# Histological analysis and transcription profiles on somatic embryogenesis in interspecific hybrids of *Elaeis guineensis* × *E. oleifera*[#]

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## ABSTRACT

*Elaeis guineensis* (African oil palm) and *E. oleifera* (American oil palm) are bred to attain high oil yields, disease resistances, and decelerated shoot elongation. We cultivated immature zygotic embryos from backcrossed and $F_1$ interspecific progenies on media containing 110, 150, or 200 mg·l⁻¹ 2,4-diclorophenoxyacetic acid (2,4-D) to obtain embryogenic cultures. These were set to multiply on medium containing 8 mg·l⁻¹ 2,4-D or lower concentrations of auxins and finally we induced plantlets regeneration, from each zygotic embryo, independently, in order to have the clones organized according to their respective origins. Reductions in auxins induced cultures to revert from highly embryogenic into competent for embryogenesis and finally to organogenic degenerated callus lines. Histology and the expression of *SOMATIC EMBRYOGENESIS RECEPTOR KINASE, DEHYDRIN, DEFENSIN, TRANSPOSASE*, and *LIPID TRANSFER PROTEIN* were analyzed on four callus lines representative of morphological aspects consistently observed. The highest number of embryogenic cultures was obtained on 150 mg·l⁻¹ 2,4-D. Maturation and multiplication of somatic embryos through secondary embryogenesis occurred simultaneously on 8 mg·l⁻¹ 2,4-D. *LIPID TRANSFER PROTEIN* expression was detected in one of the embryogenic cultures and correlated with protoderm onset. Three six-week cycles on induction medium yielded 1.5 shoots above 6 cm per poly-embryogenic complex, which performed better than embryoids individualized mechanically. Rooting was observed for 77% and 82% of shoots from these two types of explants, respectively. Rooted plantlets ready for acclimatization were obtained nine months after shoot induction had started.

**Keywords:** Amazon; Arecaceae; Dendê; Caiaué; Gene Expression

## 1. INTRODUCTION

*Elaeis guineensis* (Jacq.) tp. *tenera* cultivars are considered the best producers of oil, which is used in food, biofuels and cosmetics [1-3]. In Latin America, *tenera* plantations are harmed by the lethal yellowing anomaly. However, it does not eliminate $F_1$ hybrids between *E. guineensis* and the Amazon native species, *E. oleifera* (Kunth) Cortés, growing in the same impacted areas. In addition, *E. oleifera* has a lower rate of annual shoot growth and a higher content of unsaturated fatty-acids [4]. To maintain high yields, *guineensis* × *oleifera* hybrids are backcrossed with *E. guineensis* [4], and the progenies resulting from this complex breeding strategy present variability. Cloning can facilitate comparison among plants grown in different experimental areas. A few thousand clones for each progeny—tens of clones for some hundreds of zygotic embryos per progeny—with their individual identities preserved would guarantee representation and equal distribution of the geno-

#Part of the Pos-Doctoral activities of the first author.

types/embryos in field trials.

*In vitro* culture of *E. guineensis* tissues was first reported in the 1970s [5,6], and the techniques were improved in the 1980s and 1990s [7-10]. Massive multiplication was reported late in the 1990s and in the 2000s [11-13]. Nevertheless, many aspects of *in vitro* research, including induction and maintenance of embryogenic cultures, are still poorly understood.

Analyses of gene expression have added knowledge to this matter. One of the genes firstly associated with embryogenesis was *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) [14]. Among palms, expression of *SERK* was recently observed at the onset of embryogenic "ear"-like structures in coconut palm [15]. *LIPID TRANSFER PROTEIN* was expressed in carrot pro-embryogenic masses and somatic embryos [16]. Expressed sequence tags (ESTs) identified as *LIPID TRANSFER PROTEIN* [17], *DEFENSIN* and *DEHYDRIN* were preferentially expressed in *E. guineensis* embryogenic cultures when compared with nonembryogenic cultures. However, the expression rates of these two last genes diverged among callus lines with different genotypes and it was concluded that transcription had been modulated principally by stress [18]. At last, a *TRANSPOSASE* was among the ESTs expressed exclusively in actively proliferating *E. guineensis* embryogenic cell cultures and was not expressed in nonmultiplying cultures [19].

In the present study, embryogenic cultures were induced from single immature zygotic embryos randomly taken from backcrossed and $F_1$ progenies of *E. guineensis* × *E. oleifera*. Cultures were multiplied on 8 mg·l$^{-1}$ 2,4-D, and we also tested multiplication in lower concentrations of auxins. Four of the callus lines obtained during the trials were selected for further analyses, because they were representative of aspects observed repeatedly and also reported elsewhere for *E. guineensis* and other palm species. We applied histological techniques and reverse transcription polymerase chain reaction (RT-PCR) to analyze the expression of *SERK*, *DEHYDRIN*, *DEFENSIN*, *LIPID TRANSFER PROTEIN* and *TRANSPOSASE* genes, and examined the relationships among the morphology and histology of those four callus lines and the results of RT. The expression of those genes has been investigated in *E. guineensis*, but the associations between gene expression and histology have not yet been studied for most of them. We tested different media to regenerate shoots from somatic embryos individualized mechanically or clustered in poly-embryogenic complexes (as defined in [8]). We applied for the first time more than one six-week cycles in two different media for shoot induction and also compared the rates of rooting for shoots obtained from individual embryoids or

poly-embryogenic complexes.

