Cyto-histological Studies on Somatic Embryos of Coffee: Ultrastructural Aspects

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Abstract: A histological and ultrastructural study of somatic embryogenesis in coffee embryos was made by light microscopy and transmission electron microscopy. A comparative study on somatic and zygotic embryos of coffee was also performed. The cells in the suspensor region of somatic embryos were filled with large-and small-sized granules containing polyphenol substances stainable in black. The granule size increased until the elongated-embryo stage and thereafter it decreased with embryo development. Such granules were very small in cotedonary embryo cells. The continuous and discontinuous cuticular layers were detected on both the somatic, globular embryos and the zygotic proembryos by treatment of iodine/potassium iodide-sulfuric acid-silver proteinate. This preparation also permitted the identification of the deposition of fine silver particles inside the polyphenol granules. In addition the plastids were filled with several starch grains of large and small sizes. Similarly, cells of the zygotic proembryos and young embryos were also filled with polyphenol granules and plastids containing starch grains. It is interesting to note that mucilaginous materials cover the surface of both the embryogenic callus and the suspensor region of somatic embryos and are also present in the intercellular spaces of these tissues. These materials were also covering zygotic embryos situated in the embryo cavity of seeds. These observations led to the conclusion that the somatic and zygotic embryos have several similarities at the ultrastructural level.

Key words: Coffee, Embryogenesis, Light microscopy, Mucilaginous material, Polyphenol, Somatic embryo, Suspensor, Transmission electron microscopy.

Coffee is a stimulating beverage of worldwide importance. In terms of traded value on the international market, coffee is one of the most important agricultural commodities. Arabica coffee is the first type of coffee consumed and today still accounts for more than 70% of the world's coffee consumption due to its high quality as a beverage. Robusta coffee and liberica coffee are two other types of coffee occupying about 25% and 1% of the world market, respectively. Accordingly, arabica was mainly used in this study.

Somatic embryogenesis in coffee plants has been examined by light microscopy10,15,16,20,21, Histological studies on somatic embryogenesis have also been reported for some other dicotyledonous plants, especially in the case of tree species7,9,12,17,18,19. In herbaceous, dicotyledonous plants somatic embryogenesis has been studied by light and transmission electron microscopy1,2,6,22.

In the present work, the developmental processes of the somatic embryos of coffee are examined in detail by light and transmission electron microscopy and compared with zygotic embryos.

Materials and Methods

The embryogenic callus and somatic embryos originated from leaf explants of Coffea arabica cvs. Mundo Novo and Yellow Catuai,
and from an interspecific cross between *C. arabica* and *C. canephora* cv. Icatu were used as the experimental materials. Aseptic cultures were made using the previously described methods\(^{13,14}\). The zygotic proembryos of cv. Yellow Catuai and the embryos excised from the very young seeds were examined.

For light microscopy, the materials were removed from culture flasks and fixed in a mixed solution of 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 6 h at 20°C. The materials were rinsed in 0.2 M cacodylate buffer solution for 6 h at 20°C and postfixed in 2% osmium tetroxide buffered in 0.1 M cacodylate for 3 h at 4°C in the dark. They were then dehydrated in a graded ethanol series. The infiltration was made by resin (Epon 812) and finally they were polymerized. The semithin sections (0.5 μm) were cut with glass knives on a Reichert Ultracut-N ultramicrotome, stained with a freshly prepared solution of 0.1% toluidine blue in 2.5% Na2CO₃ (pH 11.3)\(^{23}\) and mounted in synthetic mountant. The specimens were examined and photographed on a Nikon light microscope, Optiphot.

For transmission electron microscopy, the procedure used for material preparation before microtoming was the same as above. The ultrathin sections (80 nm) were cut with a diamond knife on a Porter Blum MT2-B ultramicrotome. The sections mounted on grids were stained with aqueous uranyl acetate for 20 min followed by lead citrate (Katayama Chemical) for 10 min. The sections were examined and photographed on a Hitachi H-600 transmission electron microscope (TEM) operated at 100 kV.

For cuticle detection, the sections were mounted on gold grids, stained with 1:1 mixture of aqueous 0.5% I₂ plus 1.5% KI solution and 60% H₂SO₄ for 20 to 30 min. After being rinsed in distilled water, the materials were washed in 10% Na₂S₂O₃ aqueous solution and next in distilled water and followed by 1% silver proteinate aqueous solution for 30 min in the dark. This is the iodine/potassium iodide-sulfuric acid-silver proteinate, I₂Kl-H₂SO₄-AgP method\(^{41}\). The sections were examined and photographed on a Hitachi H 600 TEM apparatus operated at 100 kV.

