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Research paper

# Development of coffee somatic and zygotic embryos to plants differs in the morphological, histochemical and hydration aspects

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In *Coffea arabica* L., the development of direct sowing of somatic embryos (SE) in planting substrate, with subsequent nursery production of plants, has promoted the industrialization of somatic embryogenesis. However, plant conversion rates are still low and require improvements to enhance the cost-effectiveness of commercial micropropagation. With the aim of improving plant regeneration from SE, we studied the morphological and histological criteria and water characteristics during germination and plant conversion of zygotic embryos (ZE) and SE. At the cotyledonary stage, SE produced in a 1 l RITA® temporary immersion bioreactor (area 55.8 $cm^2$) were morphologically similar in size (2–3 mm) but abnormal as compared with mature ZE. Protein and starch reserve levels were extremely low throughout germination and conversion to plantlets, while the water status remained steady [water content (WC) from 76 to 87%, $\Psi$ from −0.37 to −0.47 MPa, pressure potential from 0.69 to 0.24 MPa]. In ZE, spectacular hydration occurred during the first 3 weeks (WC from 37 to 75%; $\Psi$ from −6.24 to −1.0 MPa). Cotyledons remained undifferentiated for 10 weeks after sowing. Conversely, after only 3 weeks under germination conditions in a RITA® bioreactor, spongy and palisade parenchyma and stomata formed in SE cotyledons. The ZE plant conversion was faster than that of SE (14 vs. 22 weeks) and more efficient (rates 96 vs. 55%), with much more substantial hypocotyl and cotyledon development. The use of a new 5 l MATIS® bioreactor (area 355 $cm^2$), designed especially to favor embryo dispersion and light transmittance to SE, markedly improved the embryo-to-plantlet conversion rate (91%). These results highlight the morphological heterogeneity and lack of protein reserves in SE at the beginning of the germination phase and marked differences in water characteristics. However, they also reveal high phenotypic plasticity, leading to a highly efficient plantlet conversion rate due to better embryo dispersion and light transmittance in more horizontal bioreactors.

***Keywords***: *Coffea arabica*, embryo-to-plant conversion, germination, histology, reserve compounds, scanning electron microscopy, seed, somatic embryogenesis, temporary immersion bioreactors, water characteristics.

## Introduction

In vitro multiplication of elite plants by somatic embryogenesis overcomes the need for long and expensive pedigree selection programs, particularly in long-living tree species. In *Coffea arabica* L., somatic embryogenesis, which has been implemented on a commercial scale in Central America since 2007, enables large-scale dissemination of F1 hybrids with a high level of heterosis (Bertrand et al. 2011). Since 2006, four million plantlets derived from somatic embryos (SE) have been produced by a production unit in Nicaragua belonging to a private company (ECOM Trading). A new production unit that has been recently set up in Xalapa (Mexico) by the same company started producing in 2012. However, the cost-effectiveness of commercial coffee propagation is still unsatisfactory. The profitability could be increased by enhancing production and/or

optimizing the somatic embryogenesis process, thus decreasing the resulting production cost per somatic seedling. Among the different developmental stages, plant conversion was identified as the main bottleneck for further implementation of the technology (Etienne et al. 2012).

Comparison of SE and zygotic embryos (ZE) during germination and young plantlet development could reveal ways of overcoming this inefficient plant conversion. The tiny coffee embryo is encapsulated in a copious endosperm, which is characteristic of the plesiomorphic state in seed plants (Forbis et al. 2002). As in other albuminous dicots, coffee endosperm is solely composed of uniform living reserve cells. Morphological and biochemical events occurring during coffee seed germination and early seedling growth have been described in detail for many species. However, in coffee, such an approach was restricted to the very first steps of the seed germination process until radicle protrusion (Giorgini and Comoli 1996, Da Silva et al. 2004, 2008). The events occurring during later germination stages leading to plant regeneration have not yet been described. Contrary to seeds and whatever the species, very few studies are currently available that describe the histological and biochemical events during SE germination and embryo-to-plantlet conversion. No literature is currently available on coffee SE germination and plantlet regeneration.

In *C. arabica*, SE are mass produced in a bioreactor and the plants are subsequently regenerated in a nursery after direct planting ex vitrum (Barry-Etienne et al. 1999). This process involves planting in a horticultural soil of SE that have undergone the first germination stages in temporary immersion bioreactors. Somatic embryo-derived plants and seedlings showed the same morphology when aerial and root systems of 30 cm tall plants were compared (Menéndez-Yuffá et al. 2010). However, it took 32 weeks for seedlings and 41 weeks for somatic seedlings to generate such plants. Moreover, only about 40% of SE succeeded in developing a plant after this additional delay. Afreen et al. (2002) showed that *Coffea arabusta* SE at the cotyledonary and germinated stages could be cultured photo-autotrophically in a semi-solid medium to ensure plantlet development. However, bulk cultivation of micropropagated plantlets in a liquid medium is still difficult in large vessels because of the poor light penetration to the plant tissues (Ducos et al. 2007). Illumination of plants grown in a bioreactor is not easy because of the substantial reduction in light intensity passing through the compact biomass (Takayama and Akita 2006). It has been concluded that a new technology is required to enhance the illumination efficiency for bulk production of transplantable plantlets. In *Coffea canephora* Pierre ex. Froehner SE, Ducos et al. (2010) showed that longer and greener embryos were produced in a horizontal bioreactor, thus ensuring higher light transmittance than in a bioreactor with vertical design.

This study was aimed at improving the plant conversion efficacy of SE. The results revealed marked differences in the morphological, histological and hydration characteristics of coffee ZE and SE during germination and subsequent plant conversion. A new bioreactor specifically designed to accommodate the unique features of SE has improved the germination conditions and ensured highly efficient plant regeneration from SE.

## Materials and methods

### *Plant material and SE regeneration in a temporary immersion bioreactor RITA®*

All experiments aimed at comparing ZE vs. SE germination were conducted with the *C. arabica* L. variety Caturra. Seeds were harvested at the Centro de Investigación sobre el Café (CICAFE) research centre in Barva (Costa Rica), depulped, fermented and dried to 30% moisture content. The SE-to-plantlet conversion rate was assessed at the industrial level in the ECOM group nursery in Sebacco (Nicaragua) using the *C. arabica* intraspecific F1 hybrid T5296 × Rume Sudan. The somatic embryogenesis process involves four stages: embryogenic callus production, embryogenic aggregate proliferation in suspension, embryo development and germination (Etienne 2005). The embryogenic callus derived from leaf pieces was placed in a modified Murashige and Skoog liquid proliferation medium (MS/2) with 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.65 µM kinetin until it produced a cell suspension of embryogenic aggregates (3–4 months). A temporary immersion bioreactor (Teisson and Alvard 1995) was used for the embryo development and germination phases using the media described by Etienne (2005). The RITA® bioreactor (CIRAD, Montpellier, France) is a 1 l unit with an upper compartment in which the plant material is placed and a lower compartment filled with the culture medium. The two compartments are connected to a pump which transfers the liquid medium from the lower compartment to the upper one. Two hundred milligrams of embryogenic aggregates were placed in the bioreactor along with 200 ml of an 'R' MS/2 regeneration medium (Etienne 2005) containing 17.8 µM 6-benzylaminopurine (6-BA). Embryos were subcultured once every 2 months. The development of cotyledonary SE was achieved after 4 months in this medium, with an immersion frequency of 1 min every 12 h. At the end of the development phase, the content of each bioreactor [around 8000 cotyledonary-shaped SE (Figure 1)] was distributed into different bioreactors to achieve a final density of ~700 embryos per bioreactor to enhance germination. Germination was triggered by applying an 'EG' MS liquid germination medium containing 1.33 µM 6-BA for 2 months, and finally for 2 weeks the embryo germination culture medium was supplied with 234 mM sucrose. The immersion frequency was maintained at 1 min every 12 h during this stage. By the end of the in vitro culture stage, each bioreactor contained around 700 germinated SE with an elongated embryo axis, a