# 2. MATERIAL AND METHODS

## 2.1. Plant Material

Pollination was conducted at Rio Urubu Experimental-Station (Embrapa Western Amazon), Rio Preto da Eva, AM, Brazil (2°35′S, 59°28′W, 200 m above sea level) and fruits were collected 106 days after this. Progeny NFEC SN585 (shortly RC585) was generated backcrossing [(*E. guineensis* tp. *tenera* × *E. oleifera*) × *E. guineensis* tp. *tenera*]. Progeny $F_1$ (NFA CN1384, shortly F1) was generated crossing *E. guineensis* tp. *tenera* × *E. oleifera*. *E. guineensis* tp. *tenera* is obtained breeding tp. *dura* × tp. *pisifera*. Zygotic embryos used for reverse transcription were collected 90 days after pollination.

## 2.2. Induction and Maintainance of Embryogenic Cultures

Fruit tissues were removed and seeds washed with detergent and 50% bleach. One day after collection, zygotic embryos were excised from the endosperm, transferred for an aseptic flow chamber, immersed in 5% bleach (approximately 0.1% active chlorine) for 5 min, and washed three times in autoclaved water. We cultivated 75 embryos from RC585 and 100 embryos from the $F_1$ progeny. Ten embryos/90 mm Petri dish were set on 20 - 25 ml of *basal medium* containing MS salts and vitamins [20], 1 mM Na$^+$ provided as sodium phosphate, Phytagel (2.2 g·l$^{-1}$), polyvinylpyrrolidone (500 mg·l$^{-1}$), cysteine.HCl (500 mg·l$^{-1}$), hydrolyzed casein (500 mg·l$^{-1}$), activated charcoal (2.5 g·l$^{-1}$), sucrose (30 g·l$^{-1}$), what was supplemented with 2,4-diclorophenoxyacetic acid (2,4-D) at 110 (452 µM from [9]), 150 or 200 mg·l$^{-1}$ to be designated as *embryogenesis induction medium*. Each embryo/calli represented one experimental unit. Treatments were compared by $\chi^2$.

Embryogenic sectors on primary calli were transferred for *multiplication/maturation medium*: same as the basal medium supplemented with 8 mg·l$^{-1}$ (40 µM) 2,4-D [21]. Cultures were multiplied in this medium for at least 12 months at 25°C ± 2°C in the dark, with transfers for fresh medium every two months. We also tested alternative multiplication/maturation medium supplemented with reduced concentrations of 2,4-D (1 or 3 mg·l$^{-1}$) or 2.4 mg·l$^{-1}$ (10 µM) Picloram.

Around the eighth month, embryogenic cultures were transported from the Laboratory of Plant Biotechnology at Embrapa Western Amazon, Manaus, for the Laboratory of Developmental Physiology and Plant Genetics, in the University of Santa Catarina, Florianópolis, Brazil. Thereafter, they were kept on multiplication/maturation medium modified to contain vitamins described in [22]

instead of those from [20] plus glutamine (1 g·l$^{-1}$) and with no cysteine.

## 2.3. Origin and Morphology of Representative Callus Lines

The callus lines designated as 509, and 511A and 511B were raised from single zygotic embryos designated as 509 and as 511, respectively, from progeny RC585. The callus line designated as 002 was raised from zygotic embryo 002 from progeny F$_1$.

- Callus line 511A was a derivative of the highly embryogenic culture 511. Following 14 months on multiplication/maturation medium containing 8 mg·l$^{-1}$ 2,4-D, part of that culture was transferred for 1 mg·l$^{-1}$ 2,4-D in basal medium free of charcoal. Two months after this, we observed the reversion for calli bearing "ear"-like embryogenic structures (as defined in [15]), which were transferred to 3 mg·l$^{-1}$ 2,4-D for additional two months.
- Callus lines 509 and 002 were highly embryogenic cultures collected from multiplication/maturation medium supplemented with 8 mg·l$^{-1}$ 2,4-D 18 months after the introduction of zygotic embryos *in vitro*.
- Callus line 511B was obtained by transferring "ear"-like embryogenic structures excised from 511A for 2.4 mg·l$^{-1}$ Picloram in basal medium free of charcoal [23] for two months followed by additional three months on basal medium free of growth regulators and charcoal.

Immediately after registration of the morphological aspects through high definition digital images, biological material (calli and/or more friable cultures) was organized as individual 100 mg samples, immediately frozen in liquid nitrogen and preserved at −80°C.

## 2.4. Histological Analysis

Samples from callus lines 511A, 509, 002, and 511B were defrosted overnight at 8°C inside 3% paraformaldehyde in 0.1 M phosphate buffer (pH 6.8). The material went through a dehydration series (30 to 96.5° ethanol) and was embedded in historesin (Leica) according to the instructions of the manufacturer. Tissues were sliced in 8 - 10 µm sections and stained with 0.05% toluidine blue in 0.1 M phosphate buffer (pH 6.8) for analyses using an Olympus light microscope/documentation system.

## 2.5. Transcription Profiles

Total RNA was extracted from individual samples of 100 mg following disruption in a tissue lyser (Precellys) using the RNeasy Plant Mini kit (QIAGEN). The number of samples extracted from each callus line (511A, 509, 002 and 511B) was defined by the amount of RNA ob-

tained from each one of them. The sum of the aliquots should reach approximately 13 µg to allow replication. RNA was treated with DNase I, purified in RNeasy Mini Spin columns (QIAGEN), quantified by spectrophotometry (Nanodrop) and evaluated through denaturing agarose gel electrophoresis. Extraction was repeated from 90 days old immature zygotic embryos as well.

