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# Mechanisms of primordium formation during adventitious root development from walnut cotyledon explants

**Fabienne F. Ermel[1], Séraphine Vizoso[1], Jean-Paul Charpentier[1], Christian Jay-Allemand[1] Anne-Marie Catesson[2], Ivan Couée[3]**

[1]Unité Amélioration, Génétique et Physiologie Forestières, Institut National de la Recherche Agronomique, BP 20619 Ardon, 45166 Olivet cedex, France
[2]Laboratoire de Physiologie Végétale Appliquée, Université Pierre et Marie Curie, 4 place Jussieu, 75252 Paris cedex 05, France
[3]Université de Rennes 1, Centre National de la Recherche Scientifique, UMR 6553, Physiologie et Ecophysiologie, Campus scientifique de Beaulieu Bâtiment 14, 35042 Rennes cedex, France

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**Abstract.** In walnut (*Juglans regia* L.), an otherwise difficult-to-root species, explants of cotyledons have been shown to generate complete roots in the absence of exogenous growth regulators. In the present study, this process of root formation was shown to follow a pattern of adventitious, rather than primary or lateral, ontogeny: (i) the arrangement of vascular bundles in the region of root formation was of the petiole type; (ii) a typical root primordium was formed at the side of the procambium within a meristematic ring of actively dividing cells located around each vascular bundle; (iii) the developing root apical meristem was connected in a lateral way with the vascular bundle of the petiole. This adventitious root formation occurred in three main stages of cell division, primordium formation and organization of apical meristem. These stages were characterized by expression of *LATERAL ROOT PRIMORDIUM-1* and *CHALCONE SYNTHASE* genes, which were found to be sequentially expressed during the formation of the primordium. Activation of genes related to root cell differentiation started at the early stage of primordium formation prior to organization of the root apical meristem. The systematic development of adventitious root primordia at a precise site gave indications on the positional and biochemical cues that are necessary for adventitious root formation.

**Key words:** Adventitious root formation – Chalcone synthase – Cotyledon – *Juglans* (root formation) – Organogenesis – Primordium

## Introduction

As emphasized by Malamy and Benfey (1997), one of the fundamental questions in developmental biology is how cells proliferate and organize to form discrete organs. In plants, this involves a large degree of postembryonic organogenesis. In this respect, adventitious root formation is of utmost interest since it consists of the development of root tissues from non-root and non-meristematic tissues. The variability of adventitious rooting between species, between clones of the same species, and between developmental stages of the same clone, points to the complexity of the mechanisms involved and outlines the necessity to determine the archetypal steps that may be common to angiosperms. Difficult-to-root species or plant systems could then be characterized with respect to these steps.

Cotyledons of angiosperms usually possess a high capacity for embryogenesis and organogenesis. However, this capacity is not uniformly shared by all the different regions of the cotyledon. Significant differences thus exist between the region in contact with the embryonic axis and other parts of the cotyledon. In walnut (*Juglans regia* L.), an otherwise difficult-to-root species, cotyledon explants from the region of attachment to the embryonic axis (Fig. 1) have been shown to generate complete roots in the absence of exogenous growth regulators (Jay-Allemand et al. 1991; Gutmann et al. 1996). In contrast, explants from distal parts of the cotyledon required auxin treatment for induction of root development (Gutmann et al. 1996). In explants from the region of attachment to the embryonic axis, morphological changes occurred in a well-defined pattern with development of a petiole-like structure at the tip of the cotyledon explant and formation of roots at the tip of this petiole (Jay-Allemand et al. 1991; Gutmann et al. 1996).

This complex developmental process of root formation at the tip of the newly formed petiole remains poorly characterized with respect to primary root development (Scheres et al. 1996), to lateral root formation, which arises from the pericycle cells of the

Abbreviations: CHS = chalcone synthase; LRP1 = lateral root primordium-1; TIP = tonoplast intrinsic protein

*Correspondence to*: I. Couée; E-mail: ivan.couee@univ-rennes1.fr; Fax: 33-299281425

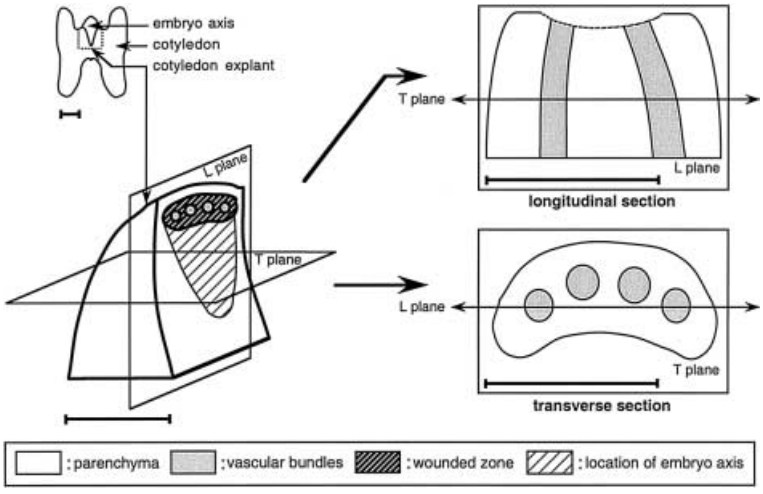


**Fig. 1.** Organization of the main tissues in walnut cotyledon explants from the region of attachment to the embryonic axis prior to in-vitro culture. After removal of the embryonic axis, cotyledon explants were dissected under axenic conditions and then cultured in-vitro as described in *Materials and methods*. Transverse (*T plane*) and longitudinal (*L plane*) sections were used for histological studies. Bars = 5 mm

primary root (Malamy and Benfey 1997), or to adventitious root formation, which typically arises from phloem or inner-cortex parenchyma cells of epicotyls or hypocotyls (Lovell and White 1986; Lund et al. 1996). Furthermore, there is little molecular information about lateral, or adventitious, root development in any plant (Smith and Fedoroff 1995; Malamy and Benfey 1997; Lorbiecke and Sauter 1999), although the key developmental stages of lateral root formation in *Arabidopsis* are slowly emerging from recent studies (Laskowski et al. 1995; Malamy and Benfey 1997).

The different developmental stages of root formation result from activation of distinct genetic programs. A small number of genes have been characterized to be specifically involved in lateral or adventitious root formation. Expression of the tomato *ROOT SYSTEM INDUCIBLE-1* (*RSI-1*) gene (Taylor and Scheuring 1994) was characterized by histolocalization of $\beta$-glucuronidase (GUS) activity in early lateral root primordia in transgenic plants containing an RSI-1-GUS gene fusion. Increased GUS activity in auxin-treated hypocotyls suggested involvement in the development of adventitious root primordia (Taylor and Scheuring 1994). Expression of the tobacco *HYDROXYPROLINE-RICH GLYCOPROTEIN* (*HRGPnt3*) gene (Vera et al. 1994) was also determined by histolocalization of GUS activity in early lateral and adventitious root primordia in transgenic plants containing an *HRGPnt3-GUS* gene fusion. Smith and Fedoroff (1995) showed by histolocalization of GUS activity in an enhancer trap line of *Arabidopsis thaliana* that a *LATERAL ROOT PRIMORDIUM-1* (*LRP1*) gene was specifically expressed in lateral and adventitious root primordia. Finally, screening and characterization of *Arabidopsis* mutants have also revealed genes which are specific to lateral root development such as *ABERRANT LATERAL ROOT FORMATION* genes (Celenza et al. 1995).

