

Induction and Morpho-Ultrastructural Analysis of Organogenic Calli of a Wild Passionfruit

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ABSTRACT

This work studied a new protocol for organogenic calli induction and characterization of the morphology and ultrastructure of callogenesis in leaf explants of *Passiflora gibertii* N. E. Brown, a native passion fruit species from Brazil. Calli induction was performed in different growth conditions (light and dark), different MS medium salt concentrations (MS and MS half strength) and the presence or absence of coconut water. The leaf explants maintained in the dark were more responsive to bud formation. In order to reduce spending on in vitro culture, the most suitable induction medium for *P. gibertii* organogenesis could, therefore be the MS half strength salt concentration medium maintained in the dark. The addition of coconut water to the culture medium was essential for both calli induction and bud formation. The morphological and ultrastructural features of the organogenic calli were isodiametric cells, characterized by an organized cellular system, nucleus with prominent nucleoli, presence of starch grains and dense cytoplasm rich in endoplasmic reticulum. The scanning electron microscopy demonstrated that buds were present on these calli.

Key words: Passifloraceae, callogenesis, electromicroscopy, optic microscope, tissue culture

INTRODUCTION

Brazil is the center of diversity for the Passifloraceae family. It hosts more than 150 native species, including some wild *Passiflora* species that are not commercially cultivated (Bernacci et al. 2008). Breeding programs may contribute to improving disease resistance in existing commercial cultivars. One native Brazilian species with considerable potential in this context is *Passiflora gibertii* N. E. Brown, a fast growing vine with attractive mauve/purple

flowers, which is apparently resistant to several important passion fruit diseases such as premature death and other diseases caused by soil-borne pathogens (Junqueira et al. 2005).

In vitro culture of native *Passiflora* spp. is a key tool for the production of clones with enhanced resistance and other desirable traits. Numerous tissue culture studies in *Passiflora* have been conducted and regeneration by organogenesis has been successfully reported from a wide range of species (Fernando et al. 2007; Lombardi et al. 2007; Pinto et al. 2010; Silva et al. 2011; Anand et

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al. 2012). However, this is not the case for *P. gibertii*, where there have been limited studies on *in vitro* cultivation (Faria et al. 2006; Faria et al. 2007; Carvalho et al. 2013).

The cytokinin 6-benzylaminopurine (benzyladenine) (BA) is very effective at promoting multiplication in several species (Lima-Brito et al. 2011; Sá et al. 2012), including *Passiflora* spp. such as *P. cincinnata* Mast. (Lombardi et al. 2007; Silva et al. 2011), *P. suberosa* L. (Garcia et al. 2011), *P. edulis* Sims (Silva et al. 2011) and *P. alata* Curtis (Pacheco et al. 2012). Coconut water contains substances that are essential for the growth and for regeneration of many species such as *P. edulis* x *P. edulis* var *flavicarpa* (Hall et al. 2000), *P. edulis* (Fernando et al. 2007) and *P. alata* Curtis (Pacheco et al. 2012). It is used as a growth supplement in plant tissue culture and its chemical composition is rich in sugars, vitamins, minerals, amino acids and phytohormones such as cytokinins that stimulate the division of mature plant cells (Yong et al. 2009).

Histological, morphological and ultrastructural analyses can be performed to monitor the regeneration and to differentiate organogenesis (Fernando et al. 2007; Silva et al. 2011; Rocha et al. 2012) from somatic embryogenesis (Pinto et al. 2011). By characterizing the regeneration path, the best growth conditions can be established allowing the development of efficient protocols for the induction and production of plants. There are no reports of organogenic calli induction in *P. gibertii* in the literature. It would, therefore, be useful to provide a detailed account of the morphological and ultrastructural changes during *in vitro* organogenic callus induction to form the seedlings from this native passion fruit. In this context, the objective of this study was to establish a protocol for organogenic calli induction on leaf explants of *P. gibertii* and to characterize the morphology and ultrastructure of these calli.

