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Explant Orientation and Polarity Determine the Morphogenic Response of Epicotyl Segments of Troyer Citrange

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The morphogenic pathway of adventitious bud and shoot regeneration at the ends of Troyer citrange epicotyl cuttings is determined by polarity and explant orientation. In explants planted vertically with the basal end inserted in the medium, bud formation at the apical end occurs by direct organogenesis. Bud growth and subsequent shoot formation is markedly increased by the addition of 6-benzyladenine (BA) to the medium. This growth regulator also increases the number of buds formed. When they come into contact with the culture medium, both the apical end and the basal end of the cuttings form a vigorous callus with many xyllary elements, more numerous in the calli from the basal end. In these calli, buds differentiate by a process of indirect organogenesis. This indirect regeneration pathway requires the addition of 6-benzyladenine to the medium, and the number of buds formed is higher at the apical end than at the basal end of the cuttings. This pathway of regeneration is reduced as the position of the cuttings during incubation deviates from the normal upright vertical position. Thus, for the basal end of the cuttings, the number of buds and shoots formed is higher when the explants are placed vertically than when they lie on the surface of the medium. For the apical end, this number is higher in explants placed horizontally than when inserted vertically in the medium in an inverted position.

Key words: Troyer citrange, *Citrus sinensis x Poncirus trifoliata*, explant orientation, histology, hormone dependence, morphogenesis, organogenesis, polarity, xylogenesis.

INTRODUCTION

Adventive nucellar embryony is a trait widespread among all citrus fruit tree genera. This constitutes a horticultural advantage since it results in seedling populations genetically identical to the maternal parent, thus providing an efficient system for multiplication of rootstocks, but it also constitutes a difficulty in the utilization of genetic diversity in sexual hybridization (Gmitter, Grosser and Moore, 1992). One option to overcome this difficulty is the genetic transformation of cells followed by the regeneration of the plant via organogenesis by means of plant tissue culture. The first step in this approach is the establishment of an efficient *in vitro* plant regeneration system.

Epicotyl and internodal stem cuttings have been used successfully for the *Agrobacterium*-mediated transformation of *Citrus*, *Poncirus* and *Citrus* x *Poncirus* hybrids (Moore *et al*., 1992; Kaneyoshi *et al*., 1994; Peña *et al*., 1995*a*, *b*). *In vitro* plant regeneration from epicotyl and internodal stem cuttings has been achieved for several citrus fruit trees (Grinblat, 1972; Chatuverdi and Mitra, 1974; Raj and Arya, 1978; Barlass and Skene, 1982; Edriss and Burger, 1984; Burger and Hackett, 1986; Moore, 1986; Durán Vila, Ortega and Navarro, 1989; Goh *et al*., 1995; Maggon and Singh, 1995; Perez-Molphe-Balch and Ochoa-Alejo, 1997; Ghorbel, Navarro and Durán-Vila, 1998). However, the proportion of regenerating explants is sometimes low, and the process of shoot development has not been thoroughly characterized. Shoot formation has been reported as a result of direct morphogenesis (Moore *et al*., 1992; Goh *et al*., 1995; Perez-Molphe-Balch and Ochoa-Alejo, 1997) or from regeneration from callus tissue formed from the primary explants (Grinblat, 1972; Chatuverdi and Mitra, 1974; Barlass and Skene, 1982; Edriss and Burger, 1984; Moore, 1986). In some publications the origin of these shoots is not reported. In the present report we show that shoot regeneration in Troyer citrange epicotyl cuttings may take place through different morphogenic pathways depending on explant position on the regeneration medium. These morphogenic pathways differ in hormone requirements and are affected differently by the ontogenic age of the explant.

MATERIALS AND METHODS

Plant material

In vitro germinated seedlings of Troyer citrange (*Citrus sinensis* [L.] Osbeck x *Poncirus trifoliata* L. Raf.) were used as tissue sources. Seeds were disinfected for 30 min in a 1·5% (v/v) solution of sodium hypochlorite, then rinsed three times with sterile distilled water and the seed coats removed under sterile conditions. The naked sterilized seeds were disinfected for 15 min in a 0·5% (v/v) solution of sodium hypochlorite, rinsed three times with sterile distilled water, and germinated in the dark at 26 ± 2 °C in 21 ×

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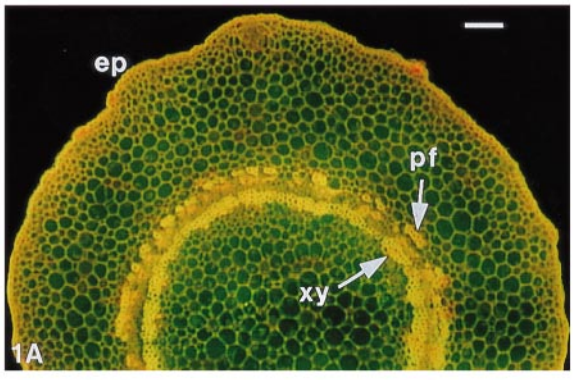
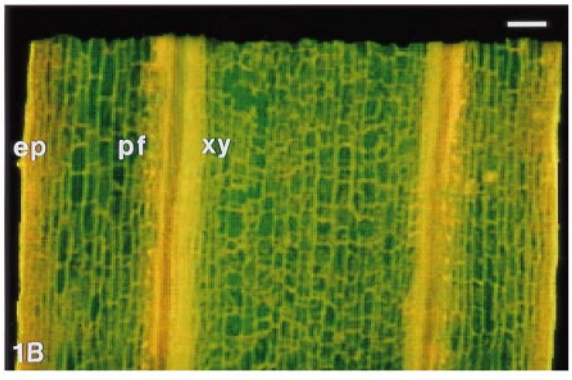
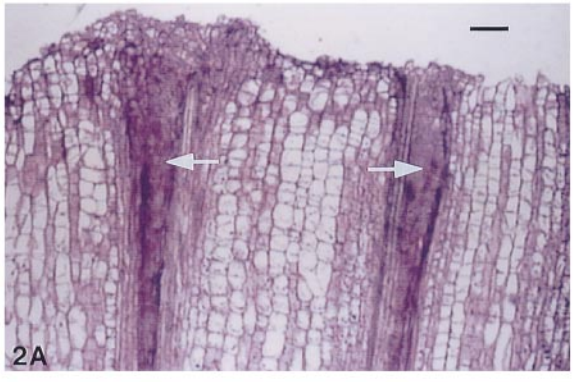
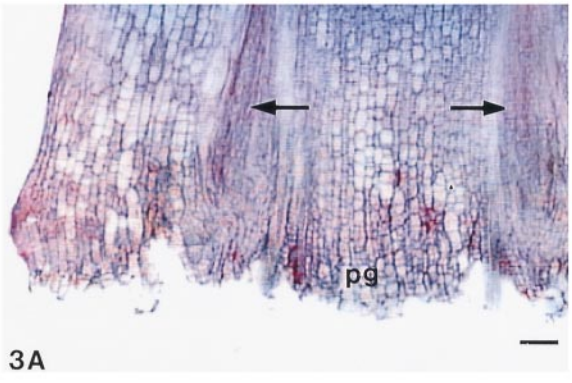
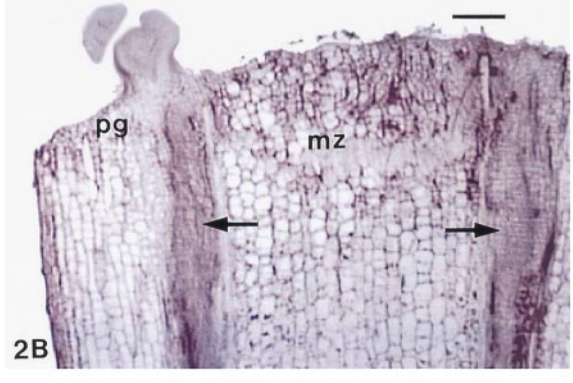
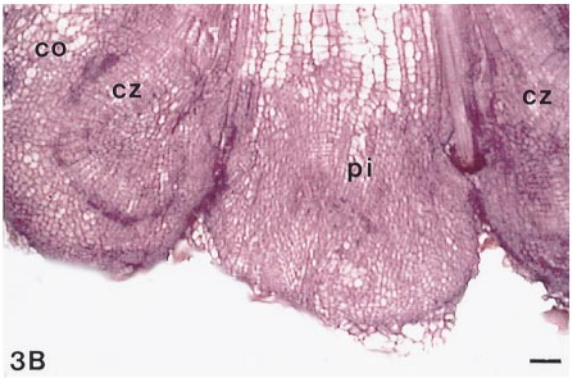


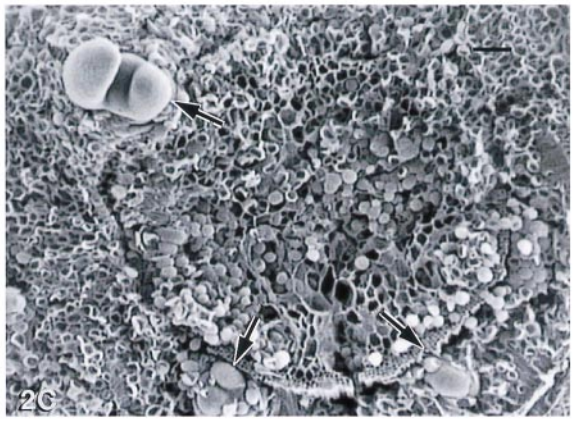












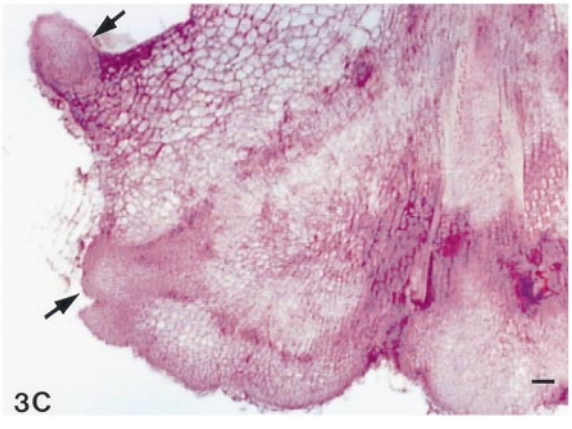


FIG. 1. A, Transverse section of a 45-d-old dark-grown epicotyl at 1 cm from the cotyledonary node. Note the initial formation of vascular tissue from the vascular cambium and the parenchymatous pith and cortex protected by an epidermis. B, Longitudinal section of the cutting at the apical end showing the uniform thickness of the tissues. Fluorescence is caused by the cutin layer at the epidermis (ep), and the lignin of the phloem fibres (pf) and the xylem (xy). Bars = 0·1 mm. FIG. 2. Apical end of epicotyl cuttings incubated in a vertical upright position. Bars = 0·1 mm.

150 mm capped test tubes containing 10 ml of Murashige and Skoog (1962) mineral medium with 30 g l^{-1} sucrose and 8 g l^{-1} agar (Sigma). Epicotyl cuttings (1 cm long) were obtained 45 to 49 d after sowing. At this time epicotyls were 5 to 9 cm long, hence five cuttings were obtained from each seedling. These cuttings were numbered according to the distance from the cotyledonary node (number 1 corresponding to the cutting nearest to this node and number 5 to that furthest from it) and used as explants. At this stage, the epicotyl was 1·5 to 2·0 mm thick and had a primary structure with an intact epidermis. In transverse sections (Fig. 1), the phloem and xylem appeared as continuous rings separated by a vascular cambium. Some periclinal divisions in the cambium could be seen in older cuttings.

Culture conditions

The culture medium contained the inorganic salts of Murashige and Skoog (1962) supplemented with 100 mg l^{-1} myo-inositol, 10 mg l^{-1} thiamine-HCl, 10 mg l^{-1} pyridoxine, 1 mg l^{-1} nicotinic acid and 30 g l^{-1} sucrose. The pH of the medium was adjusted to 5·8 before the addition of 8 g l^{-1} agar (Sigma, microbiologically tested), and the mixture was then autoclaved at 105 kPa for 30 min. Filter sterilized 6-benzyladenine and 1-naphthaleneacetic acid were added after autoclaving at a final concentration of 4·4 μM and 0·54 μM, respectively. Previous trials proved these concentrations were optimal for shoot regeneration at the two ends of the explants (data not presented). The medium was dispensed as 50 ml aliquots into 84 × 73 mm culture jars capped with polypropylene caps. Ten explants were planted in each culture jar. The explants were either (*a*) placed horizontally on the medium surface; (*b*) placed vertically in the medium in an upright position, with the basal end of the cutting inserted 2–3 mm deep and the 7–8 mm apical end protruding; or (*c*) placed vertically in the medium in an inverted position, with the apical end of the cutting inserted 2–3 mm deep and the 7–8 mm basal end protruding.

All cultures were incubated for 45 d under a 16 h daylength, with an irradiance of 56 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (L36W/21–840, Osram), at a temperature of 26 ± 2 °C. At the end of the incubation period the number of adventitious shoots was recorded and the non-growing adventitious buds counted under a dissecting microscope. The sum of these two figures is the number of buds formed. Callus size was estimated by the area of its projection on the medium. Fifty cuttings from ten seedlings were measured per treatment.

Histological examination

Explants were sampled at various intervals after culture initiation and fixed in FPA (formalin: propionic acid: ethanol; 1:1:18) for 48 h, dehydrated in a graded t-butanol series and embedded in paraffin wax. Sections (15 μm thick) were stained either with 0·1 % (w/v) Aniline Blue (Baker grade) in 0·1 M Na_2HPO_4, Schiff's reagent or Carmin Iodine Green. Where appropriate, fluorescence was observed using an excitation band of 450–490 nm on a Nikon Optimat microscope.

Explants for scanning electron microscopy were dehydrated in a graded series of ethanol, critical point dried, sputter coated with gold, and observed with a JSM 6300 Jeol scanning electron microscope operated at 15 kV.

RESULTS

Regeneration in cuttings inserted vertically in an upright position

All the cuttings inserted vertically in the medium in an upright position formed adventitious buds and shoots at both ends (Table 1). There were, however, marked differences between the two ends in the extent of cell proliferation and in the process of morphogenesis.

In the apical end, cell division was very intense during the first 5 d after planting. At the cut surface a phellogen differentiated (Fig. 2A). This phellogen formed a suberized layer two-four cells thick and a few rows of parenchyma cells beneath (Fig. 2B). The vascular cambium divided periclinally up to a depth of 2 mm from the cut surface, forming a ring of non-vacuolated small-sized meristematic cells between the phloem and the xylem. The frequency of these cell divisions was highest near the cut, resulting in a characteristic thickening of the cutting at the apex. Most of these cell divisions occurred during the first 5 d after planting (Fig. 2A), and the size of this tissue did not increase significantly thereafter (Fig. 2B). Observation of the explants using fluorescence revealed the presence of a few scattered tracheary elements in this tissue. No callose was detected in these observations.

Adventitious buds formed directly onto the cambial zone near to the cut surface (Fig. 2B). Most of these buds differentiated within the first 15 d of incubation. A variable proportion of these buds grew to form shoots up to 16 mm long after 45 d of incubation. As the buds increased in size they expanded on the tissue formed from the periclinal divisions in the vascular cambium, and the growing shoots were located in a ring on this tissue (Fig. 2C).

The initial stages of cell proliferation in the basal end of the cuttings were similar to those described for the apical end, resulting from the activity of a phellogen at the cutting and periclinal cell divisions of the vascular cambium (Fig. 3A). From the beginning, the rate of cell division was higher in the basal end than in the apical end, and also it lasted for

A, A ring of meristematic cells, derived from cambial activity, is present (arrows) after 5 d of incubation. B, Explant after 14 d of incubation. The cut surface is protected by a suberized cell layer derived from a phellogen (pg). No further growth has occurred in the cambial zone (arrows). A meristematic zone (mz) has formed parallel to the cut surface, some 0·2 mm deep, but the rate of cell proliferation is very low. A well developed bud has started to sprout. C, Scanning electron micrograph after 10 d of incubation. Three buds (arrows) have formed in the cambial zone. FIG. 3. Basal end of epicotyl cuttings incubated in a vertical upright position. Bars = 0·1 mm. A, After 5 d of incubation. A ring of meristematic cells derived from cambial activity is present (arrows). The phellogen (pg) has formed a suberized cell layer. B, After 14 d of incubation. The callus at the edge of the cutting derives both from the continued cell divisions in the cambial zone (cz) and the meristematic activity beneath the edge of the cutting, both in the pith (pi) and the cortex (co). C, After 14 d of incubation showing bud formation on the surface of the callus (arrows).

TABLE 1. *Influence of explant position in the medium on callus formation and adventitious bud and shoot formation in the apical and basal ends of epicotyl cuttings*

Explant position and cutting end	Explants with adventitious buds (%)	Adventitious buds per explant (no. ± s.e.)	Adventitious shoots per explant (no. ± s.e.)	Callus size (mm^2)
Vertical upright				
Apical end	100	6·7 ± 0·5	4·6 ± 0·3	—
Basal end	100	11·0 ± 0·6	6·7 ± 0·4	63 ± 2
Horizontal				
Apical end	100	20·3 ± 0·5	10·0 ± 0·3	34 ± 1
Basal end	100	9·3 ± 0·6	4·3 ± 0·4	32 ± 2
Vertical inverted				
Apical end	100	11 ± 1	5·2 ± 0·6	55 ± 5
Basal end	58	1·0 ± 0·01	0·07 ± 0·005	—

—, No unorganized callus was visible to the naked eye.
Data are the means of 50 explants ± s.e. The number of buds and shoots per explant was calculated by dividing the total number of buds/shoots by the number of explants with buds.

a longer period. This resulted in the formation of a hard, non-friable, green callus made mostly by parenchymatous cells with numerous tracheary elements (Fig. 3B). These tracheary elements were most frequent in the portions of callus arising from the activity of the vascular cambium. No callose could be detected in the callus tissue.

After 9 d of incubation, cell clusters were distinguishable within the callus, mostly in the proximity of the phellogen. The cells of these clusters were small, having a prominent nucleus and a dense cytoplasm. Globular structures formed in the surface of the callus from these clusters (Fig. 3C). These structures, that become young buds and eventually shoots, clearly originated from the callus. In contrast to the apical end, buds in the basal end seemed randomly scattered on the callus surface. This callus overgrew the primary explant and completely covered the basal end of the cutting (Fig. 4A).

Regeneration in explants planted vertically in an inverted position

The basal end of the explants inserted vertically in the medium in an inverted position formed a small hemispheric callus with a green smooth surface (Fig. 4B). This callus arose mainly from mitotic activity of the vascular cambium at its inner side and was heavily lignified, with numerous xyllary elements (Fig. 5). The phellogen which developed at the cut formed a layer of 2–3 suberized cells but no parenchymatous cells. In some of these explants, one (rarely two) adventitious bud formed at the edge of the callus. These buds did not grow to form shoots (Table 1).

The apical end of these explants, in contact with the medium, formed a vigorous callus as a result of mitotic activity in the vascular cambium and in meristematic zones (mz) beneath the cut edge, both in the pith (pi) and the cortex (Fig. 6). Many xyllary elements formed in the tissue derived from the vascular cambium. In the surface of this callus, meristematic nodules that become adventitious buds and shoots were formed. Their number was, however, significantly smaller than in the apical end of explants incubated horizontally on the medium surface (Table 1). Callus formation and bud differentiation basically followed the pattern described above for the basal end of the cuttings inserted vertically in the medium in a normal orientation.

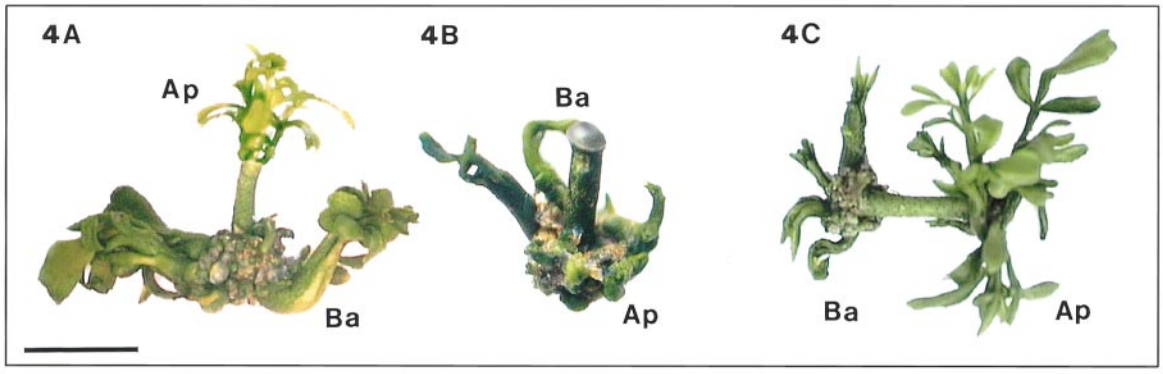
Regeneration in cuttings placed horizontally on the medium surface

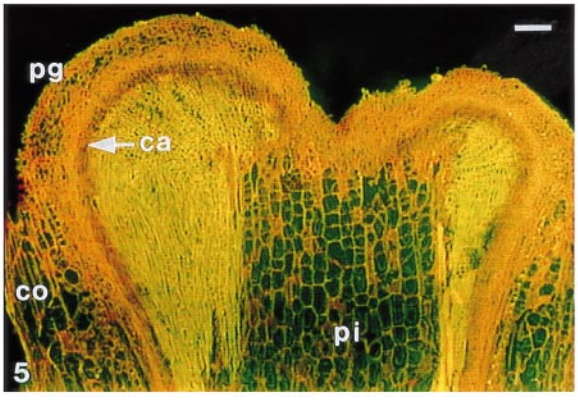
Adventitious buds and shoots formed from both cut ends in all the cuttings placed horizontally on the medium surface (Table 1). The process of regeneration was similar in both ends, and followed the pattern described above for the basal end of explants inserted vertically in the medium. A callus formed in both cut ends arising both from periclinal divisions of the vascular cambium and from the activity of a phellogen, which differentiated at the cut surface (Figs 7A and 8A). This phellogen formed a layer several cells thick of suberized tissue on the surface and parenchymatous cells beneath it (Fig. 7D). The size of these calli increased with the duration of incubation (Figs 7B and 8B). Xyllary elements differentiated mostly in the callus tissue derived from the vascular cambium (Figs 7B and 8B). These xyllary elements were much more frequent in the basal end of the cuttings. In some explants, this portion of the callus tissue at the basal end was formed almost exclusively by clusters of xyllary elements (Fig. 8D). No callose was detectable in these calli using the highly sensitive fluorescence observation in Aniline Blue stained sections. Subsequently, the tissue formed from the vascular cambium become heavily lignified. This lignification was more intense in the basal end (Fig. 8C) than in the apical end (Fig. 7C) of the cuttings.

As described above for the basal end of explants inserted vertically, these calli formed meristematic nodules that became young buds and eventually developed into shoots. These structures were more abundant in the apical end than in the basal end of the cuttings (Table 1; Fig. 4C). The number of adventitious buds and shoots in the basal end was smaller in explants placed horizontally on the medium than in those planted vertically (Table 1).

Hormone requirements for bud and shoot formation

Bud formation in the apical end of explants planted vertically in an upright position was slightly, but signifi-





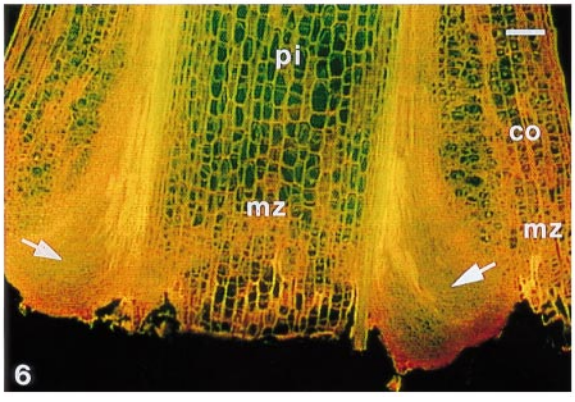


FIG. 4. Morphological aspect of the explants after 45 d of incubation. The length of the cutting was 10 mm. Bar = 10 mm. A, Explant incubated vertically in an upright position. The callus at the basal end (Ba) overgrows several-fold the initial diameter of the cutting. No callus formed at the apical end (Ap). B, Explant incubated vertically in an inverted position. A vigorous callus with buds and shoots is present in the apical end (Ap). In the basal end (Ba) there is a small hemispheric hard callus with a smooth surface. C, Explant incubated horizontally. Calli with buds and shoots formed at both ends. Both shoot number and size are larger at the apical end (Ap). FIG. 5. Basal end of an explant incubated vertically in an inverted position after 10 d of incubation. Most of the tissue derives from the activity of the vascular cambium (ca). The fluorescence reveals the intense lignification of these cells. No significant cell proliferation occurred in the cortex (co) nor in the pith (pi). At the cut surface a phellogen (pg) has formed a suberized cell layer. FIG. 6. Apical end of an explant incubated vertically in an inverted position after 10 d of incubation. The portions of the callus derived from the vascular cambium (arrows) show some lignification as revealed by its fluorescence. A meristematic zone (mz) has formed in the pith (pi) and the cortex (co) parallel to the cut edge.

cantly, reduced when growth regulators were omitted from the incubation medium (Table 2). The addition of hormones was more critical for subsequent bud growth, and in their absence very few buds grew to become shoots (Table 2). Additional trials showed that this hormone effect was due solely to the addition of 6-benzyladenine (data not presented).

When the explants were placed horizontally on the culture medium, no callus formed at the apical end in the absence of added hormones. Very few buds were visible under the dissecting microscope, and most of them remained quiescent without becoming a shoot (Table 2). The main hormone effect was due to the addition of 6-benzyladenine, with auxin having only a marginal effect (data not presented).

Hormone application was critical for callus formation and bud differentiation in the basal end of cuttings, irrespective of the position of the explants in the incubation medium (vertical upright or horizontal). Buds were not visible and shoots did not develop from this end when hormones were omitted from the medium (data not shown).

Gradients in bud and shoot regeneration

In the explants planted vertically in an upright position, both the number of buds which differentiated and the number of shoots which developed in the apical end decreased with the distance of the cutting from the cotyledonary node (Fig. 9). In many experiments the number of organs formed was linearly and inversely related to the distance from the cotyledons ($r^2 \geqslant 0{\cdot}9$), but in some cases there was a marked reduction in regeneration from cutting 1 to cutting 2, followed by a smaller, albeit significant, reduction as the distance from the node increased.

No consistent trend was found for the effect of distance of the cutting from the cotyledonary node on bud and shoot formation in the basal end of these explants, with the distance to the node accounting for less than 1 % of the total variation in the number of organs formed. However, in some experiments, regeneration was maximal in cuttings 2–4, with a significant reduction in cuttings 1 and 5 (Fig. 9).

In the explants planted in a horizontal position, the distance of the cutting from the node had no effect on organ

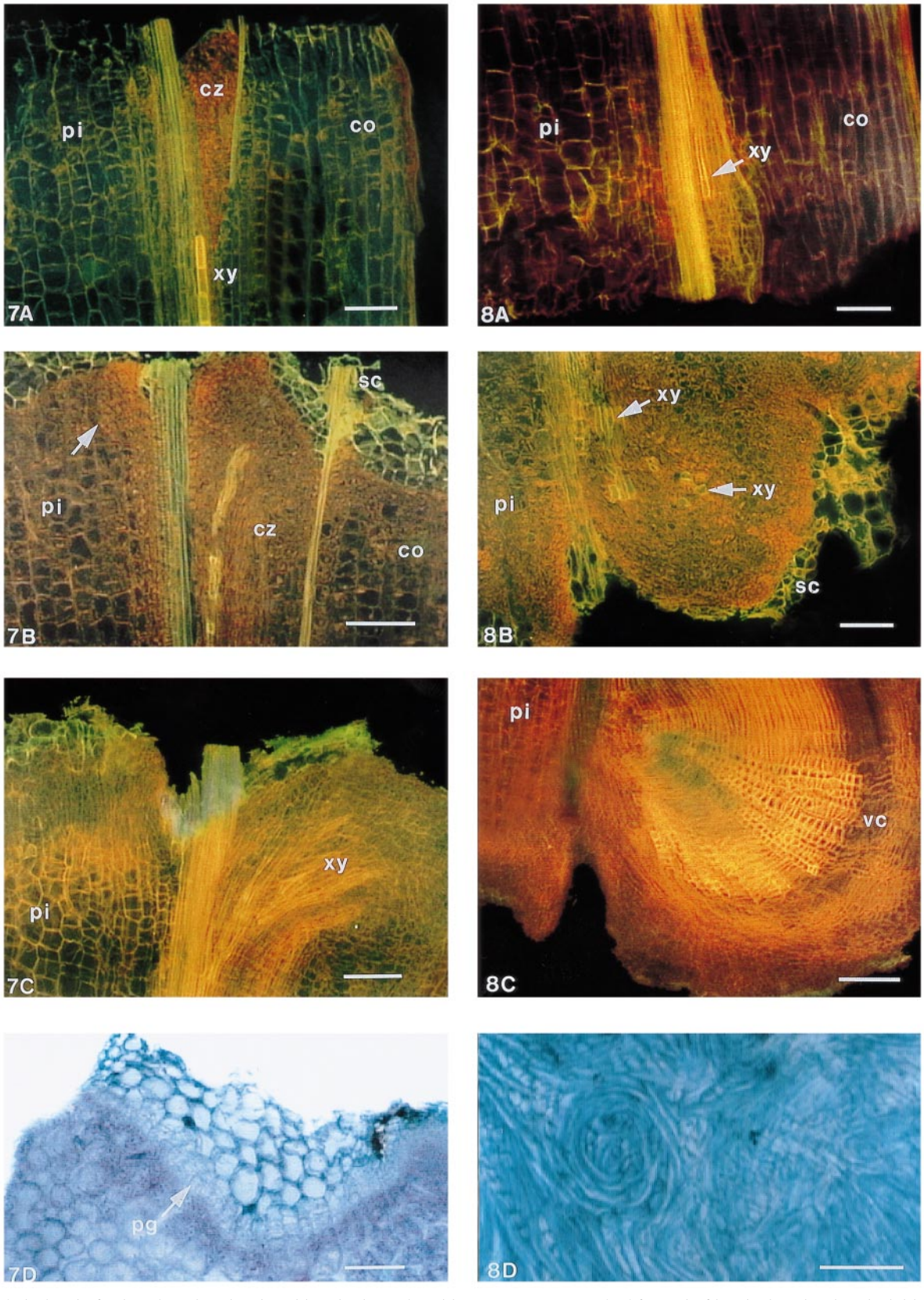


FIG. 7. Apical end of epicotyl cuttings incubated in a horizontal position. Bars = 0·1 mm. A, After 5 d of incubation showing the initial callus formation by the divisions in the vascular cambium. Xylem (xy) formation is revealed by its fluorescence. B, After 11 d of incubation, showing the continuous cell proliferation from the vascular cambium. Cell division has also started in the pith (arrow). The callus is protected by suberized cells (sc). C, After 13 d of incubation showing the partial lignification of the tissue derived from the activity of the vascular cambium and the

TABLE 2. *The influence of the addition of hormones to the culture medium on bud and shoot formation in the apical end of the cuttings*

Explant position and medium	Explants with adventitious buds (%)	Adventitious buds per explant (no. ± s.e.)	Adventitious shoots per explant (no. ± s.e.)	Callus size (mm²)
Vertical upright				
+Hormones	100	6·7 ± 0·5	4·6 ± 0·3	—
−Hormones	92	4·1 ± 0·5	1·4 ± 0·3	—
Horizontal				
+Hormones	100	20·3 ± 0·5	10·0 ± 0·3	34 ± 1
−Hormones	46	1·4 ± 0·5	0·3 ± 0·3	—

No unorganized callus was visible to the naked eye (direct regeneration pathway).

The cuttings were incubated in a complete medium with 4·4 μM 6-benzyladenine and 0·54 μM 1-naphthaleneacetic acid (+Hormones) or in the same medium without added growth regulators (−Hormones). Data are the means of 50 explants ± s.e.

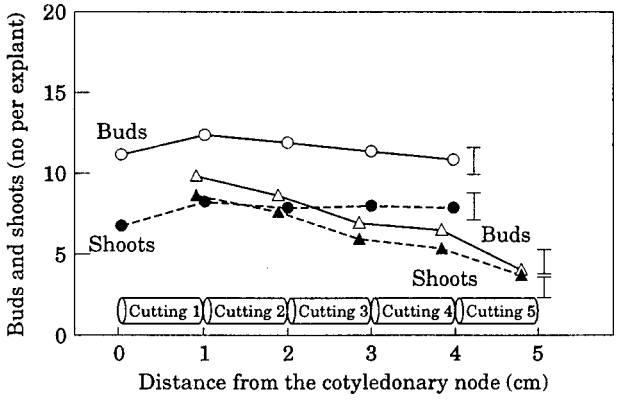


FIG. 9. Influence of the distance of the epicotyl cutting from the cotyledonary node on adventitious bud (○, △) and shoot (●, ▲) formation in the apical end (△, ▲) and the basal end (○, ●) of explants planted vertically in a normal position. Vertical bars represent least significant difference ($P = 0·05$).

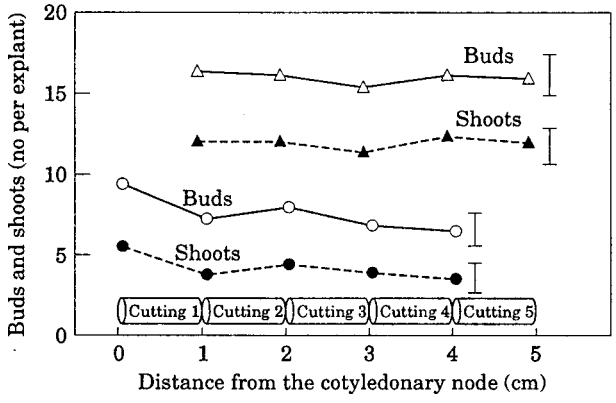


FIG. 10. Influence of the distance of the epicotyl cutting from the cotyledonary node on adventitious bud and shoot formation in explants planted horizontally. Vertical bars represent least significant difference ($P = 0·05$). Symbols as in Fig. 9.

formation except for a significantly higher number of both buds and shoots in the basal end of cutting 1 than in cuttings 2–5 (Fig. 10).

DISCUSSION

Shoot regeneration from epicotyl and internodal stem cuttings has been reported for many *Citrus* species (see Introduction), but in most cases the adventitious shoots develop from a primary callus derived from the explant. Direct shoot organogenesis without callusing has been reported for Carrizo citrange (Moore *et al.*, 1992) and *C. aurantifolia* (Pérez-Molphe-Balch and Ochoa-Alejo, 1997), but other reports state that shoot organogenesis in these genotypes occurs after callus formation (Raj and Arya, 1978; Barlass and Skene, 1982; Edriss and Burger, 1984; Moore, 1986; Durán-Vila *et al.*, 1989). Our research demonstrates that for Troyer citrange the morphogenic pathway at the apical end of the cutting depends on explant position, and that in explants planted vertically in an upright position the shoots arise from the vascular cambium by a process of direct morphogenesis (Fig. 2), while in those explants in which the apical end is in contact with the medium, regeneration is indirect from callus tissue (Fig. 3). This behaviour may explain the conflicting reports on morphogenesis quoted above, since in the experiments of Moore *et al.* (1992) and Perez-Molphe-Balch and Ochoa-Alejo (1997) the explants were planted vertically, while in other studies explants were incubated in a horizontal position. However, this effect of explant position on the morphogenic pathway may not occur in all genotypes. Goh *et al.* (1995) stated that epicotyl explants of *C. grandis* cultured horizontally showed direct shoot regeneration without callusing. There seem to be differences in the response of each species to culture conditions.

formation of xyllary elements (xy). D, Detail of the callus surface after 11 d of incubation showing the phellodermis and the cork cells formed by the phellogen (pg). FIG. 8. Basal end of explants incubated in a horizontal position. Bars = 0·1 mm. A, After 5 d of incubation showing the initial callus formation by the divisions in the vascular cambium. Xylem formation (xy) and lignification are more intense than in the apical end of the explants (Fig. 7A). B, After 11 d of incubation. The tissue derived from vascular cambium has greatly increased in size and shows scattered xyllary elements (xy). The callus surface is protected by suberized cells (sc). C, Lignification of the cells derived from the vascular cambium (vc) in explants incubated for 13 d. D, Clusters of xyllary elements in the tissue derived from the activity of the vascular cambium.

There is no simple explanation for the change in the pathway of morphogenesis when the apical end of the cutting comes into contact with the incubation medium. Possibilities include: (1) the pathway of morphogenesis is determined by hormone levels, which would be higher in tissues of explants incubated horizontally or in the end inserted into the medium, due to facilitated uptake; or (2) the vigorous callus growth in explants placed horizontally competes with direct organogenesis, which may be masked or inhibited by the growth of the callus. Neither of these possibilities, however, is fully supported by the experimental observations. The first possibility is ruled out because added hormones are readily transported to the apical end, as shown by their influence on bud formation and shoot development (Table 2). Further, increasing the amount of hormones in the medium does not induce callus formation in the apical end of explants planted vertically (Moreira-Dias *et al*., unpubl. res.). Competition between callus growth and direct shoot organogenesis (possibility 2) may not be the reason for the reduction in shoot formation through direct morphogenesis in the apical end, as this reduction also occurs in cuttings incubated in a horizontal position in a hormone-free medium, and in this medium callus is not formed (Table 2).

On the other hand, explant orientation seems to interact with polarity to affect shoot regeneration. Both direct and indirect shoot regeneration decrease as the cutting position deviates from the vertical upright position. Thus, direct shoot regeneration in the apical end of cuttings incubated on hormone-free medium is higher in a vertical upright than in a horizontal position (Table 2). Indirect shoot regeneration in the basal end of cuttings is higher in vertical upright explants (with the basal end inserted in the medium) than in horizontally incubated explants, whilst in the apical end it is higher in horizontally incubated explants than in inverted explants (with the apical end inserted in the medium; Table 1). Explant orientation, rather than contact with the medium, seems to determine the number of buds and shoots formed.

A further effect of polarity on regeneration is demonstrated by the higher number of shoots regenerated from the calli in the apical than in the basal end of explants incubated horizontally (Fig. 10). This effect was reported earlier for *C. mitis* (Sim, Goh and Loh, 1989) and Carrizo citrange (Moore *et al*., 1992), but in these reports only one cutting was used from each seedling. Therefore, the effect of polarity was not separated from the influence of the distance of the end of the explants from the cotyledonary node, which also affects morphogenesis (Figs 9 and 10). Our results are clear-cut with respect to this aspect since a cut separated the apical end of cutting (n) from the basal end of cutting ($n+1$). Thus, the apical edge of cutting 1 was less than 0·1 mm from the basal edge of cutting 2 in the epicotyl, and the same applies to the apical and basal edges of every successive pair of cuttings (2–3, 3–4 and 4–5; Figs 9 and 10). Therefore, the distance to the node may not have affected the differences in morphogenesis reported here. This effect of polarity on morphogenesis is shown despite the fact that organogenesis occurred 2–3 weeks after planting on calli which overgrow the explants (Fig. 4). However, from the early stages of growth, calli at either end of the explant differed in the amount of xyllary elements differentiated, being much higher in calli from basal ends (Figs 7B and 8B). This xyllary element differentiation occurred without the prior formation of sieve elements, as judged by the absence of callose in the preparations. This behaviour is different to the normal pattern of differentiation, since phloem differentiation is considered to precede that of the xylem (Aloni, 1995). Also, lignification was more intense in callus from the basal end (Figs 7C and 8C).

The two pathways of regeneration we have characterized differ in hormone requirements and in the influence of the distance of the cutting from the cotyledonary node. Indirect bud and shoot regeneration only occurs in the presence of benzyladenine, which is considered an essential addenda for shoot regeneration from *Citrus* stem explants (Grinblat, 1972; Barlass and Skene, 1982; Edriss and Burger, 1984; Moore, 1986; Sim *et al*., 1989), while direct bud formation occurs in the absence of this hormone which, however, markedly enhances shoot development (Table 2). In agreement with Burger and Hackett (1986) we found that direct shoot regeneration decreased with the distance of the cutting from the cotyledonary node (Fig. 9). In contrast, indirect organogenesis is not markedly influenced by this parameter (Fig. 10).

In conclusion, we have demonstrated that shoot regeneration from Troyer citrange epicotyl explants may be obtained either through direct shoot organogenesis or through indirect organogenesis depending on the conditions of explant incubation. Since the apical end of the cuttings may be transformed when inoculated with *Agrobacterium* (Moore *et al*., 1992), the direct regeneration pathway may be used for plant transformation, thus avoiding the formation of chimeric plants and the genetic variability which is possibly introduced in callus tissue-regenerated plants.

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