



Comparative ontogenesis of *Coffea arabica* L. somatic embryos reveals the efficiency of regeneration modulated by the explant source and the embryogenesis pathway

Ilse Fernanda Ferrari^{1,2,3} · Giovanna Arcolini Marques² · Wellington Luis Sachetti Junior² · Bárbara Bort Biazotti² · Matheus Pena Passos² · Julieta Andrea Silva de Almeida⁴ · Jorge Maurício Costa Mondego¹ · Juliana Lischka Sampaio Mayer²

Received: 25 January 2021 / Accepted: 17 May 2021 / Published online: 21 June 2021 / Editor: Yong Eui Choi
© The Society for In Vitro Biology 2021

Abstract

In vitro micropropagation is an important technique for coffee multiplication performed by somatic embryogenesis, which can be indirect, with the formation of calluses, or direct, when embryos are formed directly from explant cells. In the present study, we characterized the ontogenesis of somatic embryos regenerated via indirect and direct somatic embryogenesis in leaf explants from *Coffea arabica* ‘Mundo Novo’ and assessed the development of these embryos in leaf explants from adult plants maintained *ex situ* and *in vitro*. Anatomical analyses showed that leaf explants present structural differences depending on their origin. In the direct pathway, tissue develops more rapidly in explants from *in vitro* plants than in those from *ex situ* explants. In both methods, the formation of a pro-embryogenic mass was found to be essential for embryo formation. In the indirect pathway, calluses from *ex situ* material presented elongated cells and a loose external appearance. Conversely, calluses from *in vitro* material presented large regions of meristematic cells with a single large nucleus and dense cytoplasm. We propose that indirect somatic embryogenesis in *in vitro*-grown explants may accelerate genetic breeding in coffee, and it represents the most efficient condition for somatic embryogenesis, producing embryos in less time than the required by *ex situ* plants (indirect: 260 d in *ex situ* conditions, 62 d *in vitro*; direct: 270 d *ex situ*, 86 d *in vitro*).

Keywords Anatomy · Callus · Micropropagation · Plant growth regulators · Pro-embryogenic mass

Key message Ontogenesis of somatic embryos from *Coffea arabica* L. showed that indirect somatic embryogenesis in leaves from *in vitro*-grown plants produces embryos in less time, reducing tissue culture time.

✉ Juliana Lischka Sampaio Mayer
mjimayer@yahoo.com.br

Ilse Fernanda Ferrari
ilsefferrari@gmail.com

Giovanna Arcolini Marques
giovannaarcolini.m@gmail.com

Wellington Luis Sachetti Junior
wellington.sachetti.jr@gmail.com

Bárbara Bort Biazotti
barbara.bio08@gmail.com

Matheus Pena Passos
matheusp.pena@gmail.com

Julieta Andrea Silva de Almeida
julietasa@iac.sp.gov.br

Jorge Maurício Costa Mondego
jmcmondego@iac.sp.gov.br

- 1 Centro de Pesquisa e Desenvolvimento de Recursos Genéticos Vegetais, Instituto Agrônomo, Campinas, São Paulo, Brazil
- 2 Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil
- 3 Programa de Pós-graduação em Genética e Biologia Molecular Universidade Estadual de Campinas, UNICAMP, Campinas, São Paulo, Brazil
- 4 Centro de Café Alcides Carvalho, Instituto Agrônomo, Campinas, São Paulo, Brazil

Introduction

Micropropagation is an important method of multiplication that can maximize *in vitro* propagation of coffee for elite and hybrid cultivars from breeding programs (Rezende *et al.* 2012; Ahmed *et al.* 2013; Almeida *et al.* 2014). Micropropagation can be accomplished through somatic embryogenesis (SE), a process that produces significant quantities of plants that are genetically identical to the plant from which the explant was taken. This technique allows seedling production independently of climatic conditions, in reduced space and time, and it also enables a high degree of automation (Gomes *et al.* 2016). SE is based on the concept of plant cell totipotency, as already differentiated cells undergo dedifferentiation and acquire embryogenic competence when under plant growth regulators, nutrients, and other particular conditions (Xu and Huang 2014; Mendez-Hernandez *et al.* 2019; Gulzar *et al.* 2020). This process involves events similar to those that occur during zygotic embryogenesis, and can be used for most species under laboratory conditions (Gaj 2004); thus, it is an important approach for studying the development of plant embryos (Quiroz-Figueroa *et al.* 2002a).

Initially, explant cells are induced to acquire embryogenic characteristics, followed by the expression of a somatic embryo (Jiménez 2005), which is a bipolar structure disconnected from the original vascular tissue, which has a unicellular or multicellular origin (Carman 1990; Dodeman *et al.* 1997; von Arnold *et al.* 2002; Fehér *et al.* 2003; Quiroz-Figueroa *et al.* 2006). SE may occur indirectly (indirect somatic embryogenesis, ISE) or directly (direct somatic embryogenesis, DSE) (Williams and Maheswaran 1986; Almeida 2020). ISE consists of two phases: the first involves re-determination of differentiated cells, followed by division and proliferation leading to the development of a cell mass called a callus (Michaux-Ferriere 1996; Almeida *et al.* 2008; Berthouly and Rezende *et al.* 2012). 2,4-Dichlorophenoxyacetic acid (auxin 2,4-D) and the cytokinin 6-furfurylaminopurin (kinetin—KIN) are commonly used in the induction of callogenesis, as they play important roles in the cell cycle, cell division, and differentiation, thus acting as efficient plant growth regulators (Söndahl and Sharp 1977; Gulzar *et al.* 2020). The second stage in ISE is related to the initiation and development of somatic embryos from certain callus cells (Berthouly and Michaux-Ferriere 1996; Santana-Buzzy *et al.* 2007; Almeida *et al.* 2008). To induce the formation of somatic embryos from stone cells, the combination of naphthalene acetic acid (NAA) and KIN is applied (Söndahl and Sharp 1977). In DSE, embryos are formed directly from explant cells that are competent and determined to an embryogenic development, without the need of callus formation (; Dublin 1981; Yasuda *et al.* 1985; Emons 1994; Motoike *et al.* 2007; Vasconcellos *et al.* 2009; Almeida *et al.* 2016). The direct pathway occurs in a single phase, which is induced by a cytokinin, such as 2-

isopentenyl adenine (2-iP), and somatic embryos are formed without auxin addition (Alves *et al.* 2018; Almeida 2020).

SE has been successfully applied to *Coffea arabica* L.; however, some genotypes respond to either the indirect or the direct method, while others respond to both (Menéndez-Yuffá and De García 1997; Quiroz-Figueroa *et al.* 2002a). ISE is usually notable for generating a greater number of somatic embryos in *C. arabica* than DSE does (Vieira and Kobayashi 2000). The pattern of somatic embryo development shares many morphological characteristics with zygotic embryo development. The various embryonic stages of development, from the globular stage to heart and then torpedo, are morphologically and anatomically similar to related formations in *Coffea canephora* zygotic embryogenesis (Moens 1965; Almeida 2020).

Leaf tissues are usually used to generate embryos (Clarindo *et al.* 2012; Landey *et al.* 2013; Silva *et al.* 2014). However, genetic engineering studies have reported that low viability of embryonic tissues and low transformation efficiency of this tissue type are major limitations in the genetic transformation of *Coffea canephora* (cvs. Congensis x Robusta hybrid—Mishra *et al.* 2010) and *Coffea arabica* (cvs. Caturra and Catuaí—Gatica-Arias *et al.* 2008). Thus, further studies are necessary to provide a clear distinction between embryogenic and non-embryogenic tissues, to demonstrate the most suitable method for obtaining embryos with high cell viability.

We aimed to characterize the ontogenesis of somatic embryos regenerated via DSE and ISE from leaf explants of *C. arabica* ‘Mundo Novo’, from plants grown both *in vitro* and established in the field (*ex situ*). Comparisons of the indirect and the direct pathways of SE showed that ISE develops embryos in less time, besides *in vitro* explants being more efficient than *ex situ* explants also considering time. Both callus (ISE) and pro-embryogenic mass (DSE) originate from mitotically active cells present in the spongy parenchyma, near vascular bundles, while epidermal tissue and palisade parenchyma remained without anatomical changes. Calluses (ISE) from *in vitro*-grown plants exhibited large regions of meristematic cells with organized cell division, a single large nucleus, and dense cytoplasm.

Materials and Methods

Plant Materials The *Coffea arabica* ‘Mundo Novo’ cultivar is one of the most cultivated in Brazil, with a high capacity to adapt to different regions. In addition, this cultivar has excellent agronomic characteristics such as tall size, red fruits, and medium ripeness; seeds with a sieve between 16 and 17; and also great drink quality (Carvalho *et al.* 2006; Carvalho 2008).

Expanded leaves from the third pair of leaves from field plants (*ex situ*) were obtained from *C. arabica* ‘Mundo Novo’, which were maintained and produced in the experimental area

of Santa Eliza Farm, IAC, Campinas, SP (-22.873942" S 47.0777908" W). The climate of the region is Cwa according to Köppen classification, and the soil classified as clay latosol. The leaves were collected under 20°C average temperature, a dew point of 17°C, 20.4 km h⁻¹ SE winds, practically cloudless, relative humidity of 60.4%, visibility of 5 km, an atmospheric pressure of 1020 mbar, and without the occurrence of precipitation. Part of these leaves was directly applied as a source of *ex situ* explants in experiments. Another part of these leaves (*ex situ*) was applied to obtaining *in vitro* explants from 4-mo-old seedlings produced via direct somatic embryogenesis, maintained under a light intensity of 45 μmol m⁻² s⁻¹ and a photoperiod of 12-h light, at 25 ± 2°C. The leaves used as explant source from *ex situ* origin had an average size of 15 cm long by 7 cm wide. On the other hand, leaves from *in vitro*-developed plants had lower dimensions: varying between 1.6 and 2.0 cm long and 0.9 and 1.2 cm wide.

Somatic Embryogenesis in *Coffea arabica* Young leaves from plants grown under field conditions (*ex situ*; the leaf was donated by a productive 12-yr-old adult plant) were disinfected by washing in detergent solution; rinsing in running water, and then in sodium hypochlorite solution (2%) for 25 min; and rinsing three times in autoclaved distilled water. The disinfected leaves were kept in a humid chamber (80% humidity) for 24 h and then exposed to sodium hypochlorite solution (2%) (Ramos *et al.* 1993). These leaves were then cut in a laminar flow cabinet to remove the midrib and edges and obtain explants with 1 cm², which were inoculated with their adaxial side in contact with the culture medium and maintained in the dark at 25 ± 2°C, until embryo formation, when only the embryos were transferred to germination-inducing culture medium under light and at 25 ± 2°C.

In vitro plants were maintained on a medium developed by Murashige and Skoog (1962) with half the concentration of macronutrients and micronutrients (half-strength MS), until they developed three pairs of young leaves, under a light intensity of 45 μmol m⁻² s⁻¹ and a photoperiod of 12-h light at 25 ± 2°C. Leaves were used to obtain explants in a laminar flow cabinet without the need for asepsis. Explants with 1 cm² were inoculated directly *in vitro*.