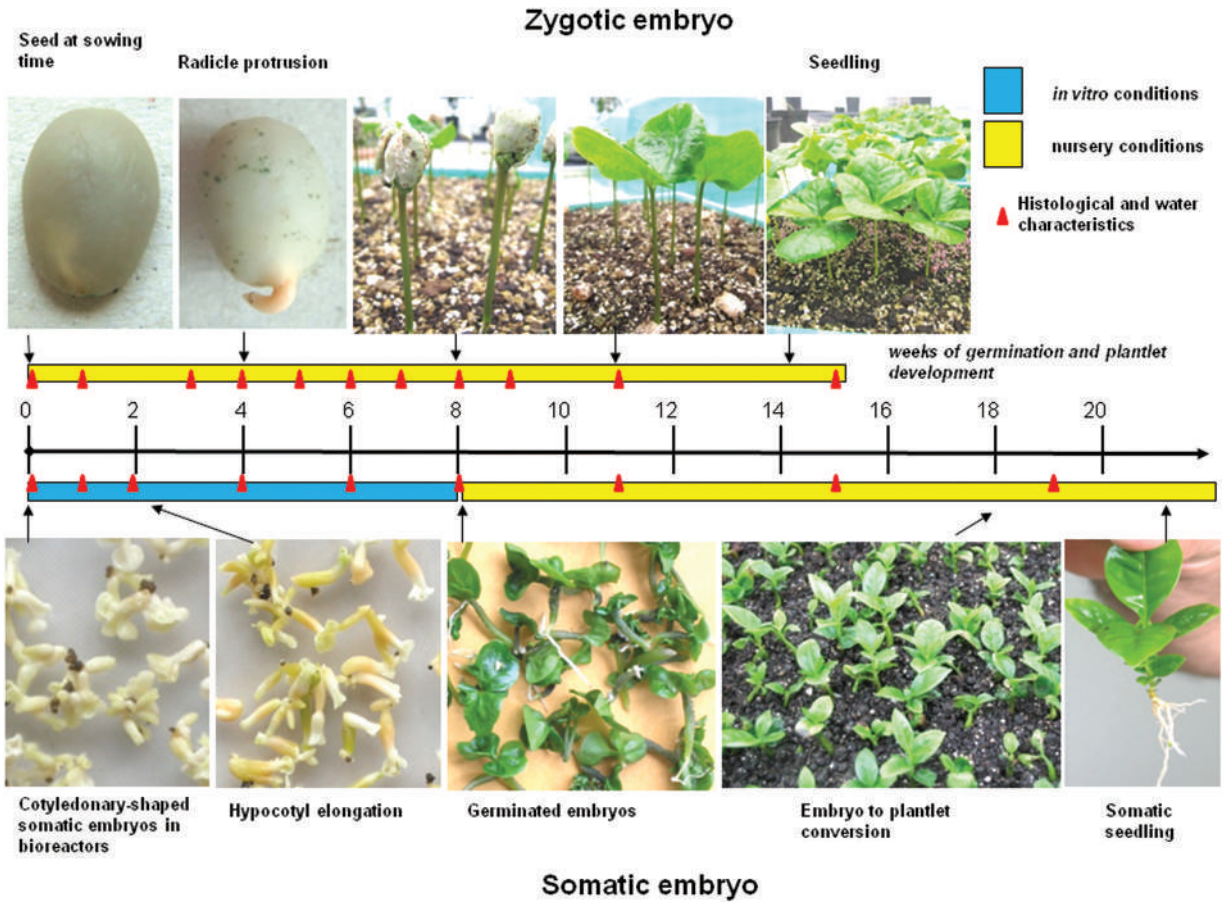


Figure 1. Different stages in the germination and plantlet regeneration of ZE (upper) and SE (lower) in *C. arabica*. The time scale is given. Samples used to compare the histological and water characteristics are indicated by red triangles.

pair of fully expanded chlorophyllous cotyledons and, for most of them, a radicle or a root tip (Figure 1). The embryo development and germination stages were achieved under light conditions (12 h photoperiod, 50 µmol photons $m^{-2}$ $s^{-1}$). All the in vitro cultures were kept at 26–27 °C.

### *Embryo sowing and plant conversion*

Germinated SE were planted vertically on the substrate surface [three parts peat moss-based growing media (PRO-MIX®, Rivière-du-Loup, Québec, Canada)/one part coffee pulp]. The culture density was ~3600 SE $m^{-2}$. The cultures were placed under a transparent roof and watered 2 × 2 min $day^{-1}$. Conversion into plantlets exhibiting one pair of leaves was generally achieved after 19–23 weeks (Figure 1). Seeds were sown in rows under 2 cm planting soil under the same culture conditions (substrate type, planting density, fertilization, shade level, watering frequency) as those used for germinated SE. Containers with SE or ZE were placed together at random.

### *Germination stages studied*

The germination and plant conversion process of seeds and SE is represented in Figure 1. Somatic embryos were studied at the cotyledonary stage, and after 1, 2, 4, 6 and 8 (germinated stage) germination weeks following transfer into RITA® bioreactors, and 11, 15 and 19 weeks of growth in planting soil. The ZE characteristics were analyzed at the mature stage in dehydrated seeds, and after 1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and 15 germination weeks following sowing in planting soil.

### *Histology and scanning electron microscopy*

Randomly chosen ZE (10 per germination stage) and SE (10 per stage) were fixed for 24 h in a solution containing 1% glutaraldehyde, 2% paraformaldehyde and 1% caffeine in 0.2 mM phosphate buffer at pH 7.2. For histological studies, the samples were dehydrated in a graded series of ethanol, embedded in 7100 resin (LKB Instruments, Mount Waverley, Vic, Australia) and cut into 3.5 µm thick longitudinal sections. The sections were stained with periodic acid-Schiff and naphthol blue black (NBB). Periodic acid-Schiff specifically stains polysaccharides red (walls and starch) and NBB stains soluble and insoluble proteins blue (Fisher 1968, Buffard-Morel et al. 1992). Sections were observed by conventional light microscopy through a DM6000 Leica microscope and photographed. Two magnification lenses were used: Lens 109 numerical

aperture = 0.30 HC PL Fluotar (Ref. Leica 11506505, Wetzlar, Germany) and Lens 209 numerical aperture = 0.70 HC Plan APO (Ref. Leica 1506166, Wetzlar, Germany). Pictures were taken with a Retiga 2000R camera (Q-Imaging Co., Surrey, BC, Canada). For scanning electron microscopy (SEM), fixed samples were dehydrated in a graded series of ethanol up to 100% ethanol and then further dried to the $CO_2$ critical point (Homès 1975). The samples were coated with platinum–gold and mounted on a slide with adhesive tape for SEM examination (Hitachi, 2360, Tokyo, Japan).

### *Quantitative starch determination*

In order to measure the starch embryo content, ZE were rapidly dissected from mature and germinating seeds. Samples of 30 SE or ZE (four samples were analyzed for each developmental stage) were either frozen in liquid nitrogen and stored at –80 °C or immediately homogenized. Samples were homogenized in ice-cold 0.7 M perchloric acid and centrifuged at 13,000 *g*, for 45 min at 4 °C. Starch was assayed on the pellets according to Smith and Zeeman (2006). Briefly, the pellets were washed once in water and twice in 80% (v : v) ethanol, then resuspended in 200 mM sodium acetate (pH 4.8), boiled for 10 min and digested overnight with α-amyloglucosidase and α-amylase. The amount of glucose released into the incubation medium was determined enzymatically.

### *Water parameters of an embryo and endosperm*

Fresh weight, dry weight (DW), water content (WC) and relative water content (RWC) were measured on SE and ZE axes and endosperm from four batches of 20–30 representative germinating SE, or 20–30 ZE, or 15 pieces of endosperm. Relative water content represents the (fresh weight–DW) : (saturated weight–DW) ratio, where DWs were determined by keeping the samples at 80 °C for 24 h and saturated weights were obtained by keeping the samples at 5 °C for 24 h between two water-soaked filter papers. The water potential ($\Psi$) was measured using a Wescor C.51 thermocouple hygrometer sample chamber (Wescor, Logan, UT, USA) and a dew point microvoltmeter (HR 33). Samples were kept in the measuring chamber for 3.5 h to attain vapor equilibrium. A 20 s cooling period was used to measure the dew point and the microvoltmeter was calibrated against NaCl standards in bars at 20 °C (Lang 1967). After $\Psi$ measurement, leaf tissues were frozen and left again for 1.5 h in the measuring chamber before recording the osmotic potential $\Pi$. The pressure potential ($P$) was calculated from the equation $\Psi = \Pi + P$. The potentials are means of five measurements on ZE or SE tissues and endosperm.