MATERIAL AND METHODS

Seeds of *P. gibertii* (access CPAC MJ-22-01) were obtained from the Embrapa Cerrados (CPAC) germplasm collection, Planaltina, DF. They were germinated in Plantmax HT[®] and the parent plants were maintained in a growth room at 25±2°C, photon irradiance of 43 µmol m⁻² s⁻¹ and subjected to a 16 h photoperiod. The plants were 52 and 109

days old for the first and second experiment, respectively. The leaves collected from mother plants were used as explants for the experiments. In a laminar growth chamber, the leaves were first decontaminated by immersion in sodium hypochlorite solution (NaOCl) containing 0.5% active chlorine and Tween 20 (one drop per 100 mL NaOCl) for 10 min and then washed three times with sterilized distilled water. After decontamination, leaves were excised (± 1 cm²) and the surfaces of the explants were given small cuts with a scalpel to induce callogenesis. The explants were then inoculated through placing the abaxial surface in contact with the medium.

In the first experiment, different growth conditions (light and dark) and different MS (Murashige and Skoog 1962) medium salt concentrations (full and half MS strengths) were tested. The media were supplemented with 8.88 µM BA, 3% sucrose, 5% coconut water and 0.5% agar (Vetec[®]). The second experiment assessed the influence of presence or absence of 5% coconut water in the MS half strength medium salt concentration supplemented with 8.88 µM BA, 3% sucrose and 0.5% agar. To obtain the organogenic calli, explants with calli from both experiments, were transferred to MS half strength salt concentration medium, supplemented with 2.22 µM BA, 3% sucrose and 0.5% agar. The organogenic calli were transferred to MSM medium (Monteiro et al. 2000a) with 3% sucrose and 2.89 µM gibberellic acid (GA₃). The pH for all the media was adjusted to 5.8 ± 0.1 before autoclaving at 120°C for 20 min. After inoculation, the explants from the first experiment were maintained under dark or in growth room under 36 µmol m⁻² s⁻¹ irradiance with 16 h photoperiod (light), both at 25 ± 2°C for 30 days. For the second experiment, the explants were maintained under dark and for calli from both the experiments and for the organogenic calli, the explants were cultivated in the light, all of them for a period of 30 days each.

For statistical analysis, the calli were classified in the following ordinal categories: 0 = no callus formation, 1 = swollen explants, 2 = early callus formation, 3 = 50% of the explants covered by callus, 4 = more than 50% of the explants covered by callus and 5 = explants completely covered by callus. A completely randomized design was used, consisting of 12 replicates per treatment for the first experiment and 45 replicates per treatment for the second experiment. Each replicate consisted of one explants. Data were analyzed by spearman

correlations and Kruskal-Wallis tests, performed in SAS (SAS Institute, 2004).

Morphological and ultrastructural analyses were performed on calli resulting from the first experiment, cultivated in the MS half strength medium salt concentration, supplemented with 8.88 μM BA, 3% sucrose, 5% coconut water and 0.5% agar, maintained 30 days on dark or light and calli from dark sub-cultured to a new medium (MS half strength salt concentration medium supplemented with 2.22 μM BA, 3% sucrose and 0.5% agar) and maintained for seven days in a growth room under 36 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, at $25 \pm 2^\circ\text{C}$ and 16 h photoperiod. In order to analyze the calli under scanning and transmission electron microscopy, they were fixed in modified Karnovsky [glutaraldehyde (2.5%) and paraformaldehyde (2.5%) in cacodylate buffer, pH 7.2] at room temperature for at least 24 h. The calli were then washed in 0.05 M cacodylate buffer (three times every 10 min) and subsequently fixed in a solution containing 1% osmium tetroxide and 0.05 M cacodylate buffer for 4 h. The calli were then dehydrated in an ascending acetone gradient (25, 50, 75 and 90%) for 10 min and three times (10 min each) in 100% acetone.