Materials collected under *in vitro* and *ex situ* conditions were subjected to ISE and DSE. To induce ISE according to Almeida *et al.* (2007, 2008), two culture media were used. First, embryogenic callus formation was induced in MS medium with the addition of sucrose (30 g L⁻¹), 2,4-D (2.5 μM), and KIN (5 μM), maintained for 50 d. Then, embryos were induced using half-strength MS medium with sucrose (20 g L⁻¹), NAA (0.5 μM), and KIN (2.5 μM). Undeveloped calluses, dark in color, and without friable appearance were discarded and not analyzed.

For DSE induction according to Ramos *et al.* (1993), a half-strength MS medium was used with sucrose (20 g L⁻¹)

and isopentenyl adenine (2-iP) (10 μM). All culture media were solidified via the addition of Vetec® agar (5 g L⁻¹; Merck KGaA®, Darmstadt, Germany), and the pH was adjusted to 5.8 using NaOH or 0.1 N HCl prior to autoclaving at 121°C and 1.5 atm for 20 min.

In both indirect and direct somatic embryogenesis treatments, the explants were individually inoculated into clear glass vials (100 mL) containing 30 mL of culture medium. Each treatment consisted of 100 repetitions with one explant in each vial. In the second phase of indirect somatic embryogenesis, callogenesis, the calluses that developed over 20 mm in size were fragmented into 2 or 3 parts. Every callus fragment was also individually transferred to flasks (100 mL) containing embryogenesis induction culture medium.

Besides, a second experiment was installed to characterize the sequence of the main morphological events occurring in DSE and ISE in plants grown in the field, also quantifying the somatic embryos formed in each SE pathway and evaluating the size of calluses and embryogenic structures formed. For this, *ex situ*-cultivated leaf explants were subjected to the same cultivation conditions described above and maintained at 25°C, ISE treatment in the absence of light, and DSE in the presence of light. Each treatment consisted of fifteen repetitions with an explant in each bottle. The explants were evaluated monthly, regarding the estimated size of calluses or embryogenic structures and the number of somatic embryos formed.

Morphological and Anatomical Analyses Analyses were performed using leaf explant samples collected at the time of *in vitro* inoculation (control), and different developmental stages 2, 8, 12, 16, 18, 28, 62, and 72 d after inoculation in the culture medium. Fully developed somatic embryos were collected (cotyledonary stage). For the anatomical analyses, samples were fixed in FAA 50 solution (formaldehyde, acetic acid, and 50% ethanol, 5:5:90) (Johansen 1940). On each sampling date, five explants under each treatment were collected for morphological and anatomical analyses. A vacuum pump was then used to remove the air contained in tissues, and the samples were dried in an ethanol series and embedded in plastic resin (Leica HistoResin®, Leica Microsystems®, Wetzlar, Germany) according to the manufacturer's instructions. The samples were sectioned in a manual rotary microtome (Leica Microsystems®) with a C-type knife. Sections (5 μm thick) were stained with toluidine blue 0.05% (Sakai 1973) in phosphate and citrate buffer, pH 4.5 (McIlvaine 1921), and mounted in Entellan® (Merck KGaA®, Darmstadt, Germany) synthetic resin. Different histochemical tests were performed: Lugol reagent to highlight the presence of starch (Berlyn *et al.* 1976); xyloidine Ponceau reagent for proteins (Vidal 1969); ferric chloride 3% reagent for phenolic compounds (Johansen 1940); ruthenium red for mucilage (Gregory and Baas 1989); Nadi reaction for terpenoids (David and Carde 1964), and Sudan IV for lipids

(Pearse 1985). Images were captured with an Olympus DP71 video camera (Olympus Corporation®, Tokyo, Japan) connected to an Olympus BX 51 microscope (Olympus Corporation®).

For scanning electron microscopy (SEM), the botanical material was fixed in FAA 50 solution, dehydrated in an ethanol series, and subjected to critical point dried with CO₂ in a Balzers model CPD 030 equipment. Then, the material was mounted on metal supports and coated with colloidal gold for 220 s in a Bal-Tec SCD model 050 equipment. Samples were analyzed and micrographs were recorded a scanning electron microscope model JSM 5800LV (Jeol®, Peabody, MA) operated at 10 kV, at the Institute of Biology, UNICAMP, SP.

Results

Anatomical Analysis of Embryogenesis Stages During DSE and ISE A pro-embryogenic mass in DSE (Fig. 1A) and a callus in ISE (Fig. 1D) first emerged at the extremity of the vascular

bundle in contact with the culture medium. The pro-embryogenic mass in DSE had a compact appearance and a smooth surface (Figs. 1B, C and 2A). Embryos at different stages of development (globular, heart, and cotyledonary stages) have apparently emerged in a restricted manner in areas within the pro-embryogenic mass (Figs. 1B, C and 2C, E, G).

The developing callus in ISE had many elongated and disorganized cells, with large intercellular spaces, and it was partially covered by a secretion (Figs. 1E and 2B). Embryos originated exclusively from callus cells (Figs. 1F and 2D), and they could be observed at various stages of development (globular, heart, torpedo, and cotyledonary stages) in the same callus region (Figs. 1H, I and 2D, F). Suspensors were detected in both DSE and ISE, connecting the embryos to the pro-embryogenic mass or callus, respectively (Fig. 1C, G). Non-embryogenic calluses had cells in a less compact arrangement, opaque aspect, and larger cells when compared to embryogenic calluses (Fig. 2H).

Anatomical Comparison of *In Vitro* and *Ex Situ* Explants Anatomical analyses revealed structural differences between

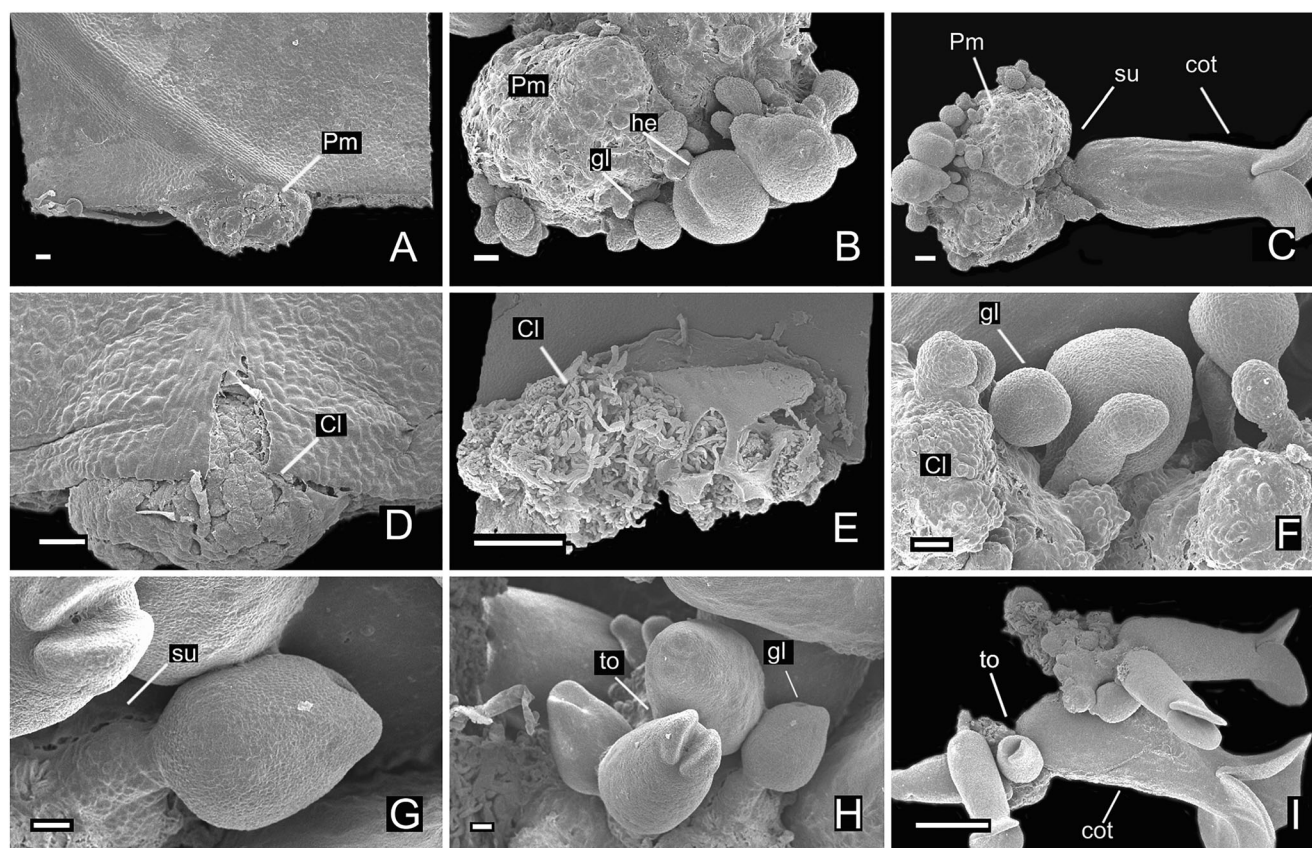
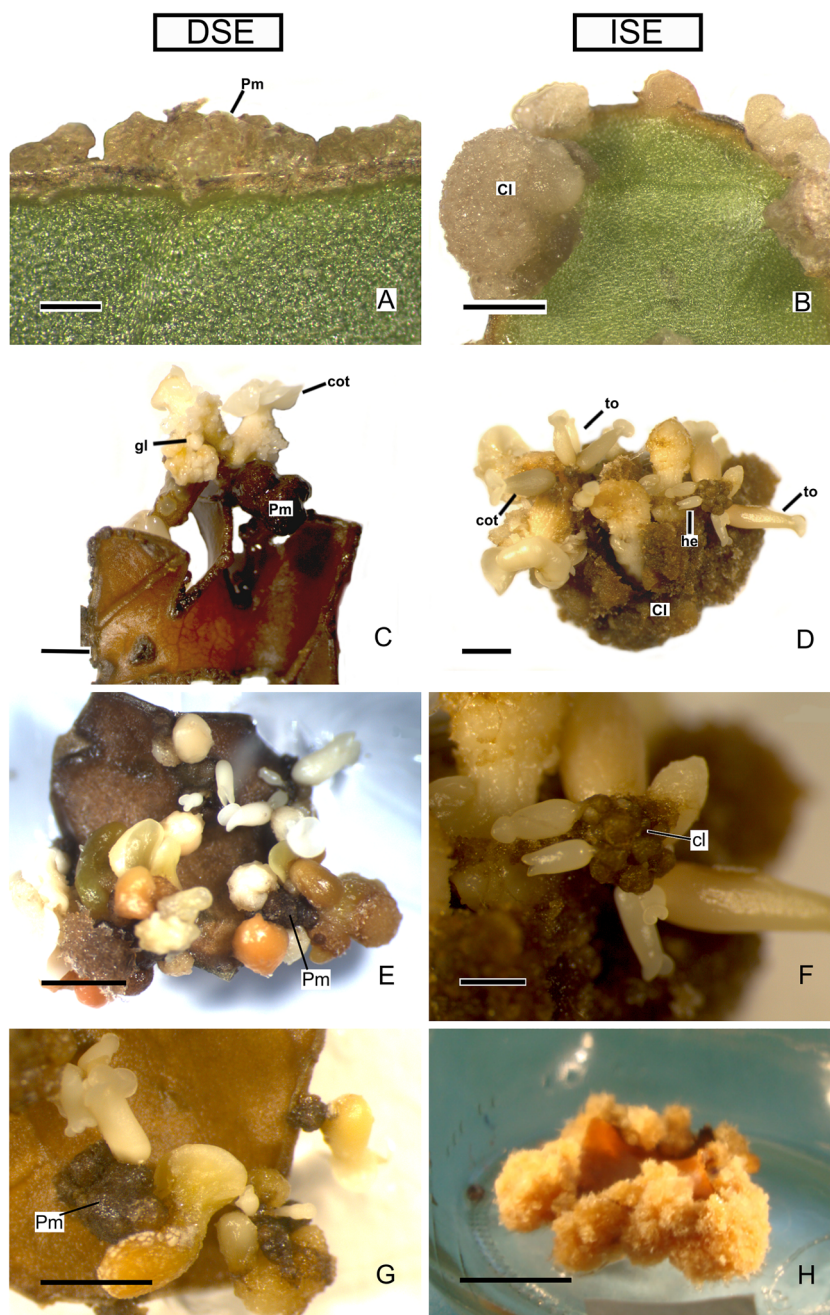


