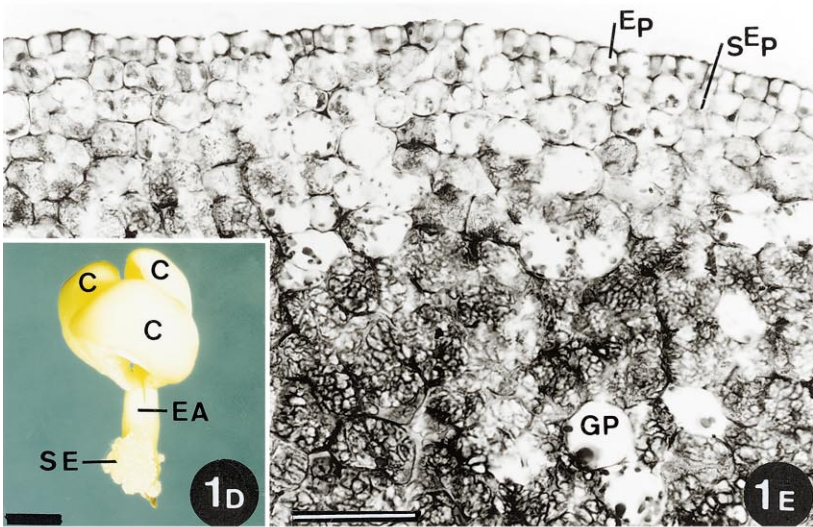
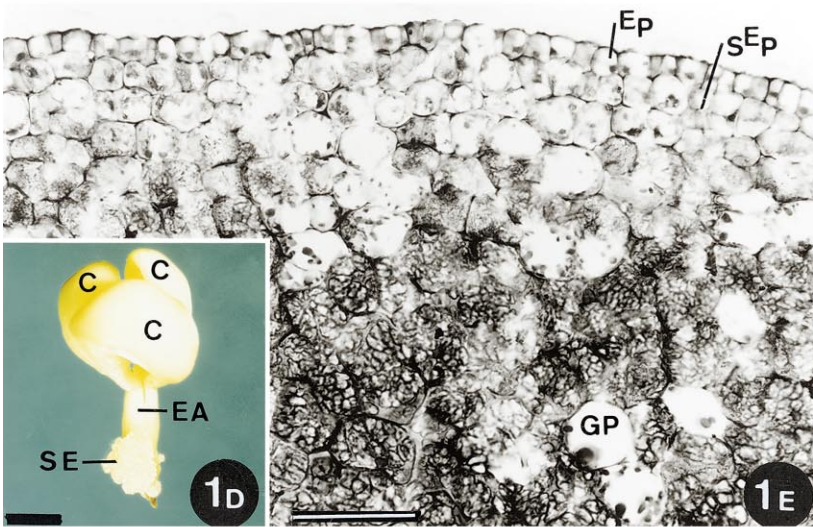
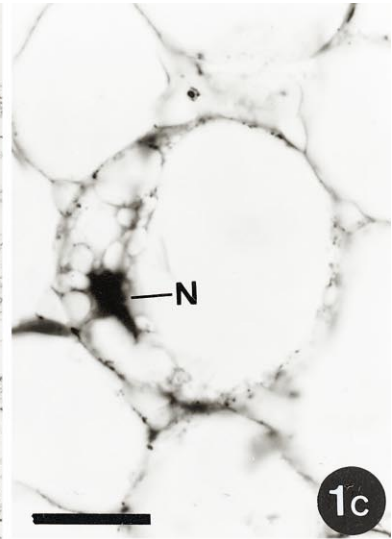
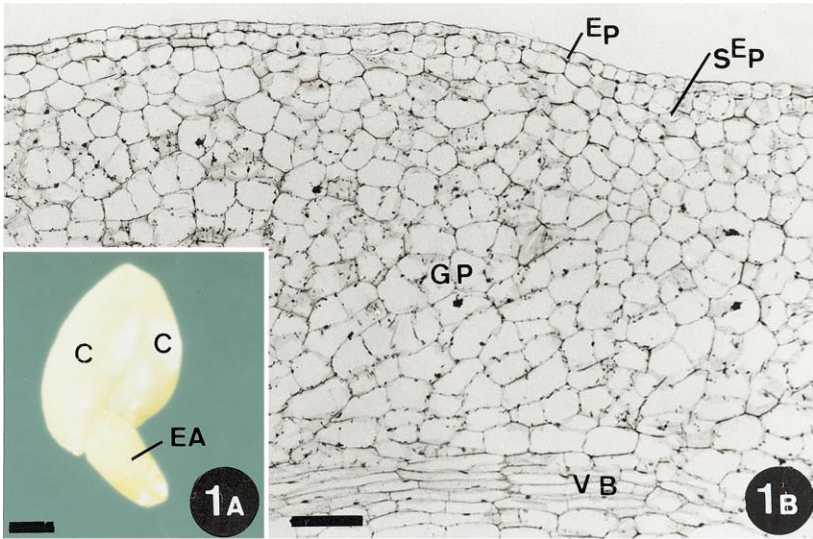
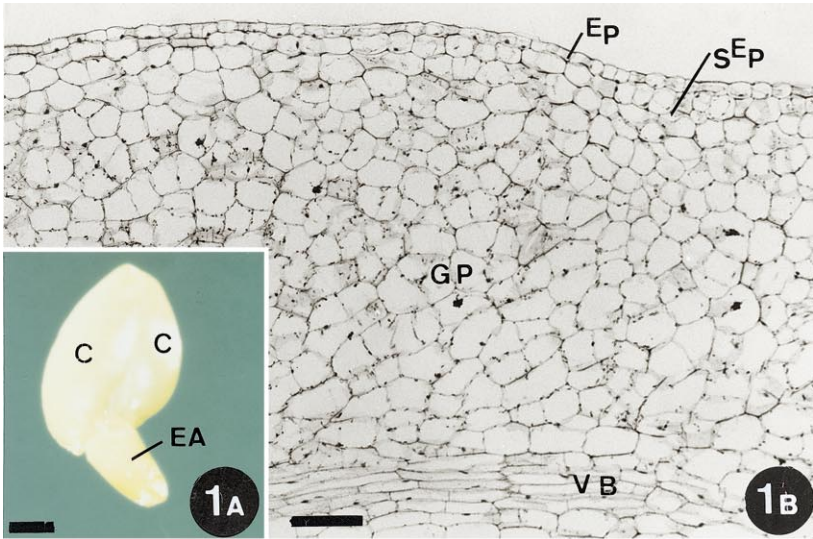
The knowledge of the mechanisms that control the morphogenic behavior of somatic embryo cotyledons may be important in controlling secondary embryogenesis and in recovering plants. This is of particular interest in the case of cork oak, because on PGR-free media that are suitable for germination, somatic cotyledons do not produce secondary embryogenesis. To explain the generally low embryogenic capacity shown by cotyledons, a possible inhibitory effect of the embryo axis, rather than the absence of competent tissues, has been suggested (Choi et al. 1997). In PGR-free medium, zygotic cotyledons of ginseng (*Panax ginseng*) gave direct embryogenesis only if they were cultured while detached from the embryo axis (Choi and Soh 1996).