Transmission Electron Microscopy (TEM) analysis was performed immediately after dehydration. The calli were put in an ascending gradient acetone/Spurr resin 30% for 8 h, 70% for 12 h and finally twice at 100% with a 24 h interval. To polymerize, the tissues were molded in pure silicon resin and dried in a forced-air oven at 70°C for 48 h. The blocks obtained were subjected to thinning, using a razor blade to section the excessive resin. Subsequently, the blocks were cut into semi-thin (1 μm) and ultrathin (<100 nm) sections by using a Reichert-Jung (ultra-cut) ultra-microtome, with a diamond blade.

The semi-thin sections were collected with a gold ring and put on glass slides and were later stained with toluidine blue (1.0 g toluidine blue, 1.0 g sodium borate and 100 mL water purified in Millipore 0.2 μm filter) and permanently mounted in Permoult medium. The ultra-thin sections were collected on formar-coated slot grids (Rowley and Moran 1975). These sections were post-stained with uranyl acetate, followed by lead acetate for 3 min and later examined in a Zeiss transmission electron microscope (EM 902 to 80Kv model).

For Scanning Electron Microscopy (SEM) analysis, after dehydration, the calli were dried in a critical point dryer CPD 030 using liquid CO_2 . The samples were then sputtered with gold prior to SEM analysis. Observations were made by using an electron microscope (LEO Evo 040) operating between 10 and 20 kV.

RESULTS AND DISCUSSION

There was no statistical difference in calli induction between the different concentrations of MS medium under different environmental conditions (light and dark), with average score of 0.5. When calli explants were transferred to the MS half strength salt concentration medium, supplemented with BA (2.22 μM) and maintained in a growth room under light, only a few explants formed in the dark showed buds after 30 days. After this period, the calli were transferred to MSM culture medium with 2.89 μM GA_3 and maintained in a growth room under light. Several replicates from the calli originated from dark and sub-cultured to this new medium presented buds (Fig. 1A), while the calli cultured in the light showed brown color and only one replicate from this calli produced the buds (Fig. 1B).

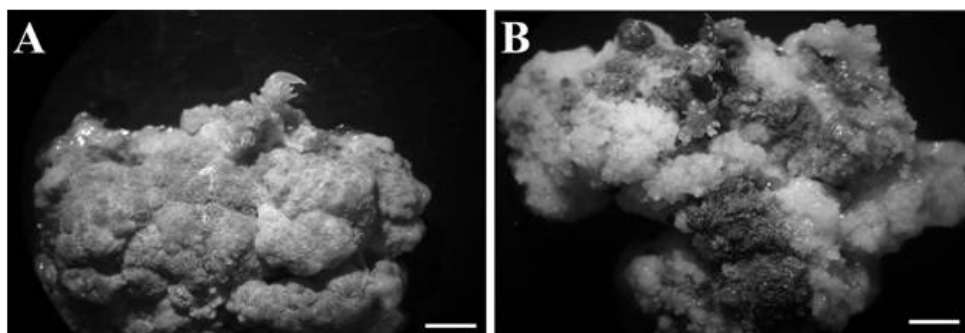


Figure 1 - Organogenic calli obtained from *Passiflora gibertii* leaf segments from dark (A) and light (B). Bar = 3 mm (A, B).

Monteiro et al. (2000b) also observed bud formation when they cultured leaves of *P. suberosa* *in vitro* in MS medium in the dark at $26 \pm 2^\circ\text{C}$ for four weeks and then transferred calli with organogenic aspects to MSM medium with $2.89 \mu\text{m GA}_3$ under light. However, when tissues of *P. suberosa* were incubated in the dark without a subsequent period in the light, no significant shoot formation occurred (Garcia et al. 2011).