**Results**

The embryogenic callus was easy to distinguish with the naked eye because of its friable yellowish appearance, by contrast with the dark brown colour of the non-embryogenic callus\(^{39}\). For a detailed study of embryogenesis, several developmental stages of somatic embryos were defined as the previous paper\(^{40}\): somatic single cell, early globular embryo, globular embryo, elongated embryo, early heart embryo, heart embryo, torpedo embryo and cotyledonary embryo.

1. **Light microscopy**

In Fig. 1, embryogenic callus composed of cytoplasmic rich, meristematic cells is observed at the somatic single cell stage. These cells with high mitotic activity are interpreted as presumptive embryo mother cells. An early globular embryo of a spherical shape and actively dividing cells of callus are clearly seen in Fig. 2. Although the callus cells are covered with mucilaginous materials (arrow), the surface of the globular embryo is clear. A typical globular embryo with conspicuous suspensor is demonstrated in Fig. 3. The embryo cells are somewhat enlarged and different from the callus cells with densely stained cytoplasm. An elongated-shape embryo with suspensor is shown in Fig. 4. The superficial cells are mostly arranged as a row. An early heart-shaped embryo with suspensor is illustrated in Fig. 5. A high magnification of the suspensor region shown in this figure clearly indicates that the embryo cells are more vacuolated than the cells on the callus side (Fig. 6).

A conspicuous suspensor of a torpedo embryo and cotyledon primordia are noted in Fig. 7 where a difference in cytoplasm density is evident among the embryo proper, suspensor and callus, and in addition, the initiation of a procambium (asterisk) in the center is seen along with the meristem (arrow) situated at the joint of two cotyledons. A high magnification view of the suspensor region in Fig. 7 indicates the presence of initial cells leading to the secondary embryo development from the suspensor region of primary embryos (Fig. 8). The border between the secondary embryo and the parent tissue is clearly discernible (arrows). Mucilaginous materials are seen on the surface of the suspensor region and secondary embryo, and further, these materials are
present in the intercellular spaces of the suspensor. A number of electron dense granules are strikingly present in cells of the suspensor and the basal region in embryo proper. They are often seen around the nuclei.

2. Transmission electron microscopy

At the top area of globular embryo, the superficial cells already have a thicker, outer compact cell wall than the anticlinal, inner wall (Fig. 9). The cells have vacuolar compartments, electron dense granules containing putative polyphenol substances and plastids with small starch grains. The cuticular layer is clearly detected on the superficial cells when treated with iodine/potassium iodide-sulfuric acid-silver proteinate (Fig. 10). The positive, strong response by the cuticular membrane is present on the outermost layer in the wall. It is seen the densely stained granules, despite of blurred images of other constitutes due to this staining procedure without uranyl and lead ions. In a high magnification view of the large-sized granules containing polyphenol substances, a strikingly positive reaction is visible as numerous deposits of fine silver particles upon the faintly stained matrix (Fig. 11). This surely proves that the granules are not homogeneous but complicated in structure, containing numerous fine particles that are evenly distributed.

Explanation of Figures

Figs. 1 to 8, 18 and 19 are light micrographs (LM). Figs. 9 to 17 are transmission electron micrographs (TEM). Figs. 1, 2, 4 to 6, 12 and 13 = cv. Icatu; Figs. 3, 9 to 11 and 14 to 19 = Catuai Amarelo; Figs. 7 and 8 = M undo Novo.

Fig. 1. The friable embryogenic callus section showing aggregation of meristematic cells (bar : 100 μm).

Fig. 2. Early globular stage of embryo (bar : 50 μm).

Fig. 3. Longitudinal section of typical globular stage of embryo (bar : 100 μm).

Fig. 4. An elongated embryo (bar : 100 μm).

Fig. 5. Early heart-shaped stage of embryo with suspensor (bar : 100 μm).

Fig. 6. High magnification of suspensor region shown in Fig. 5 (bar : 100 μm).

Fig. 7. Longitudinal section of torpedo-shaped embryo (bar : 100 μm).

Fig. 8. High magnification of suspensor region shown in Fig. 7 (bar : 50 μm).

Fig. 9. TEM micrograph of epidermal cells of globular embryo (bar : 5 μm).

