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ORIGINAL PAPER

New insights into the in vitro organogenesis process: the case of *Passiflora*

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Abstract We present evidences that ultrastructural electron microscope findings are valuable ways to understand the in vitro regeneration process, in particular in the yellow passion fruit. Shoot-regeneration was induced in hypocotyl and leaf-derived explants using 4.44 μM BAP, and the entire organogenic process was analyzed using conventional histology, scanning and transmission electronic microscopy. Both direct and indirect regeneration modes were observed in hypocotyl explants, but only direct regeneration occurred in leaf-derived cultures. In the direct pathway from both explant types, meristemoids developed into globular structures, here called protuberances. The peripheral meristematic layers of the protuberances displayed ultrastructural characteristics indicative of a high metabolic activity, and only these cells originated shoots and leaf primordia, the latter being frequent when leaf explants were used. Moreover, the peripheral cells of the protuberances derived from leaf explants lost adhesion during the culture, diminishing the regeneration rates. We recommend the use of hypocotyls as a source of explant to obtain shoots as well as a genetic transformation system for the yellow passion fruit. However, the direct pathway is preferred because a type of amitosis occurred in the peripheral cells of hypocotyl-derived calli, which has the potential to result in genetic instability of the regenerating plants/tissue.

Keywords Amitosis · Electronic microscopy · Histology · Passion fruit · Protuberances

Abbreviations

BAP	6-Benzylaminopurine
N^6	
TDZ	Thidiazuron (*N*-phenyl-*N*′-1,2,3,-thiadiazol-5-ylurea)
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

Introduction

The yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Degener) is one of the important fruit crops in Latin America where many species of *Passiflora* are native to both tropical and sub-tropical

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climates. Found in majority of Brazilian and Andean villages, it is consumed as fresh fruit. Furthermore, it produces a juice that is processed in small scale for export to European Community countries.

Passion vines are susceptible to the bacterium *Xanthomonas axonopodis* pv. *passiflorae* and to the passion fruit woodiness virus. Both pathogens cause diseases that evolved over the last two decades resulting in great losses to the juice industry and fruit producers, mostly in southeastern Brazil. So far, there is no source of resistant material in the *P. edulis flavicarpa* germplasm suggesting the use of transgenic plants. However, the recovery of transgenic shoots cultured on selective media was reported to be difficult due to the very low frequency of in vitro bud elongation (Trevisan et al. 2006).

Overall, the efficiency of the genetic transformation system depends on the rate of cell transformation and cell ability to develop into buds. It is well established that meristemoids situated internally to the surface of the explant can reduce the efficiency of *Agrobacterium* mediated transformation, while shoots originating on the surface have a better chance of being transformed. Thus, it is important to locate and identify the cells involved and responsible for the in vitro plant morphogenesis, particularly in *Passiflora*.

The organogenesis in vitro is the principal mode of regeneration in *Passiflora* and may be direct (Dornelas and Vieira 1994; Appezzato-da-Glória et al. 1999; Hall et al. 2000; Becerra et al. 2004) or indirect (Monteiro et al. 2000; Lombardi et al. 2007), depending on the source of the explant and the genotype used. Protocols for inducing organogenesis in *Passiflora* explants are well known and the use of BAP or less frequently TDZ is recommended.

Regeneration of leaf structures on the surface of *P. edulis* f. *flavicarpa* explants is very frequent (Appezzato-da-Glória et al. 2005); however, it has been erroneously interpreted in the literature as buds that do not elongate. In addition, shoots of chimeral origin, i.e., with transformed and non-transformed cells are being recovered from these explants (unpublished data), but they are not useful to transformation.

Locating the meristemoids in the target tissue, the need of the transformed cell to be involved in the morphogenic process and the genetic stability of the resultant plants are essential requirements to the success of a genetic transformation-based breeding program.

Recently, our group reported that meristemoids formed on the callus of *Bauhinia forficata* showed fragments of chromatin dispersed in the cytoplasm, indicating the occurrence of a type of amitosis. However, such nuclear fragmentations were not observed in direct organogenesis of other legumes e.g., soybean (Appezzato-da-Glória and Machado 2004). Amitotic division apparently occurs at the transition from a differentiated cell state to one of disorganized growth, and it was reported to occur mainly during the initial of the callus growth (Bregoli et al. 1997). The main significance of these studies relies on the necessity of ultrastructural analyses for characterizing the process of in vitro regeneration. Possibly, the amitotic process has implications on the genetic stability of the primary regenerants, deserving more attention.