The browning in calli occurs mainly due to the oxidation of phenolic compounds by phenol oxidase (Erland and Mahmoud 2014) and it is likely that the products of this oxidation process are formed in the light (Afshari et al. 2011; Ndakidemi et al. 2014). Phenolic secretions and other exudates in plant tissue culture systems lessen explant initiation, growth and development (Ndakidemi et al. 2014; Yildirim and Turker 2014). The light source also causes an increase in lipid peroxidation leading to lower rates of physiological processes (Tariq et al. 2014; Yildirim and Turker 2014) as observed in the present work with *P. gibertii* calli cultured in the light.

The use of coconut water to the culture medium

significantly influenced calli induction. Higher levels of calli induction were observed using coconut water (score of 0.72) compared with the absence of coconut water (score 0.28) (Fig. 2). The calli from the medium with coconut water presented clear bud formation (Fig. 3A), while calli from the medium without coconut water did not produce buds (Fig. 3B) after 30 days transferred to a new medium and maintained in the light.

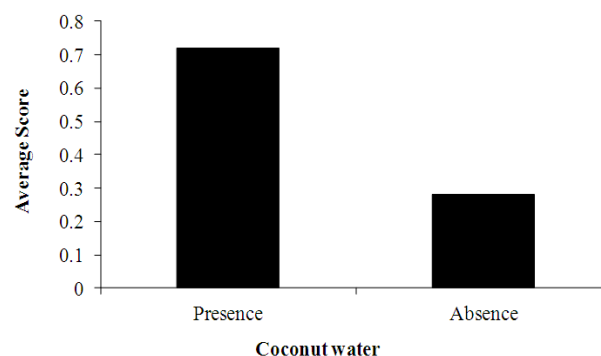


Figure 2 - Callus induction in *Passiflora gibertii* leaf segments in the presence and absence of coconut water in a MS half strength salt concentration medium with BA ($8.88 \mu\text{m}$) at 30 days of cultivation.

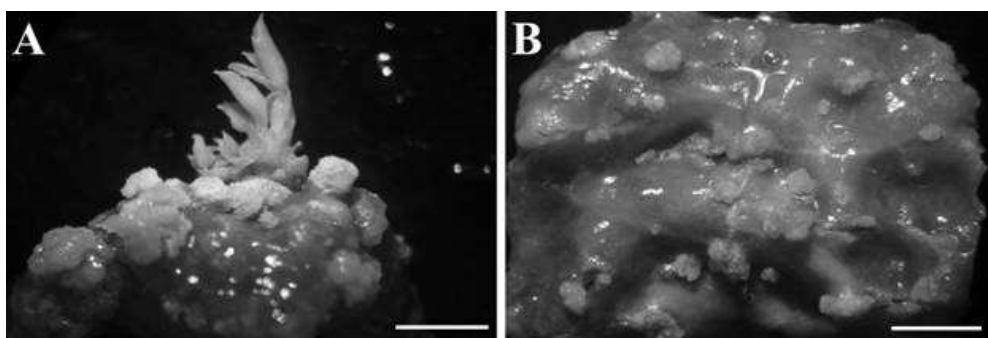


Figure 3 - Organogenic calli obtained from *Passiflora gibertii* leaf segments grown in culture medium with (A) and without (B) coconut water. Bar = 3 mm (A, B).

These results were in general agreement with several studies (Hall et al. 2000; Pacheco et al. 2012), indicating that coconut water and BA added to the culture medium of *Passiflora* spp. promoted the organogenesis. Kantharajah and Dodd (1990) reported that the addition of 20% coconut water to MS medium with BA $8.88 \mu\text{m}$ significantly increased bud production of *P. edulis* var. Norfolk Island. Similarly, Fernando et al. (2007) reported that the direct organogenesis from leaf discs of *P. edulis* occurred only when leaf explants were cultured on MS medium supplemented with 4.44

μm BA and 5% of coconut water. Coconut water may also be beneficial for the *in vitro* cultivation of other species. For example, Al-Khayri et al. (1992) observed that the addition of 15% coconut water to the culture medium increased callus growth, regenerative potential and bud growth in spinach (*Spinacia oleracea* L.).