### *Effect of bioreactor size and design on SE production and plantlet conversion*

Two types of bioreactors were tested: RITA® and MATIS®. A new polycarbonate MATIS® bioreactor (CIRAD, Montpellier, France) was specially designed and patented with the following main features: (i) a larger size than RITA® (5 vs. 1 l) in order to scale up embryo production, and (ii) a larger area (355 vs. 55.8 $cm^2$) to spread out the biomass better to obtain greater light transmittance. The 1 l RITA® and 5 l MATIS® are both round temporary immersion bioreactors that differ in their diameters (9.5 and 35 cm, respectively) and vessel sizes, but with similar heights (14 vs. 15 cm, respectively). RITA® and MATIS® were inoculated respectively with 10 and 25 g cotyledonary-shaped embryos from the *C. arabica* intraspecific hybrid T5296 × Rume Sudan. The other in vitro culture and nursery conditions were identical to those described above. The evaluation of embryo numbers and developmental stage distributions were conducted at the end of an 8-week subculture. The final embryo-to-plantlet conversion rates were assessed after 20 weeks in the nursery. All the values are means ± SD calculated from eight measurements in different bioreactors.

### *Statistical analysis*

Most of the data were analyzed by analysis of variance followed by Tukey's honestly significant difference test. Asterisks in figures and different small letters in tables indicate significant differences for each parameter between means ($P \leq 0.001$, Tukey's test).

## Results

### *Embryo-to-plantlet conversion*

The whole germination process—from the fully developed embryo to the first pair of leaves—was about 19–23 weeks for SE and about 15 weeks for ZE (Figure 1). Germination, characterized by radicle emergence, was high for both embryo types after 4 weeks (96 ± 3.2% for ZE vs. 91 ± 4.0% for SE). However, the plant conversion frequency of SE in planting substrate was only 55% on average after 22 weeks, while plant conversion from seeds was almost always at 96% under the same nursery conditions (Table 1). Nevertheless, high variability in conversion rates was noted among SE batches since values can vary within a range of 30–75%.

### *Morphological changes during germination*

Zygotic embryos and SE growth curves were comparable during the first 4 weeks of germination, but the patterns differed considerably thereafter (Figure 2a and b). Zygotic embryos began growing very rapidly by the 6th week (Figure 2a and b). Conversely, SE development was very slow and stunted, so plantlets generated between the 15th and the 20th week were small in size (Figure 1). The stunting was due to a very short hypocotyl (~1 cm) compared with seedlings (~8 cm) as well as a reduced leaf area, with atrophied cotyledons and small initial pairs of leaves. Moreover, ZE early root development was much more vigorous than that of SE, when comparing total

Table 1. Plantlet conversion efficiency of coffee SE produced in RITA® in an industrial nursery for the *C. arabica* intraspecific F1 hybrid T5296 × Rume Sudan.

| Date of sowing of somatic embryo batches | No. of sown embryos | No. of regenerated plantlets | Embryo-to-plantlet conversion rate (%) | Plant conversion duration (weeks) |
|---|---|---|---|---|
| 02/11/2008 | 2330 | 1328 | 57 | 20.2 |
| 04/11/2008 | 9170 | 6887 | 75 | 21.0 |
| 05/11/2008 | 7800 | 3820 | 49 | 22.0 |
| 07/12/2008 | 9300 | 2964 | 32 | 22.7 |
| 18/12/2008 | 9000 | 4983 | 55 | 23.6 |
| 19/12/2008 | 10,800 | 7451 | 69 | 22.5 |
| 24/12/2008 | 5100 | 2642 | 52 | 21.8 |
| 26/12/2008 | 1260 | 708 | 56 | 23.7 |
| 27/12/2008 | 10,500 | 6348 | 60 | 22.0 |
| 06/01/2009 | 10,200 | 4335 | 43 | 21.1 |
| Total | 75,460 | 41,465 | | |
| Means | | | 54.8 ± 12.2 | 22.1 ± 1.1 |
| Control seed | 700 | 672 | 96 | 14.0 |

Data were obtained from 10 independent SE batches from the commercial production of Year 2008. Seeds from the *C. arabica* var. Caturra were used as control.

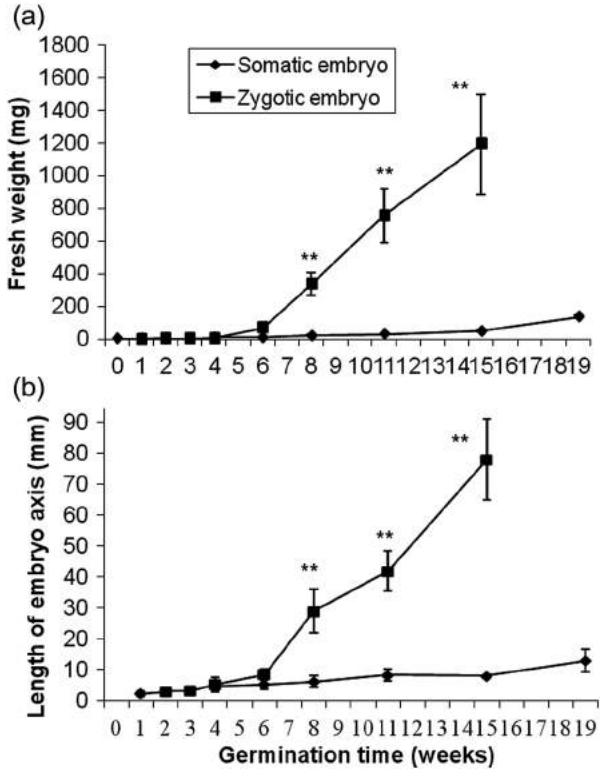


Figure 2. Growth curves of ZE and SE during germination and plantlet regeneration. (a) Variations in fresh weight (mg); (b) variations in embryo hypocotyl length (mm). Fresh weight and embryo hypocotyl length are means of measurements of 100 representative germinating embryos. Asterisks indicate significant differences for each parameter between means obtained for SE and ZE ($P \leq 0.001$, Tukey's test).

root lengths of young plantlets at the two-leaf stage (129 ± 21 cm vs. 29 ± 17 cm, respectively). At the cotyledonary stage, SE and ZE were similar and very small in size (~2–3 mm) and weight (~2 mg) (Figure 2). However, ZE were quite uniform in shape (Figure 3a), whereas SE were very heterogeneous and usually clustered, so their embryo axis and cotyledon dimensions were highly variable (Figures 1 and 3d). Contrary to SE, ZE consisted of a cylindrical axis with a smooth epidermis and two adherent cordiform cotyledons (Figure 3a). In contrast to SE, the epidermal cells of the ZE were aligned perfectly (Figure 3f vs. c). During the first 4 weeks of germination, ZE growth was the result of a greater cell elongation than in SE (Figure 3c vs. f), with cell lengths of 19.9 ± 2.9 and 10.9 ± 1.5 µm, respectively. This elongation was greater in the basal part of the hypocotyl, where it was accompanied by spectacular thickening and curving (Figure 3b), which then facilitated the radicle protrusion noted by the 4th week, whereas the cotyledons remained folded in the seed for a long time (until the 9th week). Stomata were observed on the cotyledons at the 9th week. Cotyledons of SE showed characteristics of differentiated leaves much earlier because many stomata were detected by the 4th week of germination in a bioreactor (Figure 3e vs. 3b, inset).

## *Histological changes during germination*

### *Zygotic embryos*

The endosperm of a mature coffee seed was composed of hard tissue with polyhedral cells (Figure 4a). These were irregular in shape, having a visible nucleus with very thick walls with a high amount of red-stained polysaccharides, mainly containing NBB-stained proteins (Figure 4c). The embryo developed within a cavity surrounded by endosperm prior to radicle protrusion (Figure 4a). Embryo axis cells were highly differentiated, with a regular polyhedral shape, and were aligned in rows (Figure 4b). Around the embryo axis and cotyledons, the cells were plasmolyzed and had convoluted cell walls (inset). High amounts of protein reserves were observed in both the embryo and endosperm. A week after sowing, although it had very little prior contact with the endosperm, the embryo was now in close contact with the endosperm reserves via a mucilage coating (Figure 4f). These protein reserves were mainly concentrated around the axis (Figure 4e) and cotyledons. Plasmolysis decreased in the embryo, the cells had become larger and the vacuoles were more voluminous (Figure 4e vs. b). The cotyledons and root and shoot meristems were not at all differentiated (Figure 4d and g). During the second and third week of germination, two or three endosperm cell layers closest to the embryo had been depleted of their polysaccharide and protein reserves and collapsed (Figure 4h and i). This phenomenon mainly occurred from the first week in the area closest to the future root tip (Figure 4d). The embryo protein and starch reserves had also changed. The embryo axis and cotyledons