RNA aliquots of 4 µg (biological replicas) from callus lines or 2 µg from immature zygotic embryos were used for 20 µl standard reactions of first strand polymerization with the Long Range 2-Step RT-PCR kit and oligo-dT primers (QIAGEN). From those, 3 µl were used for second-step PCR amplification reactions (technical replicates) using specific primers for *SERK* (5'-GGCGTCGT CGTAAGCCGCAAG-3' and 5'-TGGCCAAGCCAAAG TCTCCAACA-3'), designed from conserved regions in the coconut *SERK* mRNA (GI90891655). Primers for *DEFENSIN* (5'-TGGGGACGAAGGTGGCGGA-3' and 5'-AAAGCATCGGCGCCGGACT-3'), *DEHYDRIN* (5'-GACGAGTACGGCAACCCGATC-3' and 5'-CTTTTCC ATCATCCCCTTCTT-3') and *TRANSLATION ELONGATION FACTOR* 1, the internal reference gene (5'-GGTGTGAAGCAGATGATTTGC-3' and 5'-CCTG GATCATGTCAAGAGCC-3') were reported in [24]. Primers for the *LIPID TRANSFER PROTEIN* (5'-TG-GAGAAAGGTGCACCGAAGCCGT-3' and 5'-CCGG-CCAGTCTTACAGCCTGCACA-3') and the *TRANSPOSASE* (5'-GCCTTGCTAGATCTAGGAGCTA GT-3' and 5'-CCGTAGCTAAGAATGGTCTTCCT-3') were designed for conserved regions in corresponding *E. guineensis* ESTs (GI193220058/DW248433 and GI221144290, respectively). All the cDNA amplicons were produced simultaneously using a step-down procedure [25] of −0.8°C·s$^{-1}$ from 78°C to 54°C for annealing in the first 13 PCR cycles. Anneling temperature was fixed in 54°C for the next 32 cycles. Synthesis was fixed in 68°C for 1 minute and denaturation was fixed in 93°C for 10 seconds. Amplicons were stained with GelRed (Biotium), resolved in 1.5% agarose gels and exposed to UV illumination. The images (TIF) were used to calculate the calibrated quantity of fluorescence using linear regression equations developed with Quantity One 4.6 (BioRad) taking the *ELONGATION FACTOR* 1 as calibrator. Reverse transcription (with three biological replicas), cDNA amplification, electrophoresis and quantification (in three technical replicas) were performed for each callus line. The identity of the amplicons was confirmed by the proper size in electrophoresis, sequencing (MegaBace) and, for the *LIPID TRANSFER PROTEIN*, by amplification using nested (5'-TGCTGCAGTGACC TGACACGGGCT-3' and 5'-TCGCAGGGGCTAAGAG CGTCGGA-3') primers. The mean values for the calibrated quantity of fluorescence for the five amplicons in

the four callus lines were compared using ANOVA and Tukey tests.

## 2.6. Plantlet Regeneration

For shoot induction, we used nondisrupted poly-embryogenic complexes (156 explants) or mechanically individualized pear-shaped somatic embryos (180 explants) taken from callus lines 509 and 002. Cultivation was conducted on basal medium free of casein, glutamine, and charcoal, supplemented with 0.56 mg·l$^{-1}$ (3 μM) naphthaleneacetic acid (NAA) and 2.25 mg·l$^{-1}$ (10 μM) 6-benzyladenine (BA) [6] that was designated as NB, or with 0.08 mg·l$^{-1}$ (0.44 μM) NAA and 5.00 mg·l$^{-1}$ (24.6 μM) 2-isopentenyladenine (2-iP) [26], designated as NI. Explants were exposed to 16 h of photoperiod under 40 - 50 μmol·m$^{-2}$·s$^{-1}$ irradiance. Poly-embryogenic complexes were submitted to three cycles of shoot induction, six weeks long each. Comparison between percentages of leaf-like structures obtained from each type of explant was performed only at the end of the first cycle because individualized embryos were not submitted to more than one cycle of shoot induction. Shoots were allowed to reach 6 cm on in half-strength basal medium supplemented with 30 g·l$^{-1}$ sucrose before mechanical individualization for rooting.

Rooting was tested on 76 shoots from poly-embryogenic complexes and on 38 shoots from mechanically individualized somatic embryos, in basal medium free of casein or glutamine, containing half the concentration of iron, supplemented with 30 or 60 g·l$^{-1}$ sucrose [6] and NAA (11.1 mg·l$^{-1}$ or 16.7 mg·l$^{-1}$). Explants were cultivated on 50 ml medium/500 ml flask or 15 ml medium/ 22.5 × 2.5 cm culture tubes. Treatments were under.

All the statistical analyses ($\chi^2$ tests, Z tests, ANOVA and Tukey tests) were performed using SigmaPlot, version 11.2, $P \leq 0.05$.

## 3. RESULTS

### 3.1. Induction of Embryogenesis

*E. guineensis* × *E. oleifera* immature zygotic embryos were maintained for more than 12 months on induction medium, because embryogenesis in palms often depends on long-term cultivation [27,28]. By the sixth month, 20% and 39% of the cultures from F$_1$ progenies and RC585, respectively, were active and responsive to changes in the cultivation conditions. The explants were primary compact calli or calli that had given raise to embryogenic cultures, which were first observed in the second month (**Table 1**). Up to this point, discards were caused by oxidation, hardening, rooting, or contamination (around 5%), in this order. The use of 150 mg·l$^{-1}$ 2,4-D was valuable in inducing embryogenesis for both

**Table 1.** Classification of calli induced on immature zygotic embryos from two progenies of *Elaeis guineensis* × *E. oleifera*, six months after inoculation on induction media supplemented with different concentrations of 2,4-D.

| Progeny | $n$ | 2,4-D (mg·l$^{-1}$) | Calli (%) | |
|---|---|---|---|---|
| | | | Primary | Embryogenic |
| F$_1$ | 100 | 110 | 25.0 | 5.0 |
| | | 150 | 45.0 | 15.0 |
| | | 200 | 5.0 | 5.0 |
| RC585 | 75 | 110 | 31.0 | 3.4 |
| | | 150 | 17.3 | 24.1 |
| | | 200 | 20.7 | 3.5 |

progenies but it was significantly superior for RC585 ($\chi^2$ = 7.16; $P = 0.0278$).

### 3.2. Morphology and Histology of Representative Callus Lines

Callus line 511A derived from 511 following a reduction in 2,4-D concentration. Two months were enough for the reversion of the cultures from friable and highly embryogenic back to compact calli displaying superficial "ear"-like embryogenic structures (**Figure 1A**). This structures had already been observed during the transition from primary to embryogenic calli, in the first months of *in vitro* cultivation, more than a year before. The highly embryogenic features of 511 was not maintained on 1 mg·l$^{-1}$ 2,4-D or recovered on 3 mg·l$^{-1}$ 2,4-D, but we could still observe displacement of internal meristematic cores for the surface of the embryogenic structures, where segmentation and spatial reorientation of the external cell layers took place (**Figure 1B**). Simultaneously, there was the extrusion of tiny pro-embryos with a small number of cells (**Figure 1C**), which would possibly mature and emerge through or detach from the surface of the calli (**Figure 1D**).

Lines 509 (**Figure 1E**) and 002 (**Figure 1I**) diverged in important aspects by the time of collection despite both were highly embryogenic. Somatic embryos in callus line 509 erupted in clusters from a compact basal callus. Some of them were intermediary, translucent and elongating (**Figure 1E**, arrow), with protoderm ongoing differentiation (**Figure 1F**, arrows). This phase of development was observed solely in this callus line. Other sectors in the same calli and culture produced pear-shaped somatic embryos with procambium and periderm, which entered secondary embryogenesis, characterized by the arising of multiple clumps of meristematic cells at the junctions to the basal callus (**Figure 1H**).

In contrast, line 002 from F$_1$ hybrids had friable, pearly colored sectors (**Figure 1I**, arrow) that were finely

fragmented (**Figure 1J**), and multiplied fast. This characteristic was exclusive of this callus line. In comparison to 509, by the time they were collected for histological and reverse transcription analyses, 002 displayed fewer sectors of fully developed/clustered somatic embryos on compact basal calli.

Callus line 511B was raised from "ear"-like embryogenic structures excised from 511A, cultivated in reduced auxin concentration (2.4 mg·l$^{-1}$ Picloram) and in absence of growth regulators. Concomitant with reductions in auxin concentration inflicted to 511 (from 110 for 8 mg·l$^{-1}$), again to 511A (from 8 to 3 mg·l$^{-1}$) and finally to 511B (as stated above) we observed the continuous degeneration of a highly embryogenic culture until reach the sectored, hairy, calli displaying root-like structures and embryogenic sectors, producing a few peripheral yellowish pear-shaped somatic embryos (**Figure 1M**). These would be associated with residual meristem segmentation (**Figure 1N**). Xylem elements presenting secondary wall deposition (**Figure 1P**) were present but no organized vascular bundle was observed.

As stated for 511A, lines 509 and 002 also produced very small isolated pro-embryos, several of them observed as four-celled structures in the histological sections, surrounded by extra-cellular matrix and thickened outer walls (**Figures 1D**, **G** and **K**). These barriers would promote the isolation of pro-embryos in coconut and date palm [28,29] and contribute to maturation. A thickened surrounding matrix can be observed surrounding larger portions of cells which are presumably in course of organization to differentiate in somatic embryos in callus line 002 as well (**Figures 1J** and **L**). This process was not observed in callus line 509. It is part of the difference between 509 and 002 with possible repercussions to the origin of the embryos coming from each one of these callus lines: from big compact basal callus in 509 and from more friable meristematic cores in 002.

### 3.3. Transcription Profiles

We detected the expression of the targeted genes through the presence of cDNA amplicons of expected sizes. Except for the *LIPID TRANSFER PROTEIN*, which was observed only in callus line 509 (**Figure 2A**, LT), amplicons coming from the other genes were present in all the callus lines and also in immature zygotic embryos (**Figure 2B**). There was no statistically significant difference among callus lines (Tukey tests, $P > 0.05$) regarding the mean values of calibrated quantity of fluorescence recorded for *SERK*, *DEHYDRIN*, *DEFENSIN* or *TRANSPOSASE* transcripts. The highest quantity of fluorescence was recorded for *DEHYDRIN*, followed by *DEFENSIN*, *TRANSPOSASE*, *SERK* and finally *LIPID TRANSFER PROTEIN* in callus line 509, which was approximately half that of *SERK* (**Figure 2C**).

We obtained good quality sequence reads covering 432 of the 579 base pairs (bp) expected for the *SERK* amplicon, including the catalytic domain of the correspondent enzyme. When that read was used to screen the GenBank, the closest match [E$^{-value}$ = 5e$^{-143}$ and 91% (368/408) identity] was to a *Cocos nucifera SERK* mRNA (AY791293.2/GI 90891655), as expected since at that moment there were no entries designated as *SERK* from *Elaeis* in the nonredundant nucleotide collection. The similarity among sequences from different palm species had already been reported [24].

Accordingly, the next best hit was a *SERK* from *Areca catechu* (GU584945.1), another Arecaceae. The third and fourth closest matches were, respectively, to a not yet named mRNA and to an mRNA for a *SERK-FAMILY RECEPTOR-LIKE KINASE* (AB188247.1), both from *Oryza sativa*, which is a monocot. *SERK* primers also prompted the amplification of a 550 bp band (**Figures 2A** and **B**), which we could not isolate and was considered an artifact of the PCR.

The sequenced portion of the cDNA amplicon corresponding to the *LIPID TRANSFER PROTEIN* was 80% identical to the *E. guineensis* DW248433 (GI 193220058) mentioned in [17]. The reads covered a domain (cd-00010-AAI/LTSS) conserved in proteins involved in plant defense against insects and pathogens, lipid transport between intracellular membranes and, nutrient storage. This same domain is present in the polypeptide deduced from DW248433 when translated in frame +1 from the first ATG codon. The amplicon produced by nested primers had around 156 bp (result not shown).

The nucleotide sequences obtained for *DEHYDRIN*, *DEFENSIN* and *TRANSPOSASE* were around 97% similar to those published for *E. guineensis*.

### 3.4. Plantlet Regeneration

Haustorium-like structures were not included in **Table 2**, despite the presence of chlorophyll. The two types of explants—poly-embryogenic complexes and somatic embryos individualized mechanically from the complexes

**Table 2.** Percentage of *Elaeis guineensis* × *E. oleifera* individualized somatic embryoids (ISE) and poly-embryogenic complexes (PEC) producing shoots only, roots only or displaying no reaction after a period of six weeks on induction media supplemented with NAA and BA (NB) or NAA and 2-iP (NI) and submitted to a 16 h photoperiod.

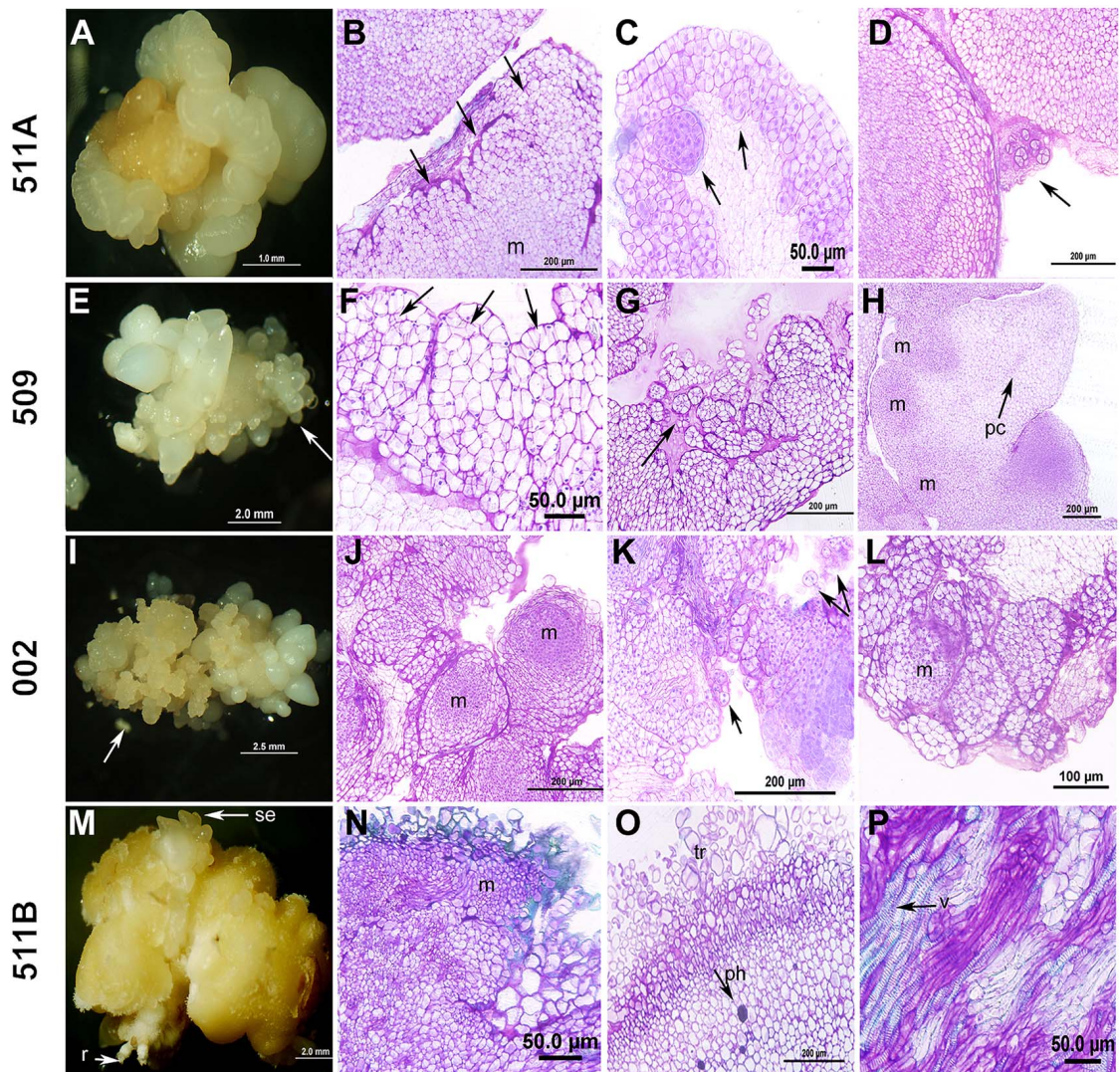| Explant | $n$ | IM | Shoots | Roots | No reaction |
|---------|-----|-----|--------|-------|-------------|
| ISE | 90 | NB | 11.5 | 21.5 | 0 |
|  | 90 | NI | 16.5 | 34.0 | 1.5 |
| PEC | 78 | NB | 15.5 | 5.5 | 7.5 |
|  | 78 | NI | 33.0 | 2.5 | 14.0 |