Here we report on the morphogenic responses in somatic cotyledons of cork oak after they were cultured, either while joined to or detached from the main axis and either with or without added PGRs. The competence of somatic cotyledons for secondary embryogenesis and the influence of the embryo axis in the morphogenic responses of cotyledons are discussed.

Material and Methods

Somatic embryogenesis was initially produced in zygotic embryos removed from cork oak acorns that were gathered in an agroforest stand in Valdelosa (Salamanca, Spain). Embryogenic lines were maintained by recurrent embryogenesis simply through monthly subculture on PGR-free medium. From these embryogenic lines, whole immature somatic embryos (trans-

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lucent dicotyledonary structures ca. 5 mm in length), semi-mature embryos (white opaque structures ca. 1 cm in length), or excised cotyledons were used as starting explants in the different experiments. For a more detailed description, see Puigderrajols et al. (1996).

Explants were cultured in baby-food jars (60-mm diameter × 66-mm height) containing 50 mL of culture medium. The macronutrients were those from Schenk and Hildebrandt (1972) medium, and the micronutrients, vitamins, and Fe-EDTA were from Murashige and Skoog (1962), including the 3% sucrose. The culture medium was solidified with 0.6% agar (type E; Sigma Chemical, St. Louis), and the pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. The cultures were incubated in a growth chamber at 25° ± 2°C, and a 16-h photoperiod (50 μmol s⁻¹ m⁻²) was provided by cool-white plus Gro-lux fluorescent lamps. Entire embryos were cultured with their root pole inserted in the medium in baby-food jars (four per jar). Cotyledons were excised at the distal portion of the cotyledonary petiole from semimature embryos. Detached cotyledons were arranged with their abaxial surfaces upward in 60-mm-diameter petri dishes (four per dish) containing 10 mL of medium. Petri dishes and baby-food jars were sealed with Parafilm.

To investigate the influence of exogenous PGRs, whole semimature somatic embryos and isolated cotyledons were cultured following a two-stage induction method known to induce somatic embryogenesis in cork oak leaves (Fernández-Guijarro et al. 1995). In one experiment with PGRs, isolated cotyledons lacking petioles were cultured. For the two-stage induction, explants were placed on the basal medium described above and supplemented with 10 μM benzyladenine (BA) and 10 μM naphthaleneacetic acid (NAA); explants were then cultured in darkness at 25° ± 2°C for 20 d. They were then transferred to the basal medium with 0.5 μM BA and 0.5 μM NAA and placed in the growth chamber at 25° ± 2°C (with a 16-h photoperiod) for another 20 d. This was followed by culture on a PGR-free basal medium (also in the growth chamber).

For each type of explant and culture condition, at least 50 explants were cultured. Samples were collected every 4 to 5 d following the initiation of treatments. For light microscopy, samples were fixed in 10% buffered formalin (pH 7.2), dehydrated through an isopropyl alcohol series, and embedded in glycol-methacrylate. The sections were stained with the periodic acid-Schiff reaction, toluidine blue, or thionin (Puigderrajols et al. 1996). Fluorescence was ob-

served using a Vanox epifluorescence microscope equipped with a HBO200 mercury burner. A combination 330–400-nm exciter filter–dichroic beam splitter DM445 and 420-nm barrier filter was used for observation with ultraviolet light.

Results

Cotyledons Joined to the Embryo Axis Cultured on PGR-Free Medium (Figs. 1, 2)

On PGR-free medium, cork oak somatic embryos produced a large number of secondary embryos, which appeared all around the hypocotyl of the embryo axis but not on the cotyledons. The cotyledons were at first translucent (fig. 1A), then became white opaque (fig. 1D), and later, green (fig. 1G). The presence of more than two cotyledons per embryo was not rare, and cotyledons often displayed a distorted morphology, but secondary embryo formation was not evident. However, in long-term cultures, cotyledons became difficult to observe because of the increasing size of the secondary embryos growing from the hypocotyl. On the surface of the cotyledons, the formation of compact outgrowths (fig. 2A) and/or the development, on rare occasions, of soft mucilaginous masses (fig. 2D) was observed.

At the histological level, early translucent cotyledons (fig. 1B) showed a single-layered epidermis formed by small cells with conspicuous nuclei, a single-layered subepidermis with regularly arranged cells, and a ground parenchyma of larger vacuolated cells with small intercellular spaces (fig. 1C). Bundles of procambium traversed the ground parenchyma. At the white opaque stage (fig. 1E), the epidermis cells were vacuolated and more cuboidal; the subepidermis consisted of two to three cell layers that were smaller than the underlying parenchyma cells. The ground parenchyma consisted of large cells with large amounts of starch grains and proteolipidic bodies (fig. 1F). Later, when the cotyledons turned greenish (fig. 1H), the content of starch and proteolipidic bodies decreased in the parenchyma cells (fig. 1I); chloroplasts were more prevalent, mainly in the parenchyma, next to the vascular bundles. At this stage, most subepidermal cells showed deposits of vacuolar phenolics.