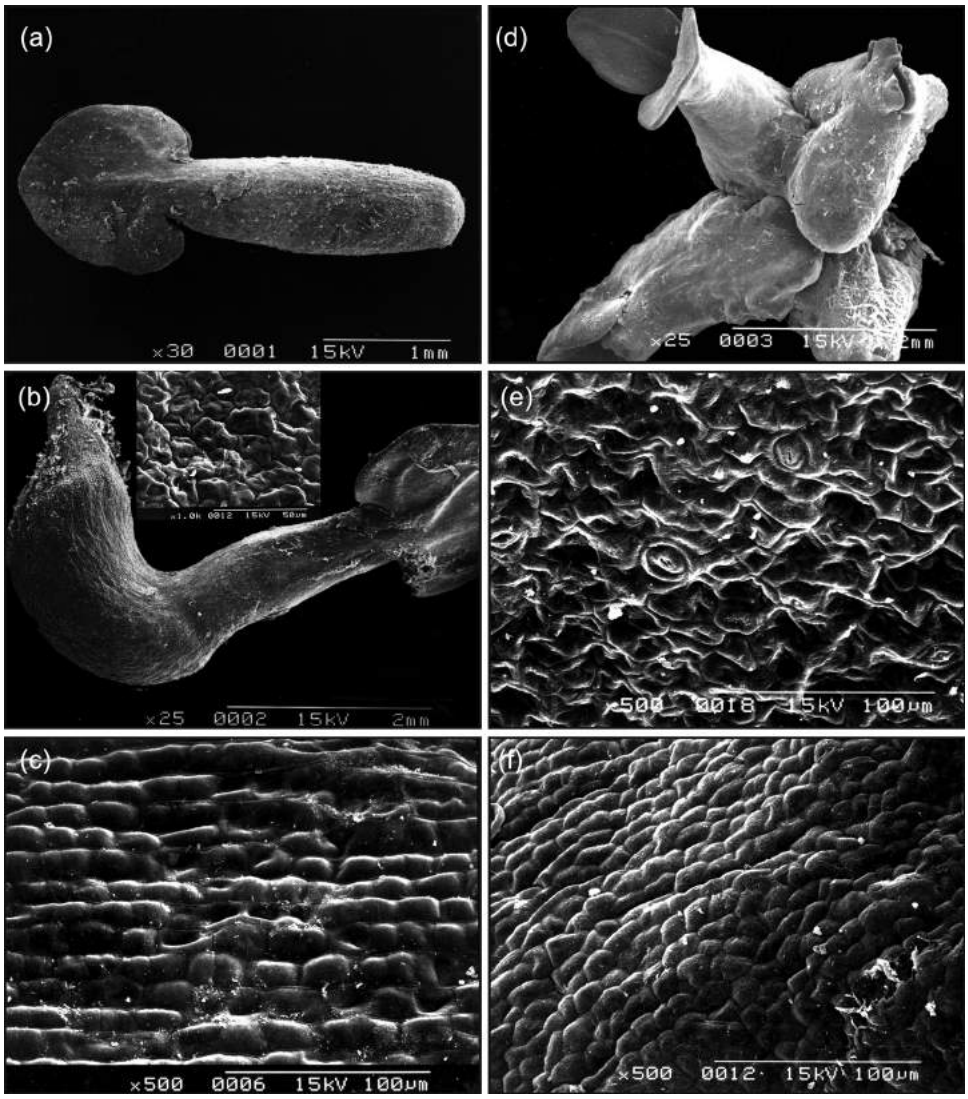


Figure 3. Scanning electron microscopy micrographs of germinating zygotic (a–c) and somatic (d–f) embryos of *C. arabica*. Zygotic embryo: (a) embryo from mature seed, bar = 1 mm; (b) embryo after 4 weeks of germination, bar = 2 mm; inset, abaxial surface of a cotyledon from 6-week-germinating ZE, bar = 50 µm; (c) surface of the hypocotyl from 4-week-germinated ZE, bar = 100 µm. Somatic embryo: (d) cluster of cotyledonary-shaped embryos, bar = 2 mm; (e) abaxial surface of a cotyledon from 4-week-germinating SE, bar = 100 µm; (f) surface of the hypocotyl from 4 week-germinating ZE, bar = 100 µm.

had lower protein content while being enriched in many starch grains located around the nucleus (Figure 4h). An analysis of the starch content confirmed a marked but not steady increase (from 12 to 30 mg $g^{-1}$ DW) during this period (Figure 5). During the 4th week of germination, the starch content was high in the embryo axis and cotyledons, which had generally not differentiated (Figure 6a). The vascular tissue developed when xylem began forming (Figure 6b). The shoot apical meristem was still not organized (Figure 6b), while radicle protrusion occurred during the 3rd week. After 6 weeks of germination, the endosperm still contained high quantities of storage proteins and polysaccharides, and continued to be depleted of these reserves in the area adjacent to the embryo (Figure 6c). The part of the hypocotyl in contact with the endosperm had a very high starch content, whereas the part that had developed outside of the seed had cells with markedly reduced storage protein content and with very little starch (Figure 6d). The cotyledons were still compact (Figure 6c) and contained high amounts of small starch grains. The vascular tissues were developing. There were also no stomata (data not shown). Seven weeks after sowing, endosperm cells had lost almost all of their protein and polysaccharide reserves (Figure 6e). The cotyledons had developed considerably and were tightly folded inside the seed and had a high starch content (see inset) and vascular tissues (Figure 6f and g). The palisade parenchyma was forming, along with the spongy parenchyma. Hypocotyl cells had elongated considerably and had lost a substantial amount of their starch content (Figure 6h and i).

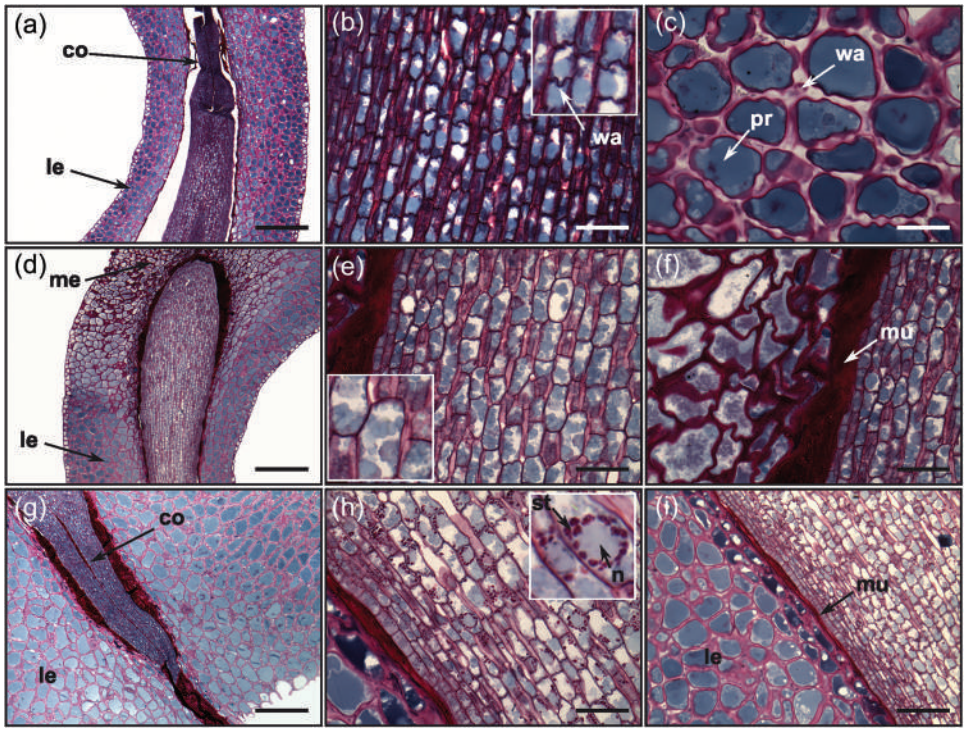


Figure 4. Light micrographs of cross-sections of ZE and endosperm during early germination (from Weeks 1 to 3). Mature seed: (a) endosperm and embryo, scale bar = 390 μm; (b) embryo axis cells with high contents of reserve (protein and starch) compounds and cell plasmolysis, scale bar = 50 μm; inset, illustration of plasmolysis and twisted cell walls; (c) endosperm cells with high levels of storage proteins (stained in blue) and polysaccharide reserves in the cell walls (stained in red), scale bar = 50 μm. After 1 week of germination: (d) endosperm digestion around the root pole, scale bar = 390 μm; (e) embryo axis cells, scale bar = 50 μm; inset, large cells with voluminous vacuoles; (f) mucilage around the embryo : endosperm interface and collapse of endosperm cells (left), scale bar = 50 μm; (g) cauline pole and cotyledons, scale bar = 390 μm. After 3 weeks of germination: (h) accumulation of starch reserves (inset, starch grains around the nucleus) and reduction in protein reserves in embryonic axis cells, scale bar = 50 μm; (i) embryo : endosperm interface, scale bar = 100 μm. co, cotyledon; le, lateral endosperm; me, micropylar endosperm; mu, mucilage coating; n, nucleus; pr, protein; st, starch; wa, cell wall.