The composition of coconut water, the liquid endosperm of coconut (*Cocos nucifera* L.), depends on factors such as the coconut variety, stage of maturity and cultivation practices (Prades et al. 2012). Coconut water presents a unique

chemical composition of sugars, vitamins, minerals, proteins, amino acids and phytohormones and it is extensively used as a growth-promoting supplement in plant tissue culture (Ma et al. 2008; Yong et al. 2009). Coconut water, contains zeatin cytokinin, normally used to induce plantlet regeneration from the callus in plant tissue culture by stimulating the division of mature plant cells (Yong et al. 2009). It also contains indole-3-acetic acid auxin (Ma et al. 2008) that has the function of patterned differentiation of cells in meristems and immature organs (Uphade et al. 2008) and the vitamin C, which prevents the oxidation and protects the calli and shoot tissues and vitamins that are water-

soluble and are required as coenzymes for enzymatic reactions essential for cellular function (Ma et al. 2008; Prades et al. 2012). The above could explain the organogenic calli obtained in the present study.

Morphological and ultrastructural differences were observed among the cells from different calli. Calli grown on the medium with BA and maintained in the light were characterized by the cells with both isodiametric (Fig. 4A) and elongated shape (Fig. 4B). In contrast, calli cultured on the medium with BA and maintained in the dark showed well-organized cell proliferation with an isodiametric structure (Fig. 4C).

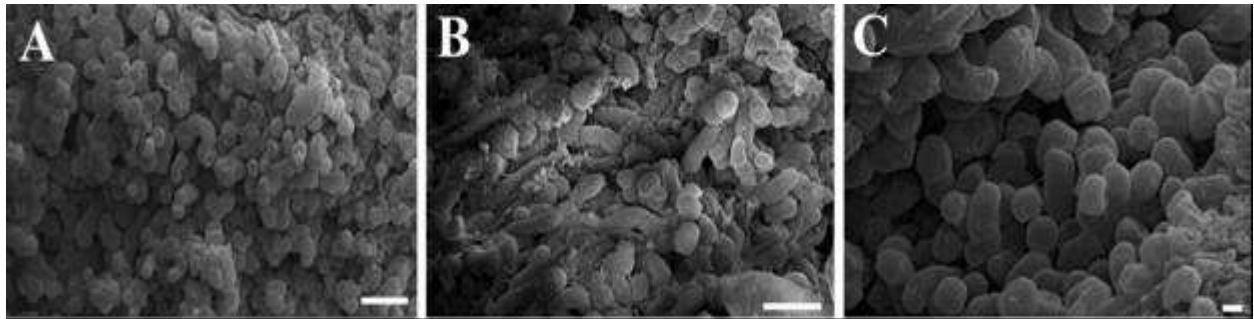


Figure 4 - Scanning electron micrographs of *Passiflora gibertii* calli grown in MS half strength salt concentrations medium with BA, and maintained in the light (A, B) and in the dark (C). Bars = 100 μ m (A, B), 20 μ m (C).

Studies using light microscopy (LM) and transmission electron microscopy (TEM) indicated that calli cells were dividing in an organized system in the calli cultured in the light (Fig. 5A and B) and in the dark (Fig. 6A and B). These calli also showed some common characteristics: a nucleus with prominent nucleoli (Figs. 5C and 6C), granular vacuole (Figs. 5D and F and 6C and D), dense cytoplasm rich in endoplasmic reticulum (Figs. 5C and D and 6C and F), starch grains (Figs. 5B and 5F and 6B and D), lipid bodies (Figs. 5D and F and 6F) and phenolic compounds (Figs. 5A and B and 6A, B and E). However, calli cultured in the light also had a cytoplasm rich in mitochondria and immature chloroplasts (Figs. 5C and E).