The compact outgrowths were found mainly on the abaxial surface of the cotyledons, but they never became organogenic or embryogenic. The outgrowths were formed by the proliferation of the subepidermal cells, and the initial growth was

Fig. 1 Cotyledons joined to the embryo axis during 40 d of culture on PGR-free medium (Schenk and Hildebrandt macronutrients; micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog). C = cotyledon; EA = embryo axis; EP = epidermis; SEP = subepidermis; GP = ground parenchyma; N = nucleus; SE = secondary embryogenesis; VB = vascular bundles. A, Somatic embryo at the beginning of the culture showing cotyledons at translucent stage. Bar = 1 mm. B, Section of a translucent cotyledon at the abaxial surface showing a single-layered epidermis, a single-layered subepidermis, and a ground parenchyma traversed by bundles of procambium. Bar = 100 μm. C, Detail of a vacuolated cell in ground parenchyma. Note the absence of reserve substances. Bar = 20 μm. D, Somatic embryo after 20 d of culture with white opaque cotyledons showing secondary embryogenesis at the root tip. Bar = 3 mm. E, Section of a white opaque cotyledon at the abaxial surface. Note cuboidal vacuolated cells forming the single-layered epidermis, the two- to three-layered subepidermis, and the ground parenchyma cells filled with reserve substances. Bar = 100 μm. F, Detail of a ground parenchyma cell showing starch grains (arrowheads) and proteolipidic bodies (arrow). Bar = 20 μm. G, Somatic embryo after 40 d of culture with greenish cotyledons and growing secondary embryos at the axis level. Bar = 3 mm. H, Section of a dark-green cotyledon at the abaxial surface showing phenolics in subepidermal cells. Bar = 100 μm. I, Detail of ground parenchyma cell with chloroplasts (arrowheads). Bar = 20 μm.

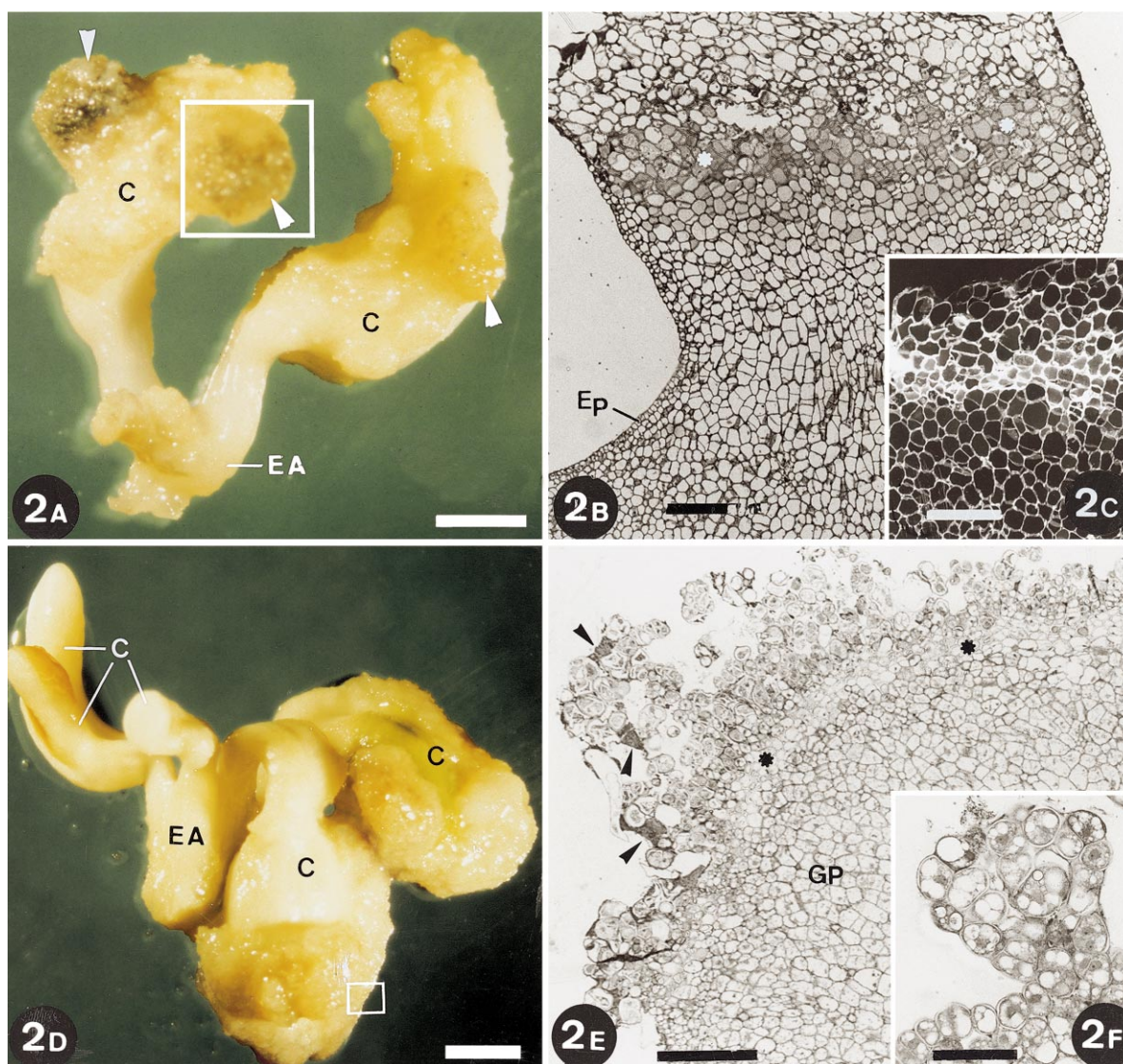


Fig. 2 Cotyledons joined to the embryo axis after 40 d of culture on PGR-free medium (Schenk and Hildebrandt macronutrients; micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog). C = cotyledon; EA = embryo axis; EP = epidermis; GP = ground parenchyma. A, Cotyledons with compact outgrowths on the abaxial surface (arrowheads). Boxed area shown in fig. 2B. Bar = 2 mm. B, Longitudinal section of a compact outgrowth (fig. 2A box) showing epidermis breakage and cells with phenolic contents (asterisks). Bar = 200 mm. C, Detail of the cell layer with autofluorescent cell wall under UV light. Bar = 200 mm. D, Soft mucilaginous masses at the distal portion of the cotyledons. Boxed area shown in fig. 2E. Bar = 2 mm. E, Histology a mucilaginous mass (fig. 2D box). Note the presence of isolated cells and group of cells surrounded by extracellular dark substance (arrowheads). Asterisks indicate cells with a pearly appearance underlying mucilaginous mass. Bar = 200 mm. F, Detail of a globular structure placed in mucilaginous mass proliferation. Bar = 50 mm.

