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Anatomical and ultrastructural examination of adventitious root formation in stem slices of apple

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Abstract

Adventitious root formation *in vitro* in 1-mm stem slices cut from microshoots of apple cv. Jork 9 was studied using light and electron microscopy. When indole-3-butyric acid (IBA) had been added to the medium, starch grains accumulated during the first 24 h of culture in cells of the cambial region and in cells in the vicinity of vascular tissue and in the primary rays. This accumulation occurred only in the basal part of explants. After that, the nuclei in these cells were activated, and the density of the cytoplasm and the number of cell organelles increased, whereas starch was broken down. Cambium cells started to divide transversely and at 96 h, after several divisions, a continuous ring of isodiametric cytoplasmic cells had appeared around the xylem near the basal cutting surface. The cells in this ring were rich in cell structures, and did not contain large starch grains and a central vacuole. Root meristemoids regenerated from the portions of the ring that were localized in the primary rays. From the other cells in the ring, callus developed. The meristemoids did not grow into the direction of the epidermis as in shoots, but along the vascular bundles. After emergence from the cutting surface, the meristemoids were transformed into small, dome-like primordia. They developed a typical root apex with root cap, root ground meristem and tracheid connection with shoot vascular tissue.

Additional key words: auxin, cambial region, *Malus*, regeneration, rooting.

Introduction

Adventitious root formation is a developmental process comprising a cascade of steps. By giving pulses with cytokinin (which may be considered as an auxin antagonist) or auxin, De Klerk *et al.* (1995) distinguished three main phases in the rooting process in microshoots of apple cv. Jork 9: 1) dedifferentiation (0 - 24 h: cytokinins are only slightly inhibitory), 2) induction (24 - 96 h: auxin is strongly

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promotory and cytokinin strongly inhibitory), and 3) morphological differentiation (96 h onwards: both auxin and cytokinin are slightly inhibitory). The same time schedule holds for rooting of thin 1-mm slices cut from *in vitro* grown shoots (De Klerk *et al.* 1993). These slices are an excellent tool to study adventitious root formation (Van der Krieken *et al.* 1993). First, the system has been simplified omitting complex interactions between stem, apex and leaves. Second, because a relatively large portion of the cells is involved in the rooting process, slices are more suitable for biochemical and molecular examinations than complete shoots. Third, because a large number of slices can be obtained from one shoot (10 or more), the system is economical requiring only small numbers of shoots. Possible pitfalls have been examined. When the proper part of the stem is used, all slices have the same capability to root and there is only a weak correlation between rooting of adjacent slices (De Klerk and Caillat 1994). Several research groups have adopted this system (Welander and Pawlicki 1993, Seifert *et al.* 1994).

In the present study, the phases in the rooting process are characterised anatomically and ultrastructurally.

Materials and methods

Plants: Shoot production of apple (*Malus domestica* Borkh. cv. Jork 9) was maintained as described previously (De Klerk *et al.* 1995). Briefly, shoot tips of 1 cm in length were cultured at 25 °C with a 16-h photoperiod (irradiance of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps) in tubes (diameter 2.2 cm) on 15 cm^3 of a modified Murashige and Skoog medium, 4.4 μM benzylaminopurine (BAP) and 0.5 μM indole-3-butyric acid (IBA). After 4 weeks, clusters of 5 to 10 shoots of 1 to 3 cm in length had been formed by axillary branching. Short shoots (< 2 cm) were subcultured on the same propagation medium and long shoots (2 - 3 cm) were used for rooting experiments.

The rooting treatment of slices has been described in detail previously (Van der Krieken *et al.* 1993, De Klerk *et al.* 1995) and was briefly as follows. Ten 1-mm stem slices were cut from defoliated shoots of 2 - 3 cm with a special device consisting of ten 1-mm metal plates and 11 razor blades. The slices were cultured for 5 d with the apical side down on a nylon mesh (4 × 4 cm) on a modified Murashige and Skoog medium with 3 μM IBA in 9-cm Petri dishes. In each Petri dish, 30 slices were cultured, originating from six shoots (from each shoot five nonadjacent slices per Petri dish). The Petri dishes were incubated upside down in the dark in a culture room at 25 °C. After the IBA treatment, the nylon mesh with the slices attached, was taken from the Petri dish, put for 5 s on filter paper to remove excess liquid and then transferred to a Petri dish with hormone-free medium. Slices were taken daily for microscopical examinations.

