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JOÃO PAULO DE MORAIS OLIVEIRA

EMBRIOGÊNESE SOMÁTICA INDIRETA EM *COFFEA*: ASPECTOS GENÉTICOS, EPIGENÉTICOS E DO AMBIENTE *IN VITRO*

ALEGRE-ES
2020

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Tese apresentada ao Programa de Pós-Graduação em Produção Vegetal do Centro de Ciências Agrárias e Engenharias da Universidade Federal do Espírito Santo, como parte das exigências para obtenção do Título de Doutor em Produção Vegetal, na linha de pesquisa em Biotecnologia e Ecofisiologia do Desenvolvimento de Plantas.
Doutorando: João Paulo de Morais Oliviera
Orientador: Prof. Dr. Wellington Ronildo Clarindo

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Aprovada em 28 de fevereiro de 2020.

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Prof. Dr. Wellington Ronildo Clarindo
Orientador

Dedico aos meus pais Geraldo Oliveira Pinto e
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"*Feliz aquele que transfere o que sabe e aprende o que ensina.*"

Cora Coralina

BIOGRAFIA

João Paulo de Morais Oliveira, filho de Geraldo Oliveira Pinto e Sônia Maria de Morais Oliveira e irmão de Camerino Ferreira de Oliveira Neto e Diana Aquino de Oliveira, nasceu em 06 de outubro de 1991 na cidade de São Domingos, Estado de Goiás, Brasil.

Em 2005, concluiu o Ensino Fundamental no Colégio Estadual João Honorato, em São Domingos, Goiás, Brasil. Em 2006, mudou-se para Goiânia, Goiás, Brasil para se preparar para o vestibular, concluindo o Ensino Médio em 2008 no Colégio Protágoras. No ano de 2009, ingressou na Universidade Estadual de Goiás, Campus Palmeiras de Goiás, Goiás, Brasil, graduando-se como Engenheiro Agrônomo no ano de 2014.

Em 2014, ingressou no Programa de Pós-graduação em Produção Vegetal, na linha de pesquisa em Fitotecnia da Universidade Estadual de Goiás, Campus Ipameri, Goiás, Brasil, obtendo o título de Mestre em Produção Vegetal no ano de 2016. No mesmo ano, ingressou no Programa de Pós-Graduação em Produção Vegetal, na Linha de Pesquisa Biotecnologia e Ecofisiologia do Desenvolvimento de Plantas, do Centro de Ciências Agrárias e Engenharias da Universidade Federal do Espírito Santo, com obtenção do título de Doutor em Produção Vegetal em 28 de fevereiro de 2020.

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O gênero *Coffea* compreende cerca de 103 espécies com diferentes níveis de ploidia e histórias evolutivas. As espécies do gênero diversificaram-se principalmente por meio de mudanças na sequência de DNA (mutações), e também por eventos de alopoliploidia e outras modificações genômicas envolvendo alterações cromossômicas estruturais. Essas alterações na estrutura do genoma resultaram em diferentes fenótipos para algumas características morfológicas, fisiológicas e reprodutivas. A embriogênese somática indireta (ESI) é uma via de expressão morfogenética que tem sido utilizada para investigar o desenvolvimento vegetal e os eventos "ômicos" (genoma, epigenoma, transcriptoma, metaboloma) relacionados, e também para propagação massal e conservação de germoplasma. Considerando que fatores genéticos, epigenéticos e/ou fisiológicos das plantas doadoras de explantes e que o ambiente in vitro interferem no estabelecimento da ESI, o presente estudo teve como objetivos: (a) estabelecer a ESI em *Coffea arabica* (2C = 2,62 pg e 2n = 4x = 44 cromossomos), *Coffea canephora* (2C = 1,41 pg e 2n = 2x = 22 cromossomos) "Híbrido de Timor" (HT) alotriploide (1C = 2,10 pg e 2n = 3x = 33) e hexaploide sintético (2C = 4,20 pg e 2n = 6x = 66); (b) investigar e comparar a influência da história evolutiva (origem e tempo de formação), das características cariotípicas (número de cromossomos, nível de ploidia e valor 2C nuclear) e dos níveis globais de citosina metilada (5-mC%) na ESI; e (c) avaliar o efeito do ambiente in vitro no estabelecimento da ESI. No primeiro estudo, o sistema líquido e semissólido influenciaram o estabelecimento da ESI em HT alotriploide. O sistema semissólido resultou em maior número médio de explantes responsivos (valor médio de ~100%) e massa celular (valor médio de 1,21 g), num menor período de tempo. Embriões somáticos (ES) foram regenerados quando os calos do sistema líquido e do semissólido foram transferidos para o meio de regeneração de ES em sistema líquido. Portanto, o estabelecimento da ESI em sistema líquido foi mais eficaz, uma vez que apresentou resposta embriogênica com 45 dias e maior número médio de ES (38,85 por calo). Além disso, variações cariotípicas não foram observadas entre as plântulas regeneradas. No segundo estudo, a ESI foi estabelecida para o HT alotriploide e hexaploide, sendo que a origem evolutiva, as divergências cariotípicas, o nível global de 5-mC% e as concentrações de carvão ativado influenciaram a resposta in vitro. O HT