**Figure 1.** Morpho-histological aspects of callus lines obtained from *Elaeis guineensis* × *E. oleifera* immature zygotic embryos. **A-D**, callus line 511A. **A**, superficial "ear"-like embryogenic structures (bar = 1.0 mm). **B**, strands of cells spatially re-oriented reaching the callus surface and tissue segmentation (bar = 200 μm). **C**, organization and extrusion of pro-embryos (arrows); the largest one shows a thickened outer wall (bar = 50 μm). **D**, cluster of isolated pro-embryos covered with extracellular material (arrow) (bar = 200 μm). **E-H**, callus line 509. **E**, sector of callus with somatic embryos at intermediary developmental phases (arrow) observed only in this line (bar = 2.0 mm). **F**, protoderm differentiation (arrow) in intermediary developed somatic embryos (bar = 50 μm). **G**, isolated pro-embryo (arrow) covered with extracellular material (bar = 200 μm). **H**, meristematic cells clumps at the base of a somatic embryo undergoing secondary embryogenesis (bar = 200 μm). **I-L**, callus line 002. **I**, sector of very friable tissue (arrow) observed only in this line (bar = 2.5 mm). This sector comprised meristematic cores undergoing recurrent fragmentation as detailed in **J** (bar = 200 μm). **K**, isolated pro-embryos (arrows) covered with extracellular material (bar = 200 μm). **L**, organization of pro-embryos and fragmentation of meristems observed simultaneously (bar = 100 μm). **M-P**, callus line 511B. **M**, compact callus displaying hairy sectors, roots, and somatic embryos (bar = 2 mm). **N**, reminiscent meristematic layer fragmentation (bar = 50 μm). **O**, trichomes and phenolic inclusion that were observed only in this line (bar = 200 μm). **P**, disorganized vascular elements (bar = 50 μm). **m** = meristematic tissue; **pc** = procambium; **ph** = phenolic inclusion; **r** = root; **se** = somatic embryo; **tr** = trichome; **v** = vascular element.

—had reached different ($z$ = 3.758; $P$ < 0.001) rates of shoot formation at the end of one cycle of cultivation in shoot induction media (IM) and exposition to light, but poly-embryogenic complexes produced more shoots. A difference between NB and NI was observed too. For the first cycle of induction (**Table 2**), the produced with two cycles of induction (204/241), even when compared with one and three cycles taken together (37/241). It is
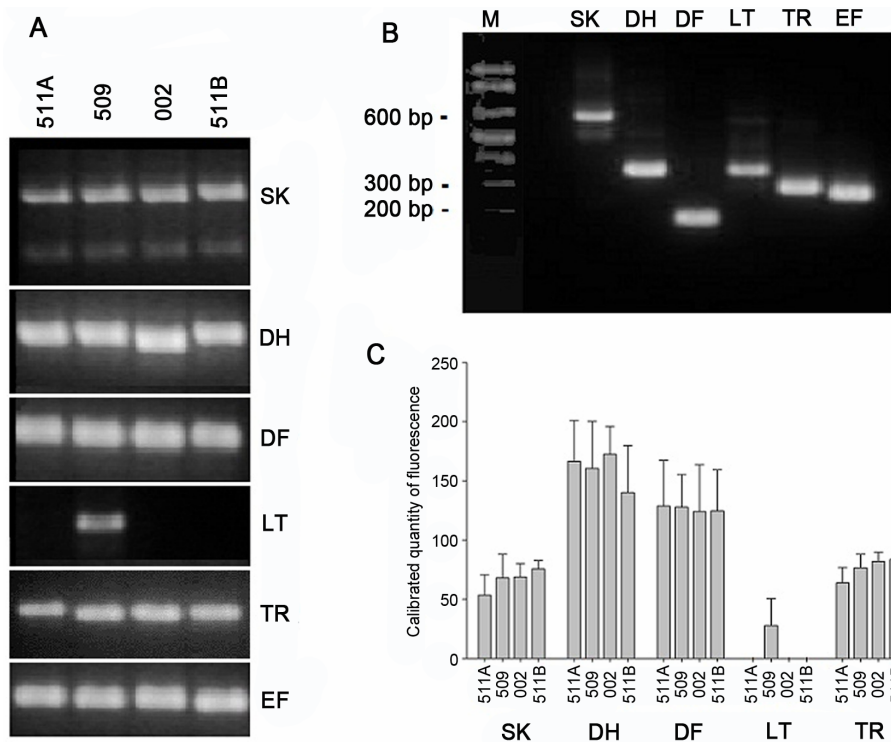
**Figure 2.** Profile of gene expression in *Elaeis guineensis × E. oleifera* hybrids. **A**, amplicons for callus lines 511A, 509, 002 and 511B. **B**, amplicons for immature zygotic embryos, collected 90 days after pollination. **C**, calibrated quantity of fluorescence to the amplicons for each callus line. Amplicons were produced by RT-PCR and fluorescence was calibrated by that of the internal reference gene. Columns represent mean values for three replicates and bars represent the standard deviations of the means. **SK** = *SERK* (*somatic embryogenesis receptor kinase*); **DH** = *DEHYDRIN*; **DF** = *DEFENSIN*; **LT** = *LIPID TRANSFER PROTEIN*; **TR** = *TRANSPOSASE*; **EF** = *ELONGATION FACTOR* 1, the internal reference gene. **M** = 1 kb Plus Ladder (Invitrogen).

interesting to note that individualized pearshaped embryos often produced multiple shoots too (**Figures 3C** and **D**), probably as consequence of secondary embryogenesis (**Figure 1H**), and this feature contributed to the final scores. We obtained 47 shoots above 6 cm from individualized somatic embryos (26% in 180, **Table 2**) and 241 from poly-embryogenic complexes (1.54 shoots/explant in average; 1.46 for callus line 509 and 1.56 for callus line 002).