Figure 1. Scanning electron micrograph of somatic embryogenesis in *Coffea arabica* 'Mundo Novo'. (A–C) Direct somatic embryogenesis; (D–I) indirect somatic embryogenesis. (A) Beginning of pro-embryogenic mass (Pm) formation. (B) Well-developed pro-embryogenic mass, with embryos in the globular (gl) and heart (he) stages. (C) Embryo in the cotyledonary stage (cot), connected to the pro-embryogenic mass

by a suspensor (su). (D) Beginning of callus (Cl) formation. (E) Well-developed callus. (F) Embryos in the globular stage (gl). (G) Suspensor (su) detailed, a structure that connects the embryo to the callus. (H) Embryos in the globular (gl) and torpedo (to) stages. (I) Somatic embryos in the torpedo (to) and cotyledonary (cot) stages. Bars: A–D, F–H = 100 μm; E and I = 1 mm.

FIGURE 2. Somatic embryogenesis in *Coffea arabica* ‘Mundo Novo’. (A, C, E, G) Direct somatic embryogenesis (DSE). (B, D, F, H) Indirect somatic embryogenesis (ISE). (A, B, D–G) Explant of *in vitro*-grown plants, (C, H) explant of *ex situ*-grown plants. (A) Presence of a pro-embryogenic mass (Pm) at the extremity of the explant. (B) Well-developed callus on the edge of an explant. (C, E, G) Somatic embryos at different stages of development: globular (gl) and cotyledonary (cot) stages connected to the pro-embryogenic mass. (D, F) Embryos at heart (he), torpedo (to), and cotyledonary (cot) stages connected to a callus. (H) Non-embryogenic callus is soft and lacks the potential for somatic embryo formation. Bars: A, B = 1 mm; C–E, G = 2 mm; H = 1 cm.



leaf explants from *ex situ*-grown plant controls (Fig. 3A) and those grown *in vitro* (Fig. 3B). The main differences were that *in vitro* explants had greater intercellular spaces in spongy parenchyma and shorter palisade cells, and the chlorenchyma cells presented generally denser cytoplasm and a more spherical shape when compared with *ex situ* explants.

Direct Somatic Embryogenesis (DSE) In the early stages of DSE, 8 d after explant inoculation, the *in vitro* conditions resulted in intense cell division from spongy parenchyma cells in the mesophyll (Fig. 3D). It was the first indication of a pro-embryogenic mass formation. In both explant sources (*in vitro*

and *ex situ*), the proliferation of mesophyll cells was observed, with cells containing evident nuclei, dense cytoplasm, and little intercellular space (Fig. 3D, E). In explants derived from *ex situ* leaves, cell division began at 8 d (Fig. 3C) but only became intense 18 d after inoculation in culture medium (Fig. 3E).

The formation of a pro-embryogenic mass was observed on the edge of *in vitro* explants, near the vascular bundle, 18 d after inoculation (Fig. 3F), with intense cell division at the surface region. Superficial mass cells exhibited meristematic characteristics, with dense cellular content and evident nucleus (Fig. 3F). After 28 d, a pro-embryogenic mass emerged in *ex situ* explants (Fig. 3G); it was delayed and had less intense mitotic activity

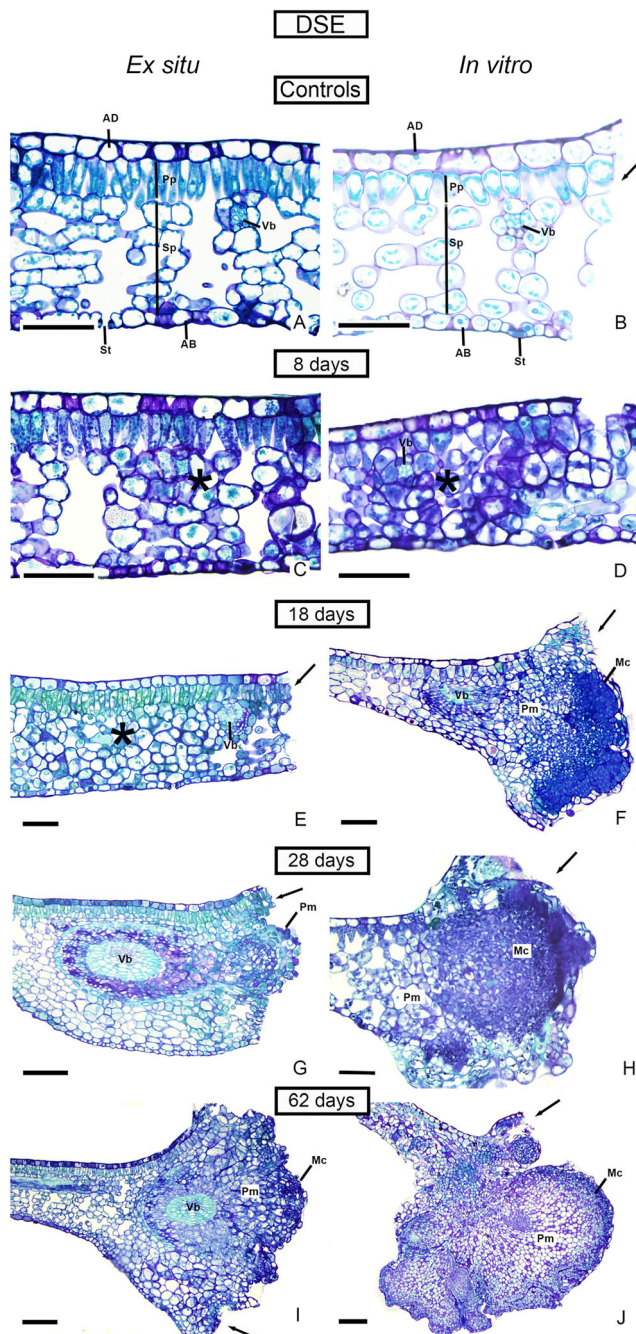


FIGURE 3. Direct somatic embryogenesis in *Coffea arabica* ‘Mundo Novo’. (A, B) Explant controls, observed with an adaxial epidermis (AD), palisade parenchyma (Pp), spongy parenchyma (Sp), vascular bundle (Vb), abaxial epidermis (AB), and stomata (St). (A, C, E, G, I) Foliar explants from *ex situ* plants. (B, D, F, H, J) Leaf explants from *in vitro* plants. (C–J) DSE: (C) 8 d, *ex situ*, early cell division in the spongy parenchyma (*); (D) 8 d, *in vitro*, high mitotic activity in the spongy parenchyma (*); (E) 18 d, *ex situ*, high mitotic activity in the spongy parenchyma (*); (F) 18 d, *in vitro*, presence of a pro-embryogenic mass (Pm) with meristematic cells (Mc) in the peripheral mass region; (G) 28 d, *ex situ*, the start of the formation of a pro-embryogenic mass (Pm); (H) 28 d, *in vitro*, pro-embryogenic mass (Pm) is formed, presence of surface meristematic cells (Mc). (I) 62 d, *ex situ*, pro-embryogenic mass (Pm) formed, presence of sparse meristematic cells (Mc). (J) 62 d, *in vitro*, pro-embryogenic mass (Pm) larger than those grown in *ex situ* conditions, presence of meristematic cells (Mc) on the surface. Arrows indicate the edge of the explant with palisade parenchyma and epidermis without anatomical changes. Bars: A–F, H = 50 μ m; E, G, I, J = 100 μ m.