hexaploide apresentou menor número médio de explantes responsivos e maior nível de 5-mC%. Durante o estágio de regeneração de ES, o HT hexaploide mostrou novamente maior nível de 5-mC%, porém ambos os híbridos exibiram valor médio de 1,3 ES normais por calo aos 330 dias. As concentrações de 8 e 16 g L^{-1} de carvão ativado aumentaram os níveis de 5-mC% e a regeneração de ES anormais no HT hexaploide. As variações de 5-mC% em calos de HT hexaploide correspondem a uma resposta adaptativa às condições in vitro e, consequentemente, correspondem à variação epigenética somaclonal. No terceiro trabalho, as linhagens de *C. arabica* diferiram em relação à resposta in vitro. ‘Oeiras’ exibiu o maior número médio de explantes foliares responsivos, seguido por ‘Caturra’ e 'Catuaí Vermelho'. Os níveis globais de 5-mC% aumentaram gradualmente ao longo do tempo nos calos friáveis de 'Catuaí Vermelho' (20,73% aos 60 dias e 30,79% aos 90 dias) e 'Caturra' (38,70% aos 60 dias e 53,40% aos 90 dias) e reduziu para ‘Oeiras’ (34,34% aos 60 dias e 33,51% aos 90 dias). 'Catuaí Vermelho' exibiu o maior número médio de ES em todos os momentos avaliados e não foi observada a regeneração de ES para 'Oeiras'. Os calos friáveis embriogênicos de 'Catuaí Vermelho' e 'Caturra' exibiram valores distintos de 5-mC%, com valores médios de 54,09% para 'Caturra' e 43,35% para ‘Catuaí Vermelho’. Com base nos resultados, o aumento global de 5-mC% é necessário para a regeneração e maturação de ES em linhagens de *C. arabica*. Este aumento global de 5-mC% está associado a uma remodelação da cromatina do estado eucromático para heterocromático. Como observado neste estudo, a metilação do DNA é um mecanismo dinâmico e variável que interfere no estabelecimento da ESI. No quarto estudo, as espécies *C. arabica* e *C. canephora* diferiram em relação às respostas in vitro. A adição de 2,4-D ao meio de indução aumentou o número de explantes responsivos em *C. arabica* e *C. canephora* e alterou os padrões de metilação global nos diferentes estágios da ESI. Além disso, o aumento da concentração de 2,4-D promoveu a regeneração de ES anormais. Os ES anormais exibiram maior nível de metilação global e núcleos com danos ao DNA. Além disso, o presente estudo mostrou que variações nos níveis globais de metilação são uma resposta adaptativa às condições ambientais in vitro.

Palavras-Chave: Café; Cultura de tecidos vegetais; Ploidia.

