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How *Agrobacterium rhizogenes* triggers *de novo* root formation in a recalcitrant woody plant: an integrated histological, ultrastructural and molecular analysis

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SUMMARY

Adventitious rooting might be induced in recalcitrant woody genotypes by infection with *Agrobacterium rhizogenes*, and, in some cases, might also require exogenous auxin. The objective of the present study was to determine how agrobacteria trigger root formation in the stem of a recalcitrant woody microcutting, which cytological events result from the combined presence of infection and exogenous auxin, and which types of roots are induced by infection. Microcuttings of a recalcitrant walnut (*Juglans regia*), infected or not with *A. rhizogenes* strain 1855, were cultured with either indolebutyric acid (IBA), IAA, or without exogenous hormones, to induce rhizogenesis. They were cytohistologically and ultrastructurally investigated at various times in culture. Southern blot and PCR analyses were performed to verify the frequency of transgenic, chimeric and bacterium-containing roots. The infection was sufficient *per se* to stimulate rhizogenesis. Rooting on the infected cuttings was enhanced by exogenous IBA, which accelerated and increased root meristemoid formation, in comparison with without-hormone treatment. Meristemoids were organized both directly by the cambial cells and indirectly by the callus, and showed a pluricellular origin. Inter and intracellular bacteria were observed in the stem throughout the culture period (30 d). They were preferentially present in the vessels, and mainly in those showing polyphenol deposition. In the infected IAA-treated cultures, a high level of secondary xylem formation occurred instead of rhizogenesis. Nontransformed roots were preferentially produced by the infected cuttings treated with the auxins. Bacterium-containing and chimeric roots were produced by infected cuttings independently of the treatment. Thus, in a recalcitrant walnut, nontransformed root meristemoids are stimulated by combining infection and exogenous indolebutyric acid. Furthermore, the persistence of bacteria in the stem during the culture and the pluricellular origin of the meristemoids explain the presence of the bacterium-containing and chimeric roots.

Key words: *Agrobacterium rhizogenes*, auxin, *in vitro* rooting, localized infection, meristemoid, plant–bacterium interaction, *Juglans regia* (walnut), root chimerism.

INTRODUCTION

The induction of adventitious roots is crucial for *in vitro* micropropagation of woody species, whose rooting capacity varies with genotype, and agronomically useful genotypes are often recalcitrant for root formation. There are two types of genesis of adventitious roots: indirect genesis, in which root primordia are produced in a callus previously formed by the cells of the primary explant; and direct genesis, in which root primordia develop directly from the cells of the primary explant, mainly those in close proximity to the vascular system. In both types of rhizogenesis, the root develops from a meristemoid (Altamura, 1996 and references therein). The lack, or the poor expression, of a macroscopic

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rooting response in a recalcitrant woody species might be associated with various histologically detectable factors, such as the inability of the explant cells to organize meristemoids (Altamura, 1996).

The English or Persian walnut (*Juglans regia*) is widely appreciated for its large and thin-shelled nuts (McGranaham *et al.*, 1987). For commercial orchards, cultivars of *J. regia* are propagated by traditional grafting on seedling rootstocks; however, this process is both costly and time-consuming. The alternative practice of grafting on black walnut seedling rootstocks makes trees vulnerable to the lethal blackline disease (Mircetich *et al.*, 1980). Furthermore, clonal propagation of commercial cultivars on their own roots by layering is very difficult (Hartmann *et al.*, 1990). Although methods for *in vitro* propagation of walnut have been published, this plant still presents problems in terms of rooting performance.

In vitro, root formation might be a direct response to wounding or it might be associated with the presence of root inducers, such as auxin. In fact, a relationship has been shown between the endogenous levels of auxin and rooting capacity (Blakesley *et al.*, 1991), though the exact role of this hormone in the rooting process has not been clearly defined. Furthermore, exogenously applied auxins (IAA, α-naphthalene acetic acid and indole-3-butyric acid (IBA)) have been shown to accelerate the rooting process in cuttings of a wide variety of plant species. However, for recalcitrant woody species, auxin alone might not induce root formation. As an alternative or complementary strategy, localized infection might be used to force the cutting to root. In fact, inoculation with the soil bacterium *Agrobacterium rhizogenes* has been reported to be highly effective in improving adventitious rooting in some woody species (Damiano *et al.*, 1995; Monticelli *et al.*, 1997).

Agrobacterium rhizogenes has the potential to induce adventitious root formation at the site of infection, causing the well known hairy root disease. This potential has been attributed to the insertion and stable integration of a portion (T-DNA) of the bacterium's root-inducing (*Ri*) plasmid into the plant genome (Chilton *et al.*, 1982). Four loci have been reported to be involved in root formation; these loci correspond to open reading frames (ORF) *10*, *11*, *12* and *15* of the TL-DNA and are referred to as root loci (*rol*) *A*, *B*, *C*, and *D* (Villaine *et al.*, 1987). In recent years, root induction by *A. rhizogenes* (strain 1855) in microcuttings of a recalcitrant genotype of walnut has been reported (Caboni *et al.*, 1996).

To date, the means by which bacteria trigger rhizogenesis in recalcitrant woody genotypes and the specific step of the process that is eventually affected have not yet been investigated. The objective of the present paper was to identify histological and ultrastructural changes occurring in the microcuttings of a difficult-to-root genotype of walnut, in relation to: the localization of the bacteria within the primary explant; the eventual contribution of a root-inductive hormonal treatment; and the type (direct or indirect) of rhizogenesis induced. A further objective was to investigate whether infection was able to enhance the formation of nontransgenic roots or to affect specifically the formation of transgenic or chimeric roots.

MATERIALS AND METHODS

Plant material

In vitro cultures of a difficult-to-root seedling of *Juglans regia* L., cv. Sorrento, were used. The axillary shoots, which originated from buds of twigs in the same physiological state, were maintained in 3-wk subculture cycles for 4 yr (multiplication phase). Microcuttings (2 cm long, consisting of the three most apical internodes and the apex) were isolated from the shoot clusters at the end of the multiplication phase and used for the rooting experiments. The culture medium consisted of DKW salts and organics (DKW1; Driver & Kuniyuki, 1984), 87.6 mM sucrose, and 0.65% agar (B and V, Parma, Italy); the pH was adjusted to 5.6 prior to autoclaving at 120°C for 20 min. For the multiplication phase, the DKW1 medium was supplemented with 0.05 μM IBA and 2.2 μM BA. For the rooting experiments, the medium was kept hormone-free (HF treatment) or supplemented with either 10 μM IBA (IBA treatment) or 10 μM IAA (IAA treatment). The light source consisted of OSRAM L40 white fluorescent tubes with a 16 h/8 h light/dark photoperiod at 24 ± 1°C (45 μE m^{-2} s^{-1}).

Agrobacterium *strain and inoculation procedure*

Agrobacterium rhizogenes Conn. (wild type 1855 NCPPB) was grown in YMB medium (Hooykaas *et al.*, 1977) at 27°C to reach an optical density of 0.6 at 600 nm; it was centrifuged at 2500 ***g*** for 10 min at 4°C and resuspended in the same amount of liquid DKW1 medium. Inoculation was carried out through the immersion of the basal part of the microcutting stem (10–15 mm) into the bacterial suspension (2 ml), contained in 15 ml sterile Falcon tubes (Becton Dickins, Plymouth, UK) and gently shaken (60 rpm) for 24 h in darkness at 24 ± 1°C. The excess bacteria were removed by using sterile filter paper, and the microcuttings were transferred to the rooting medium in darkness, for a co-cultivation period of 24 h. As a control, noninfected explants were dipped into liquid DKW1 medium without bacteria and then cultured in conditions identical to those of the infected explants. Microcuttings were then transferred onto fresh medium

containing 0.52 mM filter-sterilized cefotaxime (Roussel Laboratories, Uxbridge, UK) to inhibit further bacterial growth, and either 10 μM IBA, 10 μM IAA, or no hormones (HF). Microcuttings were cultured for 10 d in darkness and then transferred to light (under the same conditions as above). The basal part was kept in darkness by wrapping the bottom of the vessel in aluminium foil.

Rooting assay

The rooted explants were counted 30 d after the beginning of the rooting experiment by observations under Leica MZ8 stereomicroscope. For each treatment, 60 microcuttings were used. The percentage of rooted explants on the total number of explants per treatment was calculated, and the differences between the percentages were evaluated using χ^2 test. The rooting experiments were repeated twice with similar results (only the data of the second experiment are shown).

DNA isolation and Southern blot analyses

Total DNA was extracted from 0.5 g of roots of infected and noninfected microcuttings and from the same weight of the basal region of the stem of nonrooted microcuttings according to Dellaporta *et al*. (1983), as modified by Archilletti *et al*. (1995). DNA was restricted, fractionated and transferred to a nylon membrane (Zeta-Probe Biorad, Hercules, USA) for hybridization, according to Caboni *et al*. (1996). A 4.3 kb *Eco*RI – *Eco*RI fragment containing *rol* genes (*ABC*) was used to detect the presence of TL-DNA in the total DNA of the samples, and a 9.3 kb *Eco*RI – *Eco*RI fragment containing *vir* genes was used to detect the presence of bacteria, according to Caboni *et al*. (1996).

PCR amplification of bacterial genes

Total DNA was extracted root by root from 30 randomly chosen roots of microcuttings treated with *A. rhizogenes*. Roots of two randomly chosen cuttings per treatment were also longitudinally divided into two parts, with DNA extracted from each part. DNA extraction was performed as reported for the Southern blot analysis.

The primers used to amplify *rolB* gene from *A. rhizogenes* were chosen according to Hamill *et al*. (1991), and the expected size of the fragment was 0.78 kb. PCR analyses were carried out using 50 ng of plant DNA in a reaction mixture containing 200 μM dATP, dTTP, dCTP, dGTP, 50 pM primers, and 0.25 units/30 μl Taq polymerase (Amersham Pharmacia, Uppsala, Sweden). Samples were first heated to 95°C for 5 min; a programmable thermal controller (Gene Attach Controller, Pharmacia-LKB, Uppsala, Sweden) was then used to carry out 25 cycles, consisting of denaturation (95°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1.5 min), with a final elongation step at 72°C for 5 min. An additional PCR analysis was performed, as already described, on the DNA of the entire root, which contained *rolB* gene, to detect *virD*1 gene (expected size of the fragment 0.45 kb, Hamill *et al*., 1991), which indicates the presence of bacteria. The two halves of the same root were analysed by PCR, for detecting both *rolB* and *virD*1 genes.

Amplified DNA was detected by ultraviolet light fluorescence after electrophoresis on 1.5% (w/v) agarose-ethidium bromide gels.

Histological analysis

At 0, 10, 15, 20 and 30 d, the stem (15 mm long starting from the basal end) of five microcuttings not subjected to infection and of five infected microcuttings per hormonal treatment was fixed, dehydrated, embedded in paraffin, and sectioned at 10 μm according to Altamura *et al*. (1991). The sections were stained with eosin and Carazzi's haemalum for detecting meristemoids (Altamura *et al*., 1991). Alternatively, the sections were stained with safranine-fast green (Jensen, 1962) or toluidine blue-O (O'Brien *et al*., 1965), for the histochemical detection of polyphenols, or with aniline blue, for the detection of callose deposits in the sieve plates under fluorescent light (Eschrich & Currier, 1964). For fluorescence observations, an Axiolab epifluorescence microscope (Zeiss, Jena, Germany), equipped with an HBO 50-W/AC mercury lamp, a beam splitter of 395, an excitation filter of 365, and an emission filter of 397 nm, was used.

For the detection of bacteria, the cells of the safranine-fast green stained sections were also observed with a DAS LEICA DMRB microscope using a PL FLUOTAR ×100. The images were acquired in digital form with a SONY DXC-101P camera equipped with a zoom adapter (up to ×16 magnifications), digitized with the Image Grabber 24 1.2 software (Neotech, Eastleigh, Hampshire, UK) for the Power Macintosh 7100/80 computer, and analysed using Optilab/Pro 2.6.1 software (Graftek, Mirmande, France).

The presence of bacteria in the vessels showing polyphenol deposition was also detected in sections stained with safranine-fast green observed at a magnification of × 3000 with a Zeiss-LSM3 Confocal Scanning Laser Microscope (excitation lasers: He-Ne 543 nm, Argon-ion 488 nm).

Transmission electron microscopy

Microcuttings were fixed in 4% solution of F. A. A. (90 ml ethanol, 5 ml acetic acid, 5 ml formaldehyde) and post-fixed in 1% osmium tetroxide at 0, 15 and 20 d and then embedded in Spurr's low viscosity

resin (Spurr's kit, Sigma-Aldrich Co., Milan, Italy) at 70°C for 8 h. Samples were prestained with 0.5% solution of uranyl acetate, and ultrathin sections (80 nm) were stained with citric acid lead (II) salt trihydrate (Reynolds, 1963) and observed with a Zeiss EM 10/C (voltage analysis 40/60 kV) transmission electron microscope.

Counting procedures and statistical analyisis

The radial extension of the proliferating zone of cambial origin, of the xylem, and of the phloem was measured with a Power Macintosh 7100/80 using Optilab 2.6.1 software; measurements were taken at day 0 and day 20 on five infected and noninfected microcuttings (observations at intervals of 80 μm) per hormonal treatment (IAA- and IAA Ar- treatments only, for the phloem). The images were acquired with a SONY DXC-101P video camera applied to the Axiolab microscope.

In all of the sectioned microcuttings of each treatment, at days 0, 10, 15, and 20, the number of meristemoids, the number of vascular nodules, and the total of the two were counted and expressed as means ± SE. On the same explants, starting from their base, the mean (± SE) longitudinal extension in mm of the microcutting region with meristemoids and vascular nodules was calculated, multiplying the number of transections showing these structures by the interval in μm between successive sections.

Mean values were always compared using Student's *t*-test.

RESULTS

Macroscopic rooting response and molecular analysis of the entire roots

After 30 d of culture, the rooting response (i.e. the percentage of explants with roots) was very low in the absence of infection and was restricted to the IBA treatment only. In the presence of infection (Ar treatments), a conspicuous rooting response was obtained, even in the absence of exogenous hormones (HF). The highest response ($P<0.01$ in comparison with IAA Ar and $P<0.05$ in comparison with HF Ar treatments) was observed in the presence of IBA. In the IBA-treated explants, infection induced a rhizogenic response that was five times greater than that obtained in noninfected explants ($P<0.01$) (Table 1).

By contrast, root productivity, evaluated as the number of roots per explant, was not affected either by the hormonal treatment or by the combined presence of hormone and infection. In fact, the number of macroscopic roots per explant always ranged from one to three.

Southern blot analysis revealed hybridization between *rol ABC* probe and root samples from infected cuttings treated with IBA, HF, and IAA (Fig. 1a). No signal was detected either in the basal parts of noninfected microcuttings (Fig. 1a) or in the infected and noninfected samples probed with *vir* genes (data not shown). However, when single roots were analysed with PCR, transgenic roots, which did not contain bacteria (*rol*+*vir*− in Table 1) and roots containing bacteria (*rol*+*vir*+ in Table 1) were detected (Fig. 1b,c). In the IBA Ar and IAA Ar treatments, a higher number of normal (*rol*−*vir*−) roots was produced, than of the combined *rol*+*vir*− and *rol*+*vir*+ roots, whereas in the HF Ar treatment there was no difference. No significant difference was observed in the HF treatment between the roots containing bacteria and normal roots, whereas a highly ($P<0.01$) significant reduction occurred in the auxin treatments (Table 1).

Localization of the bacteria in the tissues

At the onset of the rooting experiment, the stem of the microcuttings showed the secondary vascular structure (Fig. 2a), with a 98 (±3.7) μm radial extension of the xylem and a 34.81 (± 0.53) μm extension of the phloem.

After 10 d of culture, callus, produced by the cortical cells, was already present in all of the treatments and was located at the base of the microcutting. The highest callus production was observed in the cuttings treated with IBA (both noninfected and infected). In comparison with day 0, an increase in secondary vascular growth (especially in xylem production) occurred in all the hormone-treated cuttings, and especially in those of IAA Ar treatment (Fig. 2b). Polyphenols, absent at day 0, were detected mainly in the infected cuttings and were present in both the primary and secondary xylem, as well as in the secretory cavities of the cortex. It appeared that parenchyma cells, flanking some vessels of the primary xylem or surrounding some of the largest tracheary elements of the secondary xylem, produced these compounds (Fig. 3a,c), which were then secreted into the vessels (Fig. 3b).

Polyphenol deposition greatly increased in the combined presence of infection and exogenous IAA, whereas the polyphenol deposition observed during the culture of the noninfected cuttings was not increased by exogenous IBA or IAA.

In the infected cuttings, the bacteria were localized in the secretory cavities of the cortex and in the nearby cells, especially in the tracheary elements of primary (Fig. 4a) and secondary xylem (Fig. 4b,c). The bacteria exhibited either the rod shape typical of the free form (Fig. 4d) or an irregular and highly heterogeneous shape (Figs. 4b,c,f and 5b–e). A 'flow' of bacteria through the pits of contiguous vessels was observed (Fig. 4c,f). Bacteria were present not only in the vessels but also in the xylem

Table 1. *Rooting response of walnut microcuttings after 30 d of culture under various hormonal treatments in the presence or in the absence of localized infection*

Hormonal treatment	Rooted explants (%)	*rol*−*vir*− roots (%)	*rol*+*vir*− roots (%)	*rol*+*vir*+ roots (%)
HF	0	—	—	—
IAA	0	—	—	—
IBA	12	100	—	—
HF Ar	40	50	17	33
IAA Ar	23	73	17	10
IBA Ar	62	70	17	13

The percentage of rooted explants on the total number of cultured explants per treatment ($n = 60$) is shown in the first column.
The percentages of normal roots (rol−vir−, second column), of transgenic roots (rol+vir−, third column), and of roots containing bacteria (rol+vir+, last column) were calculated on 30 randomly chosen roots per treatment. The presence of *rolB* and *vir D1* genes was evaluated by PCR.
HF, hormone-free medium; IAA, medium with 10 μM of IAA; IBA, medium with 10 μM of indolebutyric acid; Ar, presence of infection with *Agrobacterium rhizogenes*.

parenchyma cells surrounding them (Figs 4b and 5a,c) and, less frequently, in the pith ray cells. The bacteria showed not only an intracellular location but were also observed in the intercellular spaces (Fig. 5b) and between the plasma membrane and the wall (Fig. 4b). In some cases, they seemed to be incorporated in the cell through plasmalemma invagination (Fig. 5c). The parenchyma cells lost their structural integrity when they became filled with bacteria (Figs 4b and 5a–c). Fibrillar material accumulated in the parenchyma cells, and bacteria were immersed in it (Fig. 5b,d,e). This material seemed to be very different in structure in comparison with the fibrillar material observed in the vessels colonized by bacteria and located near the wall and at the borders of the secondary wall thickenings (Fig. 4c,e, and Fig. 5e, for comparison).

In the parenchyma cells filled with bacteria and fibrillar material, cell death occurred as a result of the total disorganization of the organelles and the disappearance of the nucleus. Independently of their inter or intracellular location, the bacteria were more numerous at the cutting base. Neither bacteria nor fibrillar materials were present in the noninfected cuttings, observed by both electron transmission (Fig. 5f) and laser confocal microscopy. After safranine-fast green staining, the cells containing high amounts of polyphenols became red in colour (Fig. 3a,d,e); however, some of them appeared bluish in colour (Fig. 3d,e). In these cells (especially vessels), a conspicuous presence of bacteria was detected.

Effects of IBA treatment on root meristemoid formation

Another event observed at day 10 in infected explants of all treatments, and in the noninfected explants treated with IBA only, was the formation of groups of meristematic cells (meristemoids, Altamura, 1996). The preferential localization of such structures was in close proximity to the cambium (direct genesis, Fig. 2d). When engaged in meristemoid formation, the cambial cells, or their immediate derivatives, lost their typically rectangular shape in transection and acquired more evident meristematic features (i.e. they became rounded and showed a hypertrophic and highly chromophil nucleus). In no case were single cells with these features detected; instead, groups of at least two or three cells were observed (Fig. 2c). In the treatments with IBA, indirect meristemoids and vascular nodules (containing vascular elements surrounded by meristematic cells) were present in the cortical callus (Fig. 2e,f). Also in the callus, the meristemoids originated from groups of cells (pluricellular origin).

After 15 d of culture, callogenesis highly increased, and mainly in the auxin-treated cuttings, independently of the auxin type and of the presence of infection; not only the cortical cells but also some cells of the pith and cambium were involved in callus proliferation. Inclusions of polyphenols increased in the xylem of all of the infected explants. Meristemoids and, to a lesser extent, vascular nodules, continued to be observed in the same treatments at day 10, reaching a very high value in the IBA Ar treatment (150 ± 10 per explant). In the cuttings of the latter treatment, the first root domes were observed, and they showed either an indirect genesis (from the cortical callus) (Fig. 2g) or a direct genesis (Fig. 3f). In IBA Ar-treated cuttings, meristemoids and domes were longitudinally distributed over one third of the length of the stem, starting from the base (Fig. 6a). Meristemoids occupied a shorter longitudinal stem portion in the cuttings of IAA Ar and IBA treatments (data not shown), and extended to only 2.5 mm from the basal cut end in the infected cuttings of the HF treatment (Fig. 6a).

At day 20, meristemoids and vascular nodules were present in the cuttings of all the treatments

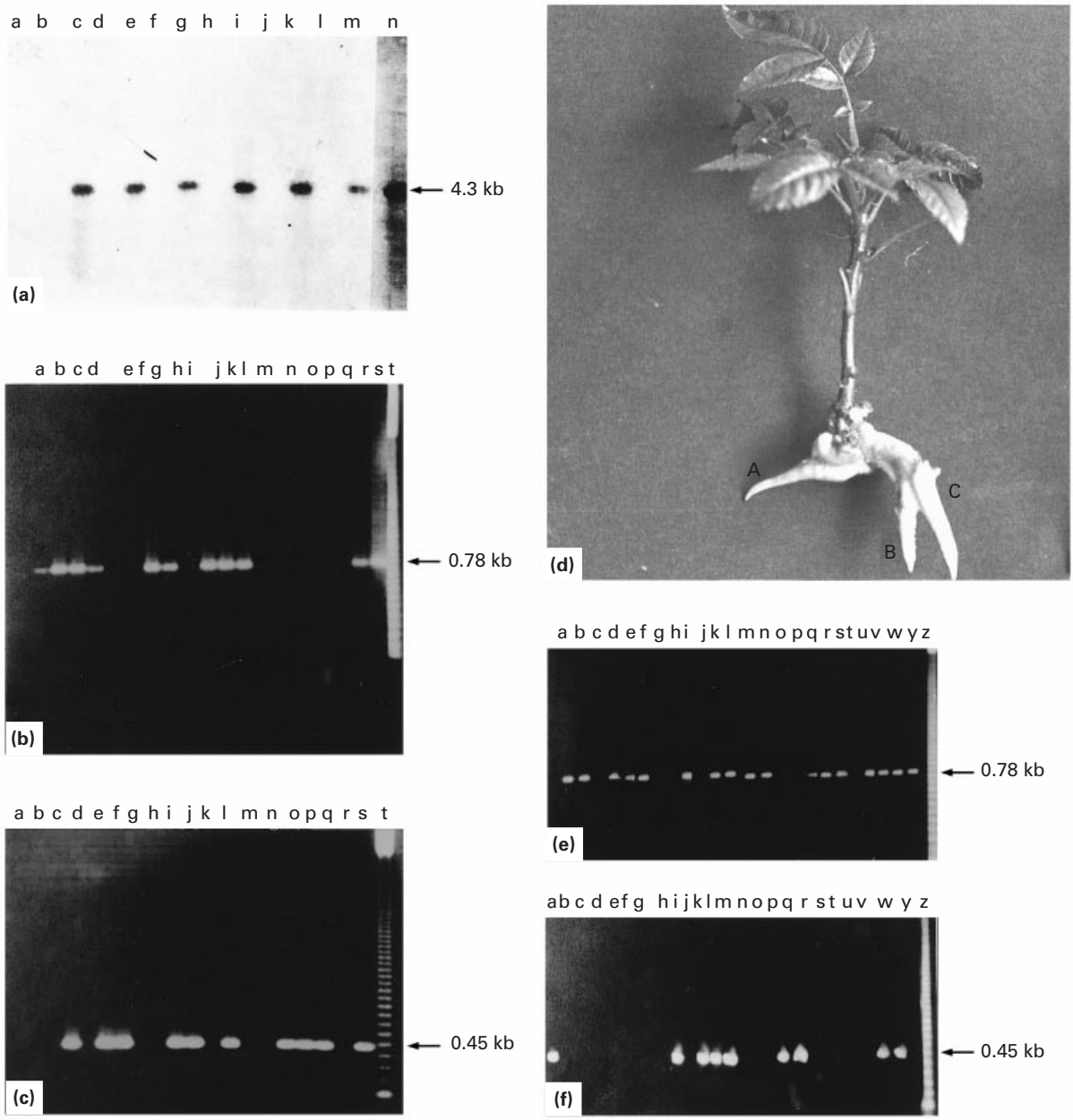

Fig. 1. (a) Southern blot analysis of roots and basal parts of infected and noninfected microcuttings of walnut treated with either IAA or indolebutyric acid (IBA) or without hormones (HF) for 30 d, performed using *Eco*RI – *Eco*RI fragments (*rolABC* genes, 4.3kb). Lane **a**, noninfected walnut cutting at day 0; lanes **b** and **d**, noninfected explants treated with IAA; lanes **c** and **e**, infected explants treated with IAA; lanes **f** and **h**, noninfected explants treated with IBA; lanes **g** and **i**, infected explants treated with IBA; lanes **j** and **l**, noninfected HF explants; lanes **k** and **m**, infected HF explants; lane **n**, positive control. (b) Agarose gel electrophoresis of *rolB* gene amplification (0.78 kb) from PCR analysis of total DNA, extracted from single roots of walnut explants infected with *Agrobacterium rhizogenes*. From lane **a** to lane **f**, IAA-treated explants; from lane **g** to **m**, indolebutyric acid-treated explants; from lane **n** to **s**, hormone-free explants. Lane **t** is a ladder of 78 bp molecular weight. (c) Agarose gel electrophoresis of *virD*1 gene amplification (0.45 kb) coming from PCR analysis of total DNA, extracted from single *rolB* + roots of walnut infected explants. From lane **a** to **f**, IAA-treated explants; from lanes **g** to **m**, indolebutyric acid-treated explants, and from lanes **n** to **s**, hormone free explants. Lane **t** is a ladder of 45 bp molecular weight. (d) *Agrobacterium rhizogenes* infected microcutting of walnut after 30 d of culture in the presence of 10 μM indolebutyric acid, showing typical results after amplification from PCR analysis of *rolB* and *virD*1 genes on the longitudinally bisected roots: A, entirely transgenic root; B, chimeric root; and C nontransformed root. (e) Agarose gel electrophoresis of *rolB* gene amplification (0.78 kb) from PCR analysis of total DNA extracted from each half of walnut roots, bisected longitudinally and representing microcuttings infected with *Agrobacterium rhizogenes* and cultured either in the presence of 10 μM indolebutyric acid (lanes **a–d**, and **e–l**) or in the absence of hormones (lanes **m–r** and **s–y**). Lane **z** is a 78 bp molecular weight ladder. The two halves of the same root are indicated by lanes with consecutive letters. (f) Agarose gel electrophoresis of *virD*1 gene amplification (0.45 kb) from PCR analysis of total DNA extracted from each half of walnut roots, bisected longitudinally and representing microcuttings infected with *Agrobacterium rhizogenes* and cultured either in the presence of 10 μM indolebutyric acid (lanes **a–d**, and **e–l**) or in the absence of hormones (lanes **m–r** and **s–y**). Lane **z** is a 45 bp molecular weight ladder. The same root portions of Fig. 1 (e) are shown by the same letters.

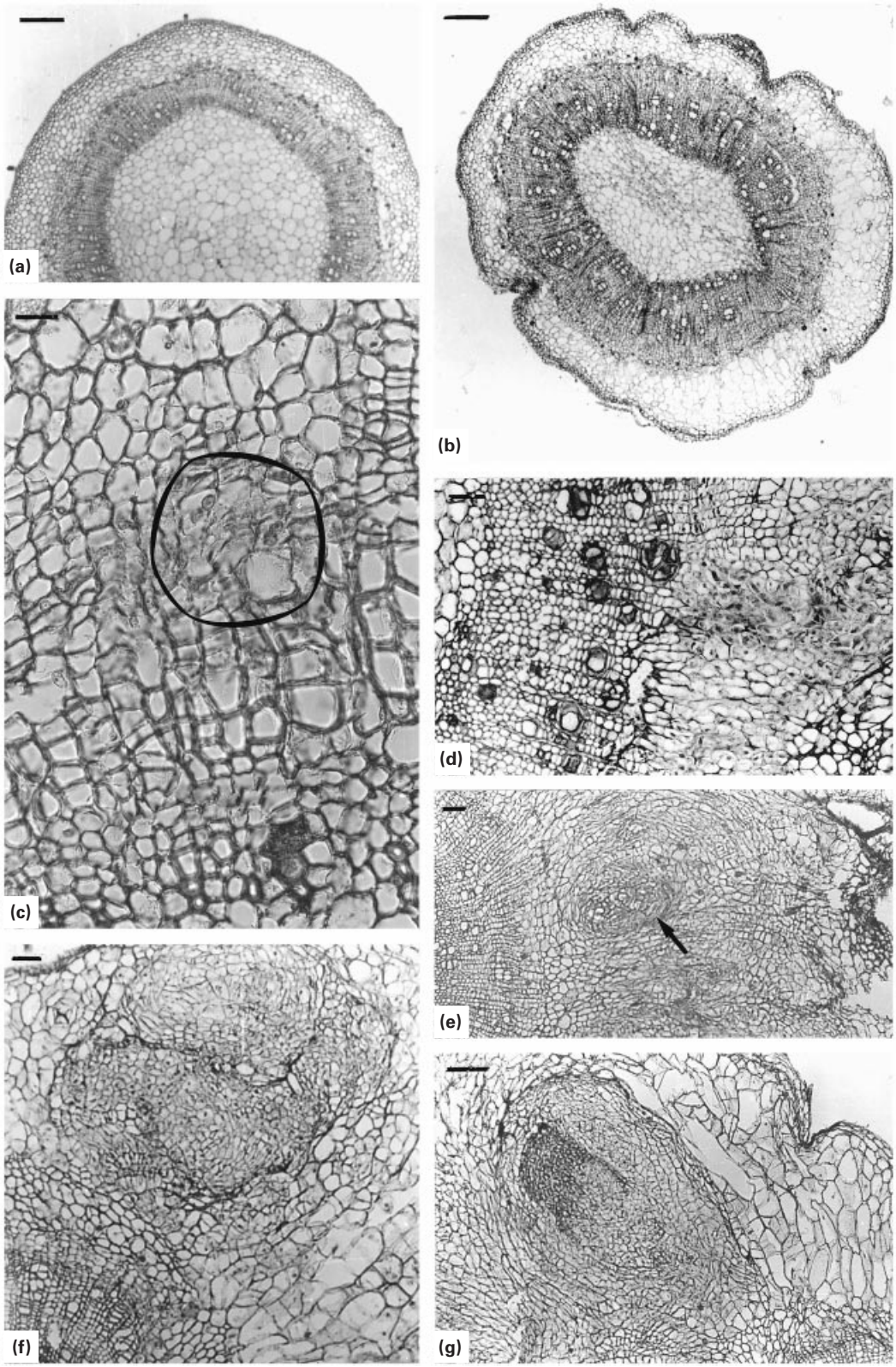

Fig. 2. Histological events occurring in walnut microcuttings during the first half of the culture on the rooting media. (Transections stained with eosin and Carazzi's haemalum. Bars = 200 μm, a–b; 100 μm, e; 50 μm, d,f, and g; 20 μm, c; IBA, indolebutyric acid; Ar, infection with *Agrobacterium rhizogenes*). (a) Stem showing the secondary vascular structure at the onset of culture. (b) Irregular shape of the stem due to callus formation from the cortical parenchyma after 10 d in culture. An increase in secondary vascular growth, especially due to xylem formation, is shown in comparison with day 0 (a) (IAA Ar-treated explant). (c) Onset of meristemoid formation from the cambium: a group of few meristematic cells with hypertrophic and chromophil nuclei is shown by the circle. (d) Meristemoid in close proximity to the cambium (direct genesis) (IBA Ar-treated explant, day 10). (e) Vascular nodules in the cortical callus. The vascular elements in the centre of the nodule are shown by the arrow (IBA-treated explant, day 10). (f) Meristemoids of indirect origin in the cortical callus (IBA Ar-treated explant, day 10). (g) Root dome *de novo* formed in the cortical callus (indirect origin) (IBA Ar-treated explant, day 15).

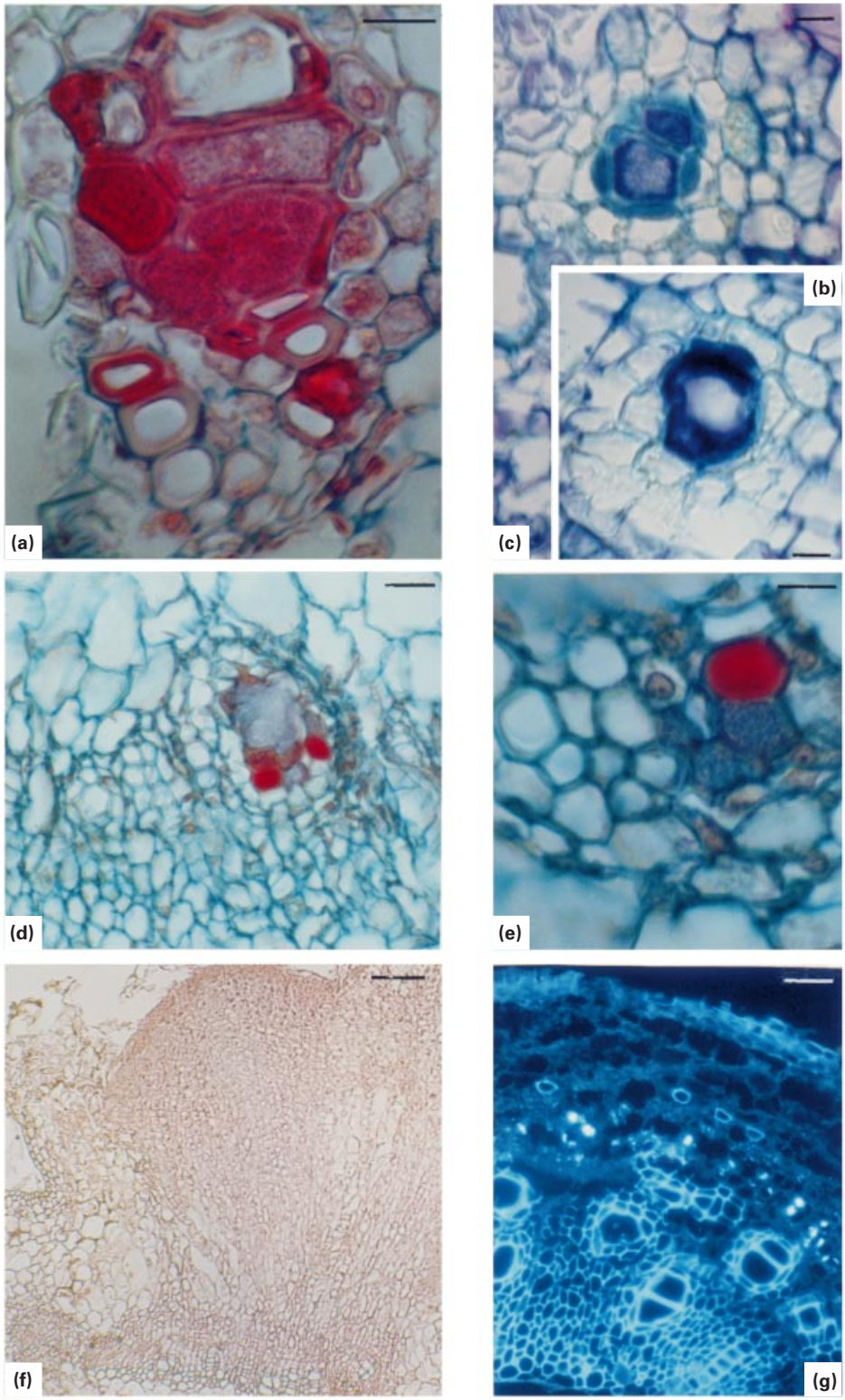

Fig. 3. Polyphenol deposition in the xylem (a–e); formation of direct root domes (f), and development of secondary vascular tissues (g) in walnut microcuttings after 10 (a–e), 15 (f), and 20 d (g), respectively, of culture on rooting media. (Transections: a, d and e stained with safranine-fast green; b and c, stained with toluidine blue-O; f with eosin and Carazzi's haemalum, and g, fluorescent micrograph of a section stained with aniline blue; IBA, indolebutyric acid; Ar, infection with *Agrobacterium rhizogenes*). Bars = 100 μm, b,c,d, and f; 50 μm, g and 10 μm, a and e. (a) Primary xylem of the stem showing polyphenol deposition (in red) in the metaxylem and in some of the surrounding xylem parenchyma cells (IBA treatment). (b) Vasicentric parenchyma cells are colourless because they have completed the secretion of phenolic compounds into a tracheary element. (c) Polyphenol deposition (in blue) in the tracheary elements of the secondary xylem and in the surrounding vasicentric parenchyma. (d,e) Details of the primary xylem in the stem of an infected cutting. The bluish instead of red colour shown by the vessels, is probably due to the presence of bacteria together with

except the noninfected cuttings of the HF treatment (Fig. 6b). The highest number of combined meristemoids and vascular nodules was observed in the infected cuttings cultured in HF treatment ($P < 0.01$ in comparison to IBA Ar- and IAA Ar-treated cuttings, and $P < 0.05$ in comparison with the other treatments). In these cuttings, the number of meristemoids, evaluated separately, was also the highest ($P < 0.01$, in comparison with the other treatments). The highest number of vascular nodules alone was found in IAA-treated cuttings; the difference was significant ($P < 0.01$) in comparison with all treatments except HF Ar (Fig. 6b). Abundant root primordia, in some cases highly developed, were observed only in IBA Ar-cuttings and showed both direct (Fig. 7a) and indirect (Fig. 7b) origin. The vasculature of the direct root primordia was continuous with the vascular system of the cutting (Fig. 7a), whereas that of the indirect primordia either arose from *de novo* formed vascular elements in the callus (Fig. 7b) or was in continuity with the vascular system of the cutting occurring by a *de novo* formed procambium-like strand (Fig. 7c).

Primordia and meristemoids, and meristemoids only, were observed in up to almost half of the stem length in IBA Ar-treated and IBA-treated cuttings, respectively (Fig. 6a). In the cuttings of the other treatments, meristemoids were located only in the basal part of the stem, becoming strictly basal in the HF Ar-treated cuttings ($P < 0.01$ in comparison with the cuttings of IBA treatments, Fig. 6a).

Effects of IAA treatment on secondary xylem formation

At day 20 in the IAA-treated, noninfected cuttings, the production of xylogenic callus was significantly higher ($P < 0.01$) than that in all of the other treatments (Figs. 6b,c, and 7 d–f). In the same cuttings, the ray parenchyma was also reactivated to cell division, altering the shape of the secondary xylem (Fig. 7g). In the infected IAA explants, the cambial cells behaved differently: proliferation of xylogenic callus was not significant in comparison with the other treatments (Fig. 6b,c), whereas xylem production was very high ($P < 0.01$ difference with all the other treatments, Fig. 6c). Furthermore, in the latter treatment only, vessels showing polyphenol deposition, frequently associated with bacteria, were observed in the entire radial extension of the xylem (Fig. 7h). To evaluate whether the IAA treatment also induced phloem production in the infected cuttings, this tissue was detected through the fluorescence of callose in the sieve plates (Fig. 3g). The radial extensions of xylem and phloem at day 20 were compared with noninfected cuttings treated with the same hormone and with those of day 0. In comparison with day 0, the cuttings of both IAA treatments showed significant increases in both phloem ($P < 0.05$) and xylem ($P < 0.01$); however, at day 20, the radial extension of the xylem was significantly ($P < 0.01$) greater (by about 33 %) in the infected cuttings than in the noninfected ones, whereas no significant difference was observed for the phloem.

Root chimerism

At day 30, in IBA, HF Ar, and IAA Ar treatments, the macroscopic roots (Table 1) exhibited the same origins and in the same explant sites as those previously described for IBA Ar-treated cuttings. In the HF Ar-treated cuttings, the number of meristemoids decreased simultaneously with the development of root primordia and macroscopic roots. In the latter treatment, bacteria were observed in the secretory cavities of the poorly developed cortical callus (Fig. 7i).

Roots of infected cuttings, randomly chosen within each treatment, were dissected from the cutting and were cut in half longitudinally. PCR analysis showed that, independently from the treatment, the roots were either normal (i.e. *rol*−*vir*− each half), or totally transgenic (i.e. *rol*+*vir*− each half), or transgenic and containing bacteria (i.e. *rol*+*vir*− one half and *rol*+*vir*+ the other), or chimeric, exhibiting one transgenic half (*rol*+*vir*−) and one normal half (*rol*−*vir*−) (Fig. 1e,f). Genetically different roots were concomitantly present on the same explant, as exemplified in Fig. 1d.

DISCUSSION

Localized infection positively interacts with IBA in enhancing rooting, and the key step in this process is the formation of the root meristemoid

This study demonstrates that, in a recalcitrant walnut, two inducers act synergistically in triggering the rooting process: the exogenous treatment with a specific auxin (IBA) and the infection with *A. rhizogenes* (wild type 1855). It further shows that the formation of the meristemoid is the key step in this process. In fact, the IBA treatment and the infection synergistically induce a very precocious and abundant formation of meristemoids, resulting

the polyphenols. (e, higher magnification of a bluish vessel; b–e, infected cuttings treated with IAA). (f) Dome of a root primordium of direct genesis (IBA Ar treatment). (g) Detail of the secondary vascular system of an IAA Ar-treated cutting. The secondary xylem is autofluorescent, and the secondary phloem is shown by the induced fluorescence in the callose plates.

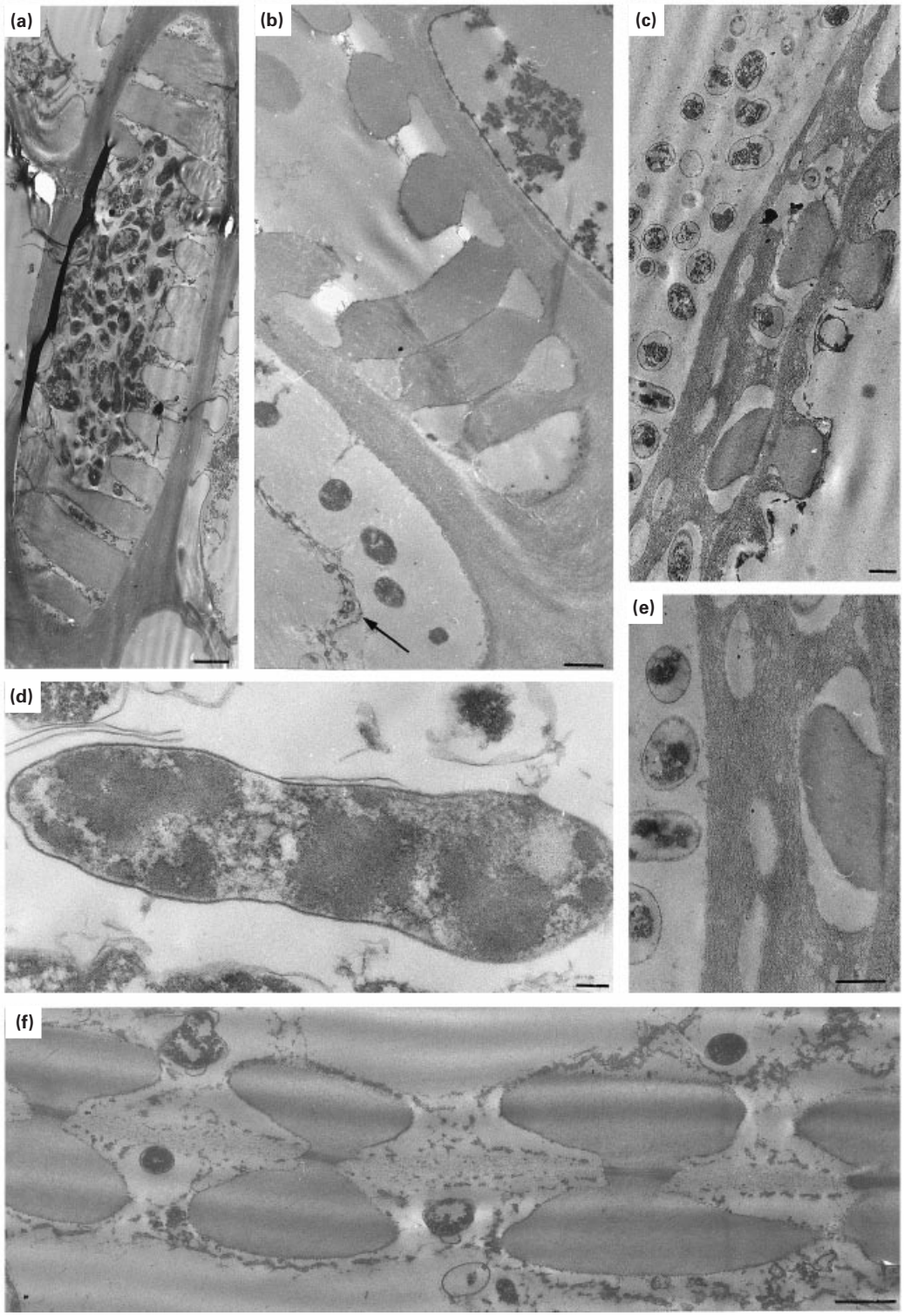

Fig. 4. Presence of *Agrobacterium rhizogenes* in the conducting elements of the xylem in the infected cuttings after 10 d of culture on the rooting media (thin longitudinal sections observed under TEM; bars = 1.5 μm, a; 1 μm, b and f; 0.5 μm, c and e; 150 nm, d; HF, hormone-free; IBA, indolebutyric acid; Ar, infection with *Agrobacterium rhizogenes*). (a) Tracheid of the primary xylem filled with bacteria (IBA Ar-treated cutting). (b) Detail of the secondary xylem showing bacteria in highly damaged xylem parenchyma cells at the flanks of a tracheary element. The arrow shows the remains of the plasma membrane in the host cell (HF Ar-treated cutting). (c) Detail of a tracheary element of the secondary xylem containing bacteria of heterogeneous shape. Fibrillar material is present near the wall and at the borders of the secondary wall thickenings. Some bacteria are located between this material and the pits (IAA Ar-treated cutting). (d) Bacterium exhibiting the rod shape typical of the free form of *Agrobacterium rhizogenes* (HF Ar-treated explant). (e) High magnification of c, showing the ultrastructure of the fibrillar material near the borders of a secondary wall thickening (IAA Ar-treated cutting). (f) Bacteria near, and passing through, the pits of contiguous vessels of the secondary xylem (IBA Ar-treated cutting).

in a high macroscopic rooting response. Both of these inducers are also able to independently affect meristemoid formation, though IBA treatment induces a poor rooting response. By contrast, infection triggers a rather consistent rooting response, as shown for infected cuttings in HF treatment. However, in these cuttings, meristemoids are produced later and in lower quantities than those obtained when IBA is added to the culture, and their location in the stem is strictly basal.

It is known that endogenous auxin positively affects rooting (see Introduction). Due to its basipetal transport in the stem (Lomax *et al*., 1995), and probably as a reaction to wounding, as shown in sweet potato tuber (Tanaka & Uritani, 1979), endogenous auxin can accumulate at the basal end of the cutting over time and, upon reaching a sufficiently high level, contribute to triggering root meristemoid formation in this site. Thus, infection might trigger the rooting process in cells with a sufficiently high auxin content. During adventitious root formation in apple, exogenous IBA has been shown to induce an increase in endogenous IBA and is partially converted into free IAA (van der Krieken *et al*., 1993). The observation that, in the infected cuttings treated with exogenous IBA, the meristemoids are distributed along an extensive part of the stem supports the hypothesis that IBA treatment increases the population of cells with the endogenous auxin content necessary for rooting. In apple, stem segments close to the apical bud of the cutting have a lower rooting capacity than the basal segments; however, increasing exogenous IBA from 0.1 to 1 μM also increases the rooting response in the most apical segments (De Klerk & Ter Brugge, 1992).

Transformation, more than the presence of bacteria, seems to be involved in enhancing rhizogenesis

It is not clear which aspect of infection (presence of bacteria or transformation) triggers rhizogenesis in walnut cuttings. It cannot be excluded that, as a reaction to bacterial infection, cells that die after being colonized by the bacteria, or some living cells close to them, might produce diffusible signals for other, healthy cells, which become capable of initiating the rooting process. Differentiation might, in fact, be activated by plant tissues in response to pathogenic stress (e.g. in potato tuber) (Altamura *et al*., 1994b). However, the results of the present study suggest that, more than the presence of bacteria, the transformation of the plant genome with the T-DNA of the root-inducing plasmid of *A. rhizogenes* plays the pivotal role in triggering rhizogenesis and, in cooperation with the signal coming from the exogenous IBA, in enhancing this process. It is well known that the T-DNA genes are transcribed in the plant cell by RNA polymerase II, and that some of them, the *rol* genes of the TL-DNA, once inserted into the plant genome, are able to induce rhizogenesis. In particular, *rolB* gene alone is able to trigger root formation, acting as a root meristem-inducing gene in various systems cultured *in vitro* (e.g. leaf minidisks and macroexplants, and thin cell layers of tobacco) (Altamura *et al*., 1994a; Bellincampi *et al*., 1996).

A number of mock infections using empty vectors (e.g. BIN19) or defective bacteria (such as those defective for, or deprived of, the *vir* region) are in progress to discriminate between the effects of transformation and bacterial presence in enhancing the rooting response of walnut.

Exogenous IBA enhances the formation of nontransformed roots in the infected explants

An important result of this study is that exogenous IBA enhances the formation of nontransformed roots in the infected cuttings and that roots containing bacteria more frequently occur in the infected cuttings of the HF treatment. Considering that agrobacteria are more numerous on the base of the cutting, the high number of roots containing bacteria in the HF treatment might simply be a consequence of the high density of root meristemoids at the stem base only. This might have facilitated the contact between the developing primordia and the bacteria, thus explaining bacterial presence in the developed roots. The presence of bacteria does not exclude that the *de novo* formed roots are also transgenic, as shown by the PCR analysis on longitudinally bisected roots, which were in part transgenic and in part colonized by bacteria. The high number of normal roots obtained in the presence of IBA might be due to the fact that by increasing callus formation, the hormone might have increased the number of potentially rhizogenic cells and might have caused an increase in auxin content sufficient to activate them for rhizogenesis.

The bacteria show an intra and intercellular localization in the cutting ; they seem to be trapped in the vessels, and their migration is impeded by polyphenol deposition

The fate of *A. rhizogenes* in inoculated tissues is still a controversial issue, especially in terms of the possible intracellular localization of the bacteria in the host. The ultrastructural analysis of the present paper shows that *A. rhizogenes* is present in the cutting throughout the culture period and that the bacterial foci are mainly located in the conducting elements of the xylem. In tobacco, with observations under SEM, *A. tumefaciens* has been reported to form microcolonies in the vascular tissue of *in vitro*

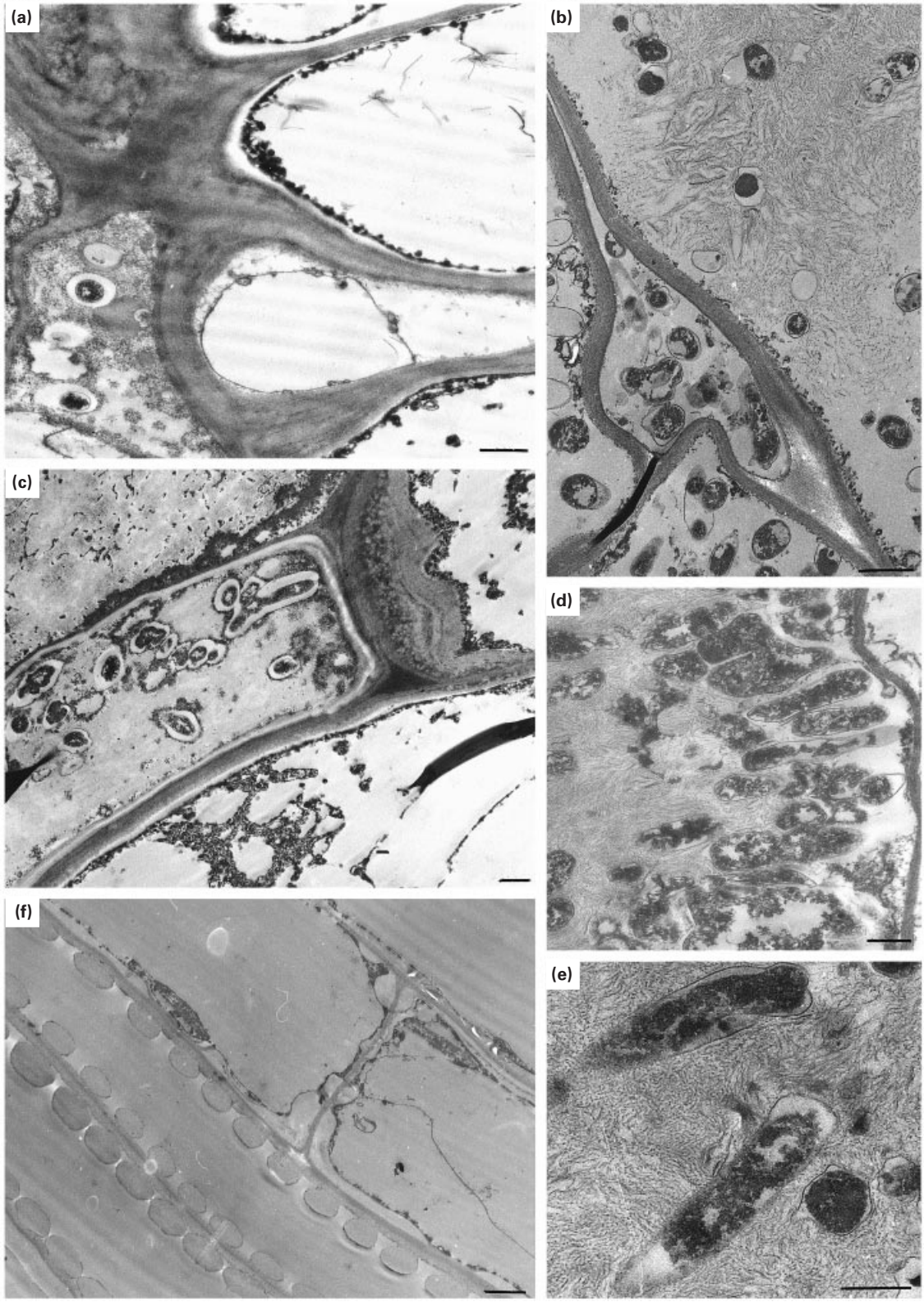

Fig. 5. Presence of *Agrobacterium rhizogenes* in the xylem parenchyma (a, c) and in the cortical parenchyma (b, d, e) of the infected cuttings after 10 d of culture on the rooting media. (Thin sections observed under TEM (a–b, transections; c–e, longitudinal sections; HF, hormone-free; IBA, indolebutyric acid; Ar, infection with *Agrobacterium rhizogenes*; bars = 1 μm, a, b, c, and d; 1.5 μm, f; and 0.5 μm, e). (a) Bacteria in a xylem parenchyma cell (left corner) near the vessels (IBA Ar-treated cutting). (b) Irregularly shaped bacteria located in a degenerating cortical cell and in the confining intercellular space (IBA Ar-treated cutting). (c) Bacteria apparently incorporated in a xylem parenchyma cell through plasmalemma invagination (IAA Ar-treated cutting). (d) Detail of a cortical cell filled with bacteria immersed in a fibrillar material (HF Ar-treated cutting). (e) Higher magnification of d, showing details of the fibrillar material including the bacteria. (f) Detail of the xylem of a noninfected cutting (IBA treatment).

shoot cultures months after the transformation (Matzk *et al*., 1996). In a rare study with TEM, in which the interaction of *A. tumefaciens* and *A. radiobacter* with *Kalanchoë daigremontiana* was investigated, bacteria were observed in damaged cells of the cortex and the pith, and in the vessels; however, some bacteria were also found in the intercellular spaces (Bogers, 1972). Similar results were obtained in the present study: *A. rhizogenes* is present in the intercellular spaces, in the vessels (mainly), and in cortical and xylem parenchyma cells. The bacteria seem to produce 'fibrillar material' near the pit cavities, probably for facilitating their migration to the still noninfected vessels. In fact, the ultrastructure of this material resembles the adhesion fibrillar material, composed of polysaccharides, produced in the host cells by other bacteria during various pathogeneses (Mount & Lacy, 1982; Brown & Mansfield, 1991). Our ultrastructural analysis also shows the presence of a different type of fibrillar material in the colonized parenchyma cells. This material seems to be produced by the host cell and could be interpreted as a protective reaction of the host. The presence of two kinds of fibrillar material, produced by the bacterium and the host, has been ultrastructurally demonstrated also during the interaction between *Pseudomonas syringae* and *Phaseolus vulgaris* (Brown & Mansfield, 1991), and during the leaf blight disease incited by *Xanthomonas oryzae* in rice cells (Horino, 1976).

Furthermore, our investigations demonstrate that when the bacteria are present in the vessels, they are preferentially located in those showing deposition of polyphenolic compounds. The deposition of phenolic inclusions has also been observed during the IBA-induced *de novo* formation of roots from walnut cotyledonary fragments (Gutmann *et al*., 1996). This result, together with our results on the presence of polyphenols also in the noninfected cuttings, lead to the hypothesis that walnut cells produce phenolic metabolities in culture as a potential protective barrier in response to wounding *per se*.

The cells that show secretory activity are in contact with the vessels. Ultrastructural analyses reported in the literature show that wounding is able to trigger secretory activity in the cells in contact with the vessels (e.g. in carnation plants), and it has been suggested that these parenchyma cells are programmed to become capable of rapidly switching on secretory activity when faced with vascular stress (Catesson & Moreau, 1985). The increase in polyphenol deposition in the vessels of walnut after infection with *A. rhizogenes* might be explained as an additional response of the microcutting to the pathogenic stress. In carnation plants infected with *Phiaolophora cinerescens*, a similar increase in secretory activity in the cells in contact with the vessels has been observed (Catesson & Moreau, 1985).

The promotive role of IAA on xylem formation might explain the failure of an IAA-induced rooting response

Though IAA is undoubtedly the major hormonal stimulant for both phloem and xylem formation (Roberts, 1988), its determining role for xylem differentiation in particular has been reported (Klee & Lanahan, 1995). IAA is also known to be a possible inducer of the rooting response; however, at least in walnut microcuttings, the hormone has an important effect on the differentiation of tracheary elements and not on rooting. It is possible that IAA triggers the sensitivity of the potential provascular cells of the cutting before activating the sensitivity of potential pre-rhizogenic cells, thus irreversibly conditioning the explant towards the 'xylogenic response'. As in animals, it is possible that the cells become canalized towards a specific developmental pathway, becoming incapable of embarking upon others. However, there exist differences between the 'xylogenic' response activated by the noninfected cuttings and that activated by the infected cuttings: the former mainly show xylogenesis from a cambium-derived callus, whereas the latter show an increased production of secondary xylem within the stem vascular system. In pea epicotyls and tobacco stems infected with *A. rhizogenes*, the cambial cells are also activated to produce xylem elements inside the initial stele (Bercetche *et al*., 1987). Furthermore, it must be stressed that though IAA is known to promote secondary vascular differentiation (i.e. not only secondary xylem but also secondary phloem in woody microcuttings, e.g. in *Malus domestica* (Auderset *et al*., 1994), in the infected explants of the present study, the promotive role of IAA is specific for secondary xylem formation. Furthermore, the results showed that the infected cuttings treated with IAA not only exhibited the highest secondary xylem formation but also the highest number of vessels with deposition of polyphenols. It is possible that the two events are related and that, in such explants, the interaction between xylem differentiation and polyphenol deposition might also be involved in the failure of the rooting response. There exists evidence that the auxin requirements of the rhizogenic process vary with different phases (i.e. a high level of endogenous auxin is indispensable for root induction, whereas a reduction in the hormone level is necessary in the successive phase of root growth (Moncousin *et al*., 1988)). By contrast, a high level of auxin seems to be necessary for both the induction and maturation of the xylem (Fukuda, 1996). Assuming that polyphenol deposition in walnut microcuttings is stimulated by bacteria (see above), the 'protective' role of such compounds on IAA (Lee *et al*., 1982) might in part explain the prolonged duration of xylem formation.

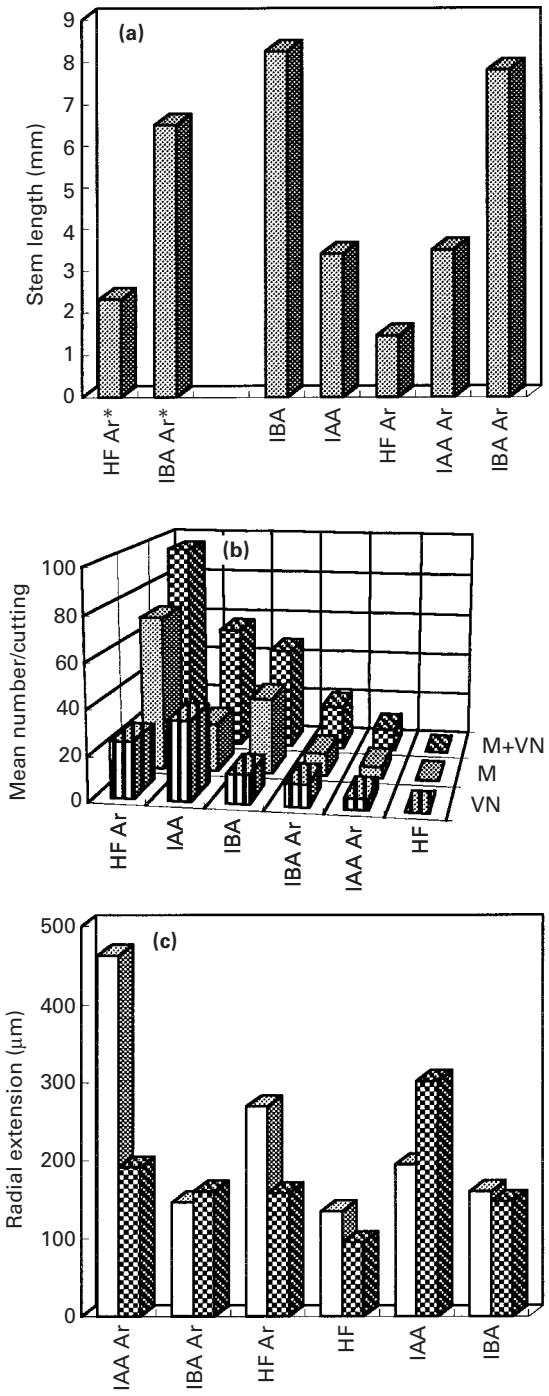

Fig. 6. (a) Mean length of the stem portion of the cutting containing meristemoids and root primordia after 15 d (HF Ar* and IBA Ar*) and 20 d (IBA, IAA, HF Ar, IAA Ar, and IBA Ar) of culture on the rooting media. Standard errors range from 0.2 to 2.1 (n = 5 for each treatment). Measurements began from the basal cut end of the stem (HF, hormone-free medium; IAA, medium with 10 μM of IAA; IBA, medium with 10 μM of indolebutyric acid; Ar, presence of infection with *Agrobacterium rhizogenes*). (b) Mean number of meristemoids (M), vascular nodules (VN), and combined meristemoids and vascular nodules (M+VN) present in the cuttings of each treatment at day 20. Standard errors range from 0.5 to 4.1 (n = 5 for each treatment; HF, hormone-free medium; IAA, medium with 10 μM of IAA; IBA, medium with 10 μM of indolebutyric acid; Ar, presence of infection with *Agrobacterium rhizogenes*). (c) Mean value of the radial extension (in transection) of the secondary xylem (open bars) and of the xylogenic callus of cambial origin (chequered bars) in the cuttings at day 20. Standard errors range from 2 to 10 (n = 5 for each treatment). (HF, hormone-free medium; IAA, medium with 10 μM of IAA; IBA, medium with 10 μM of indolebutyric acid; Ar, presence of infection with *Agrobacterium rhizogenes*).

The pluricellular origin of both the direct and the indirect root meristemoids explains root chimerism

Preliminary PCR analyses of the callus from which the roots protrude show that it might be negative to *rolB* and *virD1* and that it can produce transgenic and bacterium-containing roots next to normal roots. Thus, callus seems to be a chimera, comprising a very large population of nontransformed cells and a few highly localized foci of transgenic or bacterium-containing cells. Furthermore, based on the low number of transgenic roots obtained and the observation that roots are of direct and indirect origin, it cannot be excluded that, also in the primary explant, the number of cells that become transformed is very low compared with that of the cells that remain nontransformed.

The histological analysis shows the pluricellular origin (two to three cells) of the roots of both direct and indirect genesis. This result explains the presence of chimeric roots. Since PCR analysis was carried out on longitudinally bisected roots, those positive to *rolB* in one half only and nontransformed in the other half have to be considered as sectorial or mericlinal chimeras for transformation.

Flax plants, regenerated after infection of hypocotyl cuttings by *A. tumefaciens*, were often chimeras for GUS expression, and this has been shown to be associated with their multicellular origin (Dong & McHughen, 1993). Shoots which were chimeras for GUS expression have also been reported for woody species (e.g. in apple after culture *in vitro* of leaves infected with *A. tumefaciens* – Yao *et al.*, 1995).

However, considering the problem of evaluating the incidence of chimerism after transformation, PCR analysis, which was used for this purpose for the first time in the present paper, provides more convincing evidence than the GUS method, owing to the well known problems of substrate penetration into the tissues and the instability of GUS protein in the transformed cells (De Block & Debrouer, 1992).

In conclusion, infection with *A. rhizogenes* stimulates rhizogenesis in a recalcitrant walnut, positively affecting root meristemoid formation. The rooting response of the infected cuttings is enhanced by exogenous IBA, which accelerates and increases root meristemoids, and induces the preferential production of nontransformed roots. Chimeric roots and roots containing bacteria are also produced by the infected cuttings, as a consequence of the persistence of bacteria in the stem during the culture

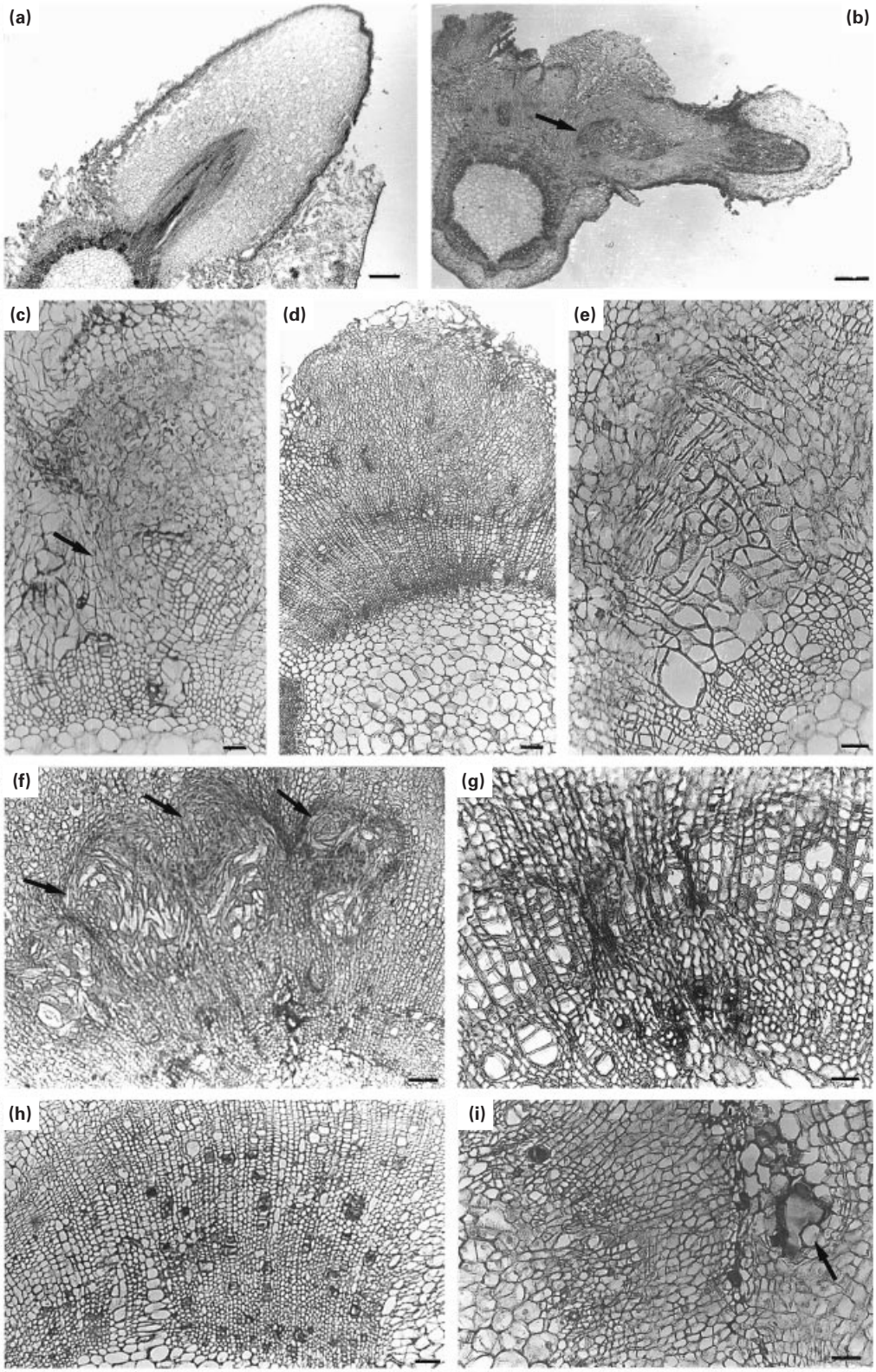

Fig. 7. Histological events in walnut microcuttings during the second half of culture on rooting media. Transections were stained with eosin and Carazzi's haemalum (HF, hormone-free; IBA, indolebutyric acid; Ar, infection with *Agrobacterium rhizogenes*; bars = 200 μm, a,b; 100 μm, d,f, and h; 60 μm, c, and 50 μm, e and g, and i; a–h, day 20; i, day 30). (a) Root primordium of direct origin (IBA Ar treatment). (b) Root primordium of indirect origin, whose vascular system (arrow) is *de novo* formed in the cortical callus (IBA Ar treatment). (c) Procambium-like strand (arrow) joining a large meristemoid of indirect origin to the vascular system of the cutting. (d) Cortex of the cutting strongly metamorphosed by conspicuous callus of cambial origin (IAA treatment). (e) Detail of a callus of cambial origin containing strands of *de novo* formed, and irregularly shaped, vascular elements (xylogenesis) (IAA treatment). (f) Callus of cambial origin containing *de novo* formed vascular nodules (arrows) (IAA-treated cutting). (g) Anomalous shape of the secondary xylem (IAA treatment). (h) Vessels showing polyphenol deposition in the entire radial extension of the xylem in an IAA Ar-treated cutting. (i) Secretory cavity (arrow) in a poorly developed cortical callus (HF Ar-treated cutting).

period, of the low occurrence of insertion of the T-DNA into the plant genome, and of the pluricellular origin of both the direct and indirect roots.

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