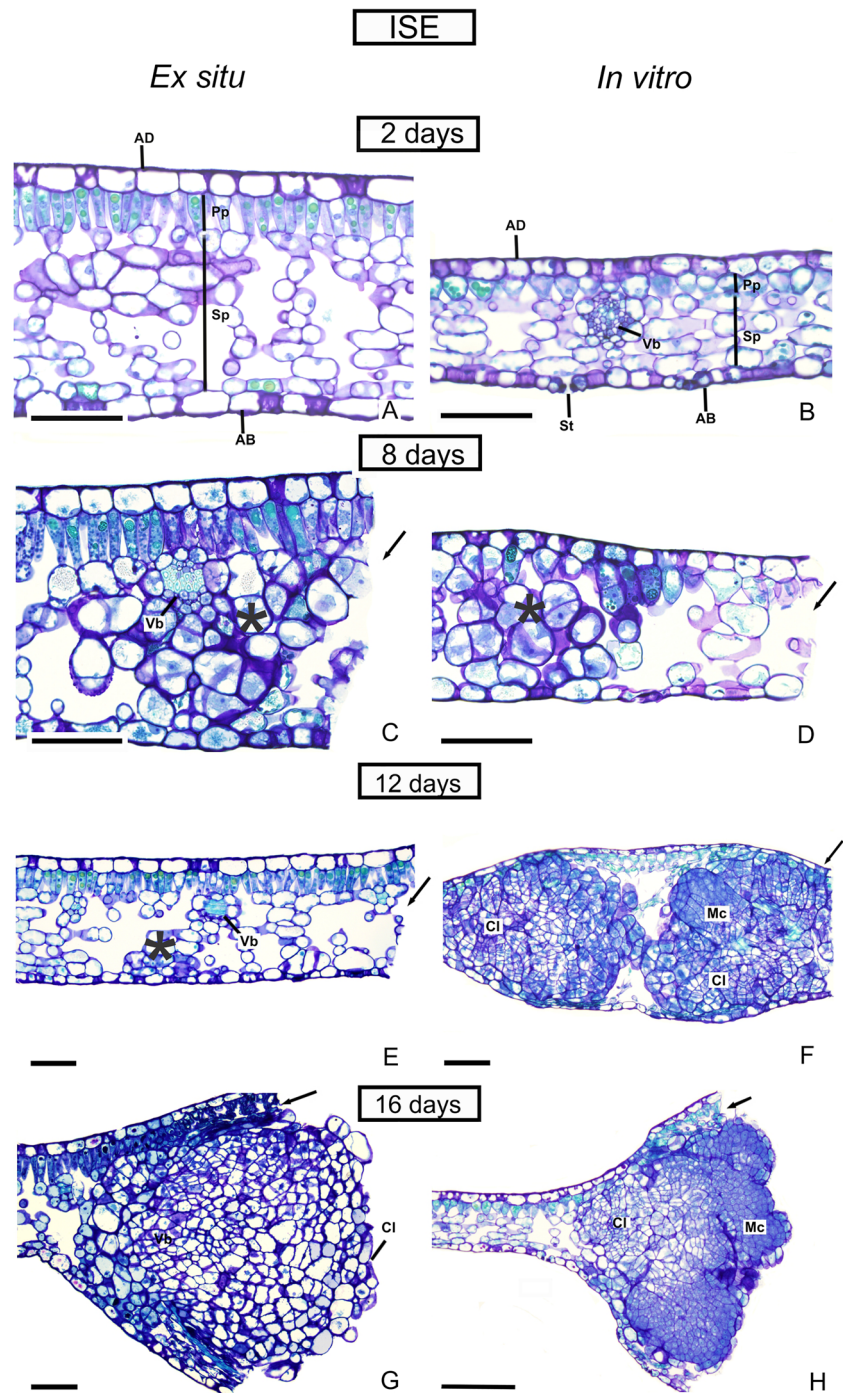
Indirect Somatic Embryogenesis (ISE) During the initial ISE process, greenish phenolic compounds were observed in the chlorenchyma cells 2 d after *in vitro* inoculation, especially in the palisade ones, in both *ex situ* and *in vitro* explants (Fig. 4A, B). Following 8 d of culture, explants displayed regions with high mitotic activity in the spongy parenchyma, mainly near the edge of the explant and next to the vascular bundles, in both *ex situ* (Fig. 4C) and *in vitro* (Fig. 4D) sources. The beginning of callus formation was seen in *in vitro* explants after 12 d; mesophyll cells had high mitotic activity along the entire explant length. These cells neither presented intercellular spaces nor formed meristems containing cells with dense cytoplasm or evident nuclei (Fig. 4F). Few differences could be observed between *ex situ* explants 8 and 12 d after inoculation (Fig. 4C, E).

Calluses were already visible at the edge of the explants 16 d after inoculation (Fig. 4G, H). In both types of explants, there was no change in the epidermis or palisade parenchyma: these cells were shifted providing space for callus growth, and the callus was derived exclusively from the spongy parenchyma cells (Figs. 4G, H and 5A, B). At this stage of callus development, the size and structure of calluses represented the greatest difference between *ex situ* and *in vitro* explants. *Ex situ* explants possessed larger, vacuolated, and rounded callus cells (Fig. 4G). *In vitro* explants had innermost callus cells that were bulkier than the surface ones, which were smaller, with dense cytoplasmic contents, and exhibiting high levels of mitosis (Fig. 4H).

After 28 d of cultivation, explant calluses from *in vitro* and *ex situ* leaves possessed very different structures (Fig. 5A, B). *Ex situ* explants had callus cells that were more elongated and vacuolated, and with larger intercellular spaces, than those from *in vitro* explants (Fig. 5A). At the edge of the explant, the vascular bundle projected toward the central portion of the callus (Fig. 5A–C). *In vitro* explants presented calluses with more vacuolated cells in their inner regions; these cells were

than did the *in vitro* explants (Fig. 3H). When the mass appeared externally in the explants, 62 d after inoculation, *ex situ* explants (Fig. 3I) seemed to have a smaller pro-embryogenic mass than *in vitro* explants (Fig. 3J), and its surface had fewer regions containing cells with dense cytoplasmic contents. *In vitro* explants presented masses with larger projections, and the innermost cells were rounded, vacuolated, and larger than the small peripheral cells, which had dense cytoplasmic contents, forming meristem regions (Fig. 3J). The same anatomical pattern was observed in subsequent samplings of both materials at 72 d after *in vitro* inoculation.

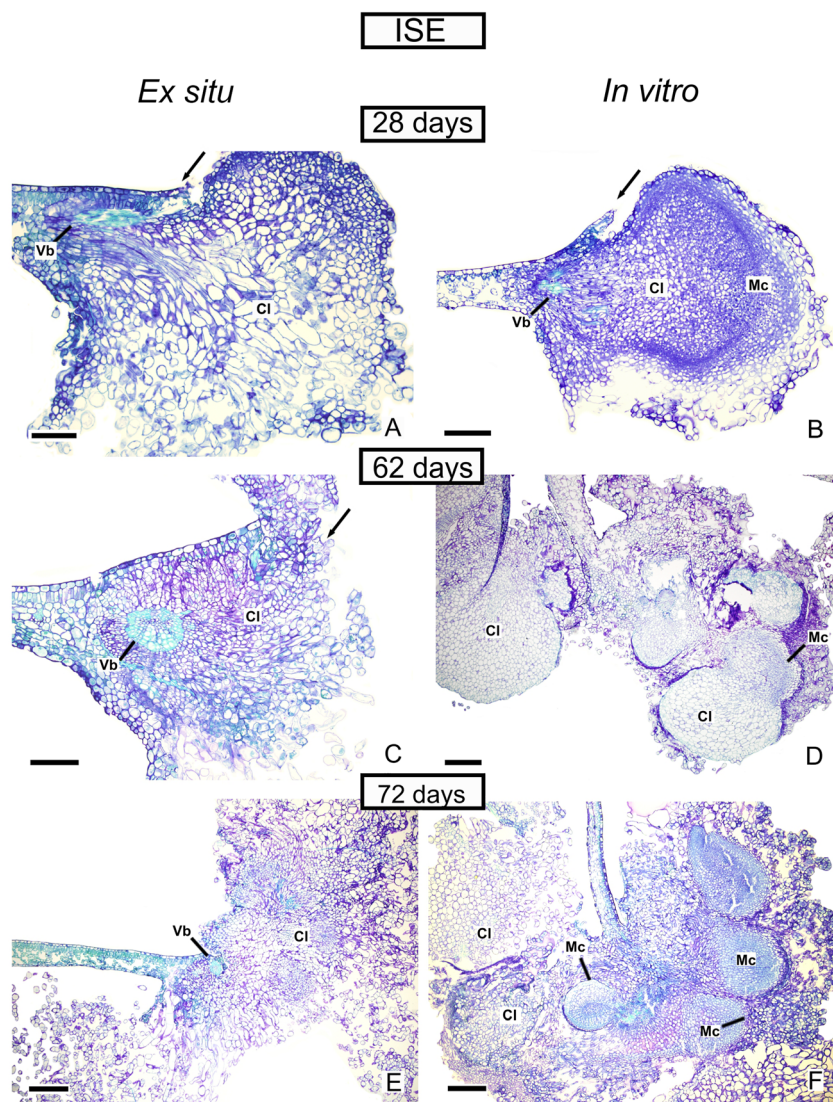
FIGURE 4. Indirect somatic embryogenesis in *Coffea arabica* ‘Mundo Novo’: (A, B) 2 d, *ex situ* explants (A) and *in vitro* explants (B), presence of adaxial epidermis (AD), palisade parenchyma (Pp), spongy parenchyma (Sp), vascular bundle (Vb), abaxial epidermis (AB), and stomata (St); (C) 8 d, *ex situ*, early cell division in the spongy parenchyma (*); (D) 8 d, *in vitro*, high mitotic activity in the spongy parenchyma (*); (E) 12 d, *ex situ*, mitotic activity in scattered cells of the spongy parenchyma (*); (F) 12 d, *in vitro*, beginning of callus formation (Cl), palisade containing meristem cells; (G) 16 d, *ex situ*, presence of a well-defined callus (Cl); (H) 16 d, *in vitro*, callus (Cl) formed, with peripheral regions containing meristematic cells (Mc). Arrow indicates the edge of the explant with the palisade parenchyma and epidermis without anatomical changes. Bars: A–G = 50 μ m; H = 100 μ m.



lined, with smaller cells arranged in continuous layers undergoing intensive cell division. More externally to these layers, cells became elongated and vacuolated, and were coated with a continuous cell layer without intercellular spaces (Fig. 5B). After 30 d of culture, these cells with meristematic characteristics and arranged in layers became more evident (Fig. 6A, B); moreover, it was possible to observe the alignment of these continuous cells with expanding outer layers containing less dense cytoplasm cells (Fig. 6B).

After 50 d of explant culture in a culture medium for embryogenic callus induction, these embryogenic calluses were transferred to an embryo formation culture medium. In *in vitro*-grown explants, samples obtained after 62 (Fig. 5D) and 72 (Fig. 5F) d presented larger calluses than those observed at earlier stages of development; however, they still contained regions with cells exhibiting meristematic characteristics and intense mitotic activity. These meristem cells contributed to the continued callus growth. Only the cells at

FIGURE 5. Indirect somatic embryogenesis in *Coffea arabica* ‘Mundo Novo’. (A) 28 d, *ex situ*, callus (Cl) formation near a vascular bundle (Vb); (B) 28 d, *in vitro*, callus (Cl) with a band of meristematic cells (Mc); (C) 62 d, *ex situ*, callus (Cl) in an advanced stage of development, with fusiform, vacuolated cells and few intercellular spaces; (D) 62 d, *in vitro*, callus (Cl) in an advanced stage of development, containing a cell mass with small, dense cells, and meristematic cells (Mc) in the outer layer of the callus; (E) 72 d, *ex situ*, callus (Cl) in an advanced stage of development, containing spindle cells with large intercellular spaces; (F) 72 d, *in vitro*, callus (Cl) with spindle and spaced cells in the central part of the callus, meristematic cells (Mc) present in different regions. Arrow indicates the edge of the explant with the palisade parenchyma and epidermis without anatomical changes. Bars: A–C = 50 μ m; D–F = 100 μ m.



the surface of calluses were bulky, with large vacuoles and intercellular spaces (Fig. 5D, F). During callus development, explants derived from *ex situ* material exhibited no obvious anatomical changes other than an increase in size, with more spaced, elongated, vacuolated, and irregularly shaped cells; the callus had a friable aspect (Fig. 5C, E).