Deposition of phenolic compounds, such as tannins on calli cultivated in the light and on the dark is mainly found in the plant vacuoles (Parham and Kaustinen 1977) where they do not interfere with cellular metabolism unless an injury or

cellular death happen. Knowledge of the biochemical makeup of the *Passiflora* genus comes from mainly studies on *P. incarnata*, *P. edulis* and *P. alata* and phytochemical studies are generally scarce for this genus (Costa and Tupinambá 2005). The chemical groups most frequently reported are indole alkaloids, flavonoids, sterols, lignans and cyanoglucosides. Since there has been reports on the presence of phenols for the *Passiflora* genus and tannins are polyphenols, there could be a possibility that this metabolite was also present in *P. gibertii*.

The ultrastructural cell characteristics observed in the present study have also been reported by other authors. Meristemoids cells in *P. edulis* (Rocha et al. 2012) and *Bauhinia forficata* and *Glycine max* (Apezzato-da-Glória and Machado 2004) presented dense cytoplasm, numerous mitochondria, prominent nuclei, concentrically arranged rough endoplasmic reticulum and plastids containing starch grains.

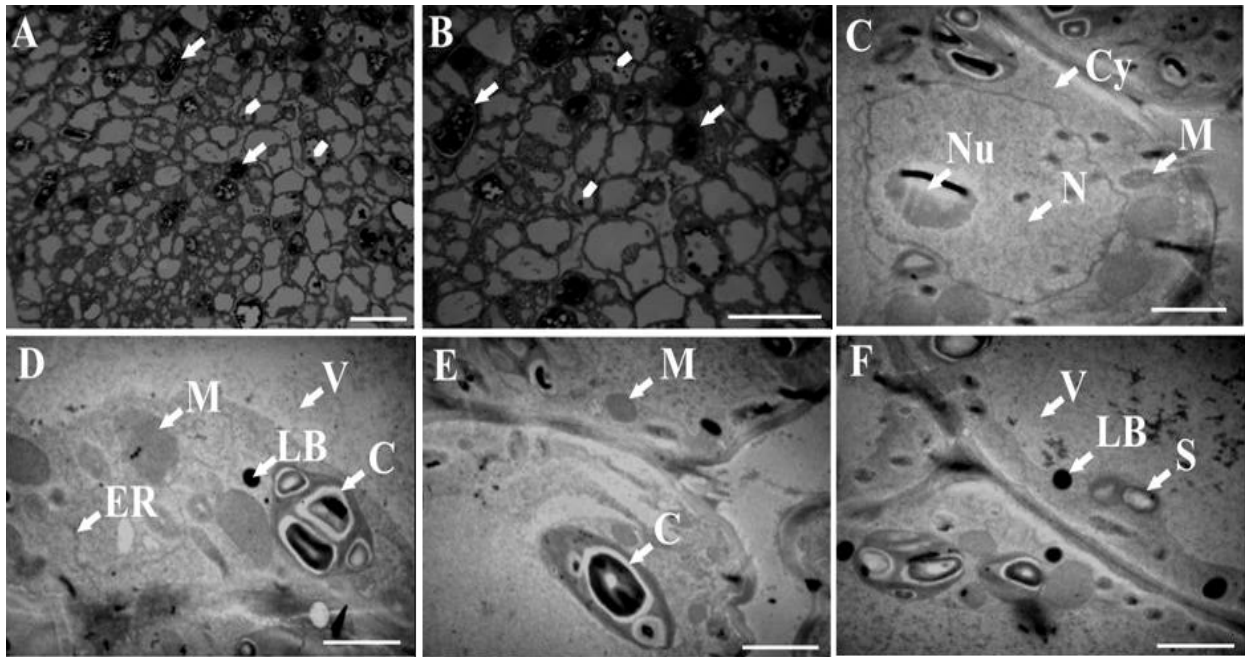


Figure 5 - Photomicrographs (A-B) and transmission electron micrographs (C-F) of *Passiflora gibertii* callus cells grown in a MS half strength salt concentrations medium with BA and maintained in the light. M = mitochondria, ER = endoplasmic reticulum, Cy = cytoplasm, V = vacuole, LB = lipid bodies, S = starch, N = nucleus, Nu = nucleolus, C = chloroplast. In A and B, cells with phenolic compound accumulation = arrows, starch grains = arrowheads. Bars = 50 μ m (A, B), 2 μ m (C, D, E, F).