Light and electron microscopy: The slices were fixed in 3 % (m/v) glutaraldehyde in 0.1 M phosphate buffer for 4 h, washed by buffer and postfixed with 1 % (m/v) osmium tetroxide for 24 h in the same buffer. After washing and dehydratation in

acetone, samples were infiltrated and embedded in Spurr medium (Spurr 1969). Semithin sections of 1 - 2 μm were stained by toluidine blue and basic fuchsine. Ultrathin sections were stained by uranyl acetate and lead citrate (Reynolds 1963) and examined in a *Tesla BS 540* electron microscope. Cells in the regions of the slice from which in the presence of IBA a ring of dividing cells developed, were analysed stereologically in longitudinal sections at various times after the start of culture. In the stereological analysis, the universal testing grid according to Weibel (1963) with 110 testing points was used. For quick orientation handmade sections stained with acridine orange, were examined in fluorescent microscope.

Results and discussion

Dedifferentiation phase (0 - 24 h): At the time of transfer to rooting medium, shoots were at the beginning of secondary growth. They had the normal organisation of epidermis, cortex and central cylinder. Strongly elongated cambial initials and their immediate derivatives were arranged in horizontal rows (Fig. 1*a*). The cambium cells were highly vacuolised. They had a narrow cytoplasm with few cell structures and a flattened, peripherally localised nucleus. Plastids were also strongly flattened and contained a well developed membrane system (Fig. 1*b*). Cells of pith and cortex were isodiametric parenchymatous and highly vacuolised. Starch grains were only present in the starch sheaths.

After 24 h of IBA treatment, large numbers of starch grains had appeared in the vascular bundles close to the basal part of the explants (in parenchymatous cells of xylem and phloem and immature tracheal and phloem elements), in adjacent cells of cortex and pith, and in primary rays and leaf gaps (Fig. 1*c,d,e*). In the cambium cells, the proportion of plastids had significantly increased and starch grains occupied a conspicuous part of the plastids (Figs. 1*f*, 5). In slices that had not been exposed to IBA, starch grains appeared in the same cells but later and in lower numbers (Fig. 4*h*, 5). Thus, IBA promoted uptake of sugar from the medium and/or conversion of sugar to starch. In conventional cuttings, auxins enhance the transport of carbohydrates to the basal part of the stem from where the roots regenerate (Haissig 1986).

Induction phase (24 - 96 h): After 2 d of IBA treatment, the cells in the cambial region showed an increased proportion of mitochondria, dictyosomes and nuclei at the expense of vacuoles (Fig. 5). The proportion of starch grains had strongly decreased. Thus, the starch grains that had accumulated during the first day of culture, functioned as a transient storage for carbohydrates before cells entered division. A similar rapid hydrolysis of starch has been found during adventitious root formation in grapevine shoots (Fabbri *et al.* 1985). At 48 h, in the cambium cells, divisions were sporadically observed (Fig. 1*g*). As a rule, divisions started in the second row from the basal cutting surface. Before division, the nucleus became round and moved to the centre of the cells. The nucleus was surrounded by cytoplasm. The dividing cells remained highly vacuolised, and caryokinesis and cell wall formation occurred in the central mass of cytoplasm. In the cambium cells,

mostly transversal divisions occurred (Fig. 1*h*) and only occasionally normal, longitudinal divisions. In slices cultured without IBA, some reduction of vacuolisation, increase of cytoplasm, multiplication of organelles and activation of nuclei was found reaching a maximum level only after 96 h of culture (Figs. 4h, 5).