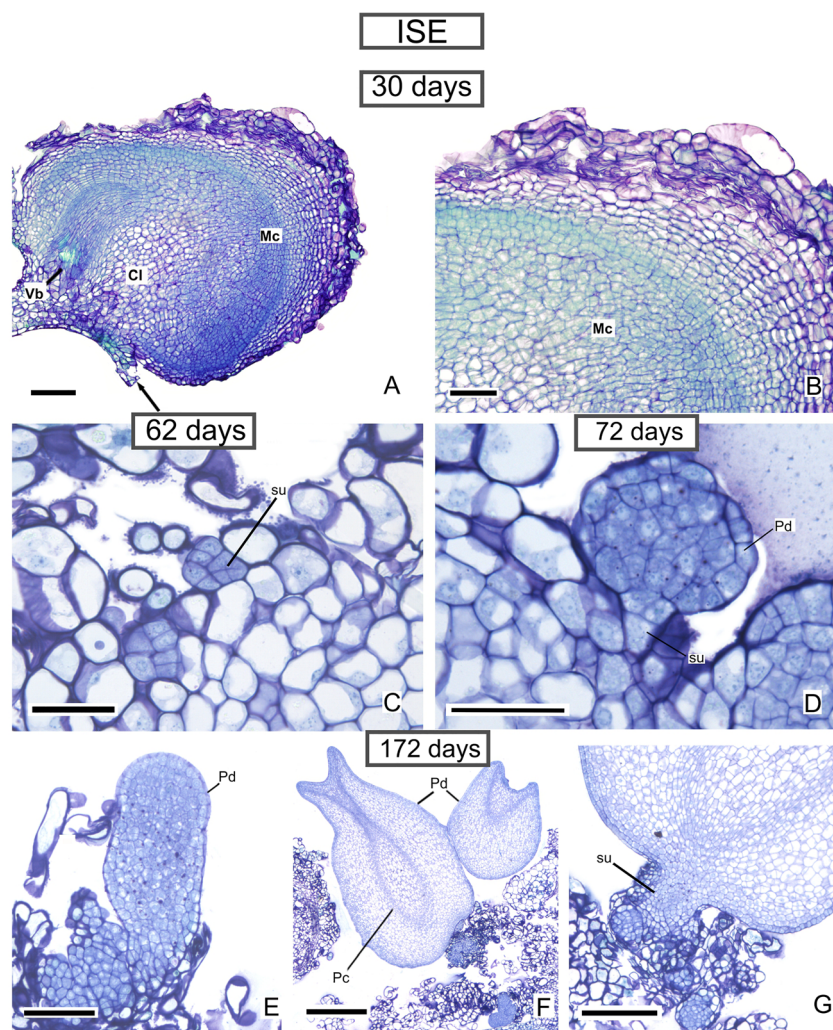
Embryo formation began after 62 d of *in vitro* culture (Fig. 6C). The pro-embryos comprised an upper portion with few dividing cells, which produces the embryo itself, and a basal portion with a set of triangular-shaped cells, which give rise to the suspensor (Fig. 6C, D). After 72 d of culture, the explants from *in vitro* sources presented globular-shaped embryos, with a well-defined protoderm, and a suspensor at the base (Fig. 6D). After 172 d of culture (Fig. 6E–G), embryos were observed at different stages of development. During the transition between embryonic stages (globular to heart stage), the embryos went through longitudinal elongation (Fig. 6E). At torpedo and heart stages, they had a well-defined protoderm

and procambium (Fig. 6F) and remained connected to the callus by the suspensor present at the embryo base (Fig. 6G). During DSE and ISE, embryonic development was similar under all studied conditions regardless of the explant origin (*ex situ* or *in vitro*), with significant variation in the timing of each ontogeny stage (Fig. 7). The embryos originated during DSE and ISE were efficient and developed into normal plants (data not shown).

The occurrence of abnormal embryos was reduced or absent in both DSE and ISE pathways (data not shown). Abnormal embryos were verified in a low percentage in the indirect route, probably due to the contact for a long time with 2,4-D. In the direct route, the occurrence of abnormal somatic embryos was even rarer.

Histochemical Analysis Histochemical tests performed during callus (Fig. 8B) and pro-embryogenic mass development (Fig. 8A, C–I) revealed the presence of phenolic compounds in

FIGURE 6. Indirect somatic embryogenesis in *Coffea arabica* ‘Mundo Novo,’ details of embryo development during ontogenesis of explants from *in vitro*-grown plants. (A) 30 d, with meristematic tissue (Mc), containing small cells with dense cytoplasm; (B) detail of the meristem region (Mc), with intense cell divisions; (C) 62 d, the beginning of embryo development, presence of a suspensor (su); (D) 72 d, detail, presence of a globular embryo in the callus end, with a well-defined protoderm (Pd) and a suspensor (su); (E–G) 172 d, (E) during the embryonic transition stage between the globular and heart stages, well-defined protoderm (Pd); (F) embryos in the heart and torpedo stages presenting a protoderm (Pd) and a well-developed procambium; (G) suspensor detail (su) at the base of the torpedo embryo. Arrow indicates the edge of the explant with the palisade parenchyma and epidermis without anatomical changes. Bars: A–D = 50 μ m; E = 100 μ m; F, G = 100 μ m.



DSE (Fig. 8A) and ISE (Fig. 8B), and lipids in the vacuoles of palisade parenchyma cells and in scattered cells bordering the callus and mass (Fig. 8D). Lipids were characterized as acidic because of their light blue color when stained with Nile Blue sulfate reagent (Fig. 8E). After 28 d of culture and callus formation, starch grains accumulated in chlorophyll parenchyma cells (Fig. 8C) and some callus cells. In DSE, starch was observed in the pro-embryogenic mass of *in vitro* explants. The presence of terpenes was observed in calluses of ISE in both *in vitro* and *ex situ* explants after 72 d of culture. Under DSE, terpenes were only observed in the *ex situ* explants after 16 d (Fig. 8F). Tests performed to indicate the presence of proteins and mucilage (Fig. 8G–I) demonstrated that proteins accumulated in calluses and pro-embryogenic mass cells (Fig. 8I).

Characterization of Morphological Events During DSE and ISE from *Ex Situ*-Cultivated Leaf Explants As observed in Fig. 9, leaf explants from *C. arabica* respond differently to the two somatic embryogenesis (SE) pathways. In the indirect one

(Fig. 9A), the explants form calluses that increase in size until 90 d after the beginning of culture. The calluses start to form somatic embryos in about 180 d after they are transferred to the embryogenesis induction medium. On the other hand, in the direct pathway, the explants form small structures called embryogenic structures, which reach less than 4 mm in size (Fig. 9B). In DSE, the formation of somatic embryos occurs after 90 d of cultivation, and they are arranged in the edges of the explants as well as on the surface of the embryogenic structures. It is also noted that in the two embryogenesis routes, the calluses and the embryogenic structures are visualized after 15 d of cultivation. These observations show that, in this species, the SE responses differ between the indirect and direct pathways.

Discussion

Somatic embryogenesis (SE) involves cell totipotency, whereby somatic cells from plant tissues undergo restructuring to

generate embryonic cells, through a series of developmental stages similar to those occurring in zygotic embryogenesis. During SE, cells go through morphological and biochemical changes that result in the formation of a somatic embryo. This process involves hormonal actions, transcription factors, and epigenetic regulation (Yang and Zhang 2010). The research here presented and discussed is innovative in comparing and analyzing structures that initiate SE pathways: the pro-embryogenic mass (in DSE) and the callus (in ISE), as well as considering the explant source: *in vitro* and *ex situ* coffee plants.

Analyses of pro-embryogenic mass development in DSE and callus development in ISE revealed that both originate from mitotically active cells present in the spongy parenchyma, while epidermal tissue and palisade parenchyma remained without anatomical changes. The callus and the pro-embryogenic mass protrude from the explants; the palisade parenchyma and the epidermis are moved, hence the mechanical pressure exerted by the protrusion. Several studies have described similar events during ISE, although only demonstrating such tissue reorganization in *ex situ*-grown plants (Pierson *et al.* 1983; Berthouly and Michaux-Ferriere 1996; Menéndez-Yuffá and de García 1997).

The development of both callus (ISE) and pro-embryogenic mass (DSE), occurring in the initial phase of SE, is notable near vascular bundles (Figs. 3E, F and 4C, G), demonstrating a correlation between the presence of vascular tissue and the early formation of these structures. The initiation of cell divisions at the bundle can be explained by the presence of totipotent pericycle cells, which form the

outermost layer of the vascular bundle (Xu and Huang 2014). The exposure of the edges of tissue after cutting the explants in regions near small vascular bundles facilitates the action of plant hormones placed in the culture medium, increasing callus and mass formation (Xu and Huang 2014).

During DSE in coffee, embryos do not emerge directly from the explant cells, as previously described for other species (Dublin 1981; Yasuda *et al.* 1985; Emons 1994; Motoike *et al.* 2007; Vasconcellos *et al.* 2009). Alternatively, embryos were observed developing from a pro-embryogenic mass formed in the spongy parenchyma, as reported by Menéndez-Yuffá and de García (1997) and Quiroz-Figueroa *et al.* (2002b), independently of the explant source. Therefore, the pro-embryogenic mass is anatomically different from the callus, as demonstrated by anatomical analyses.