Fig. 10. The positive response in the outermost layer of epidermal cell wall of globular embryo, which treated with the iodine/potassium iodide-sulfuric acid-silver proteinate (bar : 5 μm).

Fig. 11. High magnification of black stained granules containing polyphenol substances treated with silver proteinate (bar : 1 μm).

Fig. 12. A montage figure showing completely different cell constellation between suspensor and embryo cells at early heart stage (bar : 10 μm).

Fig. 13. Suspensor basal region of elongated embryo (bar : 10 μm).

Fig. 14. An anticlinal division of the epidermal cells of torpedo-shaped embryo (bar : 5 μm).

Fig. 15. Inner part of embryonic axis in zygotic embryo showing the plastids with small starch grains and granules containing polyphenol substances (bar : 5 μm).

Fig. 16. Epidermal cells at the bottom side of zygotic proembryo treated with silver proteinate method (bar : 5 μm).

Fig. 17. The formation of continuous cuticular layer on the epidermal cells of zygotic proembryo when treated with silver proteinate (bar : 5 μm).

Fig. 18. LM of immature zygotic proembryo (bar : 100 μm).

Fig. 19. LM of the basal end of zygotic embryonic axis (bar : 100 μm).

Abbreviations used in Figures

The suspensor cells of elongated embryo are filled with a great quantity of the large-sized granules and with a few small-sized granules, both of which contain polyphenol substances (Fig. 13). In addition, plastsids with large starch grains and mitochondria are also seen here. The compound starch grains are occupying most of the matrix of plastids, as if they are amyloplast in appearance. This figure further indicates a row arrangement of the superficial cells, the contents of which are similar to that of inner cells. The outer wall of the superficial cells is covered with mucilaginous materials which are gradually thinner upward. The materials are loosely stratiform with furry components, and envelop densely stained, amorphous bodies (arrows).

A montage figure of an early heart-stage embryo, which extends over two pages, represents a probable demarcation between embryo proper and suspensor (Fig. 12 arrows). Both areas are different in the constituents of the cells. In the embryo side (the upper part in the figure), the plastids are filled with rather small starch grains and the remaining rudimentary matrix. The suspensor side has plastids full of large starch grains. In addition, the cells at the embryo side have a number of small-sized and a few large-sized granules containing polyphenol substances. The suspensor side have rather few and small polyphenol granules. The size of these granules at this stage is generally smaller than at the elongated-embryo stage. In both sides, however, vacuole compartments are present in the same way, and nuclei have a nucleolus and a little amount of heterochromatin along the nuclear envelope.

At the torpedo stage, cell division is still present in the epidermal cells with thicker, outer walls (Fig. 14). Condensed chromosomes arranged in an anticlinal plane are seen to hold the activity of cell division. Starch grains are slightly enlarged in the plastids and large polyphenol granules are present.

3. Zygotic embryos
In the light microscopic examination of zygotic proembryos, meristematic cells are observed on the basal and central portions of the embryo (Fig. 18). In the TEM observation of the proembryo treated with silver proteinate, a discontinuous formation of cuticular layer is seen on the outer surface of epidermal cell wall (Fig. 16). The cuticular layer is not clear on the sites indicated by double arrows. In another region, it is ascertained the formation of a continuous cuticular layer in the wall (Fig. 17). In the light microscopic examination, it is revealed that the cells at the basal end of the zygotic young embryo axis are meristematic, but the cells of inner region of this axis are slightly elongated (Fig. 19). In the TEM observation of the inner region of axis, it is found the presence of black stainable granules containing polyphenol substances and of plastids which have small starch grains and a fair-sized matrix (Fig. 15). Mucilaginous materials were also seen over zygotic embryos in the embryo cavity. These figures could verify the similarities of zygotic embryos to the results from somatic embryos.

Discussion
1. Histological studies
Development of proembryo-like cell group and globular embryos from callus tissues of *C. canephora* were studied by light micrographs. It was found that the somatic embryos having a suspensor with a width of 1 or 2 cells are originating from the periphery of the callus. The fact that new secondary embryos from the primary somatic embryos of *C. canephora* had the broad suspensor might indicate the multicellular origin of secondary embryos. Furthermore, a study of the somatic embryos of *C. arabica* has demonstrated the development of early globular embryos near the periphery of the callus mass. Our results are indicative of the same events in the formation of primary somatic embryos with suspensor-like connections and in the secondary embryo development from the suspensor region of the primary embryos. Our figures are consistent with a scanning electron micrograph related to the multicellular suspensor of somatic embryo in *C. arabica*.

