UNIVERSIDADE FEDERAL DE VIÇOSA

LUANA WALQUÍRIA DOS SANTOS

INDIRECT SOMATIC EMBRYOGENESIS IN *Coffea canephora* AND *Coffea eugenioides*: ENDOGENOUS HORMONE LEVELS AND COPY NUMBER OF GENES RELATED TO AUXIN BIOSYNTHETIC PATHWAY AND MORPHOGENIC IN VITRO RESPONSE

VIÇOSA - MINAS GERAIS
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Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Magister Scientiae*.

Orientador: Wellington Ronildo Clarindo

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APROVADA: 16 de agosto de 2021.

Assentimento:

Luana Walquíria dos Santos
Autora

Wellington Ronildo Clarindo
Orientador

A Deus, aos meus pais Ana Lúcia e José Martins (in memoriam) e aos meus irmãos Luccas, Luciana e Lucimeire
Dedico

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A todos, meu muito obrigada!

Não vês que somos viajantes?
E tu me perguntas:
Que é viajar?
Eu respondo com uma palavra: é avançar!
Experimentais isto em ti
Que nunca te satisfaças com aquilo que és
Para que sejas um dia aquilo que ainda não és
Avança sempre! Não fiques parado no caminho.

Santo Agostinho

RESUMO

SANTOS, Luana Walquíria dos, M.Sc., Universidade Federal de Viçosa, agosto de 2021. **Embriogênese somática indireta em *Coffea canephora* e *Coffea eugenioides*: níveis endógenos de hormônios e número de cópias de genes relacionados com a via biossintética de auxina e com a resposta morfogênica in vitro**. Orientador: Wellington Ronildo Clarindo.

A embriogênese somática indireta (ESI) em *Coffea canephora* e *Coffea eugenioides* tem sida estabelecida e comparada durante a formação de calos e regeneração de embriões somáticos, evidenciando que essas espécies exibem a mesma resposta in vitro. No entanto, diferenças intraespecíficas em relação às respostas da ESI também foram relatadas. A ESI é influenciada por aspectos genéticos e fisiológicos. Nossos objetivos foram: (a) induzir a ESI em *C. canephora* e *C. eugenioides*; (b) identificar e quantificar os níveis endógenos do ácido indol-3-acético (IAA), zeatina (Z) e ácido giberélico GA_3 e GA_4 durante a indução de calo; e (c) determinar o número de cópias gênicas de *AUX/IAA33* e *YUC4* que estão envolvidos na via biosintética da auxina, e de *WOX4* e *LEC1* que estão envolvidos na via morfogênica. Utilizando cromatografia líquida acoplada à espectrometria de massas (LC-MS), a identificação e quantificação de IAA, GA_3, GA_4 e Z foram realizadas em ambos os *Coffea*. Através da hibridação in situ por fluorescência (FISH), foi verificado o número de cópias dos genes *AUX/IAA33*, *YUC4*, *WOX4* e *LEC1*. A ESI foi estabelecida para *C. canephora* e *C. eugenioides*, os quais exibiram a mesma resposta in vitro. No entanto, os dois *Coffea* diferiram em relação aos níveis dos hormônios endógenos, com exceção de IAA aos 90 dias. Essas espécies possuem duas cópias gênicas de *AUX/IAA33*, *YUC4*, *WOX4* e *LEC1*, corroborando para sua diploidia. Portanto, nossos resultados mostram que *C. canephora* e *C. eugenioides* diferem no aspecto fisiológico, mas não no aspecto genético aqui investigado. Desse modo, nossos dados contribuem para a base do entendimento da resposta in vitro e para o aprimoramento dos procedimentos de propagação.

Palavras-chave: Café. Cultura de Tecidos Vegetais. Regeneração in vitro. Embrião Somático. Genética. Fisiologia Vegetal

ABSTRACT

SANTOS, Luana Walquíria dos, M.Sc., Universidade Federal de Viçosa, August, 2021. **Indirect somatic embryogenesis in *Coffea canephora* and *Coffea eugenioides*: endogenous hormone levels and copy number of genes related to auxin biosynthetic pathway and morphogenic in vitro response**. Advisor: Wellington Ronildo Clarindo.

Coffea canephora and *Coffea eugenioides* indirect somatic embryogenesis (ISE) has been established and compared during callus formation and somatic embryo regeneration, showing that these species exhibit the same response in vitro. However, intraspecific differences regarding ISE responses have also been reported. ISE is influenced by genetic and physiological aspects. We aimed to: (a) induce ISE in *C. canephora* and *C. eugenioides*; (b) identify and quantify the endogenous levels of indole-3-acetic acid (IAA), zeatin (Z) and gibberellic acid GA_3 and GA_4 during the callus induction; and (c) determine the number of gene copies of *AUX/IAA33* and *YUC4* that are involved in the auxin biosynthetic pathway, and of *WOX4* and *LEC1* that are involved in the morphogenic pathway. Using liquid chromatography coupled to mass spectrometry (LC-MS), the identification and quantification of IAA, GA_3, GA_4 and Z was performed in both *Coffea*. From the fluorescence in situ hybridization, the copy number of the *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1* genes was verified. ISE was established for *C. canephora* and *C. eugenioides*, which exhibited the same in vitro response. However, the two *Coffea* differed in relation to endogenous hormone levels, with the exception of IAA at 90 days. These species possess two gene copies of *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1*, corroborating to their diploidy. Therefore, our results show that *C. canephora* and *C. eugenioides* differ in the physiological aspect, but not in the genetic aspect investigated here. Thus, our data contribute as a basis to understand the in vitro response and for the improvement of the in vitro propagation procedures.

Keywords: *Coffee*. Plant Tissue Culture. In vitro regeneration. Somatic Embryo. Genetic. Plant Physiology.

SUMÁRIO

RESEARCH PAPER: INDIRECT SOMATIC EMBRYOGENESIS IN *Coffea canephora* AND *Coffea eugenioides*: ENDOGENOUS HORMONE LEVELS AND COPY NUMBER OF GENES RELATED TO AUXIN BIOSYNTHETIC PATHWAY AND MORPHOGENIC IN VITRO RESPONSE

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1. Introduction

Indirect somatic embryogenesis (ISE) is established for in vitro propagation of different plant species, making a start with callus formation and subsequently proceeding for somatic embryo recovery. This regeneration pathway is widely used as an alternative for mass propagation in *Coffea* lines relevant to coffee production and/or breeding programs (Etienne et al. 2018). *Coffea* ISE has been conducted in different steps, as well as for other species, such as *Carica papaya* L. (Solórzano-Cascante et al. 2018), *Echinacea purpurea* (L.) Moench (Dehestani-Ardakani et al. 2020) and *Agave tequilana* 'Chato' (Delgado-Aceves et al. 2021).

Coffea ISE is characterized by transference and/or subculture of the propagule to the chemical and/or physical new and different tissue culture conditions (Samson et al. 2006; Sanglard et al. 2019; Amaral-Silva et al. 2021). Our research group has performed the *Coffea* ISE in two main steps. In the first moment occurs the callus formation by dedifferentiation (van Boxtel and Berthouly 1996) and/or the cell cycle activation of undifferentiated cells (Campos et al. 2017) from leaf explants. The dedifferentiation and the cell cycle proliferation are induced by in vitro conditions, highlighting the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D – synthetic auxin) and 6-benzylaminopurine (BAP – synthetic cytokinin) (Loyola-Vargas et al. 2016). The second moment is marked by somatic embryo regeneration, which is recovered after transference of the friable callus to in vitro conditions characterized by absence of 2,4-D and addition of active charcoal (Sanglard et al. 2019; Amaral-Silva et al. 2021).

During *Coffea* ISE, the cells of the explants (first moment) and friable callus (first and second moments) undergo “omics" changes, which have been identified and

measured, such as epigenetic (Landey et al. 2013; Nic-Can et al. 2013; Amaral-Silva et al. 2021), transcriptomic (gene expression, Arroyo-Herrera et al. 2008; Nic-Can et al. 2013; Quintana-Escobar et al. 2019) and physiologic (De-la-Peña et al. 2008; Amaral-Silva et al. 2021) changes. Considering these “omics” outcomes, comparative studies in the same in vitro conditions involving different species are important to evidence similarities and divergences during ISE response.

ISE is mainly established from leaf explants in diploid *Coffea canephora* Pierre ex Froehner and *Coffea eugenioides* S. Moore (2n = 2x = 22 chromosomes). We established the ISE for these two *Coffea* species and compared the in vitro response during the friable callus formation and somatic embryo regeneration (Sanglard et al. 2019). *C. canephora* and *C. eugenioides* showed the same mean values for both friable callus formation and somatic embryo regeneration, demonstrating that these *Coffea* respond equally to ISE. However, different in vitro responses in *Coffea* have been reported even for the same species (Samson et al. 2006), suggesting that genetic and physiological characteristics interfere with the ISE pathway (Loyola-Vargas and Ochoa-Alejo 2016). Based on these data, we raise the following questions: is the similarity in vitro response between these diploid *Coffea* related to their endogenous hormone? Do these species have the same number of gene copies related to the ISE establishment and auxin biosynthesis pathway?

Endogenous hormones play essential roles in ISE. Amaral-Silva et al. (2021) identified and quantified the plant hormones during ISE in *C. canephora* in liquid medium. The *C. canephora* ISE is influenced by auxins, gibberellins and cytokinins. Endogenous levels of the auxin indole-3-acetic acid (IAA) and the cytokinin zeatin (Z) were essential for the in vitro response (Jiménez 2001, 2005; Amaral-Silva et al. 2021). The balance of these endogenous hormones influences the gene expression

reprogramming, cell dedifferentiation and cell division of the friable callus, which are a prerequisite for the ISE pathway (Fehér 2015; Shimotohno et al. 2021). Still, the gibberellic acid GA_3 and GA_4 regulate the plant's antioxidant protection system by decreasing oxidative stress, which can be caused by the environment conditions necessary for in vitro culture (Ji et al. 2015; Saleem et al. 2020). Furthermore, gibberellins are related to the somatic embryo regeneration in different species, such as *Zea mays* L. (Jiménez 2001, 2005).

As well as knowledge of the physiological aspects are important for ISE, the genetic basis is also relevant. *C. canephora* and *C. eugenioides* are diploid *Coffea* species (2n = 2x = 22 chromosomes) with close nuclear DNA content (2C = 1.38 pg for *C. eugenioides*, 2C = 1.41 pg for *C. canephora*). However, the genus diversified about 5 – 25 Mya from a single ancestor (Lashermes et al. 1996; Mahé et al. 2007) and differences have been identified for the species with the same ploidy level. *C. canephora* and *C. eugenioides* showed karyotype differences, as two sites of 18S rDNA for *C. eugenioides* and one for *C. canephora*. In addition, differences about the gene copy number have been reported. For instance, *Coffea humblotiana* Baill, also diploid, did not show the gene related to the caffeine biosynthetic pathway, the *3,7-DIMETHYLXANTHINE METHYLTRANSFERASE OR CAFFEINE SYNTHASE* (*DXMT*) gene. The absence of the *DXMT* gene in *C. humblotiana* is related to failures in caffeine synthesis, giving its coffee a decaffeinated status (Raharimalala et al. 2021).

Several genes influence and determine the somatic embryogenesis responses (Nowak and Gaj 2016). Some of them have been identified and characterized. From these approaches, the fundamental role that different genes play during the somatic embryogenesis pathway has been described and, thus, they have become of great interest for tissue culture (Stone et al. 2001; Haecker et al. 2004; Lv et al. 2020; Uc-

Chuc et al. 2020). Activation of these genes triggers a series of cascade reactions that promote the somatic embryogenesis induction and, later, the somatic embryo regeneration (Nowak and Gaj 2016). Some genes that influence the somatic embryogenesis are *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA33*), *YUCCA4* (*YUC4*), *WUSCHEL RELATED HOMEOBOX* (*WOX4*) and *LEAFY COTYLEDON1* (*LEC1*).

IAA biosynthesis occurs mainly through the tryptophan-dependent (Trp) pathway, involving two gene families: *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA*) and *YUC*. In this pathway, the tryptophan is converted to indole-3-pyruvic acid (IPyA) in a reaction catalyzed by tryptophan aminotrasferase, and the IPyA is converted to IAA by YUC flavin monooxygenase (Enders and Strader 2015). Spatial and temporal changes in auxin levels lead to gene expression reprogramming and activation of auxin response genes, such as the *AUX/IAA* family and the *AUXIN RESPONSE FACTOR* (*ARF*) family (Luo et al. 2018). Members of the *AUX/IAA* gene family encode short-lived nuclear proteins, which interact with ARFs by chemical interaction between their homologous C-terminal domains.

AUX/IAA protein, at low concentrations of auxin, directly interact with ARFs, preventing ARFs from activating auxin response genes (*AUXRE*s) (Guilfoyle and Hagen 2007; Wei et al. 2021). In high concentration, auxin increases the affinity of the TRANSPORT INHIBITOR/AUXIN SIGNALING RESPONSE F-BOX (TIR1/AFB) protein complex for the AUX/IAA proteins, resulting in ubiquitination of AUX/IAA and its further degradation via the 26S proteasome (Enders and Strader 2015; Luo et al. 2018). From the free ARFs, transcription of auxin-responsive genes occurs, including genes from the somatic embryogenesis pathway (Wei et al. 2021; Su et al. 2021).

Some genes activated by auxin encode transcription factors, such as WOX4 and *LEC1*. *WOX4* and *LEC1* expression was measured in *C. canephora* somatic

embryos obtained from direct somatic embryogenesis (Nic-Can et al. 2013). *WOX4* is related with the maintenance of the pluripotent stem cells in meristems and with cell differentiation (Schoof et al. 2000; van der Graaff et al. 2009; Nic-Can et al. 2013). *LEC1* also acts in the auxin biosynthesis pathway due to it activates YUC family genes, responsible for the transcription of the YUC flavin monooxygenase enzymes, which convert IPyA to IAA (Mantovani 1999; Kumar et al. 2020).

As the in vitro ISE responses in *C. canephora* and *C. eugenioides* are statistically equal (rate of friable calli formation and cotyledonary somatic embryo recovering), these species can be used to undiscover the endogenous (genetic and physiologic) factors related. Based on the knowledge that the in vitro response is influenced by genetic and physiological factors, we aimed to establish the ISE in *C. canephora* and *C. eugenioides*, measure the IAA, Z, GA_3 and GA_4 levels during the induction of callus, and to determine the copy number of *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1*.

2. Material and methods

2.1 ISE establishment

We collected leaves of *C. canephora* and *C. eugenioides* plantlets regenerated via ISE and that have been propagated under in vitro conditions. Five leaf fragments (~2 cm^2) of the two *Coffea* were inoculated in Petri dishes (60 x 15 mm) containing a friable callus induction medium. Friable callus induction medium was prepared based on Murashige and Skoog (1962) and identical to Sanglard et al. (2019). 25 Petri dishes were inoculated for *C. canephora* and for *C. eugenioides*, which were kept in the dark

at 22 ± 2ºC for 90 days. Each resulting friable callus of each *Coffea* was transferred to Petri dishes (60 x 15 mm) containing somatic embryo regeneration medium. The composition of the somatic embryo regeneration medium was similar to the induction medium, differing in the absence of 2,4-D and supplementing 2.0 g L^{-1} activated charcoal (Isofar®) (Sanglard et al. 2019). A single friable callus was inoculated into each Petri dish, totaling 50 dishes for *C. canephora* and for *C. eugenioides*. The Petri dishes were kept in the dark at 22 ± 2ºC for 120 days.

C. canephora and *C. eugenioides* in vitro responses were compared in two moments*:* (a) at the first moment monitoring and recording of friable callus formation fortnightly for 90 days; and (b) in the second moment monitoring and recording the number of somatic embryo recovered monthly for 120 days. The data obtained during the ISE were transformed $\sqrt{(x + 0.5)}$ and exhibited normal distribution. Thus, we proceeded with the analysis of variance (ANOVA). The mean values were compared by Tukey's test ($P \leq 0.05$) and represented in box plot graphs. Then, regression analysis was performed from the best fit the mean values observed ($P \leq 0.05$). All statistical analyzes were accomplished in the R software (R Core Team 2020).

2.2 Determination of the endogenous hormones IAA, GA_3, GA_4, and Z

Coffea friable callus were collected at 60 and 90 days in callus induction medium. The extraction, identification and quantification of the endogenous hormones IAA, GA_3, GA_4, and Z were carried out following Vital et al. (2019) and Amaral-Silva et al. (2021). We identified and quantified the hormones using an Ultra Performance Liquid Chromatography (UPLC), part of the Agilent 1200 Infinity series of devices coupled with a triple quadrupole mass spectrometer (QqQ, Agilent Technologies, model 6430). The identification and quantification of IAA, GA_3, GA_4, and Z were

performed using the multiple reaction monitoring (MRM) mass spectrometry technique. According to the standard of each compound, a calibration curve from 0.1 ng to 200 ng was generated and the data analyzed in the software SkyLine 3.6. The statistical analysis regarding the quantification of the endogenous hormones was accomplished from ANOVA, followed by Tukey's test ($P \leq 0.05$), and represented by a box graphs-plot. All data were run in R software (R Core Team 2020).

2.3 Gene copy number

Based on the relevance of genes to the in vitro response, we investigated the copy number of four genes, two related to auxin biosynthetic pathway, the *AUX/IAA33* and *YUC4*, and two related to the morphogenic response of the somatic embryogenesis, *WOX4* and *LEC1*. *C. canephora* and *C. eugenioides* nuclei were obtained from the cell aggregate suspensions. Friable callus were collected and transferred to Erlenmeyers containing 30 mL of liquid medium, whose composition was similar to the callus induction medium, however, without the phytagel. The Erlenmeyers were kept in the dark at 22 ± 2ºC and under constant agitation in an orbital shaker at 110 rpm. The resulted cell aggregates were washed three times in dH_2O and macerated for 2 h at 36°C in enzymatic pool (4% cellulase Sigma®, 0.4% hemicellulase Sigma®, 1% macerozyme Onozuka R10 Yakult, 100% pectinase Sigma®) diluted in dH_2O in 1:20 ratio (enzyme: dH_2O). The cell aggregates were washed again for 10 min in dH_2O, fixed and stored at -20°C. The slides were prepared by cell dissociation and air-drying of the enzymatically macerated aggregates, and subsequently dried on a hot plate at 50°C, and stored at 36ºC for 5 days (Silva et al. 2020).

For amplification of the genes related to somatic embryogenesis in *Coffea* and the auxin metabolic pathway, we designed the primers from the nucleotide sequences recorded in the *Coffee* Genome Hub and National Center for Biotechnology Information (NCBI) databases. *LEC1*, *WOX4*, *YUCA4* and *AUX*/*IAA33* primers (Supplement 1) were produced using the PrimerQuest Tool program by Integrated DNA Technologies and analyzed by the online programs Primer-Blast and OligoAnalyzer.

Coffea genomic DNA was obtained according to Barbier et al. (2019). The DNA concentration and purity were determined through spectrophotometry using NanoDrop 1000 spectrofhometer (Thermo Scientific), and its integrity was verified by 1.5% agarose gel electrophoresis. The amplification reaction mix consisted of 0.5 µM oligonucleotide primer for each gene (Supplement 1), 200 ng of genomic DNA, 200 µM dNTPs (Promega), 1X Colorless GoTaq® Flexi Buffer (Promega), 5 u uL^{-1} GoTaq® Flexi DNA Polymerase (Promega) and 1.5 mM $MgCl_2$ (Promega). The labeling reaction consisted of 0.5 µM oligonucleotide primer for each gene (Supplement 1), 200 ng amplified genomic DNA, 200 µM each dATP, dCTP and dGTP, 150 µM dTTP, 1 µL ChromaTide™ Alexa Fluor™ 488-5-dUTP (green, Invitrogen) or Tetramethyl-Rhodamine-5-dUTP (red, Sigma®), 1X Colorless GoTaq® Flexi Buffer (Promega), 5 u uL^{-1} GoTaq® Flexi DNA Polymerase (Promega) and 1.5 mM $MgCl_2$ (Promega). All reactions were accomplished from initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95 °C for 1 min; annealing at 58ºC for *LEC1* and *WOX4*; 45ºC for *AUX/IAA33* and 43ºC for *YUCA4* for 1 min; extension at 68°C for 1 min; final extension at 68 °C for 5 min and finally hold 4°C. The labeled genomic probes were quantified again in a NanoDrop and evaluated by electrophoresis in 1.5% agarose gel.

Coffea slides were washed in 1X PBS for 5 min, 4%formalin for 10 min and in 1X PBS for 5 min. The slides were dehydrated in 70%, 85% and 100% ethanol series for 5 min in each concentration, and dried at room temperature. Nucleus denaturation was carried out in 70% formamide/2X saline-sodium citrate (SSC) buffer for 3 min at 67°C, followed by 70%, 85% and 100% ethanol series for 5 min in each concentration, and air-dried. The hybridization mix consisted of 2X SSC (Sigma ®) + 50% formamide (Sigma®) + 200 ng probe, in a total volume of 35 uL, and denaturation at 85 °C for 5 min in the MJ Research PTC-200 Thermal Cycler. The slides were incubated in the ThermoBrite system (ThermoFisher) at 37°C for 24 h. Stringency washing was performed in 2X SSC for 5 min at 40ºC. The nuclei were contrasted with 40% glycerol/PBS + 6-diamidino-2-phenylindole (DAPI). The images were captured using the Olympus BX60 Fluorescence Microscope, equipped with epifluorescence, filters WU (for DAPI), WB (ChromaTide™) and WG (for Rhodamine), and immersion objective of 100X with numerical aperture of 1.4.

2.4 Chromosome number confirmation

Chromosomes *C. canephora* and *C. eugenioides* were obtained from cell aggregate suspensions. Cell aggregates were treated with 3 µM amiprophos-methyl (antitubulin, Sigma) for 7 h at 30 °C, washed in dH_2O, fixed and stored at -20ºC. Afterwards, they were washed again and macerated for 2 h at 36º. After maceration, cell aggregates were washed, fixed and stored at -20º. The slides were prepared by cell dissociation of the enzymatically macerated aggregates, and subsequently dried on a hot plate at 50°C, and stored at 36ºC for 5 days (Clarindo et al. 2012). Metaphases were captured using the Olympus BX60 Fluorescence Microscope, equipped with

epifluorescence, filters WU (for DAPI) and immersion objective of 100X with numerical aperture of 1.4.

3. Results

C. canephora and *C. eugenioides* statistically showed the same ISE responses. *C. canephora* showed a mean value of 2.51 and *C. eugenioides* of 2.63 for friable callus formation (Figure 1a). Friable callus formation occurred from the 15th day in callus induction medium and extended up to the 90th for the two *Coffea* (Figure 1b – c). During the friable callus formation gradually occurred the cell proliferation, which was recognized by cell mass increase of the callus (Figure 1c). *C. canephora* and *C. eugenioides* callus showed pale yellow color and friable appearance. The two *Coffea* did not show significant differences in the mean number of regenerated somatic embryos. *C. canephora* presented a mean value of 0.11 and *C. eugenioides* of 0.10 for regenerated somatic embryos (Figure 1e – f). Somatic embryo regeneration started on the 60th day, and continually occurred until the 180th day in the regeneration medium (Figure 1d – f). Somatic embryo development increasingly occurred over the months for both species.

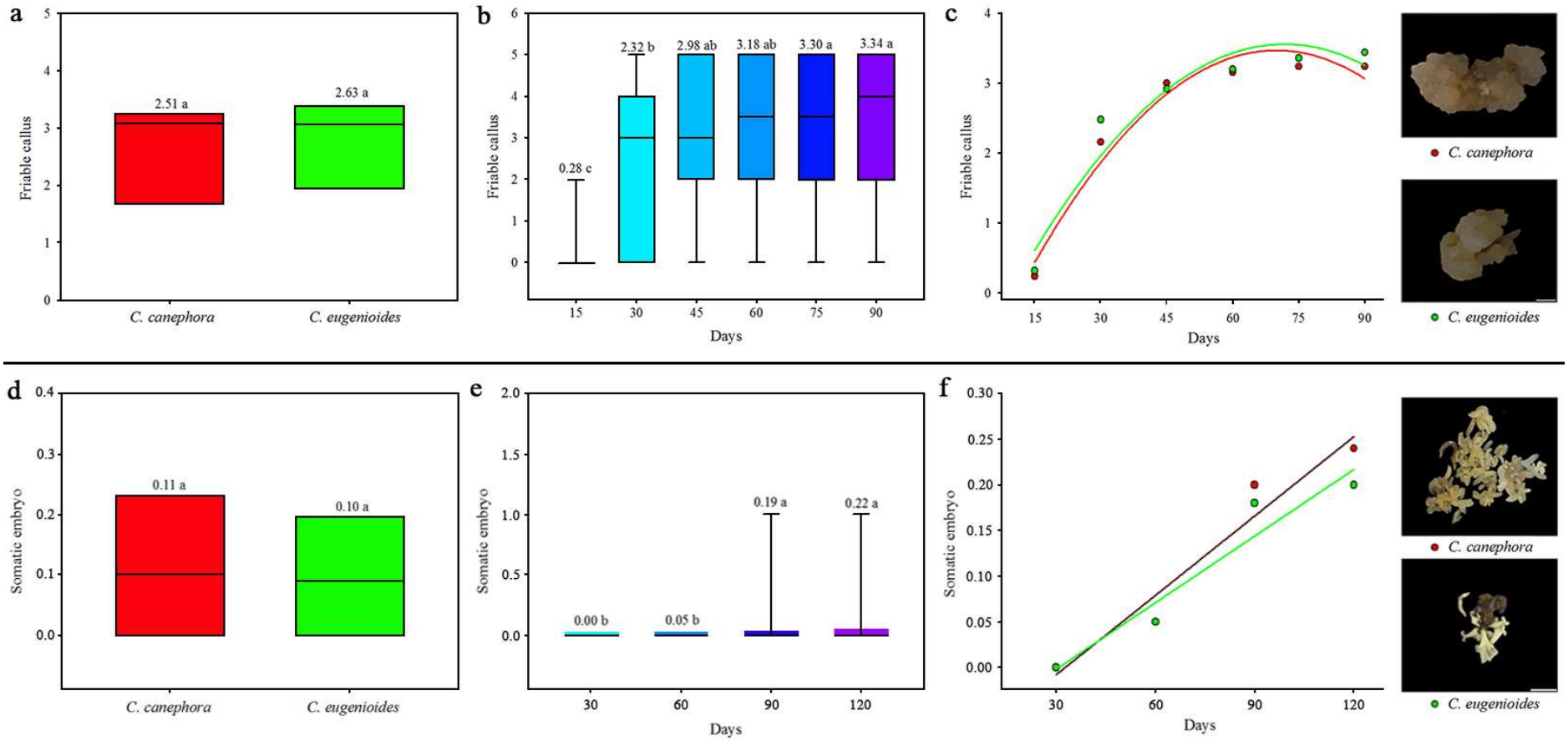

Figure 1. Mean number of friable callus and somatic embryos formed during *C. canephora* and *C. eugenioides* ISE. (a) Mean number of friable callus of *C. canephora* and *C. eugenioides*. (b) Gradual formation of friable callus over 90 days. (c) Quadratic polynomial regression analysis was significant ($P < 0.05$) for *C. canephora* ($Y = - 0.2257X^2 + 2.1057X - 1.4400$, $R^2 = 0.96$) and *C. eugenioides* ($Y = - 0.2057X^2 + 1.9691X - 1.1520$, $R^2 = 0.93$). On the right side of the regression graph, callus of *C. canephora* and *C. eugenioides* showing pale yellow color and friable appearance. (d) Mean number of somatic embryos of *C. canephora* and *C. eugenioides*. (e) Gradual formation of somatic embryos over 120 days. (f) The linear regression analysis was significant ($P < 0.05$) for *C. canephora* ($Y = - 0.0870X - 0.0950$, $R^2 = 0.94$) and *C. eugenioides* ($Y = 0.0730X - 0.0750$, $R^2 = 0.93$). Bar = 5 mm.

Hormonal levels of IAA, GA_3, GA_4 and Z were determined for *C. canephora* and *C. eugenioides* during the friable callus induction moment (Figure 2a – h). The levels of these endogenous hormones were statistically different between the *Coffea*. *C. canephora* presented mean levels of IAA, GA_3, GA_4 and Z higher than C*. eugenioides*, regardless of the time evaluated. The mean values of these endogenous hormones also oscillated for the same species over time. In *C. canephora*, the IAA, GA_3 and GA_4 mean levels reduced during friable callus induction between 60 days and 90 days: IAA from 0.66 to 0.27, GA_3 from 48.38 to 16.69, and GA_4 from 118.63 to 49.33. Differently, the Z mean values increased from 6.87 to 12.34. For *C. eugenioides*, we noticed the increase of the IAA, GA_3 and Z mean values between the same periods: IAA from 0.15 to 0.25, GA_3 from 4.91 to 11.92, and Z from 1.13 to 3.36. The mean value of GA_4 decreased from 32.41 to 13.52 in the same period. Therefore, the hormones GA_4 and Z showed the same profile during friable callus induction for the two *Coffea*. The opposite was found for IAA and GA_3. In addition, the IAA/Z ratio at 60 days was 0.10 for *C. canephora* and 0.13 for *C. eugenioides*. At 90 days, this proportion had a decline, being that *C. canephora* exhibited 0.02 and *C. eugenioides* 0.07.

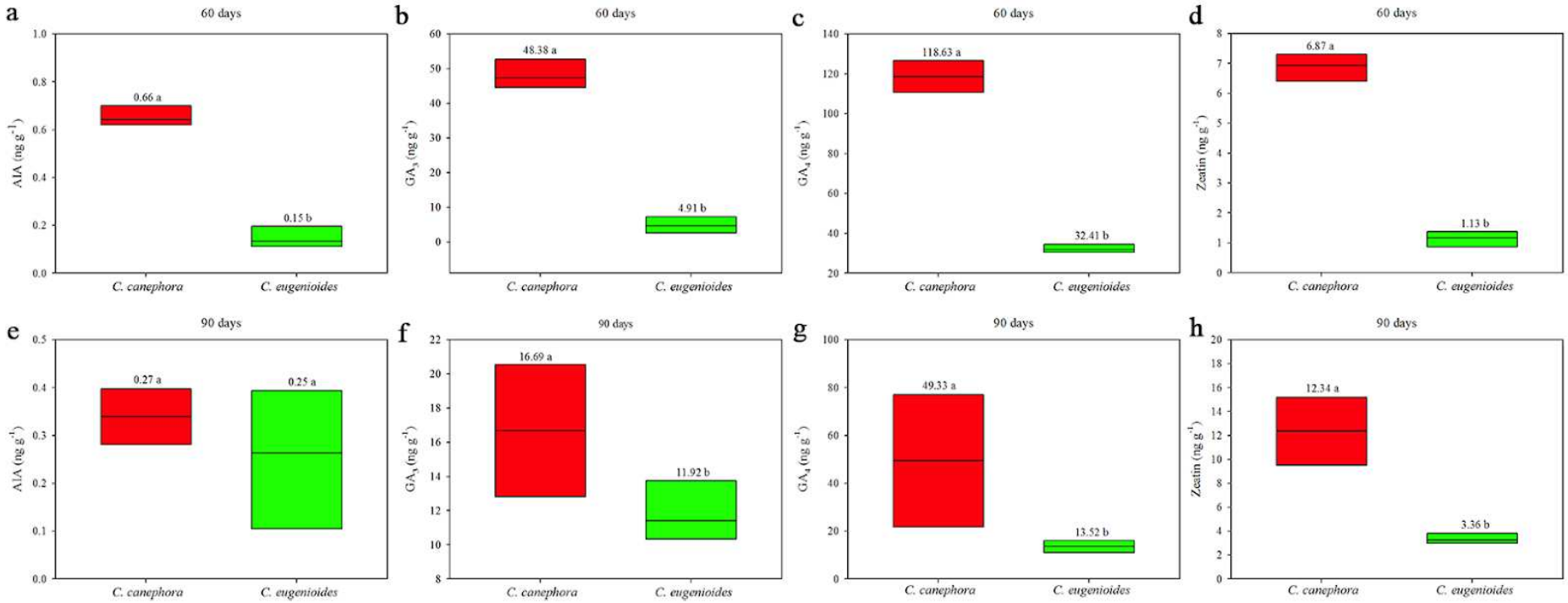

Figure 2. Identification and quantification of the endogenous hormones levels in *C. canephora* and *C. eugenioides* friable callus up to 60 days and up to 90 days: (a and e) Indole-3-acetic acid (IAA); (b and f) Gibberellic acid (GA_3); (c and g) Gibberellic acid (GA_4); and (d and h) Zeatin (Z).

After confirming the ploidy level of *C. canephora* and *C. eugenioides*, we determined the copy number of the genes related to auxin biosynthesis and the somatic embryogenesis pathway (Figure 3). *C. Canephora* and *C. eugenioides* have 2n = 2x = 22 chromosomes, evidencing that none chromosome number variation occurred during the ISE. The number copy for each gene was determined from the fluorescent signals, respective for each gene probe, in the interphasic nuclei of *C. canephora* and *C. eugenioides*. Both *Coffea* exhibited two signals for the *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1* genes. Therefore, *C. canephora* and *C. eugenioides* have one copy of the genes, considering the basic chromosome number x = 11.

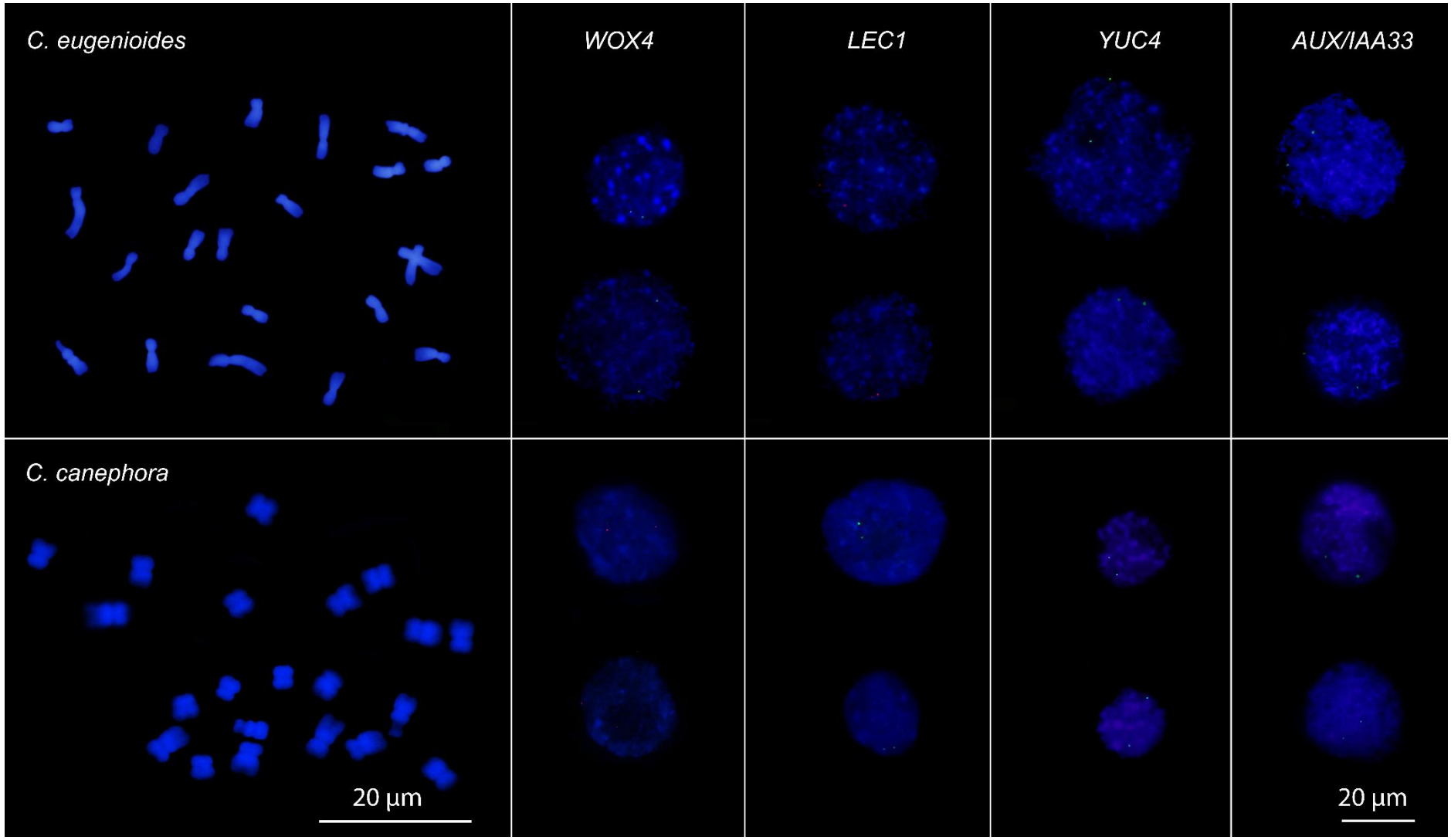
C. eugenioides
WOX4
LEC1
YUC4
AUX/IAA33
C. canephora
20 µm
20 µm

Figure 3. *C. canephora* and *C. eugenioides* chromosome number of 2n = 2x = 22. Interphase nuclei of *C. canephora* and *C. eugenioides* exhibiting two signals for *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1* genes. Bar = 20 µm.

4. Discussion

ISE establishment depends on the in vitro environmental, as well as the genetic and physiological aspects of the explant donor. Under the same in vitro conditions, we established the ISE for *C. canephora* and *C. eugenioides*, which exhibited the same ISE responses for both moments: callus induction and somatic embryo regeneration. Comparative ISE studies involving these two, as well as other *Coffea* species, under the same in vitro conditions are still limited (van Boxtel and Berthouly 1996; Samson et al. 2006; Sanglard et al. 2019). Comparing *C. canephora*, *Coffea arabica* L., *Coffea heterocalyx* and *Coffea* sp. *Moloundou*, Samson et al. (2006) found different responses for *Coffea* sp. *Moloundou*. For this, the authors designate the ISE response as being species-specific. However, in our previous study (Sanglard et al. 2019), *C. canephora* and *C. eugenioides* exhibited the same in vitro response, demonstrating that these two *Coffea* respond in the same way to ISE. Therefore, *C. canephora* and *C. eugenioides* are important models to understand the physiological and genetic aspects of the ISE.

Although *C. canephora* and *C. eugenioides* have the same ISE responses, the IAA, GA_3, GA_4 and Z levels were significantly different between the friable calli of the two *Coffea*. *C. canephora* calli had higher hormone levels than from *C. eugenioides*. However, this difference did not negatively influence the ISE establishment. The ratio of endogenous IAA to endogenous Z (IAA:Z) gradually decreased over the calli induction, and no significant difference was observed between the *Coffea*. The data

from the present study reveal that the IAA:Z ratio plays a fundamental role in the first moment of the ISE. The hormonal balance between IAA:Z is known to establish central control in the process of somatic cell dedifferentiation and subsequent cell differentiation through gene reprogramming (Iwase et al. 2011). IAA and Z activate genes that interfere in the cell's fate and trigger biochemical, physiological and morphological changes, important for ISE (Fehér 2015). Furthermore, the IAA:Z ratio together with other hormones promote cell division and proliferation, leading to callus formation (Jiménez 2005; Iwase et al. 2011; Fehér 2015).

The presence of IAA, Z, GA_3 and GA_4 hormones corresponded to the period in which there was greater formation of callus (from 60 days to 90 days), evidencing the role of IAA, Z, GA_3 and GA_4 in the reactivation of cell division and cell dedifferentiation, both essentials and prerequisites for the ISE in *C. canephora* and *C. eugenioides* (Amaral-Silva et al. 2021; Shimotohno et al. 2021). Endogenous hormones act in a multilaterally integrated way in the cell division regulation during the process of cell dedifferentiation and proliferation. The cell cycle is strictly controlled by the activity of cyclin-dependent kinases (CDK) and cyclin complexes (CYC). Auxin modulates CDKA expression and increases the availability of the CYCD-CDKA complex in *Arabdopisis thaliana* L. The increase of the CYCD-CDKA complex and the expression of CDKB, CYCA, CYCB and CYCD, induced by auxin, promotes the progression of cell cycle phases. Furthermore, cytokinins also induce the expression of CYCD and promote the transition of the G1/S and G2/M phases, and their biosynthesis is essential for entry into mitosis in callus cultures. Gibberellins, in turn, influence the degradation of the DELLA protein, known to inhibit cell proliferation by inducing the expression of CDK inhibitors (CKIs). In this way, gibberellins lead to reduction of CKIs, promote CYCB expression, and control the cell cycle (Claeys et al. 2014; Shimotohno et al. 2021).

In addition physiology, the genetic stability exhibited by species during somatic embryogenesis is relevant for tissue culture (Zhang et al. 2006). Thus, we confirm that *C. canephora* and *C. eugenioides* have 2n = 2x = 22 chromosomes, evidencing that there were no variations in their chromosome number during the ISE. Although 2,4-D is widely used in culture medium for callus formation in *Coffea* and other species, it has been associated with chromosomal alterations found during somatic embryogenesis. In vitro conditions, as the growth regulators, can promote somaclonal variation, as the aneuploidy. Aneuploidy has frequently been found in callus and suspension cultures *Hordeum vulgare* L., *Citrus sinensis* L. and *Pisum sativum* L. (Hervé et al. 2016).

The ploidy level and nuclear genome size also influence the in vitro response of somatic embryogenesis (Zhang et al. 2006; Iantcheva and Revalska 2018). In *Medicago* genus, such as *Medicago truncatula* L., embryogenic competence is directly associated with nuclear genome size. Individuals with a smaller genome size responded faster and regenerated a higher number of somatic embryos than individuals with a larger genome size (Iantcheva and Revalska 2018). In *Coffea*, studies correlating the ploidy level and nuclear genome size with the in vitro response are still initial and need to be more investigated. In a previous study, our research group found an identical ISE response for the two diploids *C. canephora* and *C. eugenioides*. However, in comparison to other polyploid species of the genus, *C. arabica* (allotetraploid) and “Hibrido de Timor" (allotriploid), the ISE differed. For callus induction, the polyploids exhibit a higher mean number of callus than diploids. However, in the second moment, *C. arabica* produces a higher number of mature cotyledonary somatic embryo in relation to the diploid *Coffea*. These data demonstrate

that genetic factors, as the ploidy level, influence the in vitro responses (Sanglard et al. 2019).

So, the knowledge of the genetic aspect has been crucial for the in vitro response. Balanced gene dosage is essential for the normal functioning of most eukaryotic genomes. Unbalanced gene dosage can cause severe phenotypic changes in plants, compromising the development (Birchler and Veitia 2007; Hervé et al. 2016). Based on this, we determined the copy number of the *AUX/IAA33*, *YUC4*, *WOX4* and *LEC1* genes. Corroborating with the diploidy of these species, we verified that *C. canephora* and *C. eugenioides* have the same copy number of the investigated genes. Changes in the copy number of a gene usually are related to changes in the gene expression level, which can result in different phenotypes, and thus influencing the in vitro response (Guo et al. 1996; Hastings et al. 2009).

C. canephora and *C. eugenioides* possess the expected copy number for diploid species, demonstrating that these genes were conserved over time. Therefore, the hormonal difference found in the two *Coffea* can be the result of several factors, such as: the genotype and physiological state of the explant donor and/or the age of the collected leaves. Furthermore, cellular and biochemical mechanisms involved in the perception of extra- and intracellular signals, in the translation and hormonal synthesis of each species (Neumann et al. 2009; Mostafa et al. 2020). These elements may have contributed to the hormonal differences found between *C. canephora* and *C. eugenioides*.

5. Conclusions

In this study, we established the ISE in *C. canephora* and *C. eugenioides* and observed that these *Coffea* respond in the same way, both in callus formation and in the regeneration of somatic embryos. We identified and quantified the IAA, GA_3, GA_4 and Z levels, and found significant differences regarding the endogenous content between these *Coffea*. *C. canephora* and *C. eugenioides* have the same gene copy number of *WOX4*, *LEC1*, *YUC4* and *AUX/IAA33*. We know that ISE is a complex pathway and that its establishment involves several factors.

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7. Supplement

Table I. Primers used to build probes in *Coffea*.

Gene	Primer Sequence	Amplicon Length (bp)	Amplicon TM (°C)	Labelling	
				C.canephora	***C. eugenioides***
LEC1	F 5'-CGAAAGCGGTGGAGATATAG-3' R 5'-GAAAGACTATCACTGCTGAGG-3'	158	58°C	ChromaTide	Rhodamine
WOX4	F 5'-CCAACCCAAGAGCAAATAGG-3' R 5'-GTGCTTTGTGGTTTTGGAAC-3'	179	58°C	Rhodamine	ChromaTide
AUX/IAA33	F 5'-GCGATTCCTGGTCATCTCATT-3' R 5'-AGTTCTCTACCTTTCCCTTTCTTG-3'	450	45°C	ChromaTide	ChromaTide
YUC4	F 5'-CGGAAGGTGTGAGAGAGATAAC-3' R 5'-CTACTCTGGCAGCTTGGATAAG-3'	708	43°C	ChromaTide	ChromaTide

8. References

Amaral-Silva PM, Clarindo WR, Guilhen JHS, Passos ABRJ, Sanglard NA, Ferreira A (2021) Global 5-methylcytosine and physiological changes are triggers of indirect somatic embryogenesis in *Coffea canephora*. Protoplasma 258:45-57. https://doi.org/10.1007/s00709-020-01551-8

Arroyo-Herrera A, Ku Gonzalez A, Canche Moo R, Quiroz-Figueroa FR, Loyola-Vargas VM, Rodriguez-Zapata LC, Rodriguez-Zapata C, Burgueff D'Hondt VM, Suárez-Solis EC (2008) Expressão de *WUSCHEL* em *Coffea canephora* causa morfogênese ectópica e aumenta a embriogênese somática. Plant Cell Tissue Organ Cult 94:171-180. https://doi.org/10.1007/s11240-008-9401-1

Barbier FF, Chabikwa TG, Ahsan MU, Cook SE, Powell P, Tanurdzic M, Beveridgeet CA (2019) A phenol/chloroform-free method to extract nucleic acids from recalcitrant, woody tropical species for gene expression and sequencing. Plant Methods 15:62. https://doi.org/10.1186/s13007-019-0447-3

Birchler JA, Veitia RA (2007) The gene balance hypothesis: from classical genetics to modern genomics. Plant Cell 19:395-402. https://doi.org/10.1105/tpc.106.049338

Blázquez MA, Nelson DC, Weijers D (2020) Evolution of Plant Hormone Response Pathways. Annual Review of Plant Biology 71:327-353. https://doi.org/10.1146/annurev-arplant-050718-100309

Braybrook SA, Harada JJ (2008) *LECs* go crazy in embryo development. Trends in Plant Science 13:624-630. https://doi.org/10.1016/j.tplants.2008.09.008

Campos NA, Panis B, Carpentier SC (2017) Somatic embryogenesis in *coffee*: the evolution of biotechnology and the integration of omics technologies offer great opportunities. Front Plant Sci 8:1460. https://doi.org/10.3389/fpls.2017.01460

Claeys H, De Bodt S, Inzé D (2014) Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends in Plant Science 19(4):231-239. https://doi.org/10.1016/j.tplants.2013.10.001

Clarindo WR, Carvalho CR, Mendonça MAC (2012) Cytogenetic and flow cytometry data expand knowledge of genome evolution in three *Coffea* species. Plant Syst Evol 298:835–844. https://doi.org/10.1007/s00606-012-0595-7

Delgado-Aceves L, González-Arnao MT, Santacruz-Ruvalcaba F, Folgado R, Portillo L Indirect Somatic Embryogenesis and Cryopreservation of *Agave tequilana* Weber Cultivar 'Chato'. Plants 10(2):249. https://doi.org/10.3390/plants10020249

Dehestani-Ardakani M, Hejazi M, Aliabad KK (2020) Indirect somatic embryogenesis of purple coneflower (*Echinacea purpurea* (L.) Moench): a medicinal-ornamental plant: evaluation of antioxidant enzymes activity and histological

study. Mol Biol Rep 47, 6621–6633. https://doi.org/10.1007/s11033-020-05713-y

De-la-Peña C, Galaz-Avalos RM, Loyola-Vargas VM (2008) Possible role of light and polyamines in the onset of somatic embryogenesis of *Coffea canephora*. Mol Biotechnol 39:215–224. https://doi.org/10.1007/s12033-008-9037-8

Denoeud F, Carretero-Paulet L, Dereeper A, Droc G, Guyot R, Pietrella M, Lashermes P (2014) The coffee genome provides insight into the convergent evolution of caffeine biosynthesis. Science 345:1181-1184. https://doi.org/10.1126/science.1255274

Enders TA, Strader LC (2015) Auxin activity: Past, present, and future. American Journal of Botany 102(2):180-196. https://doi.org/10.3732/ajb.1400285

Etienne H, Breton D, Breitler JC, Bertrand B, Déchamp E, Awada R, Marraccini P, Léran S, Alpizar E, Campa C, Courte F, Georget F, Ducos JP (2018) Coffee Somatic Embryogenesis: How Did Research, Experience Gained and Innovations Promote the Commercial Propagation of Elite Clones From the Two Cultivated Species?. Front Plant Sci 9:1630. https://doi.org/10.3389/fpls.2018.01630

Fehér A (2015) Somatic embryogenesis - stress - induced remodeling of plant cell fate. Biochimica Biophysica Acta 1849:385–402. https://doi.org/10.1016/j.bbagrm.2014.07.005

Guilfoyle TJ, Hagen G (2007) Auxin response factors. Current Opinion in Plant Biology 10(5):453-460. https://doi.org/10.1016/j.pbi.2007.08.014

Guo M, Davis D, Birchler JA (1996) Dosage effects on gene expression in a maize ploidy series. Genetics 142(4):1349-1355

Haecker A, Groß-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131(3):657-668. https://doi.org/10.1242/dev.00963

Hastings PJ, Lupski JR, Rosenberg SM, Ira G (2009) Mechanisms of change in gene copy number. Nature Reviews Genetics 10:551-564. https://doi.org/10.1038/nrg2593

Hervé E, Romain G, Thierry B, Jean-Christophe B, Estelle J (2016) Plant Fidelity in Somatic: Embryogenesis-Regenerated Plants. An Overview. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic Embryogenesis: Fundamental Aspects and Applications. Springer, pp 121-150

Iantcheva A, Revalska M (2018) Early events during the induction of somatic embryogenesis in genera *Medicago*. Bulgarian Journal of Agricultural Science, 24(6):1042-1052

Iwase A, Ohme-Takagi M, Sugimoto K (2011) WIND1: a key molecular switch for plant cell dedifferentiation. Plant Signal Behav. 6(12):1943-5. https://doi.org/10.4161/psb.6.12.18266

Ji P, Tang X, Jiang Y, Ya T, Gao P, Han W (2015) Potential of gibberellic acid 3 (GA3) for enhancing the phytoremediation efficiency of *Solanum nigrum* L. Bull Environ Contam Toxicol 95:810–814. https://doi.org/10.1007/s00128-015-1670-x

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regulation 47:91-110. https://doi.org/10.1007/s10725-005-3478-x

Jiménez VM, Bangerth F (2001) Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis in vitro. Plant Sci 160(2):247-257. https://doi.org/10.1016/S0168-9452(00)00382-4

Kumar V, Jha P, Van Staden J (2020) *LEAFY COTYLEDONS* (*LECs*): master regulators in plant embryo development. Plant Cell, Tissue and Organ Culture 140:475-487. https://doi.org/10.1007/s11240-019-01752-x

Landey RB, Cenci A, Georget F, Bertrand B, Camayo G, Dechamp E, Herrera JC, Santoni S, Lashermes P, Simpson J, Etienne H (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

Lashermes P, Trouslot P, Anthony F, Combes MC, Charrier A (1996) Genetic diversity for RAPD markers between cultvated and wild accessions of *Coffea arabica*. Euphytica 87:59-64. https://doi.org/10.1007/BF00022965

Loyola-Vargas VM, Avilez-Montalvo JR, Avilés-Montalvo RN, Márquez-López RE, Galaz-Ávalos RM, Mellado-Mojica E (2016) Somatic Embryogenesis in *Coffea* spp. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic Embryogenesis: Fundamental Aspects and Applications. Springer, pp 246-266

Loyola-Vargas VM, Ochoa-Alejo N (2016) Somatic Embryogenesis. An Overview. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic Embryogenesis: Fundamental Aspects and Applications. Springer, pp 1-8

Luo J, Zhou JJ, Zhang JZ (2018) *AUX/IAA* Gene Family in Plants: Molecular Structure, Regulation, and Function. Int J Mol Sci 19(1):259. https://doi.org/10.3390/ijms19010259

Lv B, Yu Q, Liu J, Wen X, Yan Z, Hu K, Li H, Kong X, Li C, Tian H, De Smet I, Zhang XS, Ding Z (2020) Non-canonical AUX/IAA protein *IAA33* competes with canonical AUX/IAA repressor *IAA5* to negatively regulate auxin signaling. EMBO J 39(1):e101515. https://doi.org/10.15252/embj.2019101515

Lye ZN, Purugganan ND (2019) Copy Number Variation in domestication. Trends in Plant Science 24(4):352-365. https://doi.org/10.1016/j.tplants.2019.01.003

Mahé L, Combes M, Lashermes P (2007) Comparison between a coffee single copy chromosomal region and *Arabidopsis* duplicated counterparts evidenced high level synteny between the coffee genome and the ancestral *Arabidopsis* genome. Plant Molecular Biology 64:699-711. https://doi.org/10.1007/s11103-007-9191-6

Mantovani R (1999) The molecular biology of the CCAAT-binding factor NF-Y. Gene 239(1):15-27. https://doi.org/10.1016/S0378-1119(99)00368-6

Meinke DW (1992) Homoeotic Mutant of *Arabidopsis thaliana*, with Leafy Cotyledons. Science 258(5088):1647-1650. https://doi.org/10.1126/science.258.5088.1647

Mostafa HHA, Wang H, Song J, Li X (2020) Effects of genotypes and explants on garlic callus production and endogenous hormones. Sci Rep 10:4867. https://doi.org/10.1038/s41598-020-61564-4

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Neumann KH, Kumar A, Imani J (2009) Plant cell and tissue culture - A tool in biotechnology: Principles and practice. Springer-Verlag Berlin Heidelberg

Nic-Can GI, López-Torres A, Barredo-Pool F, Wrobel K, Loyola-Vargas VM, Rojas-Herrera R, De-la-Peña C (2013) New Insights into Somatic Embryogenesis: *LEAFY COTYLEDON1*, *BABY BOOM1* and *WUSCHEL-RELATED HOMEOBOX4* Are Epigenetically Regulated in *Coffea canephora*. PLOS ONE 8(8):e72160. https://doi.org/10.1371/journal.pone.0072160

Nowak K, Gaj MD (2016) Transcription Factors in the Regulation of Somatic Embryogenesis. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic Embryogenesis: Fundamental Aspects and Applications. Springer, pp 53-80

Oliveira JPM, Ferreira A, Clarindo WR (2021) *In Vitro* Regeneration of Stable Allotriploid Plantlets of the “Híbrido de Timor” (*Coffea*). Cytologia 86(3):1-6

Quintana-Escobar AO, Nic-Can GI, Galaz Avalos RM, Loyola-Vargas VM, Gongora-Castillo E (2019) Transcriptome analysis of the induction of somatic embryogenesis in *Coffea canephora* and the participation of ARF and Aux/IAA genes. PeerJ 7:e7752. doi: 10.7717/peerj.7752

Raharimalala N, Rombauts S, McCarthy A, Garavito A, Orozco-Arias S, Bellanger L, Morales-Correa AY et al (2021) The absence of the caffeine synthase gene is involved in the naturally decaffeinated status of *Coffea humblotiana*, a wild species from Comoro archipelago. Scientific Reports 11:8119. https://doi.org/10.1038/s41598-021-87419-0

Rubio V, Bustos R, Irigoyen ML, Cardona-López X, Rojas-Triana M, Paz-Ares J (2008) Plant hormones and nutrient signaling. Plant Molecular Biology 69(4):361-373. https://doi.org/10.1007/s11103-008-9380-y

Saleem, M. H., Fahad, S., Adnan, M., Ali, M., Rana, M. S., Kamran, M., Ali, Q., Hashem, I.A., Barashuram, P., Ali, M., Hussain, M.R. (2020). Foliar application of gibberellic acid endorsed phytoextraction of copper and alleviates oxidative stress in jute (*Corchorus capsularis* L.) plant grown in highly copper-contaminated soil of China. Environ Sci Pollut Res 27:37121–37133. https://doi.org/10.1007/s11356-020-09764-3

Samson NP, Campa C, Gal LL, Noirot M, Thomas G, Lokeswari TS, De Kochko A (2006) Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species. Plant Cell Tissue Org Cult 86:37–45. https://doi.org/10.1007/s11240-006-9094-2

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Cesário LM, Ferreira A, Carvalho CR, Clarindo WR (2019) Indirect somatic embryogenesis in *Coffea* with different ploidy levels: a revisiting and updating study. Plant Cell, Tissue and Organ Culture 136:255-267. https://doi.org/10.1007/s11240-018-1511-9

Schoof H, Lenhard M, Haecker A, Mayer KFX, Jurgens G, Laux T (2000) The Stem Cell Population of *Arabidopsis* Shoot Meristems Is Maintained by a Regulatory Loop between the *CLAVATA* and *WUSCHEL* Genes. Cell 100(6):635-644. https://doi.org/10.1016/S0092-8674(00)80700-X

Shimotohno A, Aki SS, Takahashi N, Umeda M (2021) Regulation of the Plant Cell Cycle in Response to Hormones and the Environment. Annu Rev Plant Biol 72:273-296. https://doi.org/10.1146/annurev-arplant-080720-103739

Silva JC, Soares FAF, Sattler MC, Clarindo WR (2020) Repetitive sequences and structural chromosome alterations promote intraspecific variations in *Zea mays* L. karyotype. Scientific Reports 10:8866. https://doi.org/10.1038/s41598-020-65779-3

Solórzano-Cascante P, Sánchez-Chiang N and Jiménez VM (2018) Explant Type, Culture System, 6-Benzyladenine, Meta-Topolin and Encapsulation Affect Indirect Somatic Embryogenesis and Regeneration in *Carica papaya* L.. Front. Plant Sci. 9:1769. https://doi.org/10.3389/fpls.2018.01769

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. PNAS 98(20):11806-11811. https://doi.org/10.1073/pnas.201413498

Su YH, Tang LP, Zhao XY, Zhang XS (2021) Plant cell totipotency: Insights into cellular reprogramming. J Integr Plant Biol 63(1):228-243. https://doi.org/10.1111/jipb.12972

Uc-Chuc MA, Pérez-Hernández C, Galaz-Ávalos RM, Brito-Argaez L, Aguilar-Hernández V, Loyola-Vargas VM (2020) *YUCCA*-Mediated Biosynthesis of the Auxin IAA Is Required during the Somatic Embryogenic Induction Process in *Coffea canephora*. Int J Mol Sci 21(13):4751. https://doi.org/10.3390/ijms21134751

van Boxtel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. Plant Cell Tiss Org 44:7-17. https://doi.org/10.1007/BF00045907

van Der Graaff E, Laux T, Rensing ST (2009) Protein family review The *WUS* homeobox-containing (*WOX*) protein Family. Genome Biology 10:248. https://doi.org/10.1186/gb-2009-10-12-248

Vital CE, Gómez JD, Vidigal PM, Barros E, Pontes CSL, Vieira NM, Ramos HJO (2019) Phytohormone profiling by liquid chromatography coupled to mass spectrometry (LC/MS). Protocols.io online. dx.doi.org/10.17504/protocols.io.zgff3tn. Accessed 26 June 2021

Wei S, Chen Y, Hou J, Yang Y, Yin T (2021) *AUX/IAA* and *ARF* Gene Families in *Salix suchowensis*: Identification, Evolution, and Dynamic Transcriptome Profiling During the Plant Growth Process. Front Plant Sci 12:666310. https://doi.org/10.3389/fpls.2021.666310

Weyers JDB, Paterson NW (2002) Plant hormones and the control of physiological processes. New Phytogist 152(3):375-407. https://doi.org/10.1046/j.0028-646X.2001.00281.x

Zhang J-E, Guo W-W, Deng X-X (2006) Relationship Between Ploidy Variation of *Citrus* Calli and Competence for Somatic Embryogenesis. Acta Genetica Sinica, 33(7):647-654. https://doi.org/10.1016/S0379-4172(06)60095-4