In DSE, tissue develops more rapidly in explants from *in vitro*-grown plants when compared to *ex situ* explants (Fig. 7). It can be compared by determining the time required for the beginning of cell division in the spongy parenchyma and the emergence of the pro-embryogenic mass. Parenchyma from *in vitro* explants is less differentiated than parenchyma in explants derived from *ex situ*-grown plants, and it possibly possesses greater totipotency. Plants grown *in vitro* may present rejuvenated tissues, which explains the anatomical and temporal differences between explants obtained from *ex situ* and *in vitro* materials (Brand and Lineberger 1992; Ruaud *et al.* 1992; von Aderkas and Bonga 2000). Physiological changes, along with the acquisition of juvenile morphology, are translated into a faster development in *in vitro*-grown plants when compared to *ex situ* plants, which are

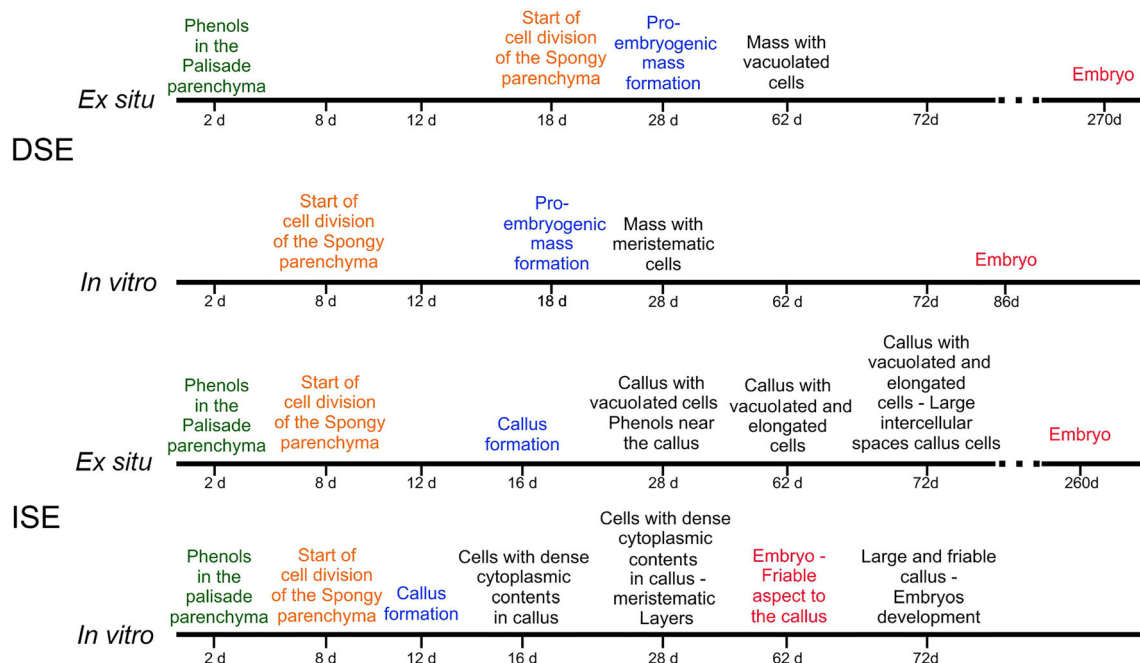


FIGURE 7. Representation of ontogenesis in *Coffea arabica* 'Mundo Novo' during direct (DSE) and indirect (ISE) somatic embryogenesis in explants from plants cultivated under experimental field conditions (*ex situ*) and in *in vitro* plant gene banks (*in vitro*).

physiologically older. The most significant difference between *ex situ* and *in vitro* materials was the time of embryo development, which occurred after 270 and 86 d of culture, respectively (Fig. 7).

Studies with cassava cultivars suggest that more juvenile tissues result in the more efficient production of somatic embryos (Ravindran *et al.* 2015). According to Claudot *et al.* (1993), mature trees can be rejuvenated by *in vitro* culture, and it is associated with the reappearance of features observed in seedlings. In some *in vitro* cultures, tissue rejuvenation is observed with the acquisition of juvenile morphology (Brand and Lineberger 1992; Ruaud *et al.* 1992). Reversion to a juvenile state has the potential to induce embryogenesis in cultures of recalcitrant species (von Aderkas and Bonga 2000). Considering these results, it is possible to suggest that in studies with materials of *ex situ* origin, it would be important to select donor tissues from younger explants for application of

somatic embryogenesis, as it tends to favor greater efficiency in the process of somatic embryo formation.

According to Kahia *et al.* (2016), DSE in *Coffea arabica-robusta x canephora* var. “Ruiru 11” leaf explants from *in vitro*-germinated seedlings produced the highest embryogenic cultures (90%) and the highest mean number of embryos (19.36) per explant when compared to leaf explants from field-grown trees. In the present work, we demonstrate the structural differences between these two types of explant sources, *ex situ* and *in vitro*; additionally, the ontogenesis throughout embryonic development both by direct and indirect somatic embryogenesis is analyzed. For this purpose, explants were evaluated at different developmental stages to identify the histological origin of callus or pro-embryogenic mass, the beginning of embryo formation, the presence of suspensor, and the integrity of formed embryos. The indication of using *in vitro* seedling tissue for a more efficient

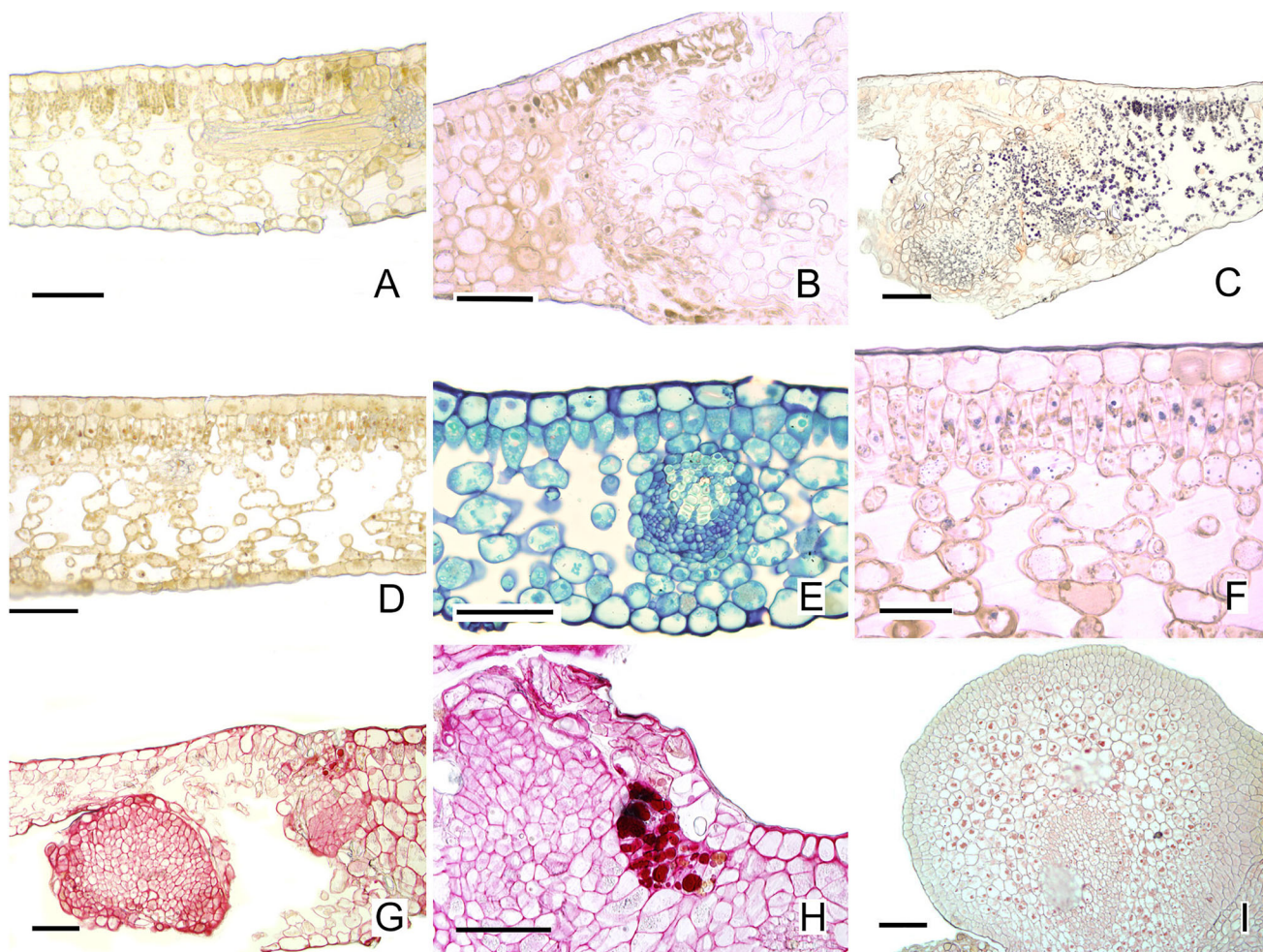
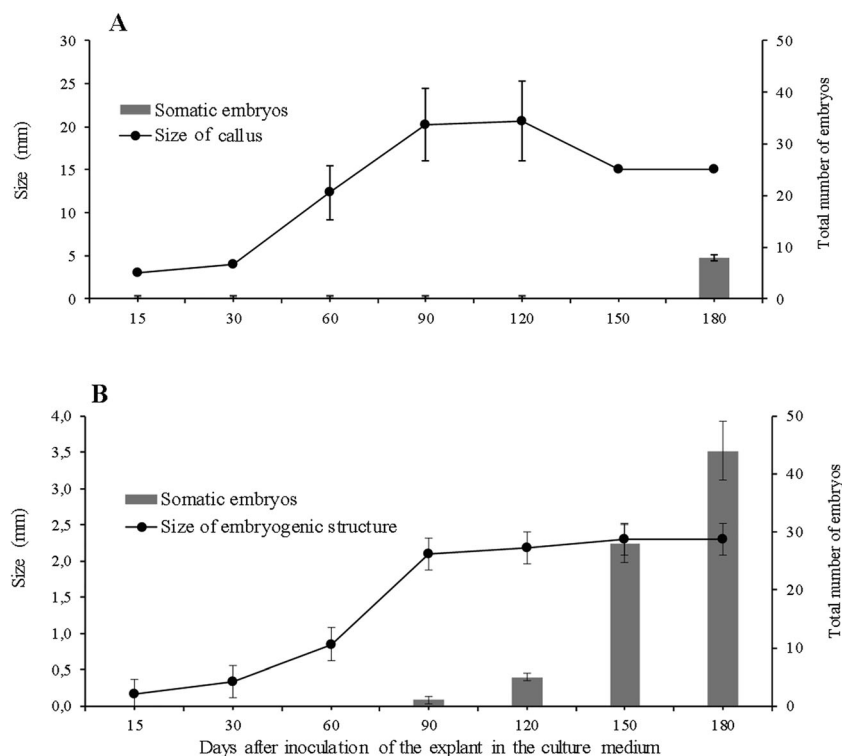


Figure 8. Somatic embryogenesis in *Coffea arabica* observed using histochemical tests. (A, D, and F) Direct somatic embryogenesis (DSE) in *ex situ*-grown plants; (C, E, G–I) DSE in *in vitro*-grown plants; (B) indirect somatic embryogenesis (ISE) in *ex situ*-grown plants. (A, B) Ferric chloride 3%, positive for phenols, brown coloration; (C) Lugol,

positive for starch, dark blue color; (D) Sudan IV, positive for lipids, yellow coloring; (E) Nile Blue sulfate, positive for acid lipids, blue color; (F) Nadi reaction, positive for terpenoids, light blue color; (G, H) Red ruthenium, pectins and mucilage, red color; (I) Xylidine Ponceau reagent, positive for proteins, pink color. Bars: A, B = 100 μ m; C–I = 50 μ m.

FIGURE 9. Characterization of morphological events that occur during indirect (A) and direct (B) somatic embryogenesis in leaf explants from ‘Mundo Novo’ cultivar of *Coffea arabica*, grown in the field.



application of somatic embryogenesis in *Coffea* is a great contribution to future studies. It allows maintaining a bank of plants that can be used as explant source regardless of climatic conditions, reducing recalcitrance.

Although ISE is more applied to *Coffea* genotypes than the direct pathway, there is a tendency to believe that somatic embryogenesis of the coffee tree via the direct pathway would be faster than via the indirect one. The results obtained in this study indicate the opposite, as embryos were obtained via ISE with 62 d in *in vitro*-grown plants, against 86 d until the development of *in vitro*-grown embryos via the DSE pathway (Fig. 7). During ISE, there were few temporal differences between the source of explants regarding the initial stages of callus development (Fig. 7). Histological observations showed that the explants presented regions with high mitotic activity near the edge of the explant and next to the vascular bundles. According to Bartos *et al.* (2018b), this mitotic activity is caused by the greater contact these regions have with nutrient medium components. However, based on complete callus formation, an anatomical difference was observed between ISE in explants from *ex situ* and *in vitro* plants: calluses from *ex situ* materials resemble those described by Pierson *et al.* (1983), with elongated cells and a loose external appearance, whereas calluses from *in vitro* materials have large regions of meristematic cells with organized cell division, a single large nucleus, and dense cytoplasm (Fig. 6A, B). These results differ from those reported by Quiroz-Figueroa

et al. (2002a) and Silva *et al.* (2014). In our study, callus differentiation was detailed under different conditions, also reporting regions with aligned layers of meristematic cells, probably with totipotent ones. When determining the total number of embryos obtained in experiments in which explants from plants in the field were inoculated in the culture medium, these conditions showed greater efficiency in somatic embryogenesis via the direct pathway when compared to ISE (Fig. 9). Another aspect to be highlighted is that the embryogenic structures, which are visualized at the edge of the explants subjected to direct somatic embryogenesis, as described by Almeida (2020), correspond to the pro-embryogenic mass characterized in the anatomical analysis of the explants submitted to the direct route.

Bartos *et al.* (2018b) reported the presence of embryogenic callus consisting entirely of meristematic regions with isodiametric cells, dense cytoplasm, evident nuclei, and thinner cell walls. Conversely, in the present study, embryogenic calluses have cells with meristematic features as well as regions with elongated and vacuolated cells. What varies is the proportion of the latter cell type inside the callus, depending on the origin of the explant: from *ex situ* or *in vitro* plants. Also, according to the differentiation made by Bartos *et al.* (2018b), calluses can be classified into two types: type 1 (primary calluses with embryogenic competence) and type 2 (primary calluses with no embryogenic competence), the second type, or non-embryogenic calluses, being less

compact, watery, and whitish; growing disorderly; and composed predominantly of elongated parenchyma cells with large intercellular spaces.

Histochemical studies revealed greater expression of phenolic compounds in explants from *ex situ* leaves than in those from *in vitro* leaves. This can be partly explained by the mechanical disinfection process and/or by the response of leaves to injury and strain during *in vitro* inoculation (Alemanno *et al.* 2003; van Boxtel and Berthouly 1996). Furthermore, leaves of adult plants may exhibit a greater accumulation of phenols (Claudot *et al.* 1993). Conversely, the low levels of phenols in explants from *in vitro* leaves may be related to the controlled conditions to which the plants were exposed, resulting in little differentiation of tissues and low levels of products derived from secondary metabolism. Starch grains were observed both in callus cells and in pro-embryogenic masses, in regions of intense cell division, in the proximity of future embryo formation regions. Starch accumulation may be associated with the embryogenic potential of explants; starch is required for the formation of somatic embryos, and it is consumed during the development of meristematic tissue (Apezzato-da-Glória and Machado 2004; Claudot *et al.* 1993). Bartos *et al.* (2018b) observed that after primary callus formation during ISE in *C. arabica* cv. Catuaí Vermelho, the starch contents increased gradually until the completion of the culture. Protein reserves appear to be crucial to the formation of embryogenic tissue during ontogenesis, as the analyses show accumulation in calluses and pro-embryogenic masses. Berthouly and Michaux-Ferriere (1996) showed that embryonic tissue does not develop when in absence of protein sources. In ISE of *C. arabica*, protein rates were observed gradually increasing during callus induction (Bartos *et al.* 2018a; Santana-Buzzy *et al.* 2007). Sharifi *et al.* (2012) identify proteins whose expression is necessary for somatic to embryogenic transition in *Crocus sativus*, as well as demonstrating some differences in the proteome pattern between non-embryogenic and embryogenic calluses.

Embryos in DSE and ISE were observed at four developmental stages (globular, heart, torpedo, and cotyledonary), and whether they were connected to the callus or pro-embryogenic mass by the suspensor. In the basal region of the embryo, the suspensor connects it to the mother tissue, as reported for other varieties of *Coffea* by Quiroz-Figueroa *et al.* (2006). Although unicellular formation was not observed at the beginning of embryo development, the attachment of embryos to mother tissue by the suspensor indicates a unicellular origin (Quiroz-Figueroa *et al.* 2002a; Williams and Maheswaran 1986). Quiroz-Figueroa *et al.* (2002a) showed that somatic embryos in leaf explants of *C. arabica*, both by DSE or ISE, originate from a single cell. In this study, it was not possible to confirm this unicellular origin despite the analysis of numerous explants and sequential sections.

The application of somatic embryogenesis in *Coffea* has been occurring since the 1970s. Since then, most studies have presented separate results to indirect and direct pathways. Currently, the present study submits a significant comparison between both somatic embryogenesis pathways in *Coffea*. Most manuscripts published regarding somatic embryogenesis in *Coffea* present anatomical analyses of one or two stages of the development in one of the applied pathways, DSE or ISE. In this study, the anatomical analyses of direct and indirect pathways were performed at different stages of development. In addition, this study was also performed on explants from *in vitro* seedling leaves and on natural environment plants (*ex situ*), which provides a broad comparison between direct and indirect pathway application in *Coffea*.

We showed that independently of the explant source, the callus and the pro-embryogenic mass originate exclusively from cells of the palisade parenchyma, which undergo cellular reprogramming to become mitotically active. Furthermore, throughout somatic embryogenesis, *in vitro* materials produced meristematic cells faster than *ex situ* materials. The present study contributes to applied research regarding the indication of using *Coffea in vitro* seedling for a more efficient application of somatic embryogenesis, an explant source that was proved to be more suitable than explant tissue from the natural environment. Besides, these results contribute positively to clarify the assumption made by some studies that younger tissues may be more appropriate to achieve greater efficiency in the application of somatic embryogenesis in *Coffea*. During somatic embryogenesis, ISE using leaves from *in vitro*-grown plants proved to be more promising than those from other evaluated conditions, generating calluses after 12 d and embryos after 62 d of culture. Reduced time in tissue culture can generate a higher quality of embryos and decrease somaclonal variation, which is a frequent problem in cellular aggregates maintained for long periods in tissue culture (Santana-Buzzy *et al.* 2007). The results of this study provide information that helps to reduce *in vitro* cultivation time by reducing the time required to obtain clones and thus production costs, allowing commercial laboratories to increase their efficiency. Therefore, we suggest that ISE of *in vitro*-grown explants may be an important tool to support breeding programs associated with gene regulation studies of ontogenesis.

Acknowledgements The authors thank the Centro de Café Alcides Carvalho (IAC) for providing the plant material used in this study. IFF received a fellowship from CAPES (Coordination for the Improvement of Higher Education Personnel), and JLSM thanks CNPq (310184/2016-9). This study was financed in part by CAPES - Finance Code 001. We would like to thank the Fundo de Apoio ao Ensino, Pesquisa e Extensão (FAPEX/UNICAMP) for the research support. We also thank

the access to equipment and assistance provided by the Electron Microscope Laboratory (LME/UNICAMP).

Author contribution IFF, JASA, and JLSM designed the experiments. IFF, GAM, WLSJ, BBB, JASA, and JLSM performed the experiments and analyzed the data. IFF, MPP, and JLSM wrote the manuscript. JMCM revised the manuscript. This study is part of the PhD thesis of IFF (Programa de Pós-graduação em Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Brazil). All the authors read and approved the final manuscript.

Declarations

Competing interests The authors declare no competing interests.