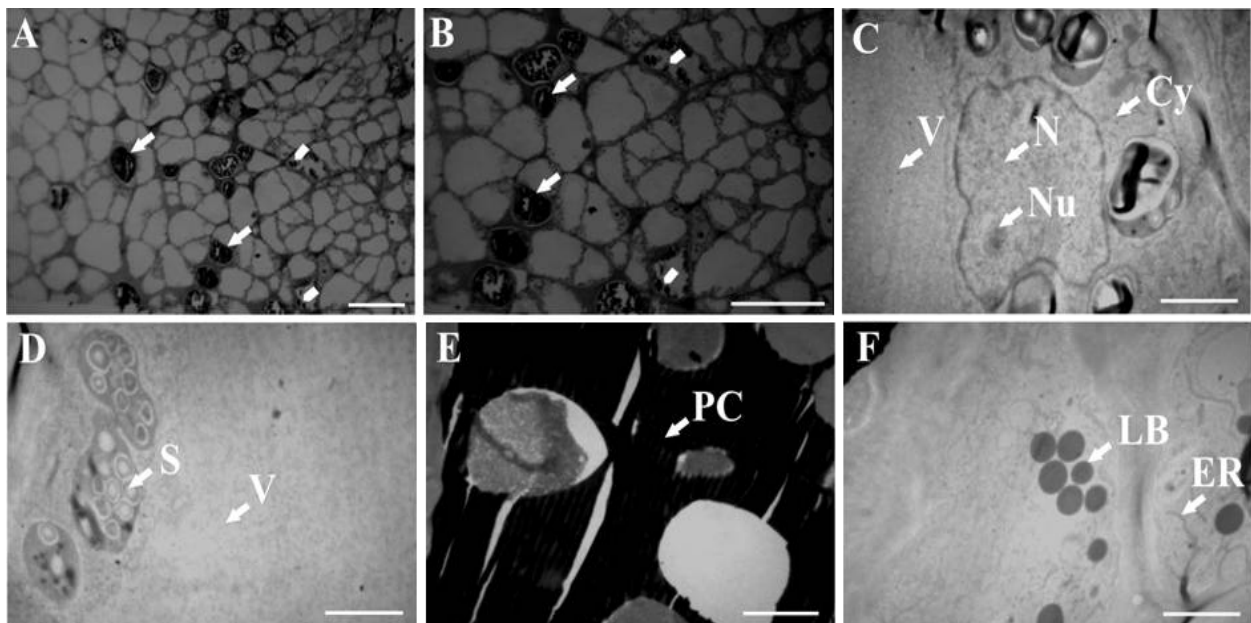


Figure 6 - Photomicrographs (A-B) and transmission electron micrographs (C-F) of *Passiflora gibertii* callus cells grown in a MS half strength salt concentrations medium with BA and maintained in the dark. ER = endoplasmic reticulum, Cy = cytoplasm, V = vacuole, LB = lipid bodies, S = starch, N = nucleus, Nu = nucleolus, PC = phenolic compounds. In A and B, cells with phenolic compound accumulation = arrows, starch grains = arrowheads. Bars = 50 μ m (A, B), 2 μ m (C, D, E, F).

The meristemoids of *B. forficata* and *G. max* did not originate from typical meristemoids cells, but rather from highly vacuolated cells (Apezzato-da-Glória and Machado 2004). Similar results were observed in the present study where cells with organogenic potential (Fig. 7A) were highly vacuolated with cytoplasm rich in organelles and starch grains (Figs. 5 and 6). These calli cells, formed in the dark and subsequently transferred to a MS half strength salt concentrations medium

with 2.22 μm BA maintained in the light, became organogenic (Fig. 7A), forming buds and foliar primordia after seven days (Fig. 7) in a new medium. The SEM results demonstrated morphological characterization of shoot buds based on emergence of the foliar primordia from a common axis (Figs. 7B, C and D) and not direct insertion of the base into the explants (isolated leaf structures).

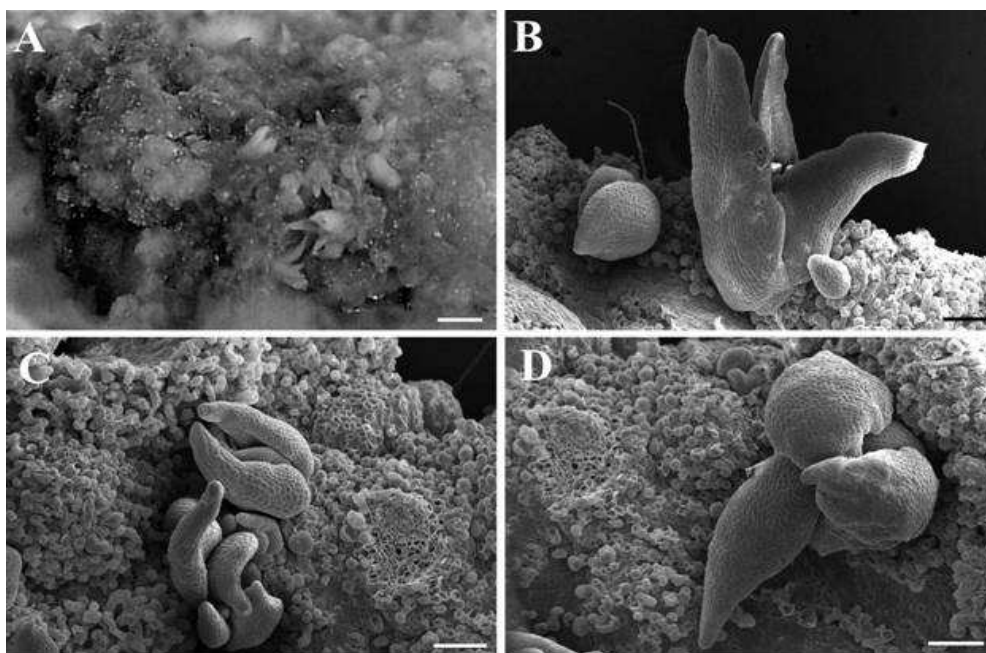


Figure 7 - Organogenic calli obtained from leaves of *Passiflora gibertii* grown in MS half strength salt concentrations medium with BA and maintained in the dark and seven days after transferring to new medium in the light. General aspect (A), scanning electron micrographs (B-D). Bars = 2 mm (A), 200 μm (B, C, D).

Apezzato-da-Glória et al. (2005) described SEM observations of *in vitro* structures that were essential for quick morphological characterization, allowing the distinction between bud and leaf primordia. According to these authors, in *Passiflora*, the formation of leaf structures was frequently misinterpreted as buds that did not elongate. Structural analysis of the *in vitro* organogenesis in *P. edulis* f. *flavicarpa* Deg. and *P. cincinnata* Mast. demonstrated the strong tendency of explants to form leaf primordial that was not observed in the present work. Apparently this organogenic path compromises the success of *in vitro* culture, because the presence of the shoot apical meristem is essential for shoot production.

CONCLUSIONS

Passiflora gibertii calli produced in the dark were more responsive to bud formation. In order to reduce the spending on *in vitro* culture, the most suitable induction medium for *P. gibertii* organogenesis could, therefore, be the MS half strength salt concentration medium maintained in the dark. However, the addition of coconut water to the culture medium was essential for both callus induction and bud formation.

The morphological and ultrastructural features of the calli with organogenic potential were isodiametric cells, organized cellular system, nucleus with prominent nucleoli, presence of

starch grains and dense cytoplasm rich in endoplasmic reticulum. The scanning electron microscopy demonstrated that the buds were present on these calli.

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