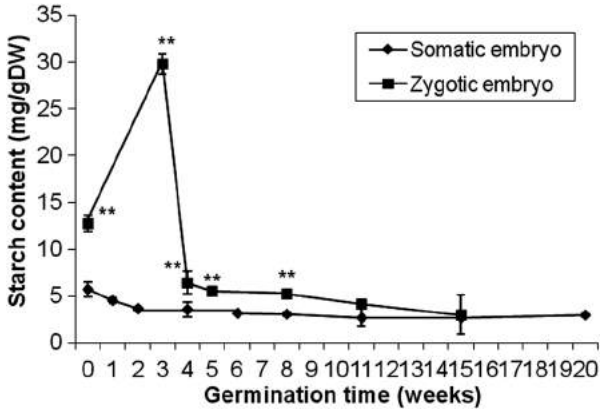


Figure 5. Variations in starch content in *C. arabica* var. Caturra ZE and SE during germination and plant conversion. Starch quantification was done with 30 SE or ZE. Starch content values are the means of four measurements ± SD on independent samples. The bars represent the standard deviations (SD). Asterisks indicate significant differences for starch content between means obtained for SE and ZE ($P \leq 0.001$, Tukey's test).

### *Somatic embryos*

The cotyledonary-stage SE, like ZE, did not develop either of the two meristems (Figure 7a). The vascular tissue was forming (procambial strands) and many dividing cells were visible in the vicinity of its future location (Figure 7a). The epidermis was well differentiated but much more irregular than in ZE (Figure 7b and c). The embryo axis, contrary to what was observed in ZE, had many intercellular spaces in which spongy tissue was forming, and the cells were not aligned (Figure 7c). Another difference was that cotyledonary-shaped SE contained very little starch and even less protein (Figure 7c). These reserves were more abundant in the epidermal region (Figure 7b and c). Cotyledons were forming and contained high amounts of starch and protein (Figure 7d). After a week of germination in a bioreactor and under light conditions, SE had elongated but their surfaces were still rough (Figure 7e). At this point, cotyledons were better structured but the tissues were still tight and had high starch and protein contents (Figure 7f). Some embryos had a forming shoot and/or a root meristem (Figure 7g). By the 2nd week of germination, the axis, especially in the vicinity of vascular bundles, contained higher quantities of protein and especially starch in the form of small tightly clustered grains (Figure 7h). Contrary to ZE, the starch content decreased during the first 3 weeks of germination (Figure 5). After 4–6 weeks, cotyledons resembled leaves

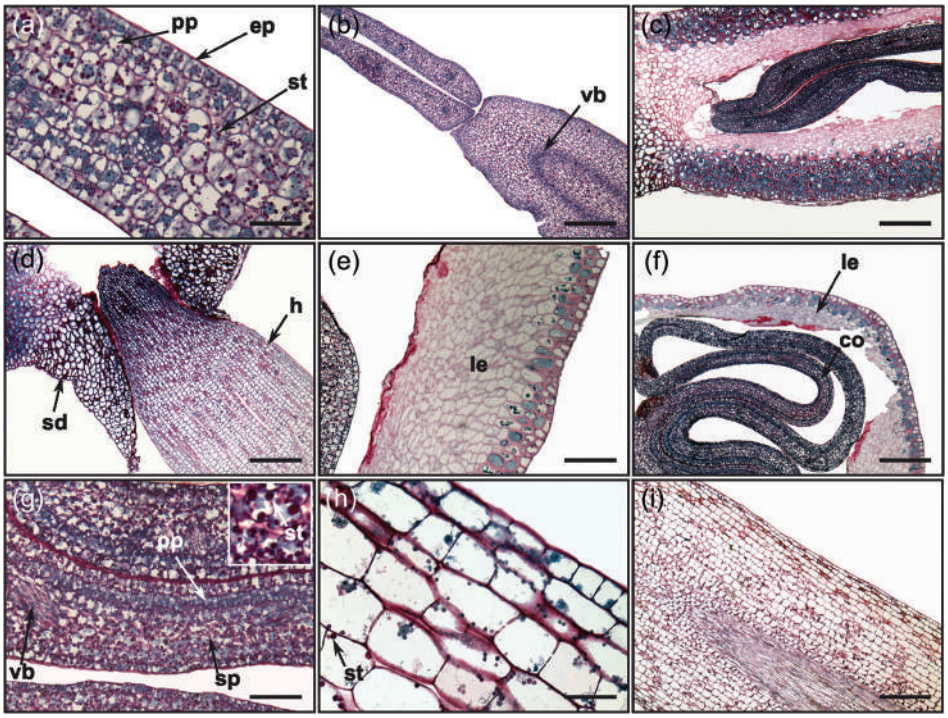


Figure 6. Light micrographs of cross-sections of ZE and endosperm during germination and plant conversion (Weeks 6 and 7). After 6 weeks of germination: (a) cotyledon, scale bar = 50 μm; (b) cauline pole of the embryo, scale bar = 390 μm; (c) cotyledon : endosperm interface, scale bar = 200 μm; (d) emerging seed hypocotyl, scale bar = 390 μm. After 7 weeks of germination: (e) endosperm depleted of its reserves, scale bar = 200 μm; (f) folding of highly developed cotyledons, scale bar = 390 μm; (g) cotyledons, scale bar = 100 μm (inset, starch grains); (h) hypocotyl cells, scale bar = 50 μm; (i) hypocotyl, scale bar = 100 μm. co, cotyledon; ep, epidermis; h, hypocotyl; le, lateral endosperm; pp, palisade parenchyma; sd, seed; sp, spongy parenchyma; st, starch; vb, vascular bundles.

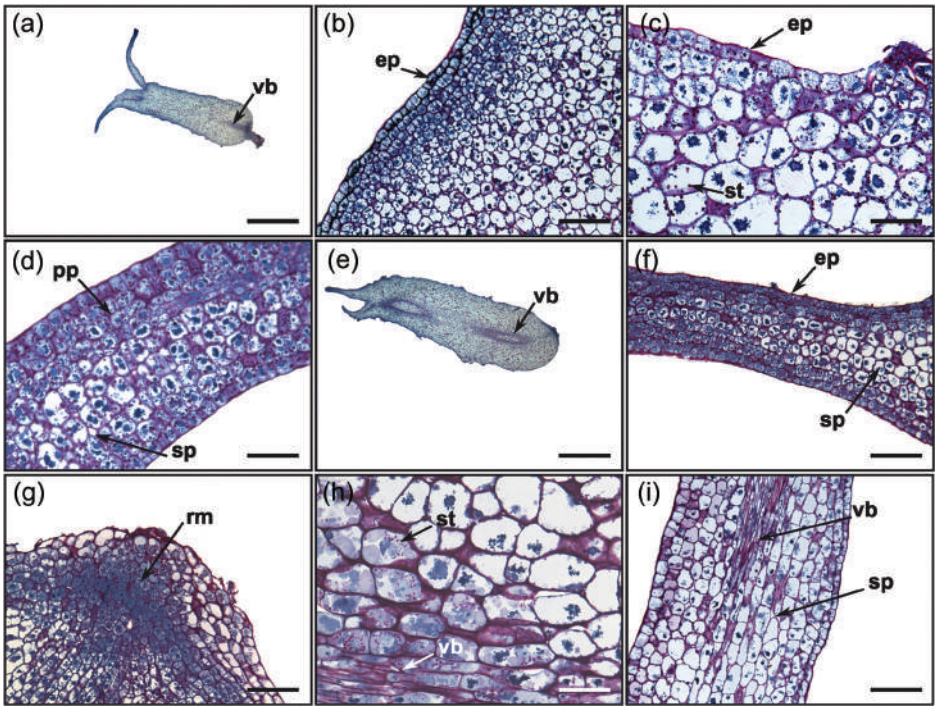


Figure 7. Light micrographs of cross-sections of SE during early germination (from Weeks 1 to 4). Cotyledonary-shaped SE: (a) embryo, scale bar = 1.2 mm; (b) embryonic axis cells, scale bar = 50 μm; (c) zone where the embryonic axis is close to the epidermis, scale bar = 120 μm; (d) cotyledon, scale bar = 50 μm. After 1 week of germination: (e) embryo, scale bar = 1.6 mm; (f) cotyledon, scale bar = 100 μm; (g) forming root meristem, scale bar = 100 μm; (h) embryo axis cells after 2 weeks of germination, scale bar = 50 μm. After 4 weeks of germination: (i) cotyledon, scale bar = 50 μm. ep, epidermis; pp, palisade parenchyma; rm, root meristem; sp, spongy parenchyma; st, starch; vb, vascular bundles.