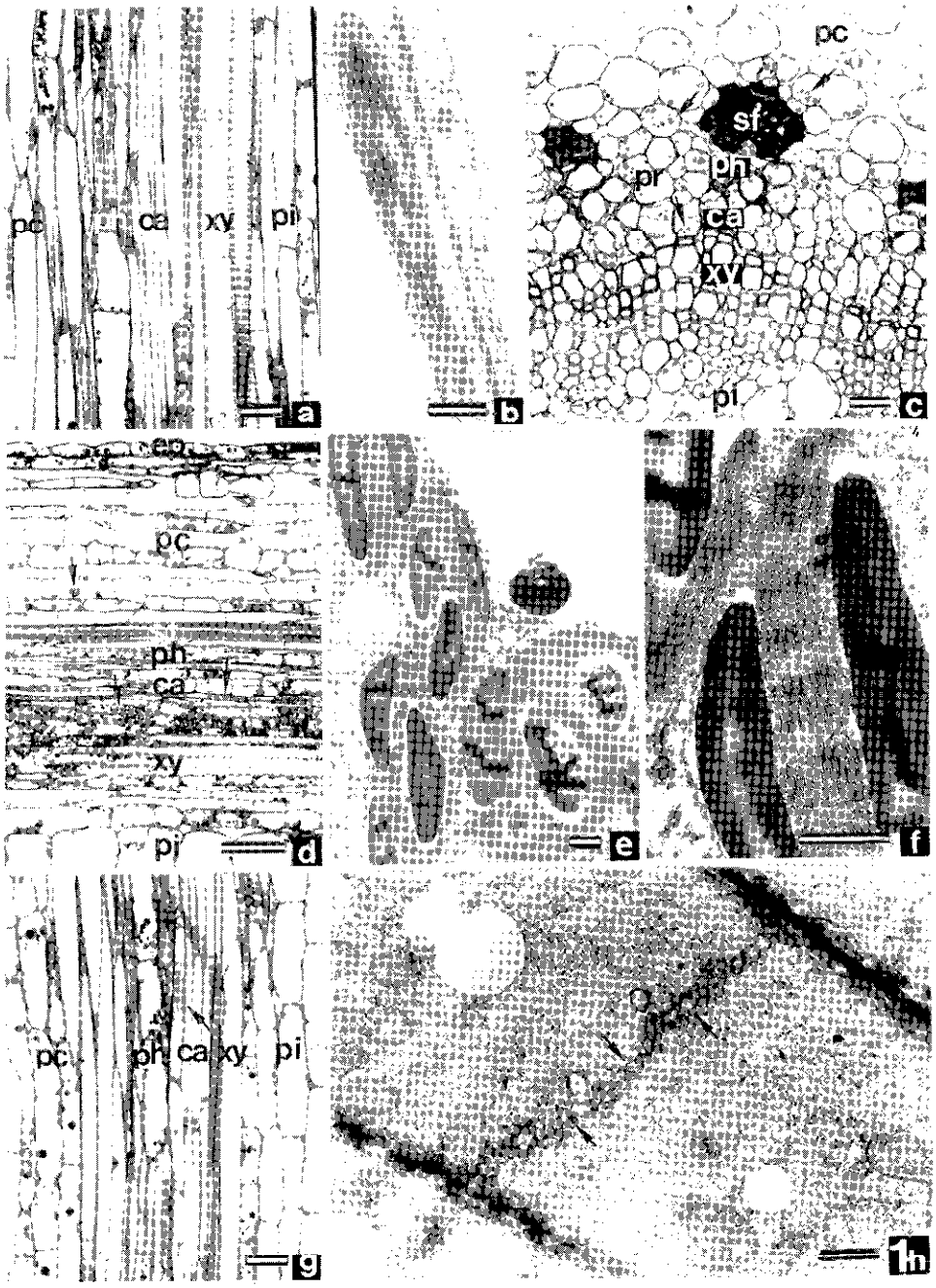
At 72 h, originally strongly elongated cambium cells in the basal part of the explants were fully segmented by transverse cell walls to more or less isodiametric cytoplasmic cells (Fig. 2*a*). The divisions of cells in rows were synchronic so that daughter cells were also arranged in rows (Fig. 2*b*). These isodiametric cells ultrastructurally resembled cells in meristems (Figs. 2*d,e*): they had a prominent, centrally localised nucleus with a large nucleolus and a strongly stained cytoplasm

Fig. 1. Micrographs of sections of stem slices of apple microshoots at various stages during the rooting treatment: ep - epidermis, ca - cambium, pc - primary cortex, ph - phloem, pi - pith, plb - prolamellar body, pr - primary ray, rgm - root ground meristem, rc - root cap, sf - sclerenchym fibers, xy - xylem (*bar* = 25 μm in *a,c,d* and 0.5 μm in *b,e,f,h*). Cells in cambial region (*a*) and chloroplast in cambium cell (*b*) at the time of transfer of the slices to rooting medium (0 h). Transversal (*c*) and longitudinal (*d*) sections of the basal part of slices at 24 h. Note the huge starch grains in the cells (*arrows*). Starch grains in the cells of xylem parenchyma (*e*) and cambium (*f*) at 24 h. Basal part of slice after 48 h of culture (*g*), first division of cambium cell can be seen (*arrow*). Transversal division of cambium cell after 48 h of culture (*h*).

Fig. 2. Micrographs of sections of stem slices of apple microshoots at various stages during the rooting treatment (continued). Abbreviations as in Fig. 1. *Bar* = 25 μm in *a,b,c,g;* 5 μm in *d,e* and 0.5 μm in *f*. Longitudinal sections of the basal part of the explant after 72 h (*a,b*). Intensively dividing cambium derivatives are indicated (*arrows*). Note the arrangement of cambium derivatives in horizontal rows (*b*). Transversal section of the basal part of the explant at 72 h (*c*). Activated cells formed first isolated areas (*arrows*). Electron-microscopical view of activated cells at 72 h (*d*). Area with obliterated phloem cells (*) at 96 h (*e*). Dense cytoplasm, rich in cell structures at 72 h (*f*). Formation of a continuous ring (*) of cytoplasmic cells around xylem at 96 h (*g*).

Fig. 3. Micrographs of sections of stem slices of apple microshoots at various stages during the rooting treatment (continued). Abbreviations as in Fig. 1. *Bar* = 25 μm with the exception of *a* and *b* (5 μm). Activated cell of xylem parenchyma at 72 h (*a*). Fine structure of isodiametric cytoplasmic derivatives of cambium at 72 h (*b*). Transversal (*c*) and longitudinal (*d*) section of area with group of cytoplasmic cells (*) giving rise to a root primordium. Surrounding cells divide in an unorganised way, remain highly vacuolized and develop callus (96 h). Group of cytoplasmic cells (*) in the inner part of cortex of young shoots with only poorly developed sclerenchymatous fibres at 96 h (*e*). Early root primordium with the tendency to form a dome-like structure at 96 h (*f*).

Fig. 4. Micrographs of sections of stem slices of apple microshoots at various stages during the standard rooting treatment (continued). Abbreviations as in Fig. 1. *Bar* = 25 μm with the exception of *e* (5 μm) and *a* (0.5 μm). Etioplast in the vicinity of a developing tracheid net (*a*). Well developed root primordium at the beginning of root-cap and root-ground-meristem formation at 120 h (*b*). Anthocyanoplasts (*arrows*) and osmiophilic layers on the inner side of the tonoplasts in cells at the periphery of roots producing anthocyanins at 144 h (*c*). Beginning of root-primordium formation in the stem (*d*). Note the normal growth of primordium top (*) perpendicularly to shoot (96 h; *ca.* 1 mm from cutting surface). Electron-microscopical view of cells root cap and root ground meristem at 120 h (*e*). Fluorescent microscopy of a root growing from cutting surface of an explant at 168 h (*f*). Tracheid net (*) of a developing root at 168 h (*g*). Note attaching of the tracheid net to the xylem of slices in the area of the first divisions (*arrow*). Cells of the cambial region (*h*) in slices cultivated 96 h on medium without IBA.



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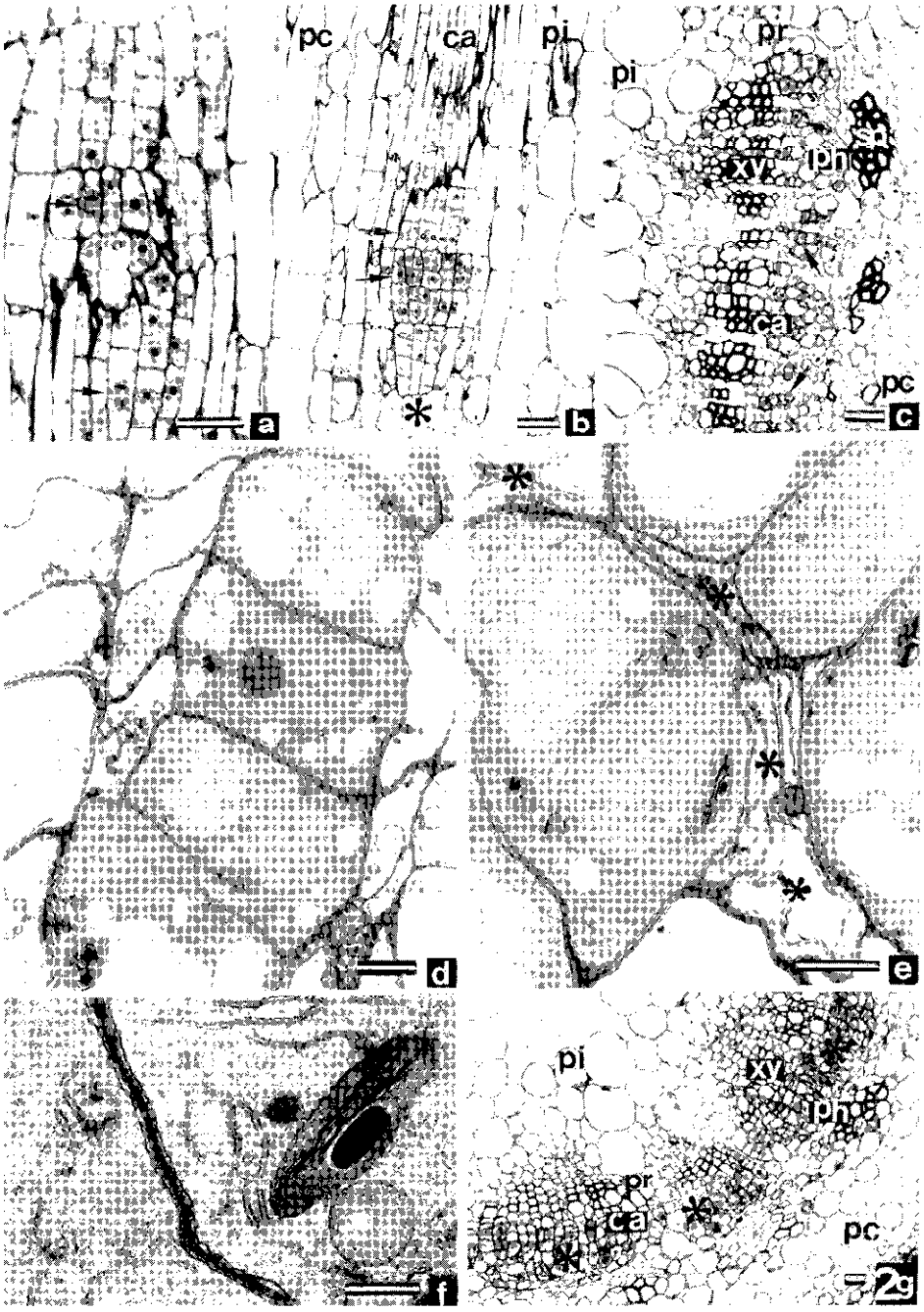

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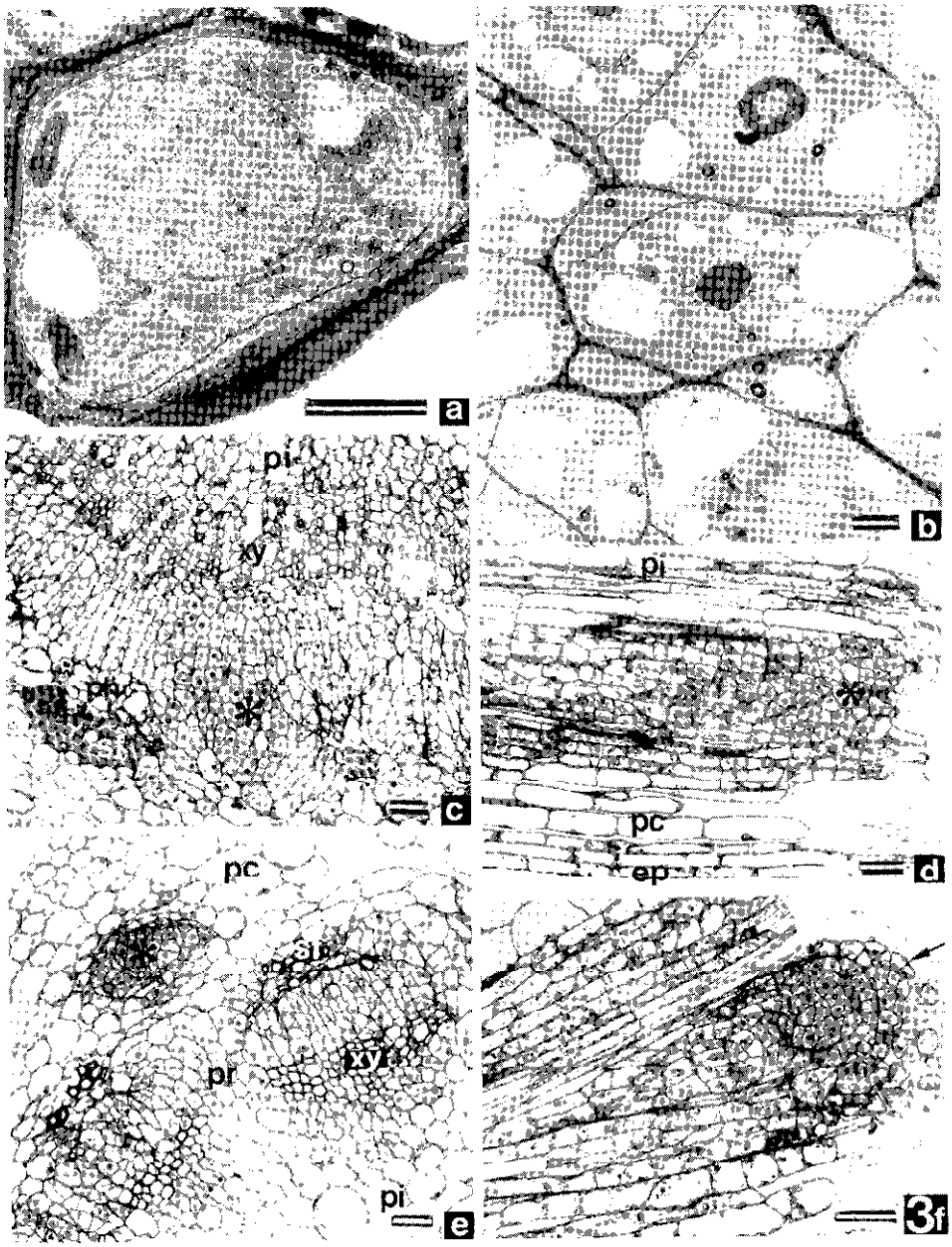

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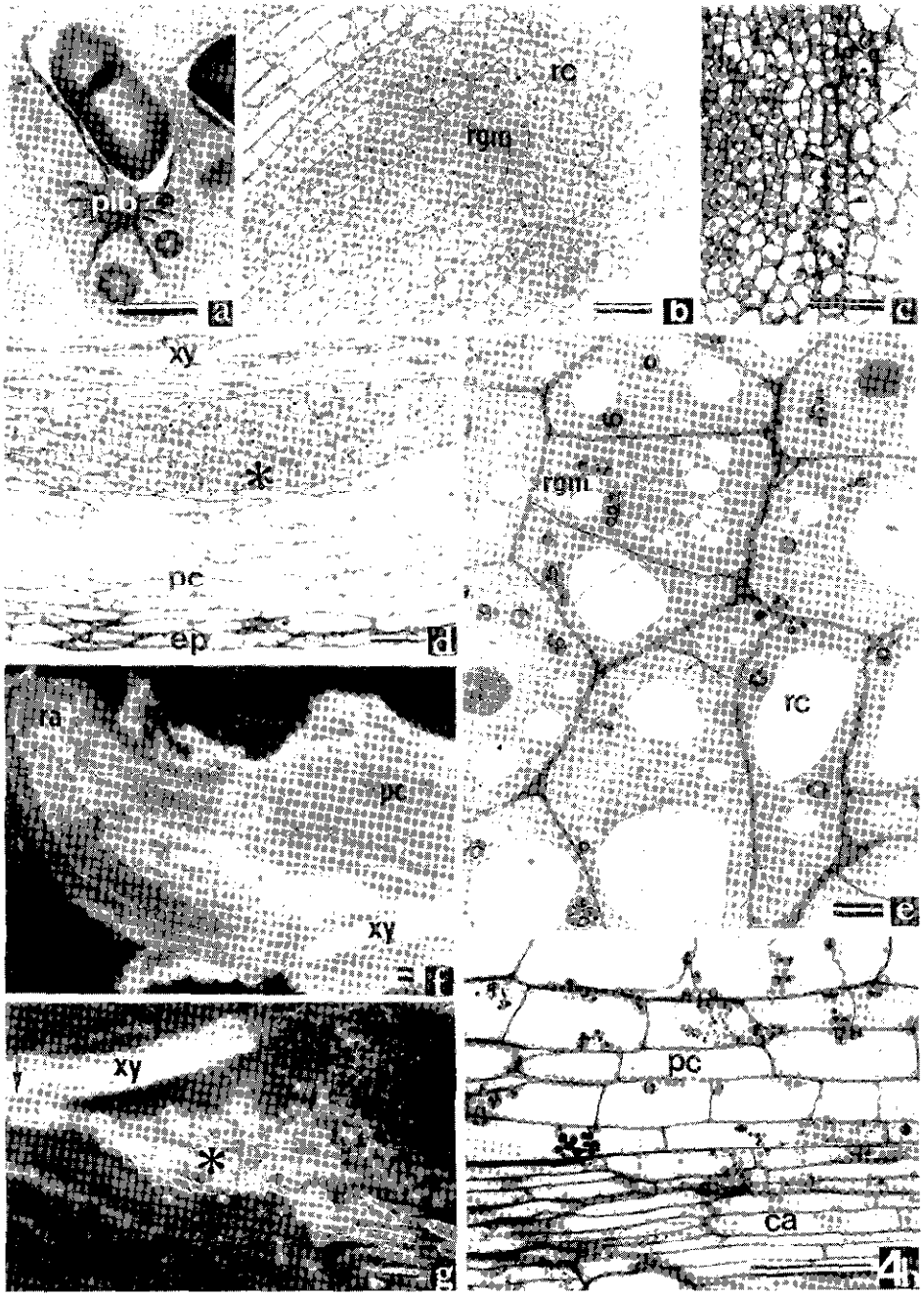

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with abundant organelles (Fig. 2*f*). Proplastids of simple organisation with sporadical starch grains and thylakoids were present (Fig. 2 *e,f*). Activated parenchymatous cells of xylem (Fig. 3*a*) and phloem exhibited a similar ultrastructure. Occurrence of highly cytoplasmic cells in the basal part of rooting shoots has been reported by many authors. These cells are localised in or close to the cambial area (Fabbri *et al.* 1985, Lovell and White 1986, Hicks 1987, Rodriguez *et al.* 1988, Darus 1989, Zhou *et al.* 1992, Harbage *et al.* 1993, Collet *et al.* 1994).

On transversal sections, cytoplasmic cells in apple slices showed at 72 h discrete areas scattered in the cambial region (Figs. 2*c,d*). At 96 h, in the basal parts of explants a more or less continuous ring had been formed around xylem by permanent divisions and activation of other cells including cells in the cambial region of primary rays (Fig. 2*g*). Phloem elements obliterated and were pushed to the sclerenchymatous fibres by intensively dividing cytoplasmic cells (Fig. 2*e*). It should be noted that many cells undergo cell divisions, but that only some of these cells did take part in root primordium formation.

Outgrowth (96 h onwards): Only some areas of the continuous ring acted as primordial initials and continued to develop roots, in particular the cambial areas of primary rays and leaf gaps (Figs. 3*c,d*) and rarely, in particular in very young shoots, areas in the inner part of the cortex (Fig. 3*e*). Only at this stage, 96 h after the start of culture, groups of meristematic cells had been formed resembling meristemoids as defined by Torrey (1966). This corresponds with the suggestion of Lovell and White (1986) that at an early stage, the cytoplasmic cells are not yet determined as root initials, and with studies using plant growth regulator pulses: until 72 to 96 h, the cells are not yet determined and the developmental programme can be easily altered by, *e.g.*, addition of cytokinin (De Klerk *et al.* 1995). The interfascicular origin of root primordia is generally found in apple shoots (Swingle 1927, Zhou *et al.* 1992, Auderset *et al.* 1994, De Klerk *et al.* 1995) and stem slices (Welander and Pawlicki 1993). The other cells in the cambial region took part in callus formation. They enlarged, became more vacuolised and divided as highly vacuolised cells in an unorganised way similar to, *e.g.*, grapevine callus cells (Jásik and Hudák 1989).

Cells taking part in root formation were cytoplasmic and contained numerous cell structures (Fig. 3*b*). Cell divisions occurred mainly close to the basal cutting surface (Fig. 3*d*). The divisions were both periclinally and longitudinally, resulting in a swelling of the basal part of the slices and in small outgrowths on the basal cutting surface. Cells in the outgrowths exhibited organised divisions resulting in typical dome-like structures (Fig. 3*f*). Such regular structures were composed of *ca.* 1500 cells and may be considered as root primordia (Lovell and White 1986).

Why primordia are formed only on the cutting surface (as opposed to the situation in stems in which the primordia are formed in the stem) is not clear. Another remarkable feature of adventitious root formation in slices is the direction of growth of the meristems in the slice that is vertically and not perpendicularly (as a rule in rooting shoots; Luxová and Lux 1981, Lovell and White 1986, Hicks 1987). In cashew stem-slices, a continuous ring of sclerenchymatous tissue blocks the perpendicular growth of primordia and directs them to the cutting surface (Bogetti, personal communication). In apple shoots, though, only discrete bounds of fibres are

present, localised under the primary phloem (Fig. 1*c*). As noted before, in stem slices, primordia developed between these bounds in the interfascicular area and not in the fascicular area below the fibre bounds. Thus, in apple stem slices the perpendicular growth of primordia was not restricted. Moreover, in whole shoots of the same material studied in the present paper, primordia developed in the normal direction (Fig. 4*d*) and roots emerged from the stem-epidermis. In *Solanum aviculare* stem slices, the direction of root primordium development depends on the thickness of the slice (unpublished results). In *Solanum*, 1-mm slices formed primordia growing perpendicularly and emerging through the cortex and epidermis. In thinner slices, though, primordia grew just as in apple slices vertically along the vascular tissues. In apple slices, maximal cell activation and division was restricted to cells close to the basal cutting surface (three rows of undamaged cambial cells). In whole shoots, however, meristemoids arose at *ca.* 1.5 mm from the basal cutting surface (Auderset *et al.* 1994). We suppose that the place of root emergence (epidermis or cutting surface) depends on the distance of the initial cells from which the meristemoids develop to the cutting surface.

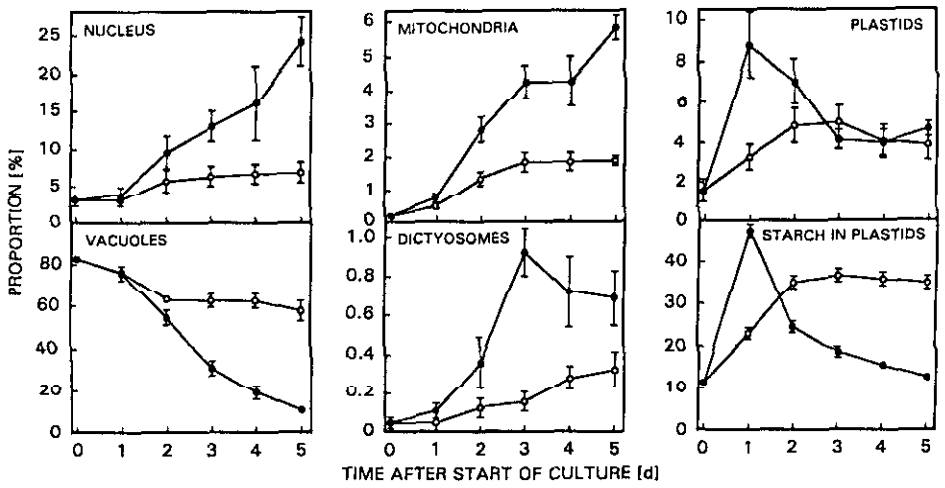

Fig. 5. Proportion of cell organelles and starch grains in cells of the cambial region in the basal part of slices cultured with (*closed circles*) or without (*open circles*) IBA. The proportion of cell organelles is presented as a percent of cell area and the proportion of starch grains as a percent of plastid area. The means ± SE are given. For each value, 30 - 40 fields were examined.

The development of roots from primordia was according to the general patterns reported previously by other authors for woody plants (Luxová and Lux 1981, Lovell and White 1986). After 5 - 6 d, a convex layer of 2 - 4 cells in the dome-like outgrowths formed a root cap (Fig. 4*b*). These cells became more vacuolised. Their cytoplasm was less densely stained and they contained more starch grains than centrally localised cells (Fig. 4*b,e*). In peripheral cells of the root cap, numerous anthocyanoplasts inside vacuoles and an osmiophilic layer on the inner side of the

tonoplast were present (Fig. 4*c*). These structures play a role in anthocyanin synthesis (Jásik and Vancová 1992). Because of the presence of these compounds, the tips of advanced primordia and young roots showed a red colour. The inner part became organised as a ground meristem and the procambial cylinder and gradually epidermis, cortex and central cylinder were formed (Fig. 4*f*). Meanwhile tracheid nets similar to those described, *e.g.*, in *Populus* stems (Luxová and Lux 1981) developed on the base of the roots (Fig. 4*f,g*). These nets connected the vascular tissues of developing roots and vascular tissue of stem slices. They were attached to the vascular tissue of the explants in the area where the first cell divisions had occurred (Fig. 4*g*). Because of the darkness during the culture, prolamellar bodies were formed in the plastids of the newly formed parenchymatous cells around tracheid nets (Fig. 4*a*). From these, typical chloroplasts were differentiated after transfer to the light.

Conclusion: A marked difference in adventitious root formation between stems and slices (the primordia emerged from the cutting surface and not from the epidermis) was found. In slices many cells did participate in the early divisions, but only from some root meristems developed. The dedifferentiation phase is characterized by a rapid increase of starch grains in cells; in the induction phase these starch reserves are used.

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