usually followed by rupture of the epidermis. In the parenchyma tissue forming the compact outgrowth, a 5–10-layer band of cells, with vacuoles filled with phenolics, was found some layers below the external surface (fig. 2B). Under the fluorescence microscope, a layer of autofluorescent cells was seen at the inner side of the band, separating it from the underlying ground parenchyma. These cells showed a blue fluorescence under UV light and a yellowish-green fluorescence under blue light (fig. 2C).

The soft mucilaginous masses found on rare occasion were

mostly on the distal portion of the cotyledons. These masses at the external side consisted of isolated meristematic cells, with a thick cell wall surrounded by a darkly staining extracellular substance (fig. 2E). Underlying the masses, three to five layers of cells showed conspicuous cell walls with a pearly appearance. These cells fluoresced strongly blue under UV light and greenish-blue under green light. Some globular structures were formed from the mucilaginous mass (fig. 2F), but no further development of these structures was observed in the histological sections.

Detached Cotyledons Cultured on PGR-Free Medium (Fig. 3)

Detached cotyledons cultured on a PGR-free medium for a few days matured in the same manner as did the cotyledons joined to the main axis. Then, although some of the cotyledons became greenish, they all became necrotic. We never obtained morphogenic responses from these explants. In only one case did we observe the formation of whitish globular structures on the abaxial surface of the cotyledon in a proximal position near the cut surface of the petiole (fig. 3A). At the histological level, these globular structures (fig. 3B) were similar to those formed on the soft mucilaginous mass.

Cotyledons Joined to the Embryo Axis Cultured on Medium with BA and NAA (Fig. 4)

Semimature embryos, selected from among those without visible external signs of secondary embryogenesis, were subjected to the two-stage induction method described above. Almost all of these embryos produced secondary embryos at the embryo axis; some 20% of treated embryos showed root elongation, and 5% displayed shoot and root elongation. The cotyledons gave a wide range of morphogenic responses, but we could not confirm the formation of true bipolar structures. Approximately 10–15 d after the initiation of the culture on a medium with 10 μ M BA and 10 μ M NAA, the cotyledonary surface appeared to be covered with compact whitish lobes, mostly on the abaxial surface (fig. 4A). When the embryos were transferred to a medium with 0.5 μ M BA and 0.5 μ M NAA, the lobes formed conspicuous phylloidal structures and a few root tips (fig. 4C).

At the histological level, we observed the formation of vascular bundles in the outer cortical parenchyma, followed by the formation of proliferating masses of epidermal and subepidermal origin (fig. 4B). These masses consisted of homogeneous vacuolated cells that were devoid of starch and that budded rapidly. Most of the initial masses formed many bud-

like structures and some adventitious roots (fig. 4D, 4F). Usually the budlike structures developed into phylloids connected with the neoformed vascular bundles. No sign of root and/or shoot-meristematic pole organization could be observed during phylloid development. New meristematic zones, which would be responsible for second-order formations, appeared in the bases of the phylloids (fig. 4E).

Detached Cotyledons Cultured on Medium with BA and NAA (Fig. 5)

The detached cotyledons on medium with BA and NAA followed a maturation process similar to that described for the attached cotyledons cultured without PGRs. Only small differences were observed. Detached cotyledons grew more regular in shape (without outgrowths) and became darker green than did the cotyledons joined to the main axis (fig. 5A). Accumulation of phenolics was observed in the subepidermal layers (fig. 5B), and the decrease in starch grains and proteolipidic bodies was less marked. Most of these cotyledons formed a small callus on the cut surface. The callus was composed of parenchymatous cells with the presence of some vascular connections with mother tissue (fig. 5C). In most cotyledons—during the ca. 60 d of observation—the calluses remained small and did not develop meristematic primordia and/or adventitious embryos. Occasionally a root developed (fig. 5D), one connected with the vascular tissue of the petiole (fig. 5E). In some cotyledons we observed the formation of secondary embryos that were attached to the cotyledonary petiole near the basal excised portions of the cotyledons (fig. 5F). These embryos originated from the peripheral parenchyma near the vascular bundles by a direct multicellular pathway. The parenchyma cells first became meristematic and then proliferated (fig. 5G–5I). In contrast to the cotyledonary tissue, the petioles did not show an accumulation of phenolics in subepidermal cell layers.

To determine whether the petiole influences the embryogen-

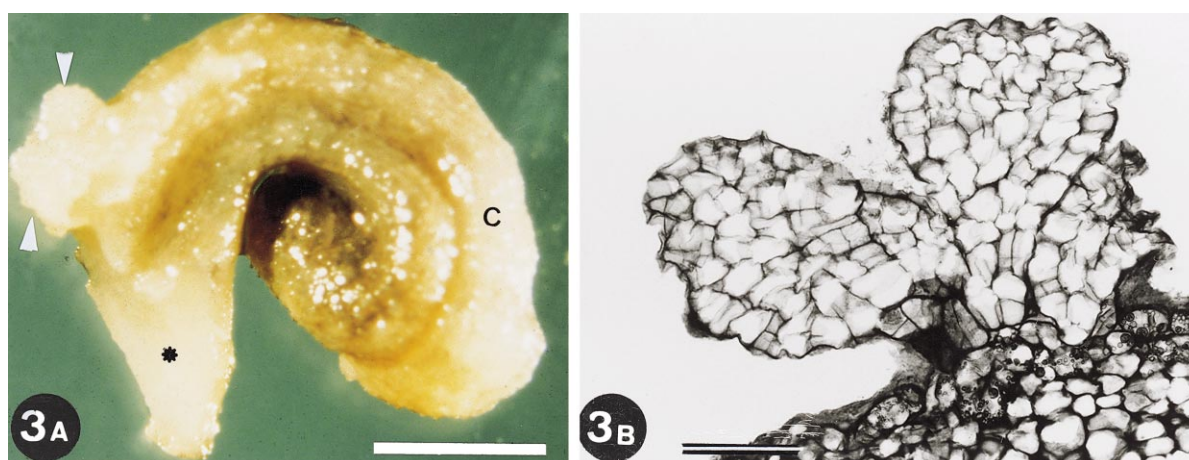


Fig. 3 Detached cotyledon on PGR-free medium (Schenk and Hildebrandt macronutrients; micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog). This was the only explant that gave morphogenic response C = cotyledon. A, Excised cotyledon showing whitish globular structure (arrowheads) on the abaxial surface. Asterisk indicates the cut surface. Bar = 2 mm. B, Histological section of white globular structures arising from epidermis. Bar = 50 μ m.

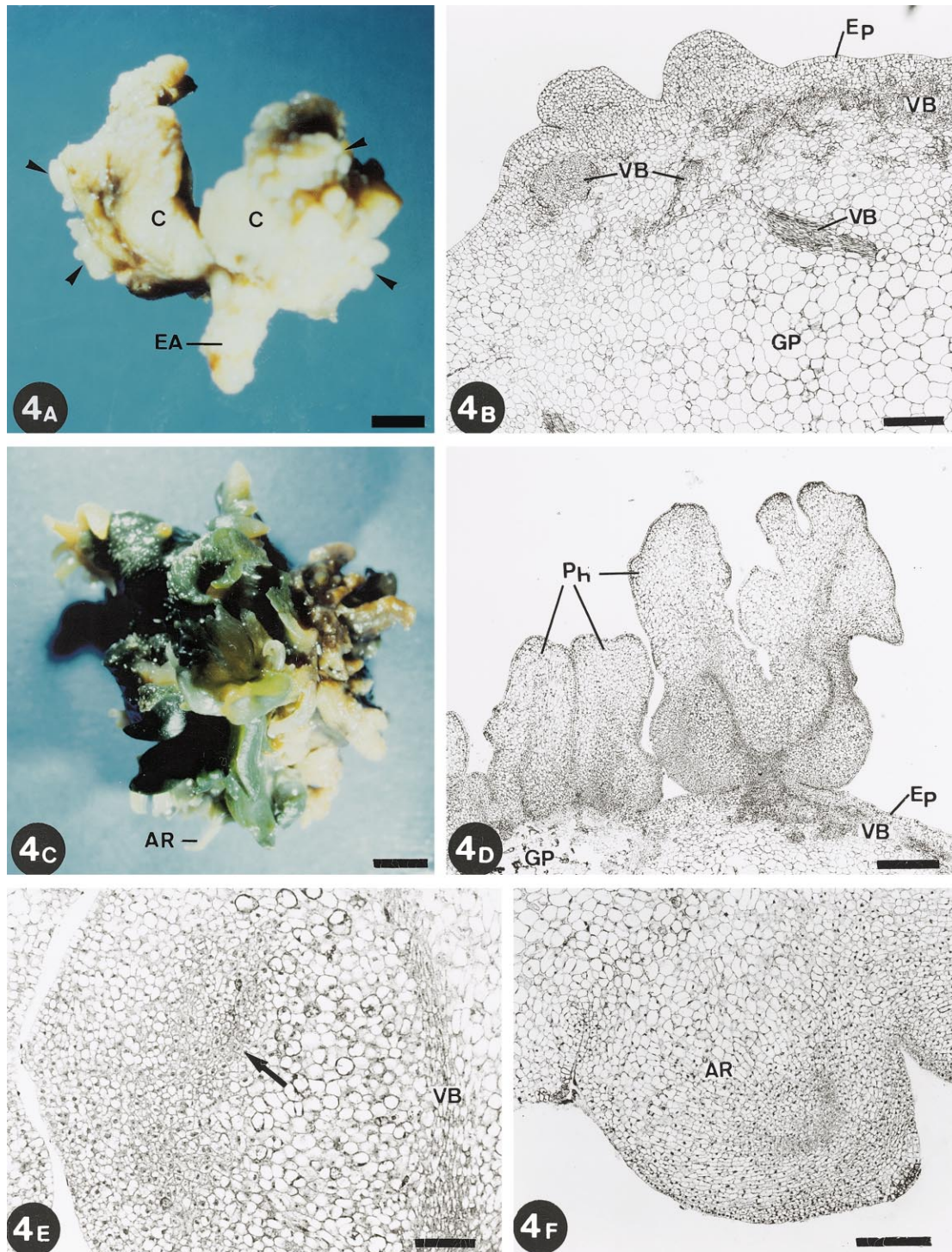
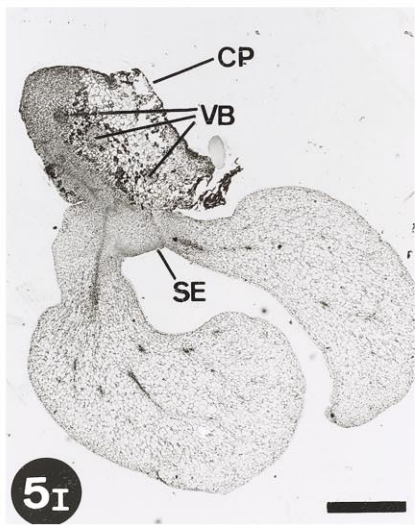
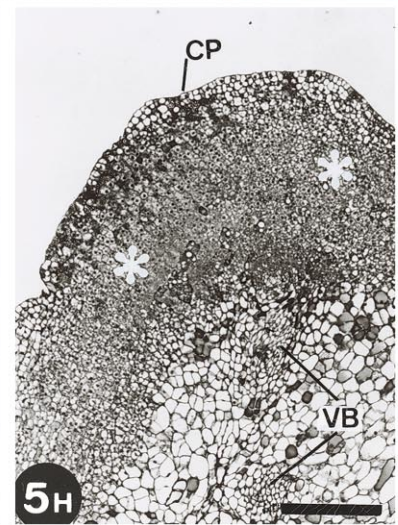
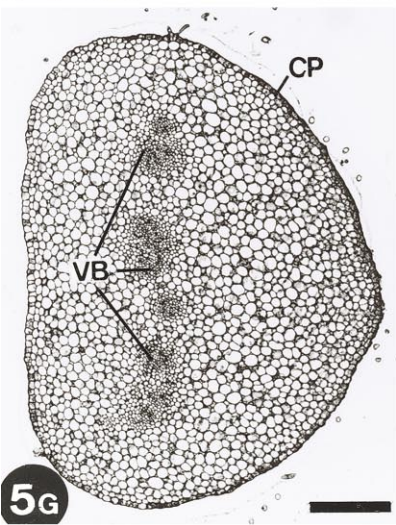
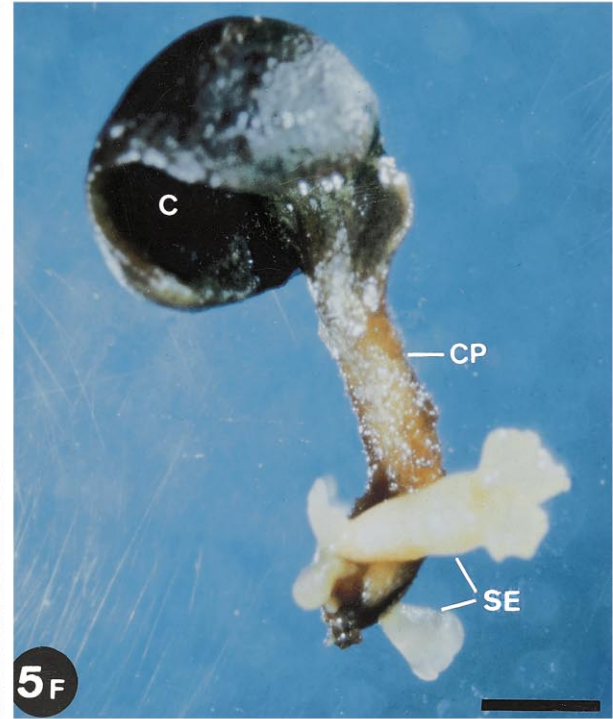
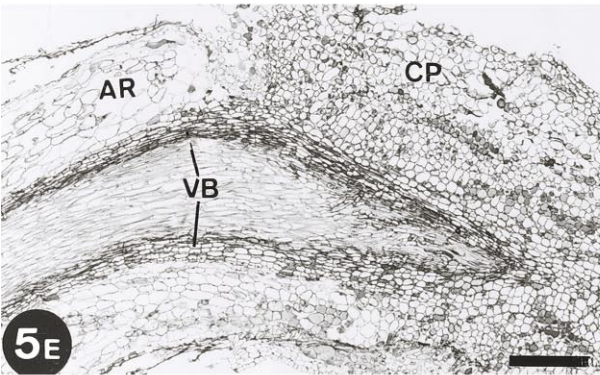
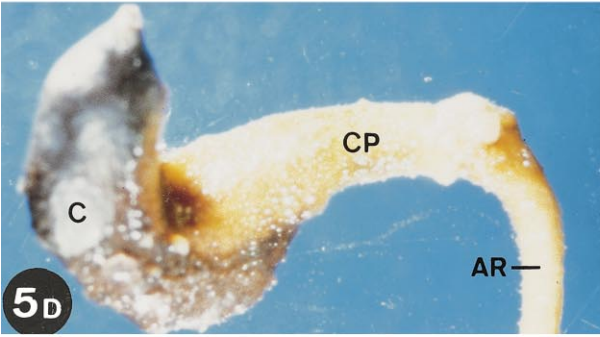
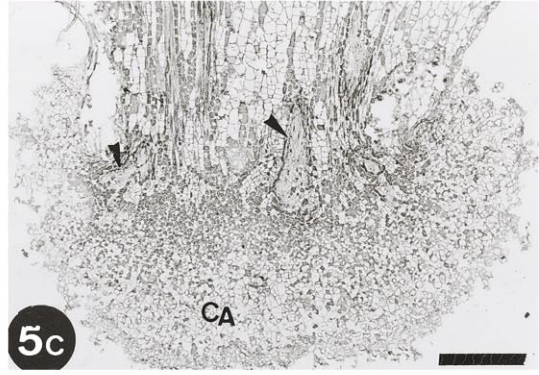
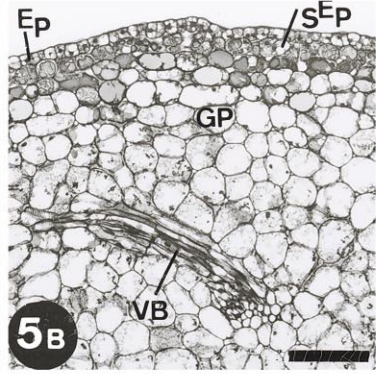
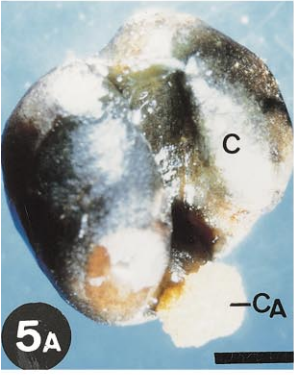


Fig. 4 Cotyledons joined to the embryo axis cultured on medium (composed of Schenk and Hildebrandt macronutrients and micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog) with $10 \mu\text{M}$ BA and $10 \mu\text{M}$ NAA in darkness for 20 d. This was followed by culture on the same medium with $0.5 \mu\text{M}$ BA and $0.5 \mu\text{M}$ NAA under a 16-h photoperiod for an additional 20 d. C = cotyledon; EA = embryo axis; EP = epidermis; GP = ground parenchyma; VB = vascular bundles; AR = adventitious root; Ph = phylloid. A, Somatic embryo after 10–15 d on the induction medium. Arrows indicate the compact whitish masses arising from abaxial cotyledonary surface. Bar = 2 mm. B, Histological section of fig. 4A showing vascular bundles in the outer cortical parenchyma and the initials of proliferating masses. Bar = 200 μm . C, Detail of a cotyledon obtained from a whole somatic embryo subjected to 20 d in dark plus 5 d in photoperiod and on a medium with low PGR concentration. Observe phylloids completely covering the cotyledon. Bar = 2 mm. D, Histological section of phylloids. Note vascular connection between phylloid and vascular bundles initially formed in outer parenchyma of cotyledon. Bar = 400 μm . E, New meristematic zone formed in the base of a phylloid. Arrow indicates formation site of new vascular bundles. Bar = 100 μm . F, Histological section of an adventitious root emerging from proliferation mass. Bar = 200 μm .



esis in explants, cotyledons with petiole, cut at proximal or distal positions, were subjected to the two-stage induction conditions. The percentage of responding explants was higher when the cotyledons bore the cotyledonary petiole, and a small percentage of these explants also displayed root or root with shoot formation (table 1).

Discussion

Although cotyledons of cork oak somatic embryos are rarely morphogenic in PGR-free medium, our results show that they are competent to give an organogenic and embryogenic response when BA and NAA are present.

In a PGR-free medium, the embryonic axis shows a high capacity for the formation of secondary embryos (Puigderrajols et al. 1996); therefore, in this tissue, the process depends on endogenous regulation. However, to yield a morphogenic response, cotyledons need an exogenous induction. By considering as well that excised cotyledons do not survive without an exogenous supply of BA and NAA, we can deduce that cotyledons do not synthesize appropriate PGRs, or they do not synthesize enough of them.

The higher percentage of cotyledonary explants exhibiting embryogenesis when the petiole was present indicates that the petiole has a greater capacity for response than does the rest of the cotyledon. The embryogenic capacity of petioles was only manifest when BA and NAA were present. So a gradient in the embryogenic response is shown between the root cap and the cotyledons.

The different morphogenic behaviors of cotyledons with the presence or absence of the main axis indicate that this part of the embryo has a clear influence. The probability of some inhibition from the axis as it relates to the morphogenic ability of cotyledons (as claimed by Choi and Soh 1996) cannot be ruled out. Considering that this effect can be overcome by the addition of exogenous BA and NAA, it can be deduced that tissues with different levels of competence require a precise balance of these substances to develop a morphogenic response.

A low percentage of detached cotyledons with a petiole showed shoot and root organogenesis on medium with PGRs. Although we could not monitor the histological origin of these shoots, it is possible that the same process of meristem initiation and development that originates somatic embryos changed into a vegetative bud, from which a shoot developed. The profuse formation of phylloids on cotyledons of somatic

embryos cultured with BA and NAA could represent an intermediate stage. This is in keeping with the idea that early stages of direct embryogenesis are similar to axillary bud formation (Halperin 1995), although further development differentiates both structures.

The formation of somatic embryos in cotyledonary petioles was independent of the wound-healing callus. Embryos originated from the cortical parenchyma opposite to the vascular bundles. There are other examples in which the internal origin of somatic embryos is reported: i.e., *Solanum aviculare*, in which embryogenesis is related to parenchyma cells associated with vascular traces close to the cut ends (Alizadeh and Mantell 1991). This origin contrasts with that found in the axis, in which the outer layers of the root cap gave rise to secondary embryos (Puigderrajols et al. 1996). This observation demonstrates that specific cells in different explants may be committed to providing the embryogenic response, at least that of multicellular origin.

Detached cotyledons on medium with BA and NAA displayed embryogenesis only in petioles. In detached cotyledons, phenolics accumulated in epidermic and subepidermic cells, except in petioles. Phenolic substances are closely related to the metabolic activity of PGRs, namely auxins, polyamines, and ethylene (Roustan et al. 1992; Cvikrova et al. 1996). In *Medicago sativa*, the total content of phenolic acids was higher in nonembryogenic cultures compared with embryogenic cultures (Hrubcova et al. 1994). Treatments that involved a relatively high concentration of BA that was administered for a short time were important in inhibiting polyphenol accumulation, thus stimulating embryo formation in *Medicago sativa* (Li and Demarly 1996). Alemanno et al. (1996) reported that in *Theobroma cacao*, cells in nonembryogenic calluses accumulated polyphenolics. Thus, their accumulation may be connected with the suppression of the embryogenic response. In fact, we had previously observed that mature somatic embryos of cork oak, which do not show secondary embryogenesis, do accumulate polyphenols in the cells of the root cap (Puigderrajols et al. 1996).

We have shown that the mucilaginous masses that infrequently formed on the distal portions of cotyledons were composed of isolated groups of cells surrounded by a dark extracellular substance. A similar pattern was observed in *Camellia japonica*, in which somatic embryogenesis appeared after a continued proliferation of cells of abaxial subepidermal layers that formed protuberances followed by cellular isolation (Barciela and Vieitez 1993). Although we have not observed somatic embryos coming from the mucilaginous

Fig. 5 Detached cotyledons cultured on medium (composed of Schenk and Hildebrandt macronutrients and micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog) with 10 μ M BA and 10 μ M NAA in darkness for 20 d. This was followed by culture on the same medium with 0.5 μ M BA and 0.5 μ M NAA under a 16-h photoperiod for an additional 20 d. C = cotyledon; CA = callus; EP = epidermis; SEP = subepidermis; GP = ground parenchyma; VB = vascular bundles; CP = cotyledonary petiole; AR = adventive root; SE = secondary embryogenesis. A, Detached dark cotyledon showing callus formation on the cut surface. Bar = 2 mm. B, Accumulation of phenolics in the subepidermal layers of dark cotyledon. Bar = 100 μ m. C, Histological section of a callus formed on the cut surface. Note the presence of some vascular connections with mother tissue (arrowheads). Bar = 400 μ m. D, Morphological aspect of a detached cotyledon that developed a root from the cut surface. Bar = 2 mm. E, Histological section of D. Note vascular connection between petiole and neofomed root. Bar = 500 μ m. F, Detached cotyledon showing secondary embryogenesis from the petiole near the cut surface. Bar = 2 mm. G, I, Cross sections of cotyledonary petioles showing sequence of somatic embryo formation. G, Cotyledonary petiole at the initial of subculture. Note the absence of phenolics. Bar = 200 μ m. H, Meristematic cells (asterisks) in ground parenchyma. Bar = 200 μ m. I, Cross section of cotyledonary petiole showing a longitudinally sectioned secondary embryo arising from it. Bar = 600 μ m.

Table 1
Morphogenic Responses of Cotyledons Detached from Somatic Embryos of Cork Oak with or without Petiole, Cultured on a Two-Stage Induction System

Explant type	Number	Percentage of explants showing			
		No response	Secondary embryogenesis	Roots	Root plus shoots
Without petiole	100	59	41	0	0
With petiole	102	24	69	4	3

Note. Contrast, Fisher's exact test: two-sided *P* value, without petiole versus with petiole = 0.0001. Data after 40 d in culture. Cotyledons were cultured on medium composed of Schenk and Hildebrandt macronutrients and of micronutrients, vitamins, and Fe-EDTA from Murashige and Skoog with 10 μM BA and 10 μM NAA in darkness for 20 d. This was followed by culture on the same medium with 0.5 μM BA and 0.5 μM NAA under a 16-h photoperiod for an additional 20 d. No response = only callus was formed at the cut surface.

masses, the globular structures that form are identical to those we previously reported in the root cap of somatic embryos of cork oak (Puigderrajols et al. 1996) and to those described in other species (Halperin 1995). Therefore, we suggest that the globular structures may be the initiation of isolated secondary embryos or proembryonic masses (of unicellular origin). The presence of autofluorescence in the cells underlying the proliferation would support this idea. Pedrosa and Pais (1995) described the same type of fluorescence in parenchyma cell walls of the embryogenic leaf regions and in the embryogenic calluses and even in the protodermis and

peripheral cells of zygotic cotyledons of several species, including *Quercus suber*.

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Literature Cited

- Alemanno L, M Berthouly, N Michaux-Ferrière 1996 Histology of somatic embryogenesis from floral tissues of cocoa. *Plant Cell Tissue Organ Cult* 46:187–194.
- Alizadeh S, SH Mantell 1991 Early cellular events during direct somatic embryogenesis in cotyledon explants of *Solanum aviculare*. *Ann Bot* 67:257–263.
- Barciela J, AM Vieitez 1993 Anatomical sequence and morphometric analysis during somatic embryogenesis on cultured cotyledon explants of *Camellia japonica* L. *Ann Bot* 71:395–404.
- Chalupa V 1990 Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). *Plant Cell Rep* 9:398–401.
- Choi YE, HS Kim, WY Soh, DC Yang 1997 Developmental and structural aspects of somatic embryos formed on medium containing 2,3,5-triiodobenzoic acid. *Plant Cell Rep* 16:738–744.
- Choi YE, WY Soh 1996 Effect of plumule and radicle on somatic embryogenesis in the cultures of ginseng zygotic embryos. *Plant Cell Tissue Organ Cult* 45:137–143.
- Cvikrova M, M Hrubcova, J Eder, P Binarova 1996 Changes in the levels of endogenous phenolics, aromatic monoamines, phenylalanine ammonia-lyase, peroxidase and auxin oxidase activities during initiation of alfalfa embryogenic and non-embryogenic calli. *Plant Physiol Biochem* 34:853–861.
- El Maâtaoui M, H Espagnac, N Michaux-Ferrière 1990 Histology of callogenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) cultured in vitro. *Ann Bot* 66:183–190.
- Fernández-Guijarro B, C Celestino, M Toribio 1995 Influence of external factors on secondary embryogenesis and germination in so-
- matic embryos from leaves of *Quercus suber*. *Plant Cell Tissue Organ Cult* 41:99–196.
- Halperin W 1995 *In vitro* embryogenesis: some historical issues and unresolved problems. Pages 1–16 in TA Thorpe, ed. *In vitro embryogenesis in plants*. Kluwer, Dordrecht.
- Hrubcova M, M Cvikrova, J Eder 1994 Peroxidase activities and contents of phenolic acids in embryogenic and nonembryogenic alfalfa cell suspension cultures. *Biol Plant* 36:175–182.
- Kysely W, HJ Jacobsen 1990 Somatic embryogenesis from pea embryos and shoot apices. *Plant Cell Tissue Organ Cult* 20:7–14.
- Li X-Q, Y Demarly 1996 Somatic embryogenesis and plant regeneration in *Medicago suffruticosa*. *Plant Cell Tissue Organ Cult* 44:79–81.
- Merkle SA 1995 Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. *Plant Tissue Cult Biotech* 1:112–121.
- Murashige T, F Skoog 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* 15:473–497.
- Pedrosa C, S Pais 1995 Factors controlling somatic embryogenesis. *Plant Cell Tissue Organ Cult* 43:147–154.
- Profumo P, P Gastaldo, RM Dameri, L Caffaro 1986 Histological study of calli and embryoids from leaf explant of *Aesculus hippocastanum* L. *J Plant Physiol* 126:97–103.
- Puigderrajols P, B Fernández-Guijarro, M Toribio, M Molinas 1996 Origin and early development of secondary embryos in *Quercus suber* L. *Int J Plant Sci* 157:674–684.
- Roustan JP, A Latche, J Fallot 1992 Influence of ethylene on the incorporation of 3,4-[¹⁴C] methionine into polyamines in *Daucus carota* cells during somatic embryogenesis. *Plant Physiol Biochem* 30:201–205.

Schenk RU, AC Hildebrandt 1972 Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can J Bot* 50:199-204.

Tulecke W, G McGranahan 1985 Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. *Plant Sci* 40:57-63.