Other genes that are known to be activated in primordia of lateral or adventitious roots are also activated in the primary root, such as *ROOT MERISTEMLESS* genes (Cheng et al. 1995), or in other developmental processes, such as *PS-IAA4/5* and *PS-IAA6* early auxin-responsive genes (Abel and Theologis 1996). Moreover, a number of genes are expressed for the normal activity of root meristems, whether primary or secondary, at early or late stages of development depending on the onset of differentiation processes (Malamy and Benfey 1997). Such is the case for some *CHALCONE SYNTHASE* (*CHS*) genes, which are preferentially expressed in the epidermis of lateral and primary root tissue (Schmid et al. 1990).

In the present work, histological and molecular studies were carried out in order to determine the stages and mechanisms of root formation in cotyledon explants from the region of attachment to the embryonic axis in the absence of exogenous growth regulators. The origin and formation of the root primordium, and the type of connection of the newly formed apical meristem with vascular bundles, were shown to follow a pattern of adventitious, rather than lateral or primary, root ontogeny. This adventitious ontogeny, which was strictly positioned in relation to vascular bundles, gave indications of the positional and biochemical cues that are necessary for adventitious root formation. Complementary molecular characterization was carried out with expression studies of potential molecular markers of primordium formation and root differentiation. The *Arabidopsis LRP1* gene (Smith and Fedoroff 1995) was used to detect *LRP1*-like transcripts in relation to primordium formation. The walnut *CHS1*, *CHS4* (El Euch 1997; El Euch et al. 1998) and *TONOPLAST INTRINSIC PROTEIN* ($\gamma$-*TIP*) genes were used to detect the levels of root-related (El Euch 1997) and elongation-related (Ludevid et al. 1992) transcripts, respectively. The comparison of histological and molecular data thus allowed us to determine different stages of development that were, at least in part, similar to those of lateral and adventitious root formation in other angiosperms (Laskowski et al. 1995; Smith and Fedoroff 1995). This adventitious process is discussed in terms of coherence with current models of adventitious root development (Lund et al. 1997).

## Materials and methods

*Plant material*. Mature seeds of walnut (*Juglans regia* L. cv. Lara) were obtained from the Station Expérimentale Nucicole Rhône-Alpes (Saint Marcellin, France). Cotyledon explants from the region of attachment to the embryonic axis (Fig. 1) were dissected under axenic conditions as previously described (Jay-Allemand et al. 1991). These explants were cultured in-vitro in a growth-regulator-free medium in the dark at 26 °C for periods of over 6 d as described in Jay-Allemand et al. (1991), and were collected at different times of culture. These explants were either fixed for histological observation as described below or frozen in liquid nitrogen and immediately freeze-dried for conservation at room temperature in the presence of silica gel. All of the experiments showed the same time-course of morphological changes as previously described (Jay-Allemand et al. 1991; Gutmann et al. 1996). At 72 h, a short petiole of approx. 1 mm was observed at the tip of the explant; from 72 to 96 h, the petiole structure elongated, up to a final length of 15–20 mm; at 120 h, roots emerged from the tip of the petiole-like structure.

*Histological analysis*. Cotyledon explants from the region of attachment to the embryonic axis (Fig. 1) were collected at different times of development (0, 8, 24, 32, 48, 56, 72, 80, 94, 120 and 144 h of in-vitro culture). At each time of development, three explants were collected. Cotyledon explants were fixed for 4 h at 4 °C in 4% (v/v) glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% (w/v) caffeine (Mueller and Greenwood 1978). After washing in 100 mM sodium phosphate buffer (pH 7.4), tissue samples were sequentially dehydrated with increasing concentrations of ethanol (from 25 to 100%) and embedded in glycolmethacrylate resin (Technovit 7100, Heraeus Kulzer, Hanau, Germany) according to the Kulzer process modified as follows: first and second pre-infiltrations were carried out for, respectively, 1 d and 1 week. Transverse and longitudinal semi-thin sections (2–3 μm, Fig. 1) were obtained with a Leitz 1400 microtome equipped with a tungsten carbide knife. Sections were stained with Toluidine Blue O (O'Brien et al. 1965), Safranin O plus Lugol, or Ponceau 2R and Azure II (Gutmann et al. 1996).

*Isolation and quantification of flavonoids*. Approximately 20 mg of freeze-dried tissue was coarsely ground. Flavonoids were then extracted by ultrasonication for 30 min at 4 °C in 2 mL of 80% (v/v) acetone containing 0.1 mM 6-methoxyflavone as internal standard, and further isolated as described in El Euch et al. (1998). Separation, characterization, and quantification by HPLC (Gold system; Beckman) were carried out as described in El Euch et al. (1998). Variation due to extraction and analytical procedures, which was estimated from the results of six independent replicates, never exceeded 6%.

*Isolation of RNA and northern blot analysis*. Three-millimetre-thick upper regions and remaining basal regions were excised from freeze-dried cotyledon explants and used for RNA isolation. The 3-mm-thick upper region corresponded to the tip of the cotyledon explant from 0 to 72 h, to the tip of the developing petiole from 80 to 96 h, and to the tip of the emerged root from 120 to 192 h. The basal region corresponded to the cotyledon, from 0 to 72 h, to the petiole structure, from 80 to 96 h, and to mature root tissue, from 120 to 192 h. Walnut tissues are characterized by a high content of phenolic compounds. Dissection of freeze-dried tissues, where the morphological structures were well-conserved, prevented the oxidation of phenolics, which is known to interfere with RNA isolation. The protocol of Chang et al. (1993) for isolating RNA from pine trees, which also have a high content of phenolics, was adapted to the isolation of RNA from walnut tissues. The use of freshly-prepared polyvinylpyrrolidone-NaCl solution was essential for the final quality of the isolated total RNA. Extractions with chloroform-isoamyl alcohol were carried out three times in order to obtain clear, colourless aqueous extracts. From 50 to 250 mg DW of 3-mm-thick upper regions from cotyledon explants, corresponding to 50–100 explants, was routinely used for RNA purification. The quantity and purity of total RNA was verified spectrophotometrically (Sambrook et al. 1989). Samples (12–20 μg) of total RNA were gel-electrophoresed and blotted to Zeta-Probe GT membranes (BioRad) by capillary transfer. Hybridization was carried out according to the instructions of the manufacturer using 500 mM sodium phosphate buffer (pH 7.2) containing 7% (w/v) SDS at 55 °C overnight. Probes labelled with [$^{32}$P]dCTP were made by random priming using the random primed DNA labeling kit of Boehringer-Mannheim. Radioactive probes were added to hybridization media at final radioactivities of at least $1.5 \times 10^6$ cpm mL$^{-1}$. Blots were washed twice at 55 °C for 20 min in 40 mM sodium phosphate buffer (pH 7.2) containing 5% (w/v) SDS. Blots were then autoradiographed. Equal loading of RNA in each lane was verified by ethidium bromide staining on the northern blot. The template for *γ-TIP* probe was a 400-bp polymerase chain reaction-(PCR)-amplified fragment of the translated region of walnut *γ-TIP* cDNA clone (I. Beritognolo and C. Breton, personal communication). The templates for *CHS1* and *CHS4* probes were PCR-amplified 630–1162 fragments of walnut *CHS1* and *CHS4* cDNA clones (El Euch 1997). These 530-bp regions of *CHS1* and *CHS4* showed 67% identity (El Euch 1997). The template for heterologous *LRP1* probe was a PCR-amplified 250-bp fragment corresponding to the 3′ exon of the *Arabidopsis thaliana LRP1* genomic clone (Smith and Fedoroff 1995; GenBank accession number U24702).