It has been demonstrated in *Theobroma cacao* that globular embryo, heart-shaped embryo and torpedo-shaped embryo with cotyledonary leaves arise from immature zygotic embryo explants. The somatic embryo development of cacao is very similar to our histological results for coffee somatic embryos, especially in the case of the heart-shaped embryo with suspensor.

In somatic embryogenesis from immature embryos of *Persea americana*, Mooney and Van
Staden have shown a rudimentary suspensor in a heart-shaped embryo, and initiation of procambium in early torpedo-shaped embryo. Globular embryo, heart-shaped embryo and torpedo-shaped embryo were demonstrated in somatic embryogenesis of *Aesculus hippocastanum*. They also reported similarities between zygotic embryos and somatic embryos through histological analysis.

Somatic embryos developed directly from embryogenic cell masses derived from anthers, ovaries, and ovules of *Vitis longis*. It was also demonstrated that embryogenic callus cells contain both starch and darkly staining lipid-like materials, whereas cells of embryos and suspensors have only the latter. Many globular embryos arose from the explant surface of the immature cotyledon of *Glycine max*. Somatic embryogenesis from hypocotyl of *Gossypium hirsutum* represented globular proembryos with suspensor devoid of filiform apparatus.

In our studies, somatic embryogenesis of *C. arabica*, which was already studied by scanning electron microscopy, is precisely traced at the histological aspect. It is noteworthy that with the development of globular embryos to elongated embryos, the superficial cells are arranged as a row, resulting in epidermal appearances.

2. Ultrastructural studies

As with many other tropical trees, coffee plants contain a wide variety of phenolic compounds, sometimes at very high concentrations. Several enzymes oxidize phenols to quinones. It has been known that the callus browning of coffee cultures is due to the formation of these quinones. Since phenols can easily cross link with proteins, phenolic compounds are generally located on the peculiar sites in cells. Localization of these compounds was demonstrated in idioblastic tannin cells of *Sambucus* shoots.

In our TEM micrographs, the black stainable granules were found remarkably in the cells at and near the suspensor region of coffee somatic embryos. The variation in granule distribution was seen during embryo development and between embryo tissues studied. The granules greatly increased in number and size at the elongated-embryo stage. At the early heart stage, they decreased slightly, and the embryo side had several large and small granules but the suspensor side had rather few and small granules. The contents of the granules could surely be interpreted as polyphenol substances because of a great amount of polyphenol compounds in coffee plants.

In addition, osmium tetroxide is a fixative which reacts particularly with lipoproteins, resulting in blacking of the tissues at the attachment sites. The postfixation with osmium tetroxide might cause the blacking of tissue, based upon the lipid-like, amorphous granules inside the cells. In coffee cells, however, along with the lipid granules, there are numerous granules which are not homogeneous and have a number of very small particles stainable with the silver proteinate method. The fine silver particles were evenly distributed in the granules. The principal contents of these granules with complicated structure are probably characterized as polyphenol substances.

3. Cuticle formation and deposition of silver particles.

The aerial parts of plants are covered by a cuticle layer which consists of a polymer of cutin embedded in waxes. The cuticular membranes of several plant species show a deposit of silver particles by the treatment of an acidic solution of iodide ions followed by silver proteinate solution. In our study, deposits of silver particles were observed in the outermost layer of both the somatic and zygotic embryos. This result is indicative of the presence of the cuticular layer over the embryo surface. However, some regions show discontinuity of the cuticular layer in both cases. This is suggestive of the progressive formation of a cuticle resulting in a continuous layer.

On the other aspect, it is very interesting to note the presence of mucilaginous materials. These materials are seen on the surface of callus and suspensor of somatic embryos but never on the area on which a cuticular layer is already formed. This result allows us to lead a conclusion that the transformation of mucilaginous materials into cuticular membranes is one of the key reactions to induce the somatic embryos from callus tissues.

In our ultrastructural detection using the silver proteinate method, besides the cuticular layer, fine particles were found in the polyphenol-containing granules. This suggests
a curious localization of polyphenol metabolism. On the other hand, a detailed ultrastructural comparison of somatic embryos with zygotic embryos is requested to more precisely analyse the cyto-histological nature of somatic embryos in *C. arabica*.

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**References**