Some shoots were used for rooting experiments (**Figure 3E**) following separation from the complexes. Despite media supplemented with 60 g·l$^{-1}$ of sucrose and 16.7 mg·l$^{-1}$ NAA promoted rooting in higher frequencies, the concentrations of sugars and NAA tested did not differ statistically. Percentages of rooting were 77 and 82 for shoots coming from poly-embryogenic complexes or individualized embryoids, respectively.

# 4. DISCUSSION

In this work, the goal was to clone *E. guineensis × E. oleifera* zygotic embryos through somatic embryogene-
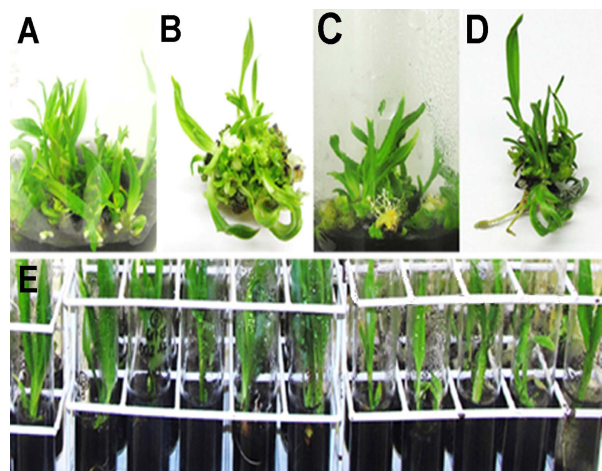


**Figure 3**. Overview of *in vitro* regenerated plantlets of *Elaeis guineensis × E. oleifera* hybrids. **A-B**, shoots emerging from poly-embryogenic complexes. **C-D**, shoots produced from individualized somatic embryos. **E**, shoots transferred for rooting media.

sis. Histological and reverse transcription techniques were used to complement morphology as support for

decisions, going deeper on the comprehension of embryogenesis and multiplication/maturation of embryogenic cultures, and to institute molecular markers to assist selection of the best callus lines, maybe among thousands if numerous progenies are going to be cloned. An example would be the *CYCLIN DEPENDENT KINASE* from coconut palm, which increased expression, marked the emergence of "ear"-like embryogenic structures [30].

"Ear"-like structures were reported decades ago for *E. guineensis* [8] and observed in coconut [15] and *Bactris* [23]. In the present study, they became visible during the initial months of cultivation and again following the reduction in auxin concentration, which induced culture 511 to revert throughout the route for embryogenesis to produce callus line 511A. For this last callus line, histological analysis revealed the migration of meristematic activity from the center of the calli to the periphery in the "ear"-like embryogenic structures, "where cells became spatially organized in strands, and the tissues entered segmentation. Rearrangements like these could be the prognostication of the development of "clusters of pro-embryos, which would remain fused to form poly-embryogenic complexes" [8]. In addition, rare clusters of isolated globular pro-embryos, with a reduced number of cells were also observed. These results all together indicated the occurrence of more than one route for embryogenesis in callus line 511A. Somatic embryos would originate from one or a few cells [28], or from the segmentation of sectors of meristematic "cells re-oriented to divide anticlinally, rather than periclinally", what was suggested to occur in barley too [8, 31]. Nevertheless, all the somatic embryos promoted to the experimental phase of shoot regeneration in this work came from poly-embryogenic complexes and not from spontaneously isolated somatic embryo.

Multiple meristematic cores were observed in 511A and the other callus lines, certainly resulting from the fragmentation of an initial meristem in each zygotic embryo. The importance of cellular injuries inflicted to the external meristematic layers and/or the central and peripheral zones of the shoot apical meristem (SAM) to induce its segmentation and the organization of multiple meristem cores has been demonstrated [32]. These processes possibly occurred in association with the appearing of "ear"-like embryogenic structures. Periclinal cellular divisions would trigger radial expansion in detriment of somatic embryos multiplication/maturation. Radial expansion could impel meristematic activity from the inner cores of the calli towards its surface, finally causing the mechanical rupture of the external cell layers, inducing fragmentation, followed by spatial reorganization and tissue segmentations. It is plausible to suppose that under ideal conditions the equilibrium between multiplication,

fragmentation and reorganization of peripheral meristematic cells is achieved and the proper number of cellular layers and rows is settled in individual neighbor apical meristems. Thereafter, photomorphogenesis and differentiation of multiple shoots from poly-embryogenic complexes become possible. The entrance in equilibrium would be interesting to mark in order to avoid unnecessary exposition of the cultures to high concentrations of auxins.

Differing from *Bactris* [26], isolated "ear"-like structures could not be induced to display secondary embryogenesis by cultivation in medium supplemented with 2.4 mg·l$^{-1}$ Picloram. Isolation and sequential reductions in the concentration of auxins resulted in a degenerated callus line with organogenic characteristics. This demonstrated that at least 8 mg·l$^{-1}$ of 2,4-D were essential to maintain the highly embryogenic features observed in callus lines 511 (not shown) and 509, at least, which were multiplied for more than a year. Once transferred back for media containing 8 mg·l$^{-1}$, calli from 511A resumed the characteristics observed in 511, produced somatic embryos and plantlets (results not show).

Cultivation in medium free of growth regulators during the stage of multiplication of the embryogenic cultures would be essential to obtain normal regenerated plantlets, when this stage is to be maintained for very long periods, as 20 years [13]. In the present work the best condition for multiplication-maturation of somatic embryos was 8 mg·l$^{-1}$ 2,4-D applied for a little more than a year [21]. Characteristics resembling the description of fast growing cultures, that would be associated with the mantled anomaly were only observed in callus line 002. In this reason, we considered that if plants affected are to be expected they will be found among regenerants from that callus line.

Expression of *SERK* genes has been related to the acquisition of cellular totipotency, which is essential for embryogenesis [33] and can be followed by pluripotency and finally organogenesis. Indeed, highly embryogenic features, competence for embryogenesis, or embryogenesis observed simultaneously to organogenesis could explain *SERK* transcription in the callus lines we studied. These conditions were observed in callus lines 509 and 002, in callus line 511A, which was very similar to the coconut calli expressing *SERK* in peripheral embryogenic structures [15] and finally in 511B, respectively. On the other hand, in *Rosa hybrida* cv. Linda two out of four *SERK* genes analyzed were good markers for somatic embryogenesis but would not be exclusively related to embryogenesis, despite no conclusion about alternative functions for those gene products had been proposed [34].

The ubiquitous expression of *DEHYDRIN and DEFENSIN* was reputed to the stresses imposed equally to

all the cultures as stated previously [18]. Dehydrins are late embryogenesis abundant (LEA) proteins related to the acquisition of desiccation tolerance but, in addition, they have been clearly related to stress responses, as well as defensins [24,35-38]. The differences among the expression profiles of those two genes and that of the *LIPID TRANSFER PROTEIN* led us to conclude that transcription of this last was not related to stress in our experiments.

We propose that the differences in those patterns of multiplication/maturation were the reasons for the exclusive expression of the *LIPID TRANSFER PROTEIN* in line 509. Callus line 509 presented mid-term pro-embryos, going through protoderm organization, fixed to a compact basal callus. Callus line 002 presented finely fragmented meristematic sectors going through cyclic multiplication. We considered the *LIPID TRANSFER PROTEIN* transcription as a transient indicator of route definition towards embryo maturation, probably going on more intensely in the poly-embryogenic complexes of callus line 509, in contrast with the prevalence of cyclic multiplication and fragmentation of meristematic cores in callus line 002. Reverse transcription was performed using the maximum amount of total RNA (4 μg) recommended for 20 μl standard reactions and a great number of PCR cycles were used for cDNA amplification in each biological replicate. These procedures assured that the results captured the differential transcription of the *LIPID TRANSFER PROTEIN*. Indeed, transcription in callus lines 002, 511A and 511B, if there was any, was so minimal that it could not be detected. In carrot, all the cell masses with diameters between 30 and 50 μm that produced somatic embryos were found to express the *LIPID TRANSFER PROTEIN*, what was not observed in the earliest stages, and expression was proposed to occur in concert with protoderm formation [39]. We detected transcription of the *LIPID TRANSFER PROTEIN* in 90 days old zygotic embryos, and this pointed out for the possibility of an extended period of expression in *Elaeis*. However, it was demonstrated in rice that expression of *LIPID TRANSFER PROTEIN* changed spatially during maturation from the protoderm, for the leaf primordium, and finally for the vascular bundles [40]. The association of a lipid transfer protein with the cytoplasm, cell walls, and spaces between protodermal and subprotodermal cells in embryogenic but not in meristematic cells of the SAM was recently reported for a lipid transfer protein in *Arabidopsis thaliana*. It was considered that some relation between the accumulation of the protein and the differentiation/dedifferentiation—named together as change of developmental direction—could exist [41]. The absence in the SAM of a protein expressed when tissues become committed to differentiation would be coherent since it is expected to last nondifferentiated for long periods [33]. Finally, a correlation between the lipid transfer protein and the accumulation of reserves in the embryos cannot be completely discarded, neither some influence of the differences among the genotypes of the callus lines on the intensity of gene expression, in reason of the complex origin of the embryos.

Transposon activation via expression of *TRANSPOSASE* has been correlated with the chromatin demethylation and remodeling in presence of 2,4-D [42], which was considered ultimately a stress factor that can influence chromatin conformation and induce embryogenesis [43]. The presence of ESTs homologous to *TRANSPOSASE* was exclusively associated with the occurrence of embryogenesis in *E. guineensis* [19] but transposons were detected in tissues of *tenera* collected in and ex vitro with no remarkable differences in distribution/localization [44].

For regeneration of plantlets, those poly-embryogenic complexes that produced green, leaf-like structures were transferred to half-strength basal medium free of growth regulators for shoot elongation up to 6 cm, and those which did not present such structures were submitted to further cycles of shoot induction. The induction of shoots on poly-embryogenic complexes was proven successful [12,13] as proposed earlier for the interspecific hybrids [45]. Cultivating poly-embryogenic complexes in medium supplemented with abscisic acid [9] before shoot induction might contribute to synchronization [46]. The procedures described above reduced the manipulation required to individualize embryos mechanically, contributing to decrease contamination rates and to obtain rooted plantlets ready for acclimatization in a year and five months.

## 5. ACKNOWLEDGEMENTS

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