References

- Ahmed W, Feyissa T, Disasa T (2013) Somatic embryogenesis of a coffee (*Coffea arabica* L.) hybrid using leaf explants. *J Hort Sci Biotechnol* 88:469–475. <https://doi.org/10.1080/14620316.2013.11512993>
- Alemanno L, Ramos T, Gargadenc A, Andary C, Ferriere N (2003) Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. *Ann Bot* 92(4):613–623. <https://doi.org/10.1093/aob/mcg177>
- Almeida JAS (2020) Observations on somatic embryogenesis in *Coffea arabica* L. In: Castanheira DT (ed) Coffee-production and research, IntechOpen, London, United Kingdom, pp 1–20. <https://doi.org/10.5772/intechopen.90853>
- Almeida JAS, Carmazini VCB, Da Silva Ramos LC (2007) Indirect effect of agar concentration on the embryogenic response of *Coffea canephora*. *Fruit, Veg Cereal Sci Biotech* 1:121–125
- Almeida JAS, Silvarolla MB, Fazuoli LC, Stancato GC (2008) Embriogênese somática em genótipos de *Coffea arabica* L. *Coffee Sci* 3:143–151
- Almeida JAS, Leal R, Carmazini V, Salomon M, Guerreiro Filho O (2014) Effect of temperature and cytokinin on the capacity of direct somatic embryogenesis in *Coffea arabica* L. genotypes. *Coffee Sci* 9:394–399. <https://doi.org/10.25186/cs.v9i3.668>
- Almeida JAS, Leal RR, Carmazini VCBM, Salomon MV, Guerreiro-Filho O (2016) Characterization of the morphological events in the somatic embryogenesis in the direct somatic of *Coffea arabica* L. genotypes. *Plant Cell Biotechnol Mol Biol* 17:393–403
- Alves IS, Carmazini VCB, Santos CD, Almeida JAS (2018) 2-Isopentenyladenine in the induction of direct somatic embryogenesis capacity of *Coffea arabica* L. *Cienc Rural* 48:1–5. <https://doi.org/10.1590/01038478cr20180001>
- Appazzato-da-Glória B, Machado SR (2004) Ultrastructural analysis of *in vitro* direct and indirect organogenesis. *Braz J Bot* 27(3):429–437. <https://doi.org/10.1590/S0100-84042004000300004>
- Bartos PMC, Gomes HT, Do Amaral LIV, Teixeira JB, Scherwinski-Pereira JE (2018a) Biochemical events during somatic embryogenesis in *Coffea arabica* L. *3 Biotech* 8:1–10. <https://doi.org/10.1007/s13205-018-1238-7>
- Bartos PMC, Gomes HT, Gomes SM, Vasconcelos Filho SC, Teixeira JB, Scherwinski-Pereira JE (2018b) Histology of somatic embryogenesis in *Coffea arabica* L. *Biologia* 73(12):1255–1265. <https://doi.org/10.2478/s11756-018-0131-5>
- Berlyn GP, Miksche JP, Sass JE (1976) Botanical microtechnique and cytochemistry. Iowa State University Press, Ames, Iowa
- Berthouly M, Michaux-Ferriere N (1996) High frequency somatic embryogenesis in *Coffea canephora*. *Plant Cell Tissue Organ Cult* 44:169–176. <https://doi.org/10.1007/BF00048196>
- Brand MH, Lineberger RD (1992) *In vitro* rejuvenation of *Betula* (Betulaceae): biochemical evaluation. *Am J Bot* 79(6):626–635. <https://doi.org/10.1002/j.1537-2197.1992.tb14604.x>
- Carman JG (1990) Embryogenic cells in plant tissue cultures: occurrence and behavior. *In Vitro Cell Dev Biol* 26:746–753. <https://doi.org/10.1007/BF02623615>
- Carvalho CHS (2008) Cultivares de café: origem, características e recomendações. Embrapa Café, Brasília
- Carvalho GR, Mendes ANG, Bartholo GF, Cereda GJ (2006) Comportamento de progênies de cafeeiro cultivar mundo novo. *Cienc Agrotec* 30(5):853–860. <https://doi.org/10.1590/S1413-70542006000500005>
- Clarindo WR, Carvalho CR, Mendonça MAC (2012) Ploidy instability in long-term *in vitro* cultures of *Coffea arabica* L. monitored by flow cytometry. *Plant Growth Regul* 68:533–538. <https://doi.org/10.1007/s10725-012-9740-0>
- Claudot A, Jay-Allemand C, Magel E, Drouet A (1993) Phenylalanine ammonia-lyase, chalcone synthase and polyphenolic compounds in adult and rejuvenated hybrid walnut tree. *Trees* 7(2):92–97. <https://doi.org/10.1007/BF00225475>
- David R, Carde J (1964) Histochimie-coloration différentielle des inclusions lipidiques et terpeniques des pseudophylles du pin maritime au moyen du reactif NADI. *C R Hebd Séances Acad Sci* 258:1338–1340
- Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. *J Exp Bot* 48:1493–1509. <https://doi.org/10.1093/jxb/48.8.1493>
- Dublin P (1981) Embryogênese somática directe sur fragments de feuilles de caféier Arabusta. *Cafe Cacao The* 25:237–242
- Emons AMC (1994) Somatic embryogenesis: cell biological aspects. *Acta Bot Neerl* 43:1–14. <https://doi.org/10.1111/j.1438-8677.1994.tb00729.x>
- Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell Tissue Organ Cult* 74:201–228. <https://doi.org/10.1023/A:1024033216561>
- Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regul* 43:27–47. <https://doi.org/10.1023/B:GROW.0000038275.29262.fb>
- Gatica-Arias AM, Arrieta-Espinoza G, Esquivel AME (2008) Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuai. *Electron J Biotechnol* 11:101–112. <https://doi.org/10.2225/vol11-issue1-fulltext-9>
- Gomes HT, Bartos PMC, Balzon TA, Scherwinski-Pereira JE (2016) Regeneration of somatic embryos of oil palm (*Elaeis guineensis*) using temporary immersion bioreactors. *Ind Crop Prod* 89:244–249. <https://doi.org/10.1016/j.indcrop.2016.05.021>
- Gregory M, Baas P (1989) A survey of mucilage cells in vegetative organs of the dicotyledons. *Israel J Bot* 38:125–174. <https://doi.org/10.1080/0021213X.1989.10677119>
- Gulzar B, Mujib A, Malik MQ, Sayeed R, Mamgain J, Ejaz B (2020) Genes, proteins and other networks regulating somatic embryogenesis in plants. *J Genet Eng Biotechnol* 18:1–15. <https://doi.org/10.1186/s43141-020-00047-5>
- Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regul* 47:91–110. <https://doi.org/10.1007/s10725-005-3478-x>
- Johansen DA (1940) *Plant microtechnique*. McGraw-Hill Book Company, London
- Kahia J, Kirika M, Lubabali H, Mantell S (2016) High-frequency direct somatic embryogenesis and plantlet regeneration from leaves derived from *in vitro*-germinated seedlings of a *Coffea arabica* hybrid cultivar. *HortScience* 51:1148–1152. <https://doi.org/10.21273/HORTSCI10771-16>

- Landey RB, Cenci A, Georget F, Bertrand B, Camayo G, Dechamp E, Herrera JC, Santoni S, Lashermes P, Simpson J (2013) High genetic and epigenetic stability in *Coffea arabica* plants derived from embryogenic suspensions and secondary embryogenesis as revealed by AFLP, MSAP and the phenotypic variation rate. *PLoS One* 8: e56372. <https://doi.org/10.1371/journal.pone.0056372>
- McIlvaine T (1921) A buffer solution for colorimetric comparison. *J Biol Chem* 49:183–186
- Mendez-Hernandez HA, Ledezma-Rodriguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A (2019) Signaling overview of plant somatic embryogenesis. *Front Plant Sci* 10:77. <https://doi.org/10.3389/fpls.2019.00077>
- Menéndez-Yuffá A, De García EG (1997) Morphogenic events during indirect somatic embryogenesis in coffee “Catimor”. *Protoplasma* 199:208–214. <https://doi.org/10.1007/BF01294507>
- Mishra MK, Devi S, McCormac A, Scott N, Chen D, Elliott M, Slater A (2010) Green fluorescent protein as a visual selection marker for coffee transformation. *Biologia* 65:639–646. <https://doi.org/10.2478/s11756-010-0078-7>
- Moens P (1965) Développement de l'ovule et embryogenèse chez *Coffea canephora*/Pierre. *Cellule* 65:127–147
- Motoike SY, Saraiva ES, Ventrella MC, Silva CV, Salomão LCC (2007) Somatic embryogenesis of *Myrciaria aureana* (Brazilian grape tree). *Plant Cell Tissue Organ Cult* 89:75–81. <https://doi.org/10.1007/s11240-007-9210-y>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Pearse AGE (1985) *Histochemistry: theoretical and applied*. Churchill-Livingstone, New York
- Pierson E, Van Lammeren A, Schel J, Staritsky G (1983) *In vitro* development of embryoids from punched leaf discs of *Coffea canephora*. *Protoplasma* 115(2-3):208–216. <https://doi.org/10.1007/BF01279811>
- Quiroz-Figueroa F, Fuentes-Cerda C, Rojas-Herrera R, Loyola-Vargas V (2002a) Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. *Plant Cell Rep* 20:1141–1149. <https://doi.org/10.1007/s00299-002-0464-x>
- Quiroz-Figueroa F, Méndez-Zeel M, Sánchez-Teyer F, Rojas-Herrera R, Loyola-Vargas VM (2002b) Differential gene expression in embryogenic and non-embryogenic clusters from cell suspension cultures of *Coffea arabica*. *J Plant Physiol* 159(11):1267–1270. <https://doi.org/10.1078/0176-1617-00878>
- Quiroz-Figueroa F, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tissue Organ Cult* 86:285–290. <https://doi.org/10.1007/s11240-006-9139-6>
- Ramos L, Yokoo E, Gonçalves W (1993) Direct somatic embryogenesis is genotype specific in coffee. *Proceedings of the 15th Colloque Scientifique International sur Le Café, ASIC*, pp 763–766
- Ravindran BM, Winter S, Thangaraj M (2015) Influence of age of explants and genotype on somatic embryogenesis in African and Indian cassava cultivars. *J Root Crops* 40(2):21–27
- Rezende JC, Carvalho CHS, Santos ACR, Pasqual M, Teixeira JB (2012) Multiplication of embryogenic calli in *Coffea arabica* L. *Acta Sci-Agron* 34:93–98. <https://doi.org/10.4025/actasciagron.v34i1.11230>
- Ruaud JN, Bercetche J, Pâques M (1992) First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. *Plant Cell Rep* 11(11):563–566. <https://doi.org/10.1007/BF00233093>
- Sakai WS (1973) Simple method for differential staining of paraffin embedded plant material using toluidine blue O. *Biotech Histochem* 48: 247–249. <https://doi.org/10.3109/10520297309116632>
- Santana-Buzzy N, Rojas-Herrera R, Galaz-Avalos RM, Ku-Cauich JR, Mijangos-Cortés J, Gutiérrez-Pacheco LC, Canto A, Quiroz-Figueroa F, Loyola-Vargas VM (2007) Advances in coffee tissue culture and its practical applications. *In Vitro Cell Dev Biol - Plant* 43:507–520. <https://doi.org/10.1007/s11627-007-9074-1>
- Sharifi G, Ebrahimzadeh H, Ghareyazie B, Gharechahi J, Vatankhah E (2012) Identification of differentially accumulated proteins associated with embryogenic and nonembryogenic calli in saffron (*Crocus sativus* L.). *Proteome Sci* 10(3):3. <https://doi.org/10.1186/1477-5956-10-3>
- Silva AT, Barduche D, Do Livramento KG, Ligterink W, Paiva LV (2014) Characterization of a putative serk-like ortholog in embryogenic cell suspension cultures of *Coffea arabica* L. *Plant Mol Biol Report* 32:176–184. <https://doi.org/10.1007/s11105-013-0632-x>
- Söndahl MR, Sharp WR (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Z Pflanzenphysiol* 81:395–408. [https://doi.org/10.1016/S0044-328X\(77\)80175-X](https://doi.org/10.1016/S0044-328X(77)80175-X)
- Van Boxtel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. *Plant Cell Tissue Organ Cult* 44:7–17. <https://doi.org/10.1007/BF00045907>
- Vasconcellos RCC, Almeida JASD, Silvarolla MB (2009) Indução de embriões somáticos em explantes foliares de genótipos de *Coffea arabica* em presença da citocinina 2-iP. *SBICafé*, Biblioteca do Café
- Vidal BC (1969) Dichroism in collagen bundles stained with Xylidine-Ponceau 2R. *Ann Histochim* 15:289–296
- Vieira LGE, Kobayashi AK (2000) Micropropagação do cafeeiro. *Proceedings of the I Simpósio de Pesquisas dos Cafés do Brasil, Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café*, pp 147–167. Available via SBICafé. <http://www.sbicafe.ufv.br/handle/123456789/536>. Cited 25 Jan 2021
- Von Aderkas P, Bonga JM (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiol* 20(14):921–928. <https://doi.org/10.1093/treephys/20.14.921>
- Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell Tissue Organ Cult* 69:233–249. <https://doi.org/10.1023/A:1015673200621>
- Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Ann Bot* 57:443–462. <https://doi.org/10.1093/oxfordjournals.aob.a087127>
- Xu L, Huang H (2014) Genetic and epigenetic controls of plant regeneration. *Curr Top Dev Biol* 108:1–33. <https://doi.org/10.1016/B978-0-12-391498-9.00009-7>
- Yang X, Zhang X (2010) Regulation of somatic embryogenesis in higher plants. *Crit Rev Plant Sci* 29(1):36–57. <https://doi.org/10.1080/07352680903436291>
- Yasuda T, Fujii Y, Yamaguchi T (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. *Plant Cell Physiol* 26:595–597. <https://doi.org/10.1093/oxfordjournals.pcp.a076946>