GENERAL ABSTRACT

The genus *Coffea* comprises about 103 species with different levels of ploidy and evolutionary histories. The species of the genus have diversified mainly through changes in the DNA sequence (mutations), and also by events of allopolyploidy and other genomic changes involving structural chromosomal changes. These changes in the structure of the genome resulted in different phenotypes for some morphological, physiological and reproductive characteristics. Indirect somatic embryogenesis (ISE) is a morphogenetic expression pathway that has been used to investigate plant development and related "omic" events (genome, epigenome, transcriptome, metabolome), and also for mass propagation and germplasm conservation. Considering that genetic, epigenetic and/or physiological factors of explant donor plants and that the in vitro environment interfere in the establishment of ISE, the present study aimed to: (a) establish ISE in *Coffea arabica* (2C = 2.62 pg and 2n = 4x = 44 chromosomes), *Coffea canephora* (2C = 1.41 pg and 2n = 2x = 22 chromosomes) "Hybrid of Timor" (HT) allotriploid (1C = 2.10 pg and 2n = 3x = 33) and synthetic hexaploid (2C = 4.20 pg and 2n = 6x = 66); (b) investigate and compare the influence of evolutionary history (origin and time of formation), karyotype characteristics (number of chromosomes, ploidy level and 2C nuclear value) and global levels of methylated cytosine (5-mC%) in ISE ; and (c) assess the effect of the in vitro environment on the establishment of ISE. In the first study, the liquid and semisolid system influenced the establishment of ISE in HT allotriploid. The semisolid system resulted in a higher mean number of responsive explants (mean value of ~100%) and cell mass (mean value of 1.21 g), in a shorter period of time. Somatic embryos (SE) were regenerated when the callus of the liquid and semisolid systems were transferred to the SE regeneration medium in a liquid system. Therefore, the establishment of ISE in a liquid system was more effective, since it showed an embryogenic response after 45 days and a higher mean number of SE (38.85 per callus). In addition, karyotype variations were not observed among the regenerated plantlets. In the second study, ISE was established for allotriploid and hexaploid HT, and the evolutionary origin, karyotypic divergences, the global level of 5-mC% and the concentrations of activated charcoal influenced the in vitro response. The hexaploid HT showed a lower mean number of responsive explants and a higher

level of 5-mC%. During the SE regeneration stage, the hexaploid HT again showed a higher level of 5-mC%, however both hybrids showed an mean value of 1.3 normal SE per callus at 330 days. The concentrations of 8 and 16 g L^{-1} of activated charcoal increased the levels of 5-mC% and the regeneration of abnormal SE in the hexaploid HT. The 5-mC% variations in hexaploid HT callus correspond to an adaptive response to in vitro conditions and, consequently, correspond to the somaclonal epigenetic variation. In the third study, *C. arabica* lines differed in relation to the in vitro response. 'Oeiras' exhibited the highest mean number of responsive leaf explants, followed by 'Caturra' and 'Catuaí Vermelho'. The global levels of 5-mC% increased gradually over time in the friable callus of 'Catuaí Vermelho' (20.73% at 60 days and 30.79% at 90 days) and 'Caturra' (38.70% at 60 days and 53.40% at 90 days) and remained constant for 'Oeiras' (34.34% at 60 days and 33.51% at 90 days). 'Catuaí Vermelho' exhibited the highest mean number of SE in all evaluated moments and no regeneration of SE was observed for 'Oeiras'. The embryogenic friable callus of 'Catuaí Vermelho' and 'Caturra' exhibited distinct values of 5-mC%, with mean values of 54.09% for 'Caturra' and 43.35% for 'Catuaí Vermelho'. Based on the results, the global increase of 5-mC% is necessary for the regeneration and maturation of SE in *C. arabica* lines. This global increase of 5-mC% is associated with a remodeling of chromatin from euchromatic to heterochromatic state. As observed in this study, DNA methylation is a dynamic and variable mechanism that interferes with the establishment of ISE. In the fourth study, the species *C. arabica* and *C. canephora* differed in relation to in vitro responses. The addition of 2,4-D to the induction medium increased the number of responsive explants in *C. arabica* and *C. canephora* and changed the patterns of global methylation in the different stages of ISE. In addition, the addition of 2,4-D promoted the regeneration of abnormal SE. The abnormal ES exhibited higher levels of global methylation and nuclei with DNA damage. In addition, the present study showed that variations in global methylation levels are an adaptive response to environmental conditions in vitro.

Keywords: Coffee; Plant tissue culture; Ploidy.

INTRODUÇÃO GERAL

O café representa uma das culturas mais relevantes do mundo e é classificado como a segunda *commodity* mais valiosa. O gênero *Coffea* pertencente à família Rubiaceae é composto por mais de 103 espécies que tem distribuição natural por toda a África tropical, em algumas Ilhas do Oceano Índico como Comores, Maurício, Reunião e Madagascar, estendendo-se para o sul e sudeste da Ásia e Australásia (Hamon et al. 2017). Esse gênero é composto por espécies que apresentam diferentes níveis de ploidia e idades evolutivas, apresentando uma série de modificações genômicas e fenotípicas. No entanto, apenas *Coffea arabica* L. e *Coffea canephora* Pierre ex Froehner são cultivadas comercialmente para consumo de bebidas e, portanto, têm mais significado econômico, representando cerca de 70 e 30% da produção mundial de café, respectivamente (De Almeida 2019). As demais espécies, como *C. eugenioides*, *C. salvatrix*, *C. racemosa*, *C. dewevrei*, *C. liberica*, *C. congensis*, *C. humilis* e Híbrido de Timor, entre outras, são importantes para programas de melhoramento como fontes de recursos genéticos, a fim de obter alelos favoráveis que conferem resistência a pragas, doenças e nematóides, tolerância à seca e melhor qualidade dos grãos para a preparação de bebidas (Charrier e Berthaud 1985).

A biotecnologia vegetal, especialmente a cultura de tecidos vegetais, aliada ao melhoramento convencional tem contribuído para o melhoramento genético em *Coffea* na tentativa de criar e desenvolver genótipos superiores, utilizando técnicas mais sofisticadas e rápidas para atender às expectativas dos agricultores por meio da propagação in vitro (Los Santos-Briones e Hernández-Sotomayor 2006; Campos et al. 2017). Além da propagação de plântulas, a cultura de tecidos vegetais cria um cenário interessante para estudar os aspectos estruturais, bioquímicos, moleculares e fisiológicos envolvidos nas diferentes vias de regeneração (organogênese e embriogênese), possibilitando uma melhor compreensão da morfogênese. Além disso, por meio de abordagens in vitro é possível analisar, verificar e comparar se as diferenças cariotípicas (nível de ploidia e número cromossômico), o tempo e a origem dos poliplóides naturais e sintéticos em *Coffea* influenciam a resposta morfogenética, fornecendo informações relevantes sobre o comportamento dos poliplóides naturais e sintéticos em ambinete in vitro.

Poliploidia em Coffea

A poliploidia é a condição hereditária de possuir mais do que dois conjuntos completos de cromossomos por núcleo da célula (Soltis e Soltis 2009). Espécies com diferentes níveis de ploidia apresentam uma série de modificações genômicas e fenotípicas quando comparadas com seus progenitores (Renny-Byfield e Wendel 2014). Os indivíduos poliploides geralmente são maiores, mais vigorosos e robustos, e se adaptam melhor às condições de estresse, em relação aos progenitores diploides (Stebbins 1971). A capacidade desses indivíduos serem superiores aos seus progenitores é denominada heterose (Van de Peer et al. 2009). Apesar disso, muitos poliploides não são superiores aos seus progenitores e nem ocorre o aumento do tamanho da planta e de seus órgãos, visto que a taxa de proliferação celular pode ser reduzida (Stebbins 1971). Sendo assim, a poliplodia tem grande impacto na evolução do genoma em plantas, por ser um mecanismo importante de adaptação, diversificação e especiação (Sattler et al 2016).

Em *Coffea*, os eventos de diversificação vêm sendo elucidados por meio de abordagens moleculares (Lashermes et al. 1999; Cenci et al. 2012; Yu et al. 2011) e citogenéticas (Bouharmont 1963, Rijo 1974; Clarindo e Carvalho 2009) mostrando que estas espécies divergiram a partir de um ancestral em comum com número básico de x = 11 cromossomos, idêntico ao reportado pelas espécies caracterizadas até o momento (Mahé et al. 2007, Yu et al. 2011). Além das espécies diploides, o gênero possui o alotetraploide verdadeiro *C. arabica*, com 2C = 2,62 picogramas (pg) e 2n = 4x = 44 cromossomos, provavelmente formado a partir da hibridização natural entre *C. eugenioides* (2C = 1,36 pg e 2n = 2x = 22 cromossomos, Noirot et al. 2003) e *C. canephora* (2C = 1,41 pg e 2n = 2x = 22 cromossomos, Clarindo e Carvalho 2009) e, seguido por um evento de poliploidização (Lashermes et al 1999, Cenci et al. 2012) (Figura 1 A, B e C).

Além de *C. arabica* outro poliploide é reconhecido entre os *Coffea*: o alotriploide Híbrido de Timor (2n = 3x = 33) representado pelo acesso 'CIFC 4106'. A primeira planta de HT 'CIFC 4106' foi encontrada em 1927 na Ilha de Timor em uma plantação de *C. arabica* 'Typica' estabelecida entre 1917 e 1918 e, possivelmente, originada a partir do cruzamento natural (Bettencourt 1973). Dados sobre o número cromossômico e o conteúdo de DNA (2,10 pg) de HT 'CIFC 4106' corroboram com a sua origem alotriploide a partir da fusão de uma célula reprodutiva reduzida de *C.*

canephora e *C. arabica* (Clarindo et al 2013) (Figura 1 D). Além disso, as plantas HT 'CIFC 4106' têm um fenótipo semelhante a *C. arabica* e *C. canephora*, mas produzem baixas quantidades de sementes e a maioria das sementes são inviáveis e, portanto, são consideradas semiférteis (Pereira et al. 2008). Apesar disso, os programas de melhoramento em *Coffea* estão interessados nesse alotriploide devido à sua resistência à ferrugem (*Hemilea vastatrix*), provavelmente herdada de *C. canephora* (Capucho et al. 2009). Sanglard et al. (2017) induziram a poliploidia do HT a partir de calos embriogênicos e regeneração via embriogênese somática indireta, caracterizando o primeiro autoalohexaploide de *Coffea* (Figura 1 E). Posteriormente, Venial et al. (2020) induziram a poliploidia de calos embriogênicos e regeneração via embriogenese somática indireta de *C. canephora* e *C. arabica*, originando os primeiro autotetraploide de *C. canephora* (4C = 2,86 pg e 2n = 4x = 44 cromossomos) e autoaloctaploide de *C. arabica* (4C = 5,24 pg e 2n = 8x = 88 cromossomos), fornecendo novo germoplasma e desenvolvendo um novo metodo de indução de poliploidia (Figura 1 F e G). Além da propagação e geração de novo germoplasma (Sanglard et al. 2017; Venial et al. 2020), esses estudos possibilitaram o surgimento de novas abordagens investigativas num âmbito genético, epigenéticos e fenotípico.

Desse modo, o gênero *Coffea* é composto por um alotetraploide verdadeiro *C. arabica* que se divergiu há aproximadamente 0,0665 milhões de anos (Cenci et al. 2012), que por sua vez, assim como *C. canephora* são progenitores do alotriploide HT 'CIFC 4106' (Clarindo et al. 2013) e recentemente por meio da poliploidização sintética induzida in vitro, este genero é composto por um autoalohexaploide (HT 'CIFC 4106', Sanglard et al. 2017), autotetraploide (*C. canephora*, Venial et al. 2020) e autoaloctaploide (*C. arabica*, Venial et al. 2020), como pode ser observado na Figura 1.

A caracterização genômica e fenotípica em neopoliploides é de grande importância, tendo em vista que após a formação de poliploides, esses indivíduos passam por instabilidade cariotípica, molecular, epigenética, bioquímica e morfofisiológica (Sattler et al. 2016). As modificações resultantes da poliploidização no genoma vegetal incluem variações em sequências únicas, número de sequências repetitivas, inversões, duplicação segmentais, translocações, inserções e mutações pontuais (Sattler et al. 2016). Segundo Dewey (1980), cada espécie responde de forma diferente à poliploidização, sendo influenciada principalmente pelo nível original de ploidia, estrutura do genoma, modo de reprodução, perenidade e o órgão que é cultivado. Em poliploides sintéticos, essas alterações têm tipicamente efeitos imediatos

no fenótipo e na aptidão de um indivíduo. Além desses efeitos imediatos, as mudanças na estrutura do genoma podem inferir transições evolutivas (Sattler et al. 2016). Portanto, o uso de poliploides sintéticos constitui uma estratégia para evidenciar a ocorrência de alterações genômicas e epigenômicas, bem como os seus efeitos sobre a expressão do gene e aparecimento de novos fenótipos (Ramsey e Ramsey 2014). Nesse sentido, há um interessante cenário a ser explorado com base na evolução poliploide e divergência destes *Coffea*.

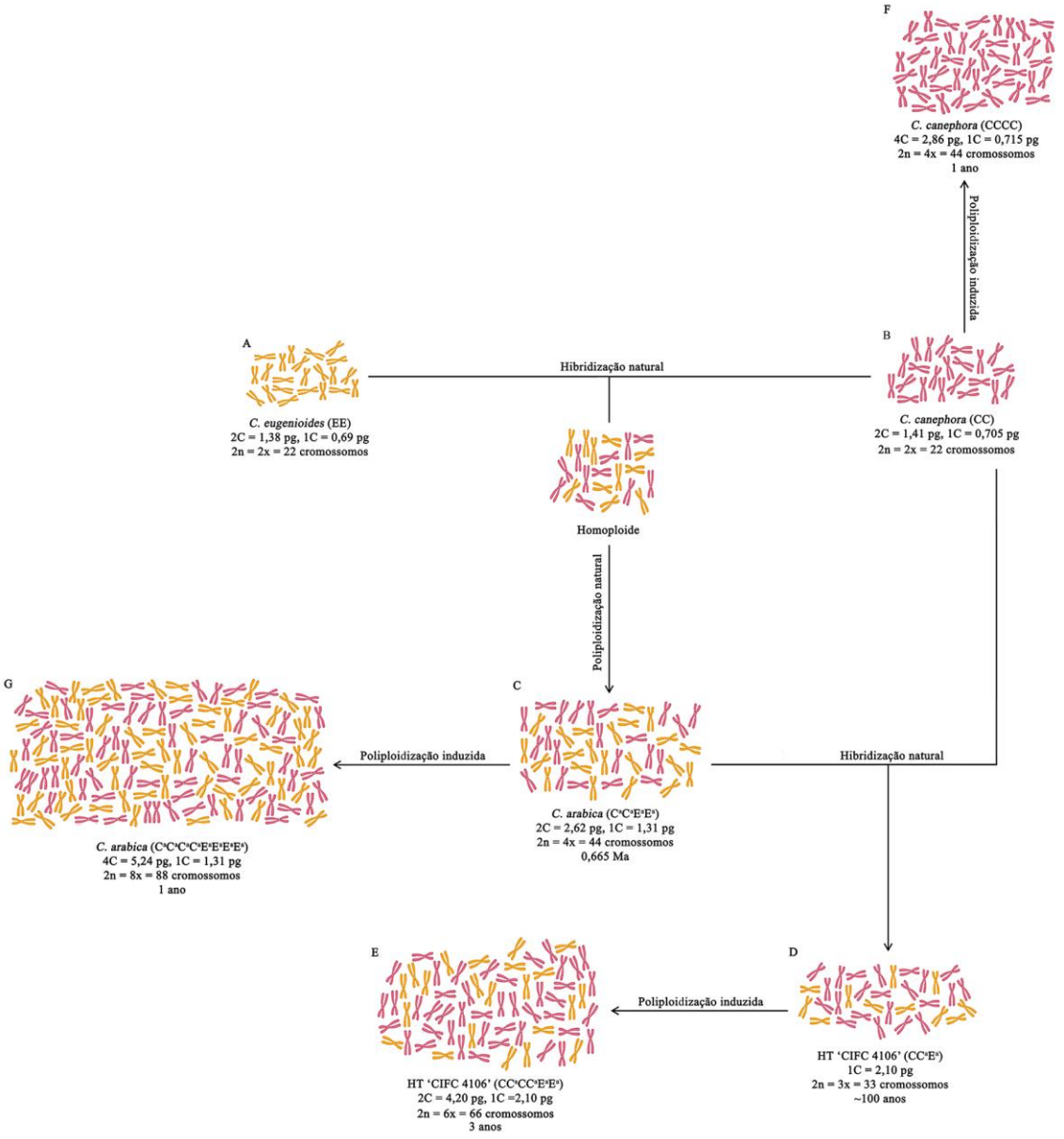

Fig. 1 – Características cariotípicas (número cromossômico e valores de conteúdo de DNA 4C, 2C e 1C), idade evolutiva e origem de poliplóides naturais e sintéticos em

Coffea. Os dados do cariótipo sobre esses *Coffea* foram registrados a partir de bibliografias citogenéticas (Pinto-Maglio e Da Cruz 1998; Noirot et al. 2003; Clarindo et al. 2006; Clarindo et al. 2009; Hamon et al. 2009; Sanglard et al. 2017; Venial et al. 2020).

Embriogênese somática indireta em Coffea

A cultura de tecidos vegetais refere-se à capacidade de uma célula vegetal de adquirir competência e assumir um novo destino de desenvolvimento após a modulação das condições de cultivo por vias organogênicas e embriogênicas (Rocha et al. 2016). A embriogênese somática é uma via morfogenética na qual células vegetais não zigóticas, incluindo células haplóides, sob condições indutivas se dividem e se diferenciam, formando uma estrutura bipolar que dará origem ao embrião somático com base na teoria da totipotência (Quiroz-Figueroa et al. 2006; Rocha et al. 2012). Embora a totipotência seja uma característica das células vegetais, nem todas as células vegetais a expressam (Germana e Lambardi 2016). De acordo com Queiroz-Figueroa et al. (2006), a capacidade de um determinado tecido gerar embriões somáticos é uma característica restrita a uma fração limitada das células. Para que as células vegetais adquiram competência, é necessário passar por uma série de eventos característicos de reprogramação de sua fisiologia, metabolismo e padrões de expressão gênica, ativação da divisão celular e desdiferenciação de células (Fehér 2008; Rose et al. 2010; Rocha et al. 2012). Depois disso, a célula ou tecido competente torna-se determinado para formar os embriões somáticos (Yang e Zhang 2010).

A embriogênese somática indireta tem contribuído para a biotecnologia vegetal, permitindo a sofisticação das práticas de clonagem e impulsionando os programas de melhoramento genético. Essa via morfogênica tem várias aplicações em *Coffea*, como: (a) propagar genótipos superiores (van Boxtel e Berthouly 1996); (b) manter, conservar e trocar germoplasma (Naidu e Sreenath 1998; Sanglard et al. 2019); (c) isolar protoplasma e regenerar plântulas (Schöpke et al. 1987); (d) induzir a poliploidização (Sanglard et al. 2017) e variações somaclonais (Sondahl et al. 1995; Sanchez-Teyer et al. 2003); e (e) transformação genética (Ogita et al. 2003; Da Silva e Menéndez-Yuffá 2003; Mishra e Slater 2012). Desde de 1970, entrenós de ramos e folhas de *C. canephora*, *C. arabica* e *C. liberica* foram utilizados como fonte de explante para o estabelecimento in vitro e propagação massal (Staritsky 1970, Sondahl e Sharp 1977).

Posteriomente, outros estudos de embriogênese somática indireta foram reportados para *Coffea*, relatando que variações intraespecíficas ocorrem nas variedades de *C. arabica*, Obatã (Maciel et al. 2003), Caturra e Catuaí (Mendes et al. 2008) e Mundo Novo (Almeida et al. 2008), e que os genótipos influenciam a resposta in vitro. Os autores têm sugerido que essas variações são decorrentes de eventos genéticos, epigenéticos e/ou fisiológicos, ressaltando a necessidade de investigações sobre os fatores que regulam/controlam essa via morfogênica. Recentemente, Sanglard et al. (2019) estabeleceram a embriogênese somática indireta e compararam as repostas in vitro entre os diploides *C. canephora* e *C. eugenioides*, o alotriploide HT 'CIFC 4106' e o alotetraploide *C. arabica*, mostrando que as divergências cariotípicas (número cromossômico e conteúdo de DNA nuclear 2C) influenciam a resposta in vitro.

Além das características genéticas/epigenéticas, as condições químicas e físicas do meio de cultura, bem como o tipo, concentração e combinação de reguladores de crescimento, são fatores determinantes para o estabelecimento da embriogênese somática indireta, uma vez que a resposta morfogenética é determinada pela ação de estímulos ambientais (Fehér et al. 2003; Zavattieri et al. 2010; Vanstraelen e Benková 2012). Abordagens in vitro comparando poliploides sintéticos com seus progenitores poliploides são escassas na literatura. Em *Coffea*, esse tipo de estudo é possível e fornece um modelo interessante para investigar as alterações genômicas, epigenômicas, transcriptoma e metaboloma durante a morfogênese in vitro. Além disso, são necessários novos estudos que busquem esclarecer as variações intraespecíficas reportadas em *Coffea*, bem como estudos que visam otimizar a propagação massal de forma mais rápida, segura e eficiente. Por exemplo, o regulador de crescimento 2,4-diclorofenoxiacético é amplamente utilizado na embriogênese somática indireta, desempenha um papel crucial no processo de desdiferenciação celular e multiplicação de embriões somáticos e está relacionado aos processos de divisão e expansão celular (Teale et al. 2006; Perrot-Rechenmann 2010). Em sistemas líquidos, agregados celulares suspensos de *C. arabica* e *C. canephora* apresentam maior captação de elementos minerais e reguladores de crescimento e, consequentemente, maiores atividades metabólicas, bem como respostas de proliferação celular e regeneração de embriões somáticos (van Boxtel & Berthouly 1996; Papanastasiou et al. 2008).

OBJETIVO GERAL

Diante do exposto, o presente estudo tem como objetivo avaliar a influência de aspectos genéticos (número de cromossomos, nível de ploidia e conteúdo de DNA nuclear), epigenéticos (metilação global da citosina) e do ambiente in vitro (sistema de cultura e adição de ácido 2,4-diclorofenoxiacético e carvão ativado) durante o estabelecimento da embriogênese somática indireta em *Coffea*.

REFERÊNCIAS BIBLIOGRÁFICAS

Almeida JAS, Silvarolla MB, Fazuoli LC, Stancato GC (2008) Embriogênese somática em genótipos de *Coffea arabica* L. Coffee Science 3:143–151.

Bettencourt AJ (1973) Considerações gerais sobre o "Híbrido de Timor". Campinas, Brasil: Instituto Agronômico de Campinas, Cicular 23.

Bouharmont J (1963) Somatic chromosomes of some *Coffea* species. *Euphytica*, *12*(3):254-257. https://doi.org/10.1007/BF00027458

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

Capucho AS, Caixeta ET, Zambolim EM, Zambolim L (2009) Herança da resistência de Híbrido de Timor UFV 443-03 à ferrugem-do-cafeeiro. Pesq. Agropec. Bras 44:276–282. https://doi.org/10.1590/S0100-204X2009000300009

Cenci A, Combes MC, Lashermes P (2012) Genome evolution in diploid and tetraploid *Coffea* species as revealed by comparative analysis of orthologous genome segments. Plant Mol Biol 78:135–145. https://doi.org/10.1007/s11103-011-9852-3

Charrier A; Berthaud J (1985) Botanical classification of coffee. In: Clifford MN; Willson LC. Coffee: botany, biochemistry and production of beans and beverage. Westport USA, The AVI Publishing Company, p.13-47.

Clarindo WR, Carvalho CR (2006). A high quality chromosome preparation from cell suspension aggregates culture of *Coffea canephora*. Cytologia 71:243–249. https://doi.org/0.1508/cytologia.71.243

Clarindo WR, Carvalho CR (2009) Comparison of the *Coffea canephora* and *C. arabica* karyotype based on chromosomal DNA content. Plant Cell Reports, *28*(1):73. https://doi.org/10.1007/s00299-008-0621-y

Clarindo WR, Carvalho CR, Caixeta ET, Koehler AD (2013) Following the track of "Híbrido de Timor" origin by cytogenetic and flow cytometry approaches. Genet Resour Crop Evol, 60:2253-2259. https://doi:10.1007/s10722-013-9990-3

Da Silva RF, Menéndez-Yuffá A (2003) Transient gene expression in secondary somatic embryos from coffee tissues electroporated with the genes gus and bar. Electronic Journal of Biotechnology 6(1):11-12.

De Almeida JAS (2019) Observations on somatic embryogenesis in *Coffea arabica* L.. In: Coffee-Production and Research. IntechOpen. https://doi.org/10.5772/intechopen.90853

Dewey DR (1980) Some applications and misapplications of induced polyploidy to plant breeding. In: Lewis WH (ed) Polyploidy: biological relevance. Plenum Press, New York, p. 445–470.

Fehér A (2008) The initiation phase of somatic embryogenesis: what we know and what we don't. Acta Biol Szeged 52:53–56.

Fehér A, Pasternak T, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss Organ Cult 74:201–228.

Gatica-Arias AM, Arrieta-Espinoza G, