Histological characterization of *in vitro* adventitious organogenesis in *Citrus sinensis*

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Abstract

The adventitious bud development was induced in epicotyl segments of Valencia sweet orange (*Citrus sinensis* L. Osbeck). Seeds were cultured *in vitro* for three weeks in the dark, followed by one week at a 16-h photoperiod. Epicotyl segments were cultured horizontally for the induction of organogenesis in Murashige and Tucker (1969, MT) culture medium supplemented with 1.0 mg dm^{-3} benzylaminopurine. Samples were observed by light and scanning electron microscopy from day zero to day 25, when buds were well grown. It was shown that the adventitious buds originated directly from the cambial region on the cut ends of the explants.

Additional key words: adventitious shoots, benzylaminopurine, cambium, direct organogenesis, epicotyl, ontogenesis, Valencia sweet orange.

Introduction

In vitro regeneration in citrus has been obtained for several genotypes, through different *in vitro* techniques, such as somatic embryogenesis from nucellar callus and protoplast cultures (for a review see Gosal *et al*. 1995) and somatic hybrids (for a review see Grosser *et al.* 2000). More recently, efforts have been made to define efficient protocols for the recovery of plants through organogenesis of citrus genotypes (Peña *et al.* 1995, Cervera *et al.* 1998, García-Luis *et al.* 1999, Almeida *et al.* 2003a,b) and related species (Hassanein and Azooz 2003/4, Hiregoudar *et al*. 2005).

In our laboratory, organogenesis has been efficiently obtained from epicotyl segments of Valencia sweet orange (*Citrus sinensis* L. Osbeck) (Almeida *et al.* 2002). The present work aims to describe the ontogenesis of adventitious buds from epicotyl explants of this cultivar, aiming to contribute to the understanding of the mechanisms controlling morphogenesis.

Materials and methods

Organogenesis was induced from epicotyl segments according to Almeida *et al*. (2002). Seeds from ripe fruits of Valencia sweet orange (*Citrus sinensis* L. Osbeck) had the integuments removed and disinfestation was done in a 25 % commercial sodium hypochlorite solution (2.5 % active chlorine) for 20 min, followed by three rinses in distilled sterile water. Seeds were introduced in Murashige and Tucker (1969, MT) medium, and maintained at 27 ± 2 ºC in the dark for three weeks, followed by one week under a 16-hour photoperiod (irradiance of 40 μmol m^{-2} s^{-1}). Epicotyl segments were introduced in MT medium solidified with 0.8 % agar and supplemented

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Abbreviations: BAP - benzylaminopurine; LM - light microscopy; SEM - scanning electron microscopy; MT medium - Murashige and Tucker medium.
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with 1.0 mg dm^{-3} benzylaminopurine (BAP). Cultures were maintained at 27 ± 2 ºC, under 16-h photoperiod (irradiance of 40 µmol m^{-2} s^{-1}) for 45 d. Samples were collected every five days, from the extremities of the epicotyl explants, processed and analyzed by light and scanning electron microscopy.

Samples for light (LM) and scanning electron microscopy (SEM) were fixed according to Rodriguez and Wetzstein (1998). For LM, fixation was done in paraformaldehyde (3 %, m/v) and glutaraldehyde (2 %, v/v) in cacodylate buffer (0.2 M, pH 7.2), dehydration in a series of methyl cellosolve, ethanol, propanol and butanol, followed by overnight infiltration at 4 °C, in butanol:infiltration medium (1:1) (*Historesin*, *Leica*, Heidelberg, Germany). Infiltration was completed with 100 % infiltration medium for 24 - 36 h. Polymerization was done at room temperature for 24 to 48 h. Transverse and longitudinal serial sections (5 µm) were stained in acid fuchsin (0.1 %) and counter stained with toluidine blue (0.05 %). For SEM, samples were fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed in buffer, dehydrated in an ethanol series, critical point dried through CO_2 and sputter coated with gold. Samples were observed and images were obtained in a *Zeiss Axiovert 35* (*Carl Zeiss*, Jena, Germany) or a *LEO 435 VP* (*Carl Zeiss*) operating at 20 keV.

Results

At explanting, the epicotyl segments (Fig. 1*A*-*E*) were green, with an approximate diameter of 200 - 300 µm (Fig. 1*A*). Transverse sections of the epicotyl segments (Fig. 1*B*) showed a distinguished single layered epidermis, where lenticels were present. Internally, the cortex was composed of 14 to 16 layers of parenchymatous cells, highly vacuolated, with intercellular spaces almost absent. The outermost and innermost layers of cortex cells were smaller than those in the medium portion of the cortex. Oil glands were frequently observed (Fig. 1*B*) in the outermost layers of the cortex. Internally to the cortex, the vascular cylinder was continuous, with three layers of meristematic cambial cells, the primary phloem external to the cambium, with discrete phloem fibers, and the xylem internal to the cambium, composed of cells with characteristic thickened walls (Fig. 1*C*-*E*). The central region, the pith, was composed of parenchyma cells with intercellular spaces more frequently observed than in the cortex region (Fig. 1*C*,*D*). Longitudinal sections of the epicotyl showed the elongated shape of most of the cortex, pith and vascular system, and more isodiametric epidermal cells (Fig. 1*D*,*E*).

The morphogenic response initiated soon after the introduction of explants in medium with BAP. After 5 d in culture, visible changes were observed in the explant (Fig. 1*F*), which became thicker, especially in the cut regions. Histological sections (Fig. 1*G*-*J*) showed structural changes, mainly in the cambium. Cortex and pith did not show significant structural alterations. At this stage, the number of cell layers in the cambium increased due to periclinal divisions of the original cambial cells (Fig. 1*H*-*J*), with only a slight increase in the number of xylem layers (Fig. 1*H*). As a consequence of these periclinal cell divisions, a rapid increase in girth was observed. The epidermal layer presented ruptures or disintegrated, especially in the region closer to the cut end of the explant.

With time, these structural alterations were intensified (Fig. 2*A*-*C*). The cambial region of the explant, at the 10 d in culture, was considerably thickened, with many cell layers, and cell divisions occurring mostly periclinally. At this stage, some anticlinal or oblique cell divisions were also observed in the cambium (Fig. 2*B*-*C*). The cortical region also showed some mitotic activity, and less organized cell distribution, with intercellular spaces irregularly distributed (Fig. 2*B*). The cambium was thicker in the region closer to the cut ends of the explants, and cambial cell proliferation was less pronounced in the more internal regions of the explant, compared to the cut ends. The intense cambial cell proliferation in the cut ends of the explants was mainly responsible for the increased girth in that region.

After 15 d in culture (Fig. 2*D*-*F*), meristematic regions, composed of small tightly packed cells, were present in the cambium of the explants (Fig. 2*E*-*F*). Less intense mitotic activity was also observed in the cortex and the presence of phloem fibers were important to distinguish between the cambial and cortical regions at this stage. The meristematic regions differentiated and developed into adventitious buds (Fig. 2*H*-*I*), which were initially observed around the 20 d in culture (Fig. 2*G*). Adventitious bud ontogenesis thus initiated from proliferation of cambial cells of the explant tissue, characterizing a direct organogenic process. Longitudinal sections of the explants (Fig. 2*C*) showed the endogenous formation of multiple buds in the cut ends of the explants. Adventitious buds were observed either in the proximal or distal end of the explant; in most cases morphogenesis was restricted to one of the cut ends, while non-organogenic callus (wound callus) formed on the other end. Only occasionally shoot buds developed in both ends of the explant. Observations on the scanning electron microscope showed details of the early stages of bud development from the cambial region (Fig. 2*I*), as well as the absence of callus, except for slight surface proliferation of callus mainly on the pith. During the process, the explant epidermis disintegrates while the tissues of the neo-formed bud will be originated from the meristematic centers. Buds showed an apical meristem, leaf primordia, numerous trichomes and visible vascular connections with the explant tissue.

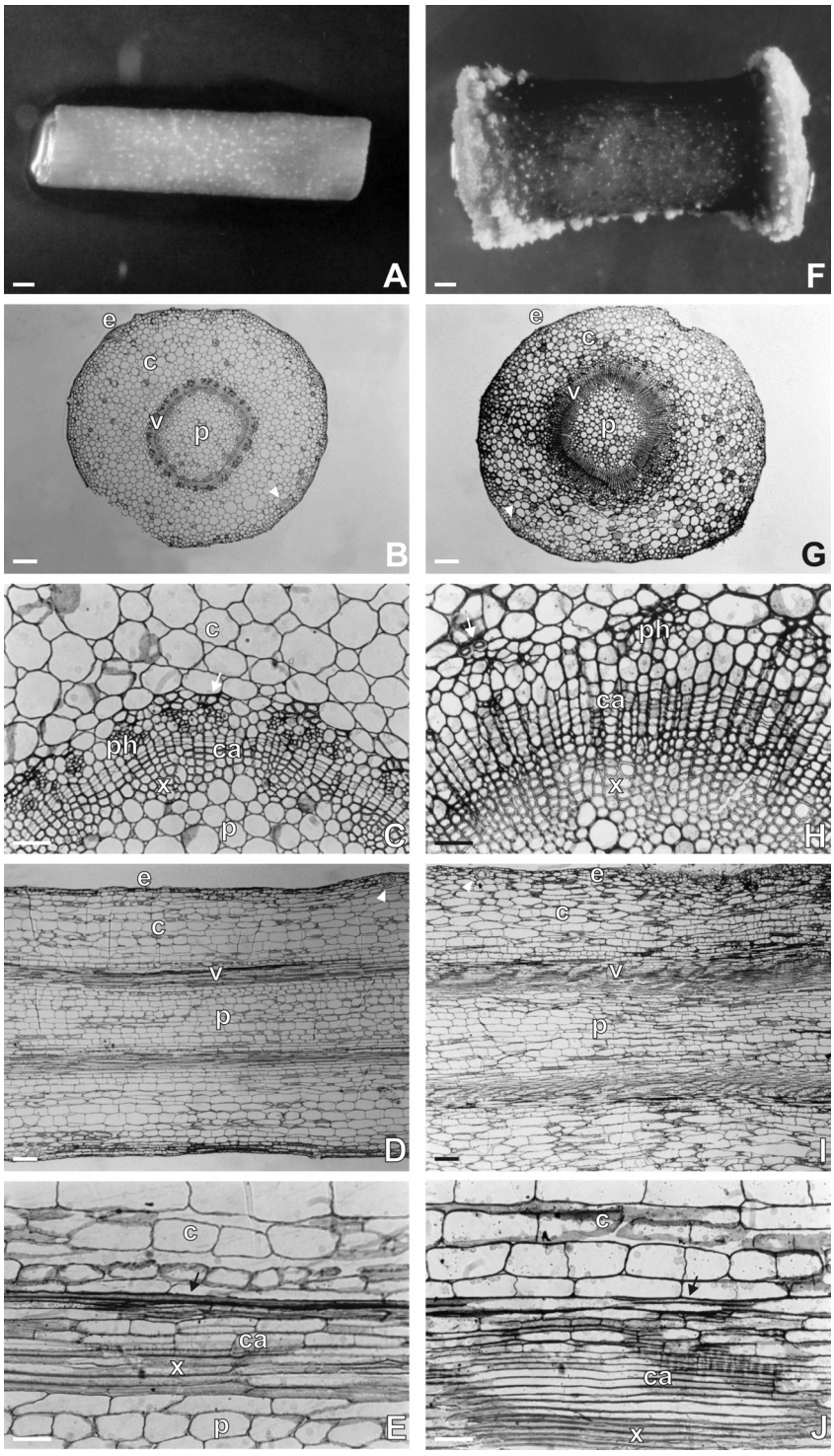

Fig. 1. Epicotyl segment of *Citrus sinensis* L. Osbeck, cv. Valencia, used as explant for organogenesis induction, four weeks after germination *in vitro* (*A*-*E*) and after 5 d in medium with 1.0 mg dm^{-3} BAP (*F*-*J*). A general view of the explant (*A*); transverse (*B*,*C*) and longitudinal (*D*,*E*) sections showing the general anatomical characteristics of the epicotyl (*B*,*D*) and detailed view of the vascular and cambial regions (*C*,*E*) of the explant; general view of the explant with cell proliferation mainly in the cut ends (*F*); transverse (*G*,*H*) and longitudinal (*I*, *J*) sections showing increase in the number of layers of vascular cambium (*H*-*J*), while cortex, xylem and pith did not show significant changes after treatment with BAP (*B*,*D*). ca - cambium, c - cortex, e - epidermis, ph - phloem, p - pith, v - vascular cylinder, x - xylem, *arrow head* - oil gland; *arrow* - phloem fibers. *Bars* = 500 µm (*A*,*F*), 200 µm (*B,D*,*G,I*), 25 µm (*C*,*E,H.J*).

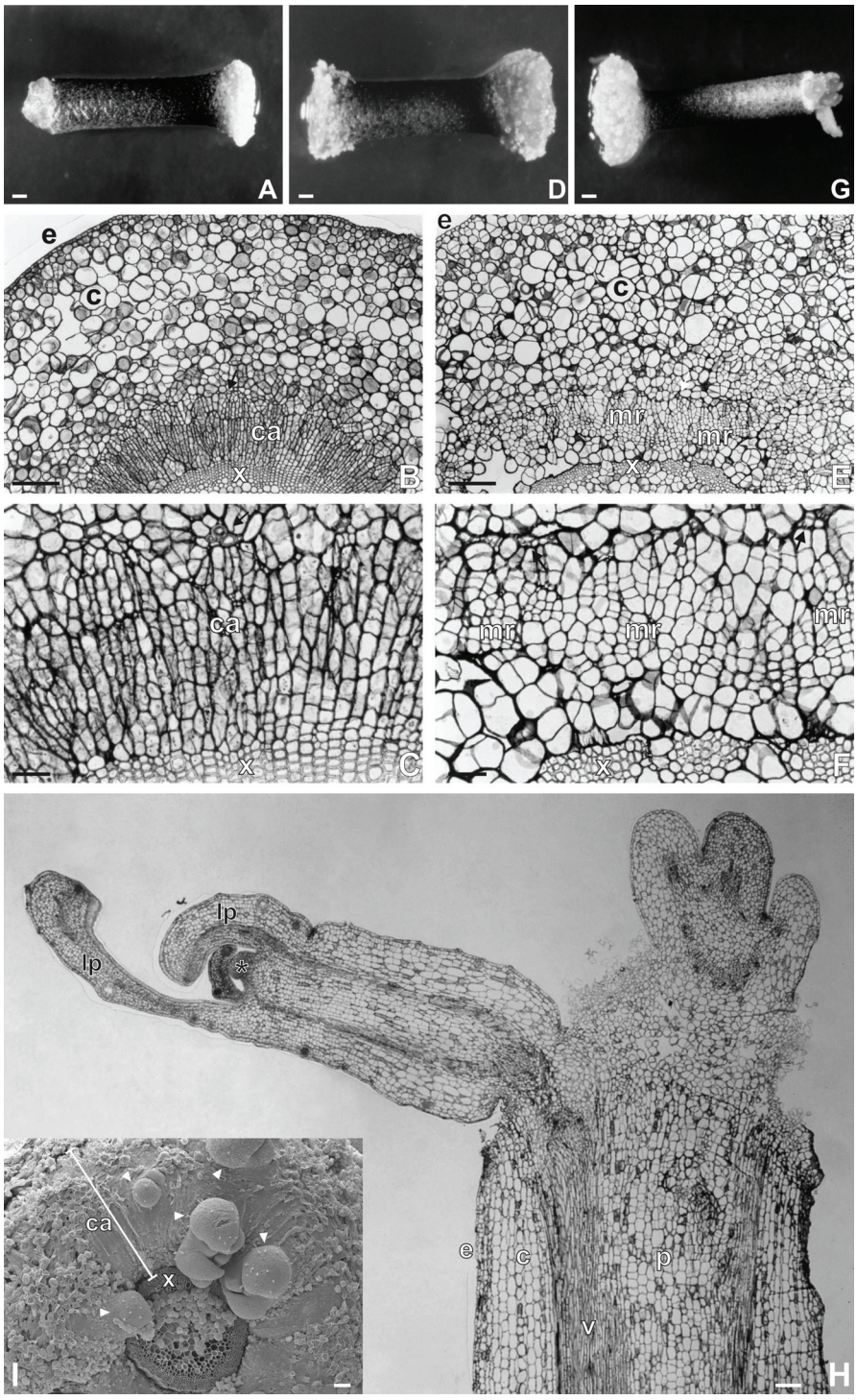

Fig. 2. Organogenesis in epicotyl segment of *Citrus sinensis* L. Osbeck, cv. Valencia after 10 (*A*-*C*), 15 (*D*-*F*) and 20 d (*G*-*I*) in medium with 1.0 mg dm^{-3} BAP. A general view of the explant at day 10 (*A*), transverse sections showing periclinal and oblique cell divisions in the cambial region, increasing the meristematic region of the explant (*B*-*C*); general view of the explant at day 15 (*D*) showing differences in response in both ends; longitudinal sections showing intense mitotic proliferation, partial tissue disintegration in some regions and meristematic centers in the cambial region (*E*-*F*); general view of the explant with adventitious buds in initial stages at day 20 (*G*); longitudinal section showing the direct origin of the shoot bud and the vascular connections with the vascular system of the explant (*H*); scanning electron micrograph showing the enlargement of the cambial region and the formation of shoot buds directly from this tissue (*I*), some slight proliferation of callus is observed on the surface of the cambium and pith; shoot buds in different stages of development (*arrow heads*). ca - cambium, c - cortex, mr - meristematic region, ph - phloem, p - pith, v - vascular cylinder, x - xylem, *arrow* - phloem fiber. *Bars* = 500 µm (*A*,*D*,*G*), 200 µm (*H*), 100 µm (*B*,*E*,*I*), 25 µm (*C*,*F*).

Discussion

During the process of increase in girth *in vivo*, the cambial cells divide periclinally. The innermost cell layer, then, will differentiate into xylem cells, contributing to the xylem, while the outermost layers of the dividing cambium will differentiate, and contribute to the phloem tissue. Since the cambium is a meristematic region of the plant, and hence, its cells maintain the ability to divide, the initiation of morphogenesis from these cells is expected, given the adequate culture conditions. In the present study, after induction with BAP, an increase in number of cambial layers was observed, with intense formation of meristematic regions and only a slight differentiation in vascular elements. In Troyer citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.), intense periclinal divisions in the cambial region have been verified by García-Luis *et al.* (1999) in epicotyl segments submitted to organogenesis induction.

García-Luis *et al.* (1999) showed that regeneration may follow different morphogenetic pathways according to explant position (horizontal or vertical) and culture medium, also differing in hormone requirement, ontogenetic age of the explant and genotype. Culture of internodal segments (mature tissue) of Valencia sweet orange, placed horizontally in medium with 1 mg dm^{-3} BAP resulted in the formation of direct organogenesis under a 16-h photoperiod, or indirect organogenesis in the dark (Almeida *et al.* 2003a), confirming the possibility of different morphogenetic pathways from the same explant.

Independently of the origin of the organogenic process in the explant tissue, the histological characterization of the process and the definition of the morphogenic regions can guide for alterations in the organogenic system to favor the contact between the *Agrobacterium* and the morphogenic region, enhancing the genetic transformation efficiency. As also mentioned by García-Luis *et al.* (1999), a direct regeneration pathway may be used for transformation purposes as a means to avoid the formation of chimeric plants and genetic variability, which can occur through regeneration from callus. Both morphogenetic pathways for Valencia sweet orange epicotyl segments (Almeida *et al.* 2003b) and mature tissue (Almeida *et al.* 2003a) have shown to be amenable for transformation through *Agrobacterium*.

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