by a uniseriate palisade parenchyma and spongy parenchyma (Figure 7i). Vascular tissue formed with phloem and xylem. In the axis, a developing shoot meristem was sometimes observed, while starch and protein levels were already significantly lower (Figure 8a and b). After 8 weeks, germinated SE produced in a RITA® bioreactor had cotyledons exhibiting a leaf-like structure (Figure 8c). At this point, embryo axis cells were elongated and had a typical long polyhedral shape (Figure 8d). Their protein and starch reserves continued to decline. Vascular bundles were visible in both shoot and root zones (Figure 8e and f). After 10 weeks, cotyledons had large intercellular spaces in the lacunose spongy parenchyma (Figure 8g). The embryo axis had a well-formed apical shoot meristem with two axillary buds (Figure 8h). After 12 weeks of germination, including 4 weeks in planting substrate, all embryos had roots and 60% of them had formed a small stem bearing two leaflets (Figure 8i).

### *Water status changes in SE and ZE during germination and plantlet conversion*

#### *Seeds*

The mature seed albumen was highly dehydrated (WC 28%, $\Psi = -7.5$ MPa) (Figure 9a and b). The first 2 weeks after sowing corresponded to a rapid and substantial hydration phase (sharp increase in WC and $\Psi$). Water content levelled off over the next 4 weeks (~50%), but the water availability ($\Psi$) increased significantly, leading to high cell turgor (*P* values of around 1.6–2.2 MPa and RWC >90%). The high RWC during this period confirmed that endosperm hydration (WC) was near its maximal value (50%) by reason of the high amount of reserve compounds. The high $\Psi$ values indicated high hydration of the endosperm. From the 4th week, the steady increase in $\Pi$ together with that of WC during the four following weeks indicated the onset of reserve mobilization (Figure 9a and b). This phenomenon was perfectly in line with the marked increase in ZE fresh weight during the same period (Figure 2). Zygotic embryo hydration (Figure 10a and b) was more gradual than that of the endosperm (Figure 9a and b) as it spanned the first 5 weeks of germination [regular increase in WC and RWC (Figure 10b) and $\Psi$ (Figure 10a)]. However, the ZE water status changed markedly over this period. During the 2nd and 3rd week of germination, embryo turgor *P* increased, thus facilitating radicle protrusion (Figure 10a). *P* then decreased, indicating cell wall relaxation, in turn generating a new $\Psi$ gradient, which was essential to support the new influx of water observed during the 4th and 5th week of germination. The embryo water status had levelled off by the 5th week of germination. The embryo was then very hydrated, with high WC and

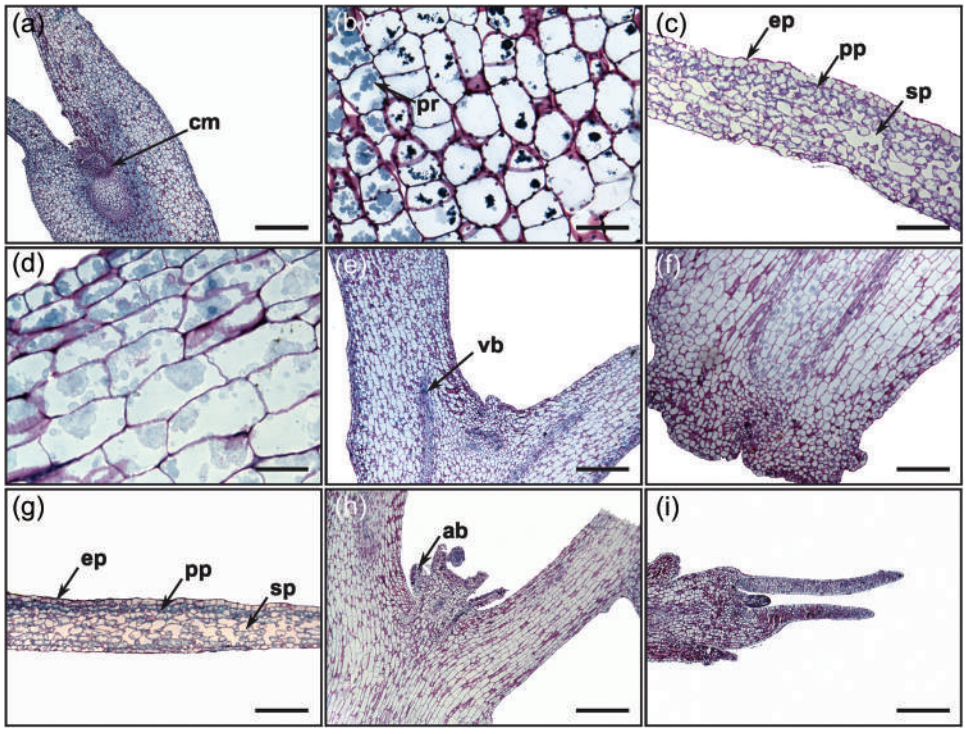


Figure 8. Light micrographs of cross-sections of SE during late germination and plant conversion (from Weeks 4 to 12). After 4–6 weeks of germination: (a) forming shoot meristem, scale bar = 390 μm; (b) embryo axis cells, scale bar = 50 μm. After 8 weeks: (c) cotyledon with a leaf-like structure, scale bar = 200 μm; (d) embryo axis cells, scale bar = 50 μm; (e) forming shoot meristem and vascular bundles, scale bar = 390 μm; (f) vascular bundles in the root pole, scale bar = 200 μm. After 10 weeks: (g) cotyledon, scale bar = 200 μm; (h) shoot meristem and axillary buds, scale bar = 390 μm. After 12 weeks: (i) stem with leaflets, scale bar = 390 μm. ab, axillary bud; cm, cauline meristem; ep, epidermis; pp, palisade parenchyma; pr, protein; sp, spongy parenchyma; vb, vascular bundles.

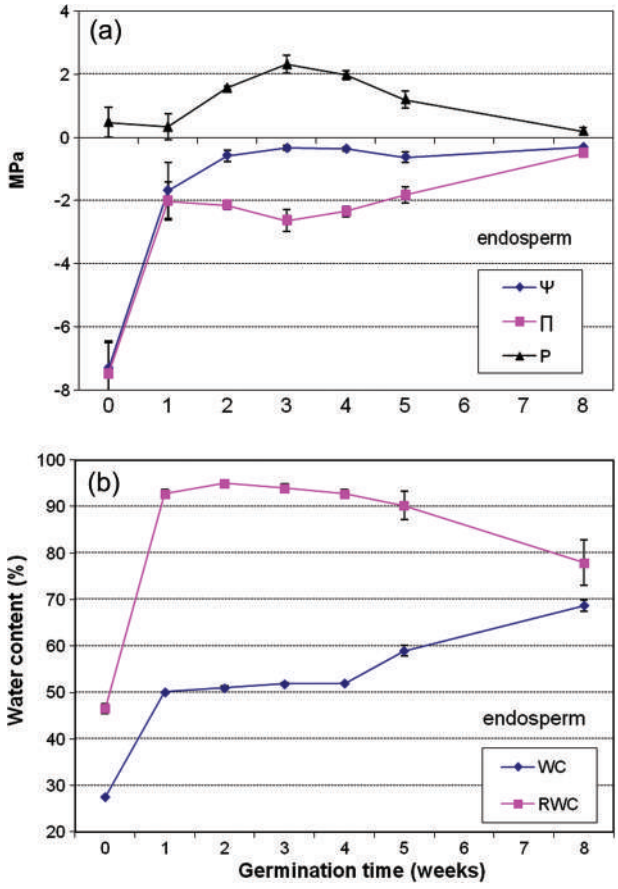


Figure 9. Variations in endosperm water status characteristics during ZE germination and embryo-to-plantlet conversion. Measurements were obtained on *C. arabica* var. Caturra during an 8-week nursery growth. The Ψ, Π and *P* values are means ± SD of five measurements. The WC and RWC values are means ± SD from four batches of 10–25 representative endosperm pieces.