Based on the above, the purpose of work reported here was to examine the cells involved in the in vitro organogenesis of hypocotyl- and leaf-derived cultures in *P. edulis* f. *flavicarpa*, comparing the direct and indirect modes of regeneration using conventional and electron microscopy.

Materials and methods

Seeds of the FB-100 population (Araguari, Brazil) of the yellow passion fruit were surface-disinfected using the following solutions: benomyl (1.0 g l^{-1}), 70% ethanol (40 s), and 2% NaOCl (30 min). After washing, seeds were planted on solidified (1.8 g l^{-1} Phytagel, Sigma) medium containing ½ MS salts (Murashige and Skoog 1962), supplemented with 3.0% (w/v) sucrose. After 15 days, segments of hypocotyls (8–10 mm) and leaf discs (8 mm) containing the midrib were excised from the seedlings and inoculated on solid MS supplemented with 4.44 μM BAP (Dornelas and Vieira 1994) and 5% coconut water. Before autoclave sterilization, pH was adjusted to 5.8. Cultures were maintained in growth room at 25 ± 2°C and 16-h photoperiod, under cool white fluorescent lamps (30 μmol m^{-2} s^{-1}).

Tissues and protuberances (0.5–2.0 mm) derived from hypocotyl and leaf explants were collected daily up to the third day of culture, and every 3 days until the lengthening of the shoots. The samples were fixed in Karnovsky solution (Karnovsky 1965). They were dehydrated in ethanol series and embedded in plastic resin (Leica Historesin), stained with 0.05% toluidine

blue (Sakai 1973) diluted in phosphate–citrate buffer titrated to pH 4.5 (McIlvaine 1921), and mounted in Entellan synthetic resin (Merck). To detect the presence of pectin, the sections were stained with coriphosphine (Weis et al. 1988) and viewed under a Leica DM LB fluorescence microscope equipped with blue (450–490 nm) and green (546 nm) filters.

After fixation, part of the protuberances was dehydrated in ethanol series and critical point-dried with CO_2 (Horridge and Tamm 1969). These samples were attached on aluminum stubs and coated with gold (30–40 nm), and examined under a LEO VP435 scanning electron microscope at 20 kV.

Hypocotyl-derived cultures were also analyzed under transmission electron microscopy. After fixation, samples were post-fixed in OsO_4 1%, incubated in an aqueous solution of uranil–acetate 0.5%, dehydrated in a rising sequence of acetone series, and embedded in Araldite resin at room temperature. Polymerization was performed at 60°C for 48 h. The ultrathin sections were contrasted with uranil acetate (Watson 1958) and lead citrate (Reynolds 1963), and examined under a Philips CM 100 electron microscope at 60 kV. To analyze the membrane systems involved in the differentiation of the hypocotyl-derived protuberances, part of the fixed samples was incubated for 17 h at 10°C, in the dark, in a solution containing Zn, I, TRIS–aminomethane and 2% OsO_4 (Reinecke and Walter 1978). The dehydration, embedding, polymerization and sectioning followed the protocol described above under TEM.

Results and discussion

Both direct and indirect regeneration pathways were observed in the hypocotyl explants. After 5 days of

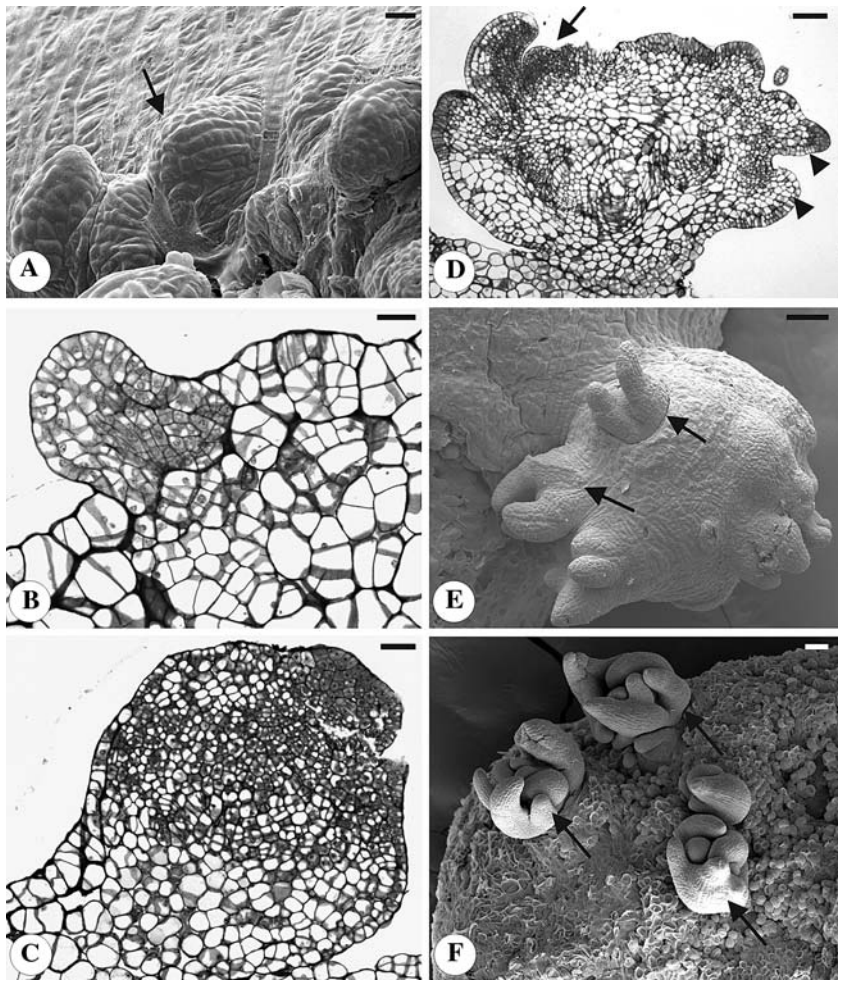

Fig. 1 Electron micrograph (**A**,**E**,**F**) and micrograph (**B**–**D**) of *Passiflora edulis* f. *flavicarpa* hypocotyls inoculated in MS medium supplemented with 4.44 μM BAP and 5% coconut water. Meristemoid (arrow) formed directly on the explant after 30 days (**A**). This meristemoid may give rise to leaf primordia (**B**), or it may form protuberances (**C**). Leaf primordia (arrowheads) and shoot (arrow) at the periphery of a protuberance after 26 days (**D**). Shoots (arrow) after 19 days (**E**). Callus-derived shoots (arrow) after 27 days (**F**). Bars (in μm): 30 (**A**,**B**); 50 (**C**); 74 (**D**); 100 (**E**,**F**)

culture, cell divisions in the cortical and pith parenchyma, near the surfaces of the sectioning of the explants, gave rise to the meristemoids (Fig. 1A). Some of these meristemoids gave rise to leaf primordia (Fig. 1B), and several others continued their development originating the protuberances (Fig. 1C–E). In a quarter of the explants, there was callus formation from cells of the cortical and pith parenchyma, the protuberances being visible on the callus surfaces (Fig. 1F) from 18 days after inoculation.

The protuberances formed directly (Fig. 1C–E) or through the callus phase (Fig. 1F) possess a continuous unistratified epidermis, which together with subepidermic layers constitute the peripheral cells of the protuberance. It is important to note that only cells of the edge of the protuberances originated shoots on their surfaces (Fig. 1D,E). This is the first report on the occurrence of protuberances and their role in the regeneration process in *Passiflora*, though the images published in the literature showed their presence (Biasi et al. 2000; Trevisan and Mendes 2005). The sole description about the formation of buds on the peripheral layers of protuberances was made in *Eucalyptus gunnii* (Hervé et al. 2001). The peripheral cells of the protuberances formed directly or indirectly during the in vitro cultivation displayed ultrastructural characteristics indicative of high metabolism (Fig. 2A,F) and presented pectin lying just outside the cell wall (Fig. 3A,B). In their central portion, the protuberances displayed parenchymatic cells and tracheary elements (Fig. 2B,C). In the vascular differentiation, the cells exhibited smooth and rough reticulum and very active dictyosomes

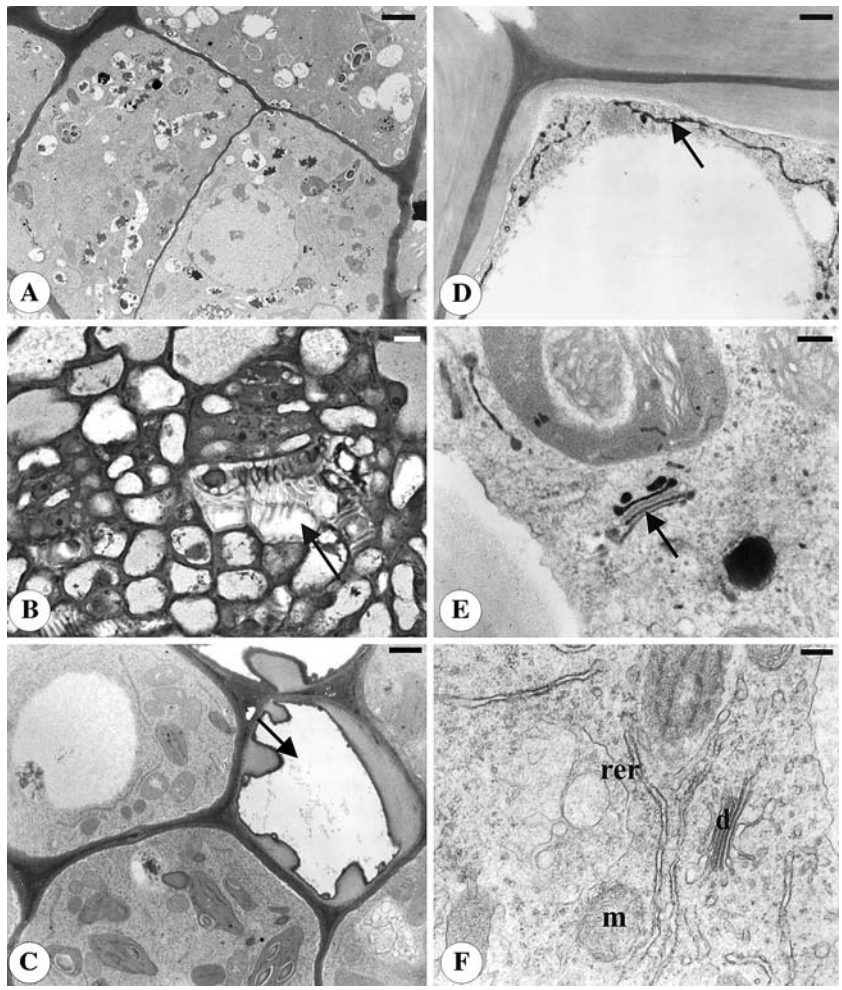

Fig. 2 Peripheral cells (**A**,**F**) and central cells (**B**–**E**) of a protuberance. Longitudinal section (**B**) and electron micrograph (**C**) showing tracheary elements (arrow). Vascular differentiation process evidenced by smooth endoplasmic reticulum (**D**) near the cell wall and the membranes of dictyosomes (**E**) with different intensities of impregnation with zinc (arrow). Mitochondria (m), dictyosomes (d), rough endoplasmic reticulum (rer) (**F**). Bars (in μm): 1.73 (**A**); 10 (**B**); 1.02 (**C**); 0.43 (**D**); 0.17 (**E**); 0.23 (**F**)

near the cell wall with different intensities in the dictyosome membrane as a result of impregnation with zinc (Fig. 2D,E). These ultrastructural characteristics are associated with parietal thickening as reported previously (Burgess and Linstead 1984).

What is noteworthy in the comparative analysis of the yellow passion fruit regeneration pathways is the ultrastructural difference in the nucleus of the peripheral cells, i.e., the cells involved in the process of regeneration. In the direct mode, the nucleus was circular in shape and no nuclear membrane invaginations were observed (Fig. 4A). In the indirect pathway, the nucleus displayed a membrane with numerous pores (Fig. 4F) and progressive formation of membrane invaginations causing the nucleus to show an irregular shape (Fig 4B,C), which eventually divided into two parts (Fig 4D). There were mitochondria in the vicinity and, occasionally, inside the nuclear invaginations (Fig. 4E). This process of nuclear fragmentation could compromise the genetic stability of transgenic buds.

Only direct regeneration pathway was observed from leaf-derived explants. After 15 days of culture, there were protuberances on the explant surfaces, especially on the midribs. The anatomical structure of the protuberances was similar to that described for the hypocotyl explant. However, throughout the culture protuberances had enlarged (Fig. 3D–F). The peripheral cells lacked their adhesion exhibiting cell discontinuity, and more

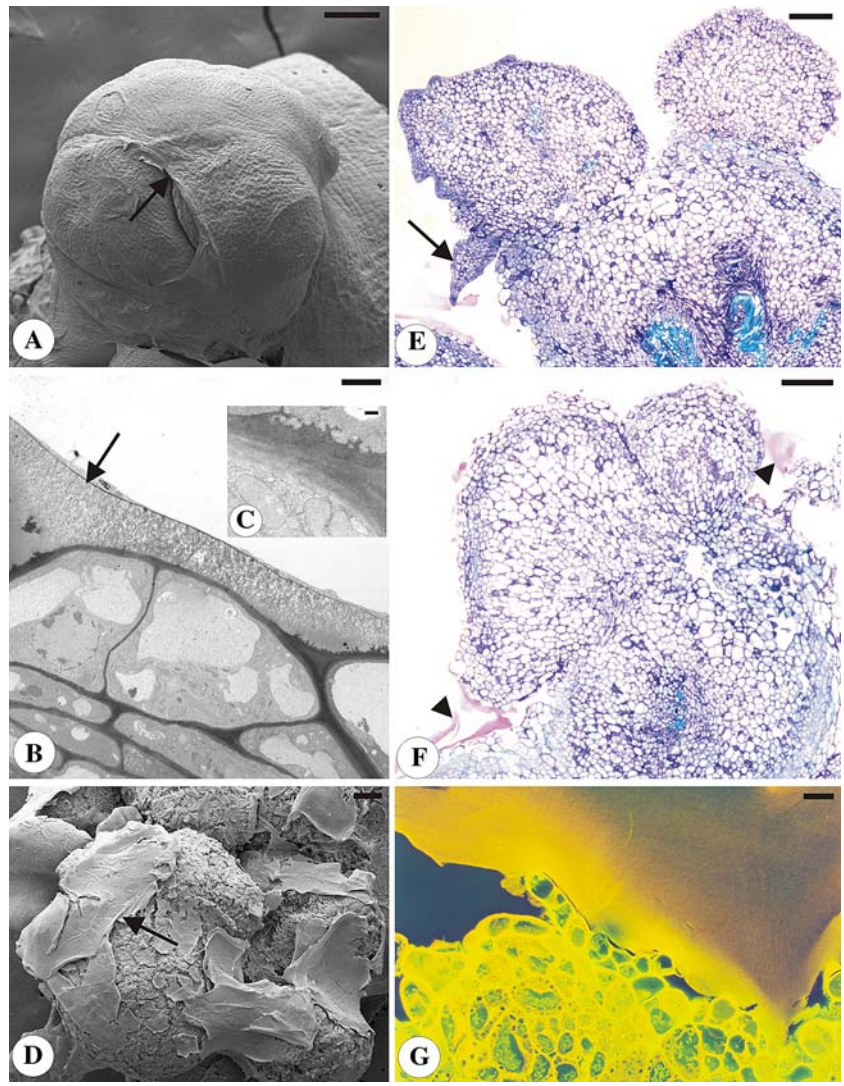

Fig. 3 Electron micrographs of a protuberance originated from hypocotyls showing the pectin (arrow) accumulated on the surface after 27 days (**A**–**C**). Electron micrograph of the protuberance originated in leaf-tissues showing pectin discontinuity (arrow) on its surface after 30 days (**D**,**F**). Longitudinal section of leaf-derived protuberances after 25 days exhibiting cell discontinuity, and a leaf primordium (arrow) originated on protuberance surface (**E**). Pectin viewed under the fluorescence microscope (**G**). Bars (in μm): 200 (**A**); 4.08 (**B**); 0.17 (**C**); 300 (**D**); 199 (**E**,**F**); 33.3 (**G**)

pectin accumulated on the protuberance surfaces (Fig. 3D–G). We can state that only cells of the protuberance edges originated a few shoots and leaf primordia (Fig. 3E).

Leaf-derived explants developed a higher number of protuberances (223/48) than the hypocotyl explants (85/48). However, the number of buds was considerably smaller. We counted 23 and 150 buds per 48 leaf-discs and hypocotyls, respectively. The few regenerated buds become hyperhydric. The smaller ratio of bud formation on protuberances from leaf-derived explants occurred due to a defect in intercellular attachment of the outermost layer of the protuberances, affecting the capability to produce shoots. The same was verified in *Nicotiana plumbaginifolia* leaf discs cultured in vitro (Lord and Mollet 2002). The defect in cell adhesion is associated with accumulation of pectic polysaccharides in the cell corners and interstitial spaces (Shevell et al. 2000), as verified in our work. As a result, the few buds originated from leaf-derived protuberances became hyperhydric as water is maintained inside their cells due to pectin sorting and accumulation on protuberance surfaces. Furthermore, several of the structures recognized as adventitious buds were in fact leaf primordia, and this explains why bud elongation was reported as problematic in *Passiflora* (Trevisan et al. 2006).

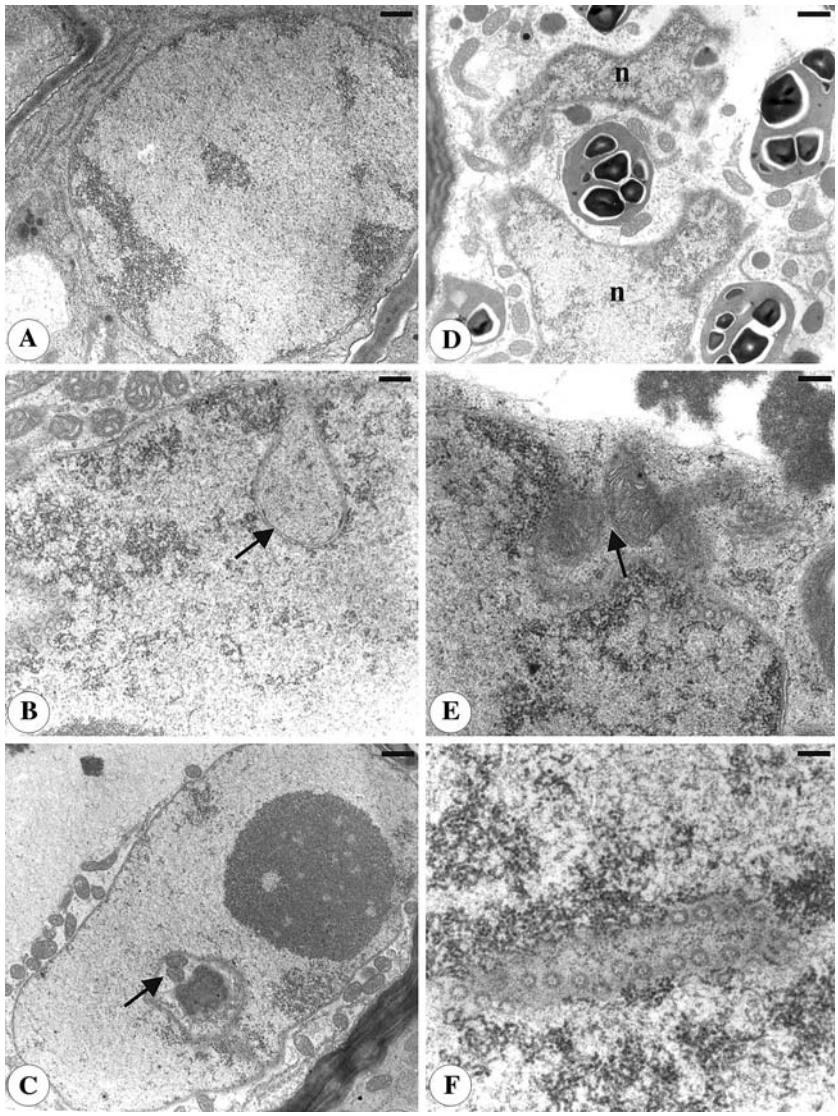

Fig. 4 Electron micrograph of the nucleus of a peripheral cell of the protuberance formed directly on hypocotyls. The nucleus is circular in shape and there are no membrane invaginations (**A**). Electron micrographs of peripheral cells of the protuberances formed on hypocotyl-callus (**B**–**F**). Nuclear envelope invagination (arrow) (**B**). Nucleus with irregular shape and mitochondria inside the invagination (arrow) (**C**). Fragmented nucleus (n) (**D**). Mitochondria (arrow) found inside the invagination of the nucleus envelope (**E**). Nuclear pore complex (**F**). Bars (in μm): 0.43 (**A**); 0.31 (**B**); 1.02 (**C**,**D**); 0.23 (**E**); 0.17 (**F**)

New approaches have recently been used in plant tissue culture studies. There were differences in general metabolism, phenolic secondary compounds and cell wall between regenerable and non-regenerable cell types (see Lozovaya et al. 2006 and references therein). Regeneration ability is associated with the expression of specific genes, which is tissue/cell-dependent. For example, leafy cotyledon genes are essential for induction of somatic embryogenesis in *Arabidopsis* (Gaj et al. 2005) as well as signaling molecules such as transcription factors are reported to be involved in control of regeneration (Srinivasan et al. 2007). Nevertheless, as shown here, comparative electron microscopy-based analysis should not be disregarded, as it is a valuable way to understand the regeneration process. Based on these reasons, we recommend the use of hypocotyl as a source of explant to obtain shoots as well as a genetic transformation system for *P. edulis* f. *flavicarpa*.

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