## Results

*Effects of wounding and germination-like modifications in the cotyledon explants*. After removal of the embryonic axis, the cotyledon explant (Fig. 1) was cultured in-vitro as described in *Materials and methods*. Removal of the embryonic axis resulted in a layer of broken cells at the surface of the explant (Fig. 1). There was neither lignification nor suberization at the site of breaking, and analysis of myricitrin and quercitrin levels (data not shown) showed no appreciable levels of these flavonoids and no increase prior to petiole elongation, which occurred between 72 and 96 h of development. Figure 2 shows the initial state of cells in the cotyledon explant. Comparison of Fig. 2 with Fig. 3C,D shows that rehydration of the cotyledon explant under in-vitro culture resulted in progressive hydrolysis of protein reserves in cells of cotyledon parenchyma. Thus, in-vitro culture effected a number of germination-like modifications in the cotyledon explant.

Superficial unwounded provascular cells became slightly enlarged, which caused their free end to bulge over the explant surface (Fig. 3B). Mitoses in the two or three superficial cell layers at the tip of provascular bundles between 24 and 48 h of development gave rise to a small dome-like callus. This limited callus formation was probably a response to wounding under conditions of 100% relative humidity (Rosenstock and Kahl 1978). At 48 h of culture, the wounded end of each young vascular bundle was covered by such a callus.

*Stage I: formation of annular meristematic zones around vascular bundles at the tip of the cotyledon explant*. No initial difference in RNA levels between the basal and upper regions of the cotyledon explant was observed (Fig. 4). Then, in the course of in-vitro culture, RNA levels increased in both regions of the explant. In the

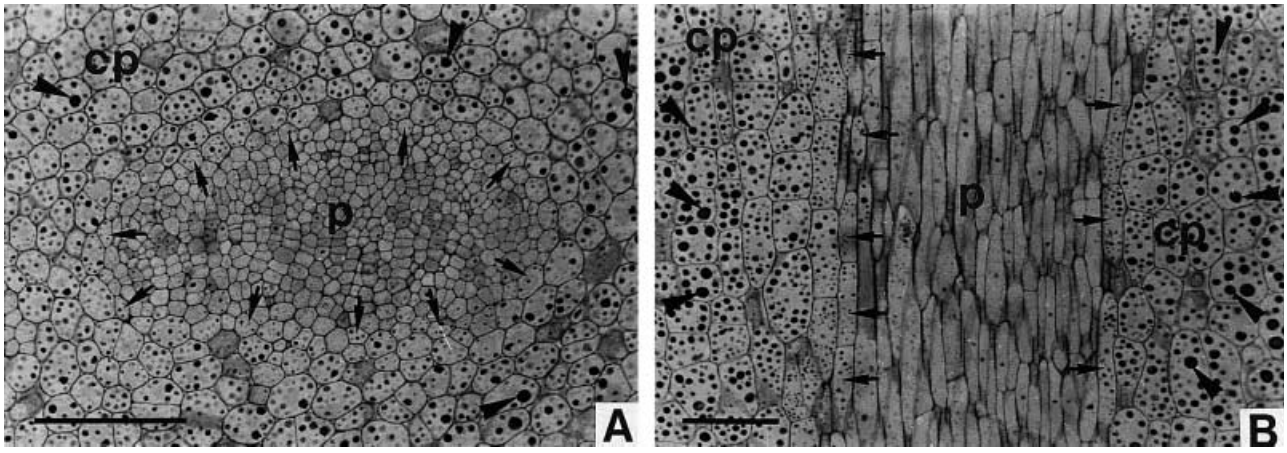


**Fig. 2A,B.** Cross-sections of the walnut cotyledon explant prior to in-vitro culture. Transverse (**A**) and longitudinal (**B**) sections were stained with Ponceau 2R and Azure II. Protein bodies (*arrowheads*) were large and abundant in cotyledonous parenchyma (*cp*) and scarce in provascular cells (*p*). The perifascicular parenchyma (*arrows*), showing narrow and elongated cells with small protein bodies, differed strikingly from the cotyledonous parenchyma. Bars = 20 μm

upper part of the explant, RNA accumulation was found to start between 0 and 24 h. Total RNA content increased up to 72 h, and remained constant until root emergence, which occurred after 120 h of development. In contrast, total RNA content in the basal part of the explant was found to remain constant between 0 and 48 h and then to increase between 48 and 72 h. This differential accumulation of RNA resulted in markedly higher levels of total RNA in the upper region than in the basal region of the explant.

The first cell divisions, which occurred in the parenchyma at the tip of the explant, started at around 20 h of in-vitro culture, thus showing good correlation with the accumulation of total RNA (Fig. 4). The number of sib-cells within the walls of mother cells in the uppermost 10–20 cell layers of the explant was strong evidence for the occurrence of three to four division cycles in cotyledonous parenchyma during the first 96 h, thus corresponding to one mitosis per 24 h. In the lower part of the explant, the start of mitoses was delayed by 24–48 h, like RNA accumulation (Fig. 4). Cell elongation, which occurred at a later stage of development, disrupted the regular pattern of cell division. Mitosis and cell elongation in the basal part of the explant resulted in the growth of a petiole from the tip of the explant, as previously described (Gutmann et al. 1996). Different rhythms and patterns of mitosis were observed between superficial cells and inner tissues of the explant (Fig. 3A,B). Differences could also be seen between provascular bundles, perifascicular parenchyma and cotyledonous parenchyma. The first tracheary elements and sieve tubes were fully differentiated at 32 h. After 32 h of development, provascular bundles showed the typical structure of vascular bundles.

The most apical cells (about 10–20 cell layers) surrounding vascular bundles began to divide actively, both transversely and periclinally (Fig. 3A,B). Transverse mitoses were frequent at first in the two uppermost layers and then spread to all of the surrounding parenchyma cells (Fig. 3B). Each vascular bundle was surrounded by an inner ring of actively dividing cells resulting from multi-directional divisions of the perifascicular parenchyma (Fig. 3A). These cells presented all the characteristics of meristematic cells such as small size and darkly stained cytoplasm (Fig. 3C,D). Cotyledonous parenchyma cells surrounding this inner ring divided periclinally, each cell dividing only once or twice. These cells thus formed an outer ring (Fig. 3A,B) of dividing cells of larger size and less darkly stained cytoplasm. The inner ring plus the outer ring constituted an annular meristematic zone (Fig. 3A,B).

First divisions in cell layers surrounding vascular bundles occurred in an asynchronous way relative to the axis of the vascular bundle. The inner meristematic ring appeared to develop before the outer meristematic ring. The first divisions were observed on each side of the procambium and then spread to the phloem side and finally to the xylem side. The outer meristematic ring started to develop before the inner ring was complete, by periclinal and transverse mitoses in cotyledonous parenchyma cells surrounding the protoxylem (Fig. 3C). Mitoses progressively extended to the whole circumference of the vascular bundle (Fig. 3A,B). The continuation of cell divisions occurred according to a radial symmetry relative to the axis of the vascular bundle, thus resulting in the final annular structure. Each of these annular meristematic zones was situated between 30 μm and 100–250 μm below the surface (Fig. 3B). The fully developed inner meristematic ring spanned a width of four to six cell layers (Table 1) and a depth of approx. 30 cell layers. The fully developed outer meristematic ring spanned a width of 6–18 concentric cell layers (Table 1) and a depth of approx. 30 cell layers. Table 1 shows that the final structure of the outer ring was not strictly symmetrical relative to the vascular bundle, with greater development at the xylem side.

*Stage II: radially-asymmetrical organization of root primordia within the annular meristematic zone*. High levels of *LRP1*-like transcripts of 1.6 kb were detected at

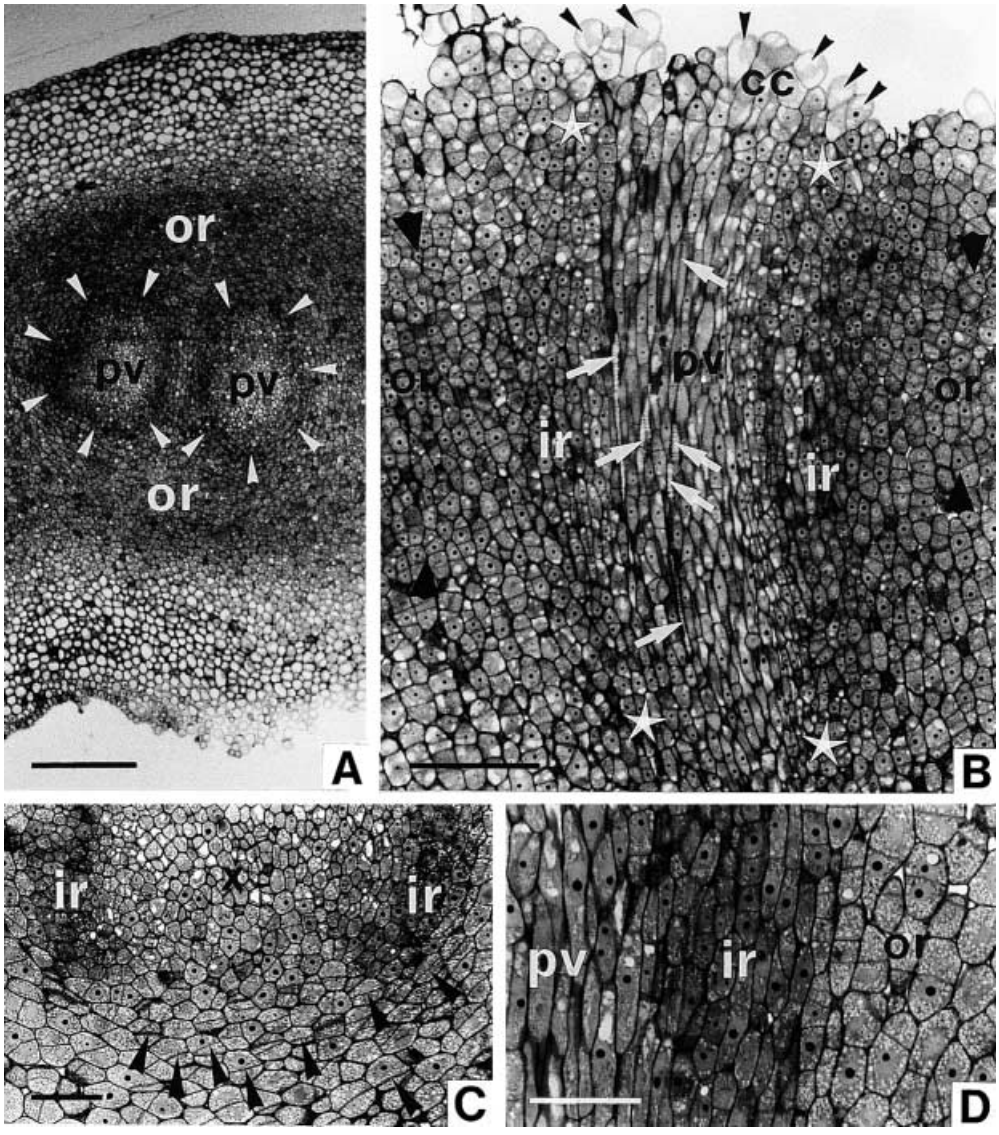


**Fig. 3A–D.** Formation and structure of the annular meristematic zones in the upper part of the walnut cotyledon explant. Transverse (**A**, **C**) and longitudinal (**B**, **D**) sections were stained with Toluidine Blue O. **A** General view showing the annular meristematic zone around two petiole vascular bundles (*pv*) at 80 h of development. Each vascular bundle was surrounded by an inner meristematic ring (*arrowheads*) and an outer meristematic ring (*or*). In the region between vascular bundles, outer meristematic rings merged together. Bar = 100 μm. **B** At 80 h of development, fully-differentiated tracheids (*arrows*) were present in the petiole vascular bundle. A dome-like callus (*cc*) with enlarged superficial cells (*small arrowheads*) could be observed at the site of wounding. All of the parenchyma cells at the top of the explant had undergone at least one division. The outer (*or*) and inner (*ir*) meristematic rings, which are respectively indicated by *large arrowheads* and *asterisks*, showed small densely stained cells. The depth of the inner meristematic ring (*asterisks*) was greater than that of the outer meristematic ring (*large arrowheads*). Bar = 50 μm. **C** At 47 h of development, initiation of the inner meristematic ring (*ir*) was observed at the procambium and phloem sides of the vascular bundle. In contrast, the outer meristematic ring started to develop at the xylem side (*x*) where periclinal divisions (*arrowheads*) were observed. The cells of the inner meristematic ring were smaller and more densely-stained than those of the outer meristematic ring. Bar = 20 μm. **D** Longitudinal section at 47 h of development showing the narrow cells of the inner meristematic ring (*ir*) with a densely-stained cytoplasm. *pv*, petiole vascular bundle. Bar = 20 μm

time 0 in all the different parts of the cotyledon, including both the basal and upper regions of the explant (Fig. 5A,B). Then, at 24 and 48 h, *LRP1*-like transcripts were detected predominantly in the upper region of the cotydedon explant (Fig. 5B). The level of *LRP1*-like transcripts in the upper region of the cotyledon explant increased from 24 h (data not shown) to 48 h. There was no detection of these transcripts at later stages of development.

After approx. 80 h of development, clusters comprising cells dividing in every direction and heavily stained with Toluidine blue progressively appeared inside inner meristematic rings (Fig. 6A). There was generally one cluster in each annular meristematic zone (Fig. 6B). These clusters were situated in a lateral position in the periphery of vascular bundles (Fig. 6B,C) at the side of the procambium (Fig. 6B). These clusters of isodiametric actively dividing cells showing an approximately

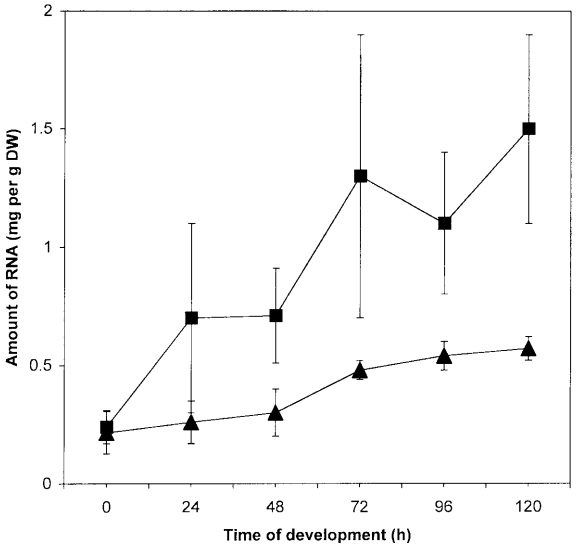


**Fig. 4.** Accumulation of total RNA during root formation from cotyledon explants of walnut. Total RNA was isolated from the basal (▲) and upper (■) regions of the cotyledon explant at different times of development and quantitated as described in *Materials and methods*. Results are the mean (±SE) from at least three independent experiments

**Table 1.** Size of the annular meristematic zone during adventitious root development from walnut cotyledon explants. Annular meristematic zones at maximal development were observed in transverse sections of walnut cotyledon explants (Fig. 3A) and cell layers in the inner and outer rings were counted. Results are the mean (±SE) of 17–34 measurements at various levels of transverse sections of 4 to 6 vascular bundles from at least 2 independent experiments

| Position | Number of cell layers | |
|---|---|---|
| | Inner ring | Outer ring |
| Xylem side | 6 ± 1 ($n$ = 17) | 18 ± 1 ($n$ = 17) |
| Procambium side | 5 ± 1 ($n$ = 34) | 6 ± 1 ($n$ = 34) |
| Phloem side | 4 ± 1 ($n$ = 17) | 8 ± 1 ($n$ = 17) |

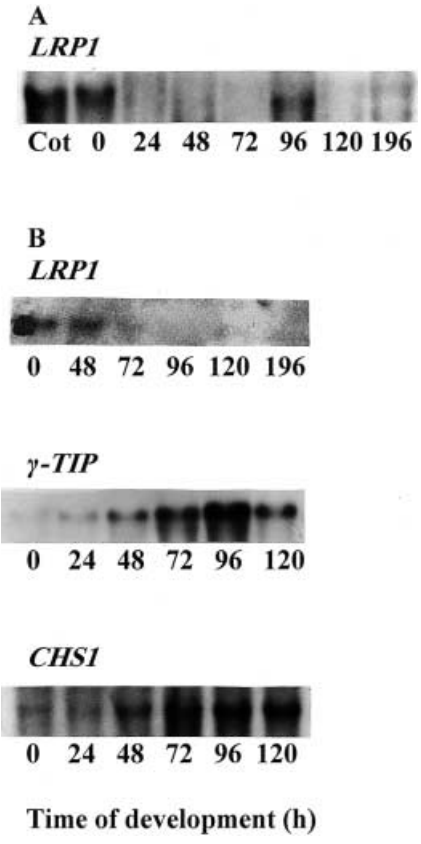


**Fig. 5.** Steady-state levels of *LRP1*-like, *γ-TIP* and *CHS1* transcripts during root formation from cotyledon explants of walnut. Basal (*A*) and upper (*B*) regions of the cotyledon explant were collected at different times of development as described in *Materials and methods*. Furthermore, samples of cotyledon tissue outside the explant (*Cot*) were also collected at time 0 h. Total RNA was isolated from these samples and RNA gel blot analysis was carried out as described in *Materials and methods*. Each lane contains 20 (*LRP1*), 12 (*γ-TIP*) or 15 (*CHS1*) μg of RNA. Equal loading of RNA in each lane was verified by ethidium bromide staining on the northern blot. Experiments using independent sets of samples were repeated at least once with similar results

spherical shape (Fig. 6D,E) were closely similar to lateral (Malamy and Benfey 1997) or adventitious (Lund et al. 1996) root primordia. The first recognizable clusters, localized in the inner meristematic ring, showed a diameter of about 6 cells, thus corresponding to a total number of cells of approx. 100. The observation of well-organized structures of this size at 80 or 94 h of in-vitro culture strongly suggested that initiation of development began earlier. These cell clusters, which increased by cell division, gave rise to fully developed primordia localized in the outer meristematic ring. At this stage, they showed a diameter of 9 or 10 cells surrounded by 2 or 3 concentric cell layers (Fig. 6E), thus corresponding to a total number of cells of about 2000.

*Stage III: organization of root apical meristems from root primordia*. Transcripts of *γ-TIP* were detected at a low level at 0 h in the upper region of the cotyledon (Fig. 5B). The level of these 1-kb transcripts then progressively increased from 24 to 96 h. The maximum of *γ-TIP* transcript accumulation was observed at 96 h, prior to the stage of emerging root apical meristem. Then, in contrast, the level of *γ-TIP* transcripts was found to decrease in the emerged root tip, as shown by the northern blot signal at 120 h (Fig. 5B).

Figure 5B shows steady-state levels of 1.6-kb transcripts of *CHS1* in the upper regions of the cotyledon explant during in-vitro culture. Transcripts of *CHS4* were found to follow the same pattern of expression (data not shown). In walnut, *CHS1* and *CHS4* are known to be exclusively or predominantly expressed in roots (El Euch 1997). The transcripts of these genes were at a low level at 0 and 24 h. Their levels then steadily increased from 48 h to 96 and 120 h. Thus, these two genes showed maximal levels of transcripts at the stages of emerging root apical meristem and emerged root tip. This increase of *CHS1* and *CHS4* transcripts in the upper region of the cotyledon from 48 h of in-vitro

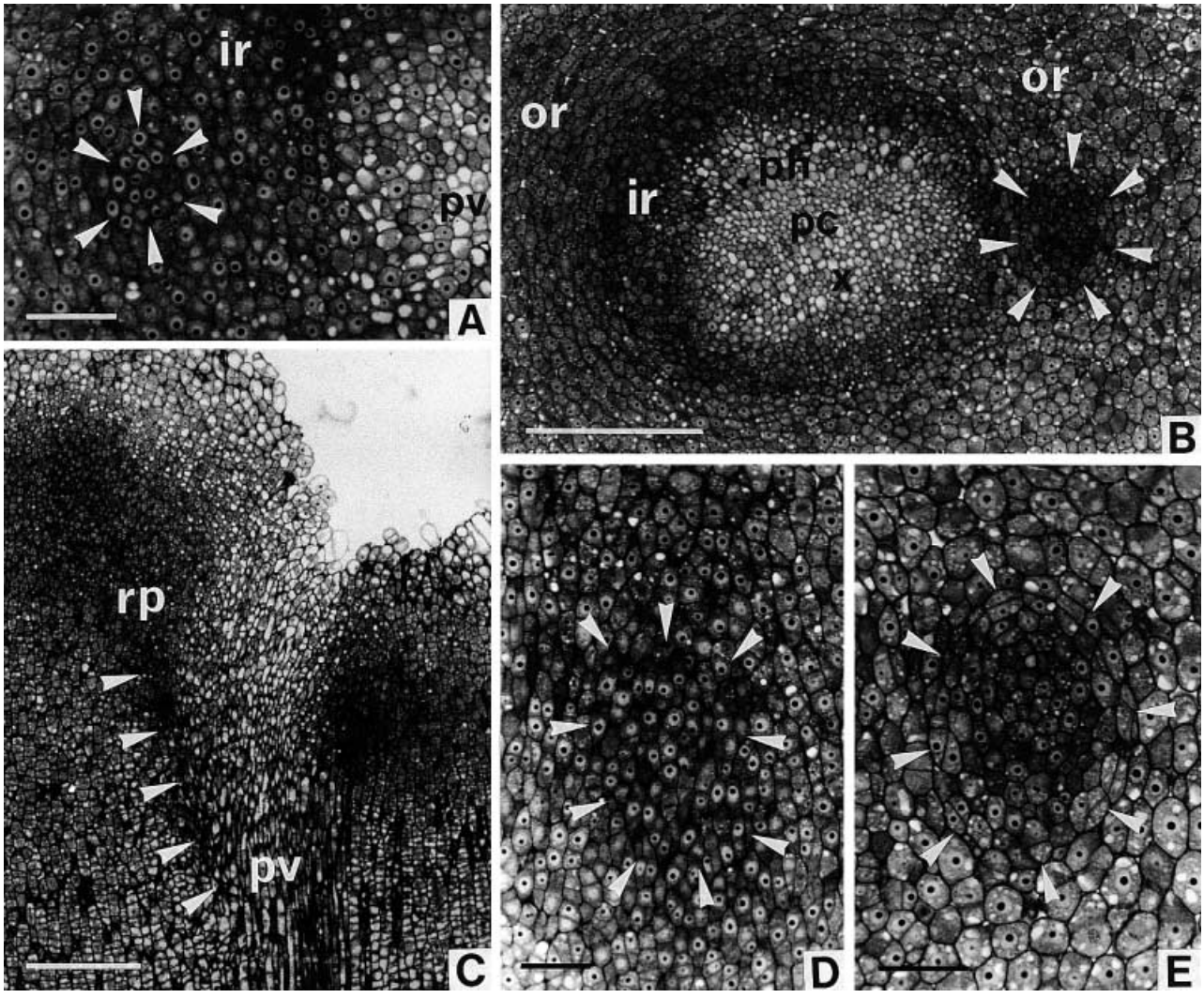


**Fig. 6A–E.** Formation and structure of adventitious root primordia within the annular meristematic zone. Transverse (**A**, **B**, **E**) and longitudinal (**C**, **D**) sections were stained with Toluidine Blue O. **A** Cluster of isodiametric, actively dividing, primordial cells (*arrowheads*) at 80 h of development near a petiole vascular bundle (*pv*) in the inner meristematic ring (*ir*). Bar = 20 μm. **B** Further development of the root primordium in the outer meristematic ring (*or*). The root primordium (*arrowheads*) was located at the side of the procambium (*pc*) in the periphery of a vascular bundle. Phloem (*ph*) and xylem (*x*) are indicated. Bar = 100 μm. **C** Development of lateral connections (*arrowheads*) at 94 h between the base of a fully-developed root primordium (*rp*) and the vascular bundle (*pv*) of the petiole. Bar = 100 μm. **D** Detail of **C** showing a fully developed root primordium (*arrowheads*) in the outer meristematic ring. Bar = 20 μm. **E** Detail of **B** showing the structure of a root primordium (*arrowheads*) with the two surrounding cell layers. Bar = 20 μm

culture was paralleled by an increase of myricitrin and quercitrin levels from 0 to 400 and 70 μg $(g\ DW)^{-1}$, respectively, during the growth of the cotyledon petiole, which occurred from 72 to 96 h of in-vitro culture. This increase of *CHS* transcripts and accumulation of flavonoids preceded the accumulation of detectable polyphenol globules, which were found to increase in the vacuoles of peripheral cells in root apical meristems at time 144 h (Fig. 7C).

Cellular connections were progressively formed between young primordia localized in the annular meristematic zones and vascular bundles of the petiole (Fig. 6C). The cells involved in these connections were more elongated than cells of the surrounding meristematic ring. It was, however, difficult to ascertain whether connections developed from the vascular bundle towards the primordium, from the primordium towards the vascular bundle, or in both directions. In parallel, the structural organization of developing root apical meristems kept pace with growth, until the typical zonation of meristems could be clearly identified at 144 h of in-vitro culture (Fig. 7A,B), with: (i) the dome-like root cap meristem and the first cells of the root cap, which lay between the root cap meristem and the disintegrating cells from the explant surface; (ii) a small group of cells, just below the root cap meristem and less densely stained than the cortex mother cells, which probably represented the future quiescent center; (iii) the ground meristem on either side of the putative quiescent center; (iv) the central procambium which could be

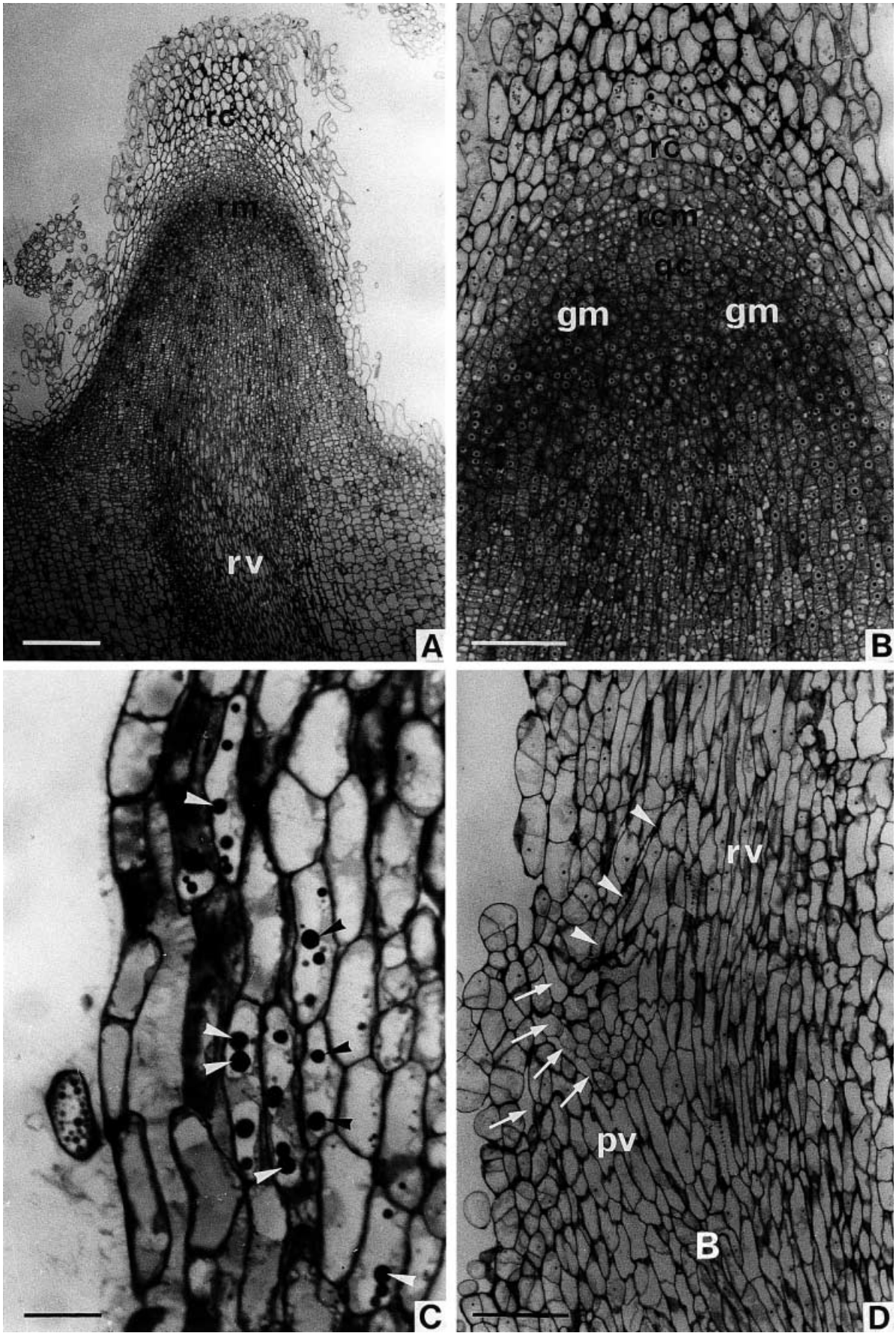
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**Fig. 7A–D.** Organization and structure of the adventitious root apical meristem at 144 h of development. Longitudinal sections were stained with Toluidine Blue O (**A**, **B**, **D**) or Safranine O plus Lugol (**C**). **A** Emergence of the adventitious root apical meristem. *rc*, root cap; *rm*, root meristem; *rv*, root vascular cylinder. Bar = 100 μm. **B** Zonation of the root apical meristem. *gm*, ground meristem; *qc*, future quiescent centre; *rc*, root cap; *rcm*, root cap meristem. Bar = 50 μm. **C** Polyphenol accumulation (*arrowheads*) in the vacuoles of newly differentiated lateral root cap cells of the emerging adventitious root apical meristem. Bar = 10 μm. **D** Lateral connections branching the vascular bundle of the petiole (*pv*) and the root vascular cylinder (*rv*). *Arrows* show differentiated vessels at the tip of the petiole vascular bundle; *arrowheads* show an oblique vessel branching the tip of the petiole vascular bundle and the root vascular cylinder. The branching junction (*B*) between the petiole vascular bundle and the base of the root vascular cylinder is indicated. Bar = 50 μm

distinguished from lateral cortex and protoderm mother cells. Cells covering the tip of this developing root were beginning to slough off as a result of the degradation of their middle lamella in a manner similar to that of mature root cap cells. Two or three layers of dividing cells separated this developing root cap from the core of the developing root apical meristem (Fig. 7A). The connections of these root apical meristems with vascular bundles of the petiole (Fig. 7A,D) showed the same position and organization as those between primordia and petiole vascular bundles (Fig. 6C), thus demonstrating that cell clusters within annular meristematic zones were root primordia and developed into root apical meristems, with one root apical meristem per primordium.

Vascular bundles in the newly-formed petiole showed the structure that is typical of petioles of angiosperms, with the alignments of phloem poles on one side and of xylem poles on the other side (Figs. 3A, 6B). At the lower part of the developing root apical meristem, continuity was observed between lateral, procambial cells of the petiole vascular bundle and some central cells of the meristem. On longitudinal sections, the continuity of vessels could be followed up to the surface of the wounded petiole bundle (Fig. 7D). During further growth of the developing root, the part of petiole vascular bundle that was just above the branching was more or less disorganized and finally pushed aside. Eventually, vessel elements from the petiole vascular bundle directly opened into the atmosphere (Fig. 7D).

## Discussion

*Root formation from cotyledon explants of walnut is an adventitious process*. The process of root formation from cotyledon explants of walnut, which is summarized in Fig. 8, occurred in the environment of a structure showing the typical organization of petiole vascular bundles. The root primordium, which was identified from its structure and from its further development into root apical meristem, bore close structural similarities to lateral (Malamy and Benfey 1997) or adventitious (Lund et al. 1996) root primordia. Primordia appeared to originate from perifascicular cells and were strictly located at the side of the phloem-xylem boundary, which is one of the known sites of adventitious root formation in angiosperms (Esau 1965; Lovell and White 1986). Furthermore, it is known to be the site of adventitious root formation in the cotyledon petiole of *Sinapis alba* (Moore and Lovell 1972). Finally, the connections between root apical meristems and vascular bundles of the petiole were clearly lateral (Fig. 9) rather than linear or perpendicular as occurs during primary and lateral root development (Esau 1965). All of this clearly demonstrated that this process of root formation followed a pattern of adventitious, rather than lateral or primary, root ontogeny.

*Initiation and early organization of adventitious root primordium*. The earliest observation of adventitious root primordia, at 80 h, revealed a spherical structure of approx. 100 cells. The fully-developed adventitious root primordium, consisting of approx. 2000 cells, was formed at the latest prior to root emergence, therefore prior to 120 h of in-vitro culture. Thus, divisions in the adventitious root primordium appeared to take place at a minimal rate of four cell cycles in 40 h, which would correspond to a maximal value of approx. 10 h for cell

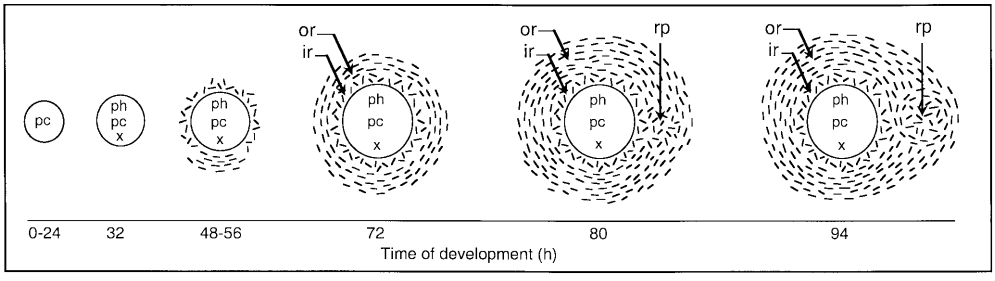


**Fig. 8.** Development of a root primordium in walnut cotyledon explants. This scheme is based on transverse sections with short lines representing the planes of division. The meristematic ring around the vascular bundle was composed of (i) an inner meristematic ring (*ir*) resulting from multi-directional divisions of perifascicular parenchyma cells (48 h) and (ii) the outer meristematic ring (*or*) resulting from periclinal divisions of cotyledonous parenchyma cells. This outer ring began at the xylem (*x*) pole of the vascular bundle (48 h) and then spread to the phloem (*ph*) pole (72 h). A root primordium (*rp*) was initiated inside the inner meristematic ring at the side of procambial cells (*pc*). This primordium appeared at first as an unorganized cell cluster (80 h). Then, the peripheral cells of the cluster divided periclinally once or twice (94 h)

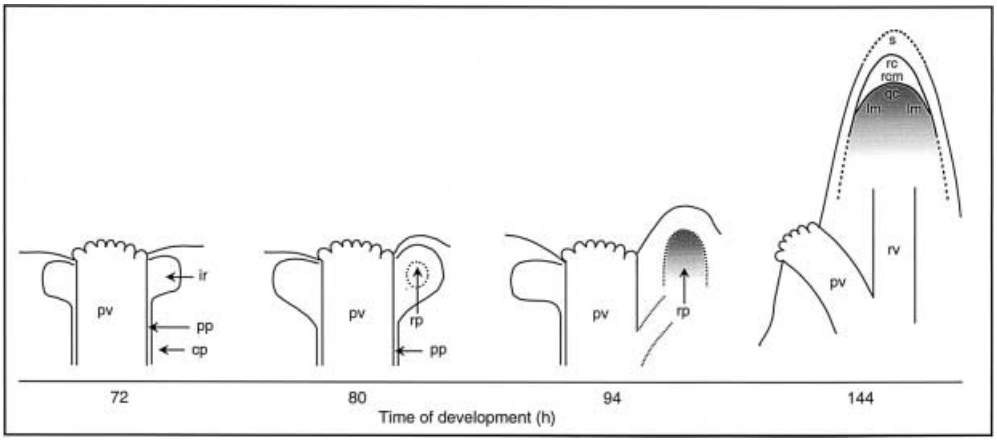


**Fig. 9.** Development of vascular connections between vascular bundles of the petiole and the root primordium in walnut cotyledon explants. This scheme is based on longitudinal sections. During root primordium development, a lateral connection was formed between the petiole vascular bundle (*pv*) and the root primordium (*rp*) inside the perifascicular ring (94 h). This connection was complete when zonation of the adventitious root meristem was clearly established at the histological level (144 h). *cp*, cotyledonous parenchyma; *ir*, inner meristematic ring; *lm*, lateral meristem; *pp*, perifascicular parenchyma; *qc*, quiescent center; *rc*, root cap; *rcm*, root cap meristem; *rv*, root vascular cylinder; *s*, superficial cells being sloughed off

doubling time. This upper value was in general agreement with previous studies (MacLeod and Thompson 1979; Laskowski et al. 1995) giving cell doubling times in lateral root primordia ranging from 2.9 to 8.2 h. Thus, the adventitious root primordium of walnut cotyledon shared a number of characteristics with previously described lateral and adventitious root primordia.

At first, no zonation was observed in the adventitious root primordium, which consisted of isodiametric cells forming a cluster of spherical shape. At a later stage, the adventitious root primordium showed an envelope of two to three cell layers surrounding a core of isodiametric cells, thus suggesting that a zonation process had started. Maximum expression of *γ-TIP* at 96 h of development (Fig. 5B), which is known to be associated with cell elongation and differentiation (Ludevid et al. 1992), preceded active elongation and emergence, which would have been consistent with differentiation occurring in relation to formation of the root apical meristem. However, substantial accumulation of *γ-TIP* transcripts and also of *CHS1* and *CHS4* transcripts occurred between 24 and 72 h of development (Fig. 5B). The *CHS1* gene in walnut has been shown to be poorly expressed in stems and preferentially expressed in root tissues (El Euch 1997; El Euch et al. 1998), thus closely resembling the expression pattern of *CHS8* in bean (Schmid et al. 1990). This pattern of expression and the succession of *CHS* expression, flavonoid accumulation and polyphenol globule formation in root apical meristems therefore suggested that induction of *CHS* genes in the upper region of the cotyledon was related to root differentiation. Taken together, accumulation of *γ-TIP*, *CHS1* and *CHS4* transcripts strongly suggested that organization and differentiation of the primordium occurred at an early stage, which was in line with the observation of well-organized structures at 80 h of development. This could be related to the demonstration, by Laskowski et al. (1995), that, in *Arabidopsis*, lateral root primordia consisting of three to five cell layers can autonomously develop into lateral roots. This early organization was confirmed by Malamy and Benfey (1997), who showed that cell-specific expression of endodermis-related genes in the lateral root primordium occurred at stages of two to four cell layers. Thus, in walnut, as in *Arabidopsis*, differentiation of root primordium cells appears to begin in the earliest stages of primordium formation, which implies that the primordia that were observed as clusters of isodiametric actively dividing cells at 80 h of development (Fig. 6) already had a substantial level of differentiation. This early process of differentiation could be interpreted as the developmental transition affecting the primordium that has been hypothesized to occur during organization of the lateral root primordium in *Arabidopsis* (Laskowski et al. 1995). This developmental transition may correspond to the organization of a root apical meristem, but definite evidence is still lacking because the early identification of initials as typical of meristem activity is difficult (Malamy and Benfey 1997). In the present case, the observation of meristem zonation was definite at the later stage of 144 h of development.

Since organization of the primordium took place within the annular meristematic zones, it was difficult to identify the origin and the founder cells of the primordium. However, the position of the adventitious root primordium in the inner ring of the annular meristematic zone strongly suggested that founder cells derived from perifascicular parenchyma. Given a cell doubling time of 10 h, as discussed above, the 100-cell primordium at 80 h of development would have resulted from an initial cluster of six cells at 25 h of development. Such a size would be in general agreement with initiation of adventitious root primordium from 5–20 founder cells in shoot cuttings (Lund et al. 1997). Smith and Fedoroff (1995) showed that, in *Arabidopsis*, *LRP1* was expressed

at a very early stage of lateral root primordium formation and that its expression decreased as the primordium grew older. Even though analysis of *LRP1*-like transcripts with the heterologous *Arabidopsis* probe must be interpreted with caution, *LRP1*-like transcripts were at first detected in the whole cotyledon (Fig. 5), then rapidly became restricted to the upper zone, where cell division and root formation took place, and finally disappeared between 48 and 72 h of development, in a correlated parallel with the increase of *CHS1* and *CHS4* transcripts. All of this was also consistent with an early stage for adventitious root primordium inception and organization.

*Mechanisms of adventitious root primordium formation and organization.* The initial stage of development was characterized by high mitotic activity affecting two different cell types around vascular bundles, i.e. cotyledonous parenchyma and perifascicular parenchyma. The radial symmetry of the annular meristematic zone indicated that the signal responsible for the activation of division radially diffused from the vascular bundle. This signal may be thought to be auxin, which is known to initiate and activate cell division during adventitious and lateral root formation (Celenza et al. 1995) and may induce *CHS* (El Euch 1997) and *LRP1* (Smith and Fedoroff 1995) gene expression. The active division of two different cell types in the present case may be related to the juvenile nature of the tissue or to high levels of the signal inducing cell division.

In contrast to the radial symmetry of the annular meristematic zone, adventitious root primordia were formed from within the annular meristematic zone and exclusively at the side of vascular bundles in alignment with the procambium. Lateral root primordium formation in *Arabidopsis* proceeds from direct division of a limited number of pericycle founder cells (Malamy and Benfey 1997). However, Laskowski et al. (1995) pointed out that, after treatment with high concentrations of auxin, all of the pericycle cells on the two xylem radii underwent division with only a subset of these dividing cells continuing to differentiate into lateral root meristems. This observation and our own data thus indicate that, in both lateral and adventitious root formation, induction of division and further primordium development may be distinct processes.

Other signals than the initial signal for cell division may therefore be involved, which would be consistent with the hypothesis that auxin is not the sole signal for lateral root formation (Celenza et al. 1995). In the case of root formation from cotyledon explants, this would imply a second signal inducing radially asymmetrical development and leading to primordium organization. The existence of at least two distinct morphogenetic gradients could also be inferred from the time-course of cell division which was found to originate from the side of the procambium during inner-ring formation and from the xylem pole during outer-ring formation (Fig. 3).

This would support the idea of a branched pathway for adventitious root formation, with induction of unorganized division as shown by the annular meristematic zone, from which a transition to primordium formation may branch. This branched pathway is also one of the models that Lund et al. (1997) proposed for adventitious root formation in tobacco. Nevertheless, it cannot be ruled out that only predetermined perifascicular cells at the side of the procambium in the upper part of the walnut explant were competent for the formation of adventitious root primordium from the very beginning and were induced to divide in parallel with other cell types. Development of in-situ hybridization in walnut tissues and further studies on the function and the cell-specific expression of *LRP1*-like transcripts during the early stages of the process should contribute to discrimination between the different possibilities.

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