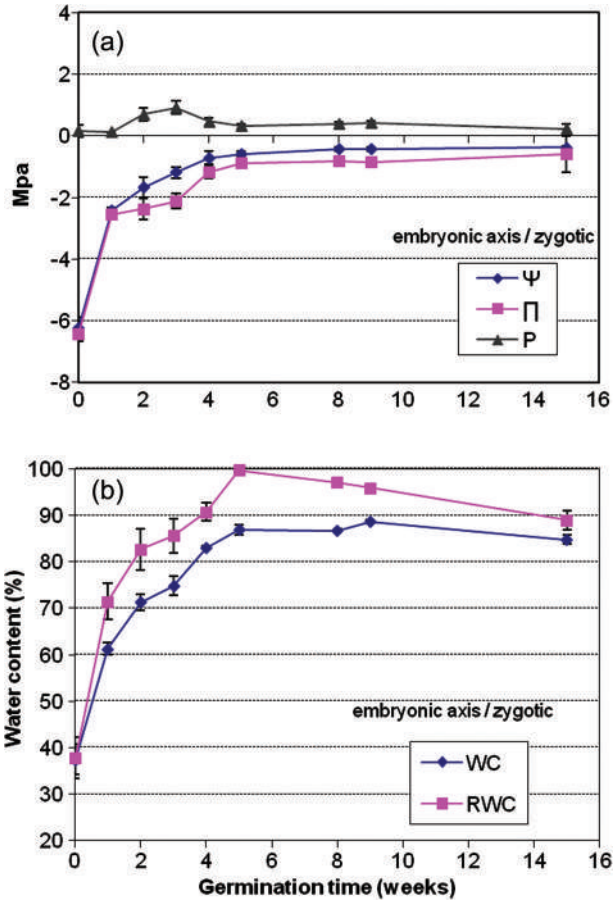


Figure 10. Variations in ZE water characteristics during germination and embryo-to-plantlet conversion. Measurements were obtained on *C. arabica* var. Caturra during a 15-week nursery period. The Ψ, Π and *P* values are means ± SD of five measurements. The WC and RWC values are means ± SD from four batches of 10–25 representative germinating ZE.

Ψ values (~88% and −0.4 MPa, respectively) (Figure 10a and b).

### *Somatic embryos*

There were few changes in the SE water status during germination and plantlet regeneration, especially with respect to the tissue water availability (Ψ; Figure 11a). There were, nevertheless, two distinct hydration phases, corresponding to the two following periods: Weeks 1–8 under in vitro culture conditions in a bioreactor, and Weeks 11–19 in planting soil. During the first 11 weeks, we noted a gradual hydration through the regular increase in the SE WC (from 70 at the cotyledonary stage to 85%; Figure 11b). During the in vitro steps, SE had significantly more negative Π values (between −1.0 and −1.5 MPa) than they did after being sown in planting soil (Figure 11a). The embryo tissues also had higher turgor than during the subsequent ex vitro plantlet regeneration phase (higher *P* values; Figure 11a). A new embryo water status was noted after transfer in planting soil (Weeks 8–19; Figure 11). This status no longer differed from that of ZE and it levelled off until the conversion into plantlets (Week 19). Under greenhouse conditions, SE thus appeared to be more hydrated but less turgid as compared with the initial in vitro germination steps [lower *P* (Figure 11a) and RWC (Figure 11b) values].

### *Influence of the bioreactor size and design on the embryo-to-plantlet conversion*

Temporary immersion bioreactors MATIS® and RITA® were compared for their ability to support germination and further plantlet conversion (Figure 12a). The germination and plant regeneration process was markedly enhanced in the larger and more horizontal MATIS® bioreactors (Table 2). After 8 weeks of culture, 65% of inoculated cotyledonary-shaped embryos regenerated plantlets directly in the MATIS® bioreactor, whereas the germinated stage was the most frequent (almost 70% embryos) in the RITA® bioreactor in which only 2.4% plantlets were obtained (Figure 12b and c). Twenty weeks

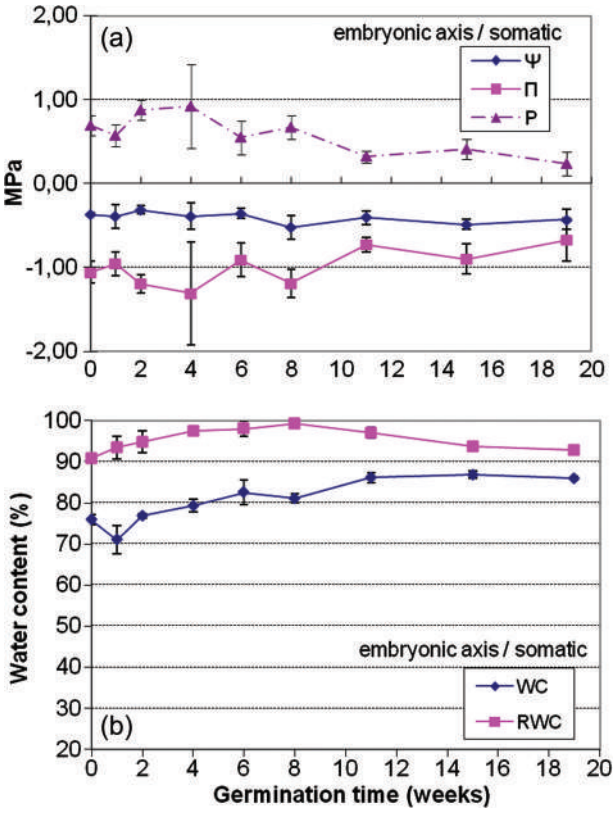


Figure 11. Variations in SE water parameters during germination and embryo-to-plantlet conversion. Measurements were obtained on *C. arabica* var. Caturra during the in vitro (Weeks 1–8) and nursery (Weeks 9–19) periods. The Ψ, Π and *P* values are means ± SD of five measurements. The WC and RWC values are means ± SD from four batches of 10–30 representative germinating SE.

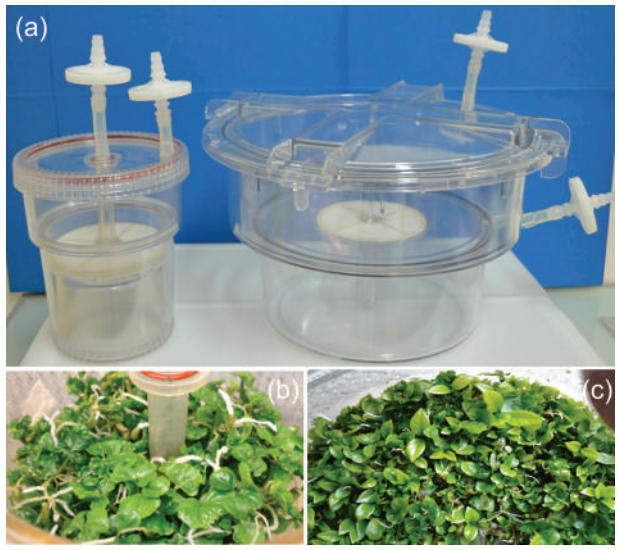


Figure 12. Improvement in coffee SE germination and further plant conversion by increasing the area of the temporary immersion bioreactor. (a) 1 l RITA® (on the left) and 5 l MATIS® (on the right) bioreactors. (b and c) Aspect of germinating embryos in the RITA® bioreactor (area 55.8 $cm^2$) (b) and the MATIS® bioreactor (area 355 $cm^2$) (c) after 8 weeks under germination conditions. All the bioreactors were inoculated with the same batch of cotyledonary-shaped embryos. Note that plantlets were more frequently produced within the larger MATIS® bioreactor (c); in spite of that germinated embryos were the more advanced developmental stages in the RITA® bioreactor (b).

after transfer in soil, the plantlet conversion rates were still much higher with embryos derived from the larger bioreactors (91 vs. 40%) (Table 2).

## Discussion

The most common strategy for developing somatic embryogenesis techniques is to try to mimic in ovulo events under the assumption that they are a model for SE development (Carman 1988). It is commonly recognized that the efficacy of ontogenesis and embryo maturation phases is a key to the success of conversion into plantlets. For this reason, in vitro culture conditions have been frequently optimized using histological, biochemical and, more recently, molecular comparisons between ZE and SE during these developmental phases (Dumont-Bédoux et al. 1996, Reidiboym-Talleux et al. 2000, Tereso et al. 2007, Sghaier et al. 2008, Hoenemann et al. 2010). There has been little interest in SE germination and plantlet conversion steps, although they may be critical for the plant regeneration frequency. This is one of the first comparative studies of coffee SE and ZE carried out during the germination and plantlet regeneration phases to optimize the embryo-to-plantlet conversion rate.

A coffee ZE, which accounts for only 1% of the seed DW, must consume abundant endosperm reserves to ensure its development. We previously showed that SE were able to germinate and convert into plantlets under ex vitro conditions (Barry-Etienne et al. 1999, 2002). However, the plant conversion rate has to be improved to ensure the commercial cost-effectiveness (Etienne et al. 2012). Under our culture conditions, seedling emergence in soil began after 50–60 days, which is in line with previous reports (Maestri and Vieira 1961). In SE, the actual germination period (root emergence) is comparable, but the time required for production of the first pair of leaflets is considerably longer. Germination is more asynchronous and the success rate is uneven and unsatisfactory. Comparisons between SE and ZE during ontogenesis and/or maturation phases in many species, including coffee, have revealed substantial qualitative differences (Etienne et al. 1993, Carasco et al. 1994, Reidiboym-Talleux et al. 2000, Stasolla and Yeung 2003). Our study also highlighted marked differences during germination and plantlet regeneration when focusing on their morphology, timing of development, tissue differentiation and heterogeneity in the different organs. At the beginning of the germination, SE like ZE were very small, but had irregular overall shape, especially with respect to certain organs such as the embryo axis, cotyledons and epidermis.

Table 2. Comparison of coffee SE germination and embryo-to-plantlet conversion rates obtained using two temporary immersion bioreactors with different sizes (l) and areas ($cm^2$).

| Bioreactor type | No. of units | No. of embryos/ bioreactor | Distribution of embryo developmental stages in the bioreactor (%) | | | Embryo-to-plantlet conversion rate in the nursery (%) |
|---|---|---|---|---|---|---|
| | | | Cotyledonary | Germinated | Plantlet | |
| 1 l RITA® (55.8 $cm^2$) | 8 | 805 ± 100b | 29.9 ± 7a | 67.7 ± 8.8a | 2.4 ± 0.9b | 40.1 ± 8b |
| 5 l MATIS® (355 $cm^2$) | 8 | 2500 ± 280a | 12.5 ± 3b | 22.4 ± 4.9b | 65.1 ± 2.6a | 91 ± 4.5a |

The RITA® (1 l) and MATIS® (5 l with a horizontal design) bioreactors were inoculated with 1000 and 2500 cotyledonary-shaped embryos, respectively. Embryo numbers and stages were evaluated at the end of an 8-week-subculture for the *C. arabica* intraspecific hybrid T5296 × Rume Sudan. The final embryo-to-plantlet conversion rates were assessed after 20 weeks in the nursery. All values are means ± SD calculated from eight measurements in different bioreactors. Different small letters indicate significant differences for each parameter between means ($P \leq 0.001$, Tukey's test).

Our findings showed that the morphological irregularity continued during germination and was previously described during SE ontogenesis (Albarran et al. 2005).

We revealed marked differences with respect to embryo starch and protein contents during the first weeks of germination. Zygotic embryos had a high protein content at the end of their maturation process. During the first 6 weeks of germination, they also benefitted from very substantial polysaccharide and protein reserves in the endosperm. Zygotic embryos assimilated cell wall polysaccharide reserves by accumulating starch in the growing tissues to be able to continue their development in the seed. These polysaccharides correspond to galactomannans (25% of the mature coffee grain fresh mass) and are associated with the secondary cell wall thickening of endosperm cells (Fisher et al. 2001, Pettollino et al. 2001) and may serve as an important food reserve for the developing seedling (Bewley and Reid 1985, Clifford 1985). The low vigour of somatic seedlings observed during early development could be caused by the initial severe lack of reserve compounds at the beginning of the germination process. Recent studies on in vitro cultured isolated coffee ZE confirmed that they developed into small-sized plantlets similar to those derived from SE when they were unable to tap endosperm reserves (Alpizar et al. 2006). Previous studies on carrot showed that SE and ZE display different protein patterns until conversion to plants, and that none of the proteins required for maturation was accumulated in SE (Dodeman et al. 1998). Similarly to coffee, in germinating *Pinus pinaster* Aitor ZE, starch accumulated in the growing preformed tissues, i.e., successively in the emerging radicle, growing hypocotyls and expanding cotyledons, closely following the spatiotemporal pattern of triglyceride depletion, whereas in SE, starch was only found in cortical cells close to the culture medium (Jordy and Favre 2003).

For most seeds, germination begins by water uptake. Seed imbibition, which directly contributes to rapid embryo elongation and root emission, is generally a triphasic process with a rapid initial water uptake in phase I (Bradford 1990, Bewley and Black 1994). Da Silva et al. (2004) confirmed that water uptake during imbibition of coffee seeds followed the common triphasic pattern. The entire SE germination and plant regeneration process took place at an almost constant water status, with some slight changes occurring after transfer to the nursery. Since the studies of Yeung and Brown (1982), it has been known that the ZE environment is characterized by the low osmotic potential of seed compartments. Coffee SE, which developed in the water-saturated environment of bioreactors, began the germination process being highly hydrated in comparison with ZE (WC 70 vs. 35%) and had a high $\Pi$. In some species, the switch from the development to germination mode depends on desiccation (Bewley et al. 1989, Bradford 1990). For a number of woody species such as conifers and European chestnut, the germination rate of mature SE was improved by slow drying under high relative humidity before transfer to the germination medium (Carman 1988, Bomal and Tremblay 1999, Malabadi and van Staden 2005, Corredoira et al. 2008). To our knowledge, coffee SE have never been tested in desiccation, osmotic or priming treatments with the aim of enhancing embryo uniformity in germination and plantlet conversion.

A mature coffee seed contains an embryo, which still grows before completion of germination, especially through the spectacular cotyledon development (Da Silva et al. 2004). This characteristic, which generally concerns seeds containing immature embryos, also applies to seeds having primitive traits such as a copious endosperm surrounding an underdeveloped embryo (Forbis et al. 2002), which may explain the extent of difference in size between ZE and SE. Our study showed that the coffee SE physiological maturation process was incomplete. Providing conditions that are conducive to maturation and desiccation in vitrum could enhance SE performance during bulk germination and ex vitro conversion into plantlets by stimulating the accumulation of specific reserve compounds and allowing more vigorous and synchronized germination. Here, we showed that it was possible to greatly improve the embryo-to-plantlet conversion rate (>90%) by using a temporary horizontally designed (6.5-fold increase in area) immersion bioreactor to favor light transmittance to SE. In coffee SE germinated under light, the histological and SEM

analysis revealed early differentiation of cotyledons into a leaflet structure, which probably gave rise to rapid photoautotrophy after ex vitro transfer. Somatic embryos thus showed high phenotypic plasticity with respect to environmental stimuli such as light exposure and a high adaptation potential. Conversely, ZE cotyledons remained folded in the seed for a long period and were only exposed to light at the end of the germination process. They became photosynthetically functional during the late germination steps prior to leaf expansion. Interestingly, previous studies of Valio (1976) and Da Silva et al. (2005) showed that coffee seeds were sensitive to white light, which delayed seed germination. They related this inhibitory effect to light induction with gibberellin biosynthesis (Hilhorst and Karssen 1992), causing a supra-optimal endogenous concentration that was toxic and delayed germination (Da Silva et al. 2005). This phenomenon might be specific to coffee seeds as, on the contrary, light has been found to strongly stimulate germination of both SE and isolated ZE (Afreen et al. 2002, Alpizar et al. 2006). Afreen et al. (2002) established in vitro photo-autotrophic cultures of *C. arabusta* SE and demonstrated the photosynthetic activity of cotyledonary and germinated embryos. Plantlet development was obtained by exposure to a high photosynthetic photon flux (100–150 $\mu mol\ m^{-2}\ s^{-1}$) in a $CO_2$-enriched atmosphere (1100 $mol\ mol^{-1}$). Recently, Ducos et al. (2009) confirmed the positive impact of $CO_2$ released by commercial coconut fiber-based substrates on ex vitro plant conversion (+20%) of bulk *C. canephora* Pierre SE. These results confirmed that it was possible to stimulate the photo-autotrophy of coffee SE during germination and further plantlet conversion.

In order to optimize plant conversion of SE in many species, it could be more effective to make use of the high phenotypic plasticity of embryos with respect to certain physiological functions like photosynthesis in order to stimulate autotrophy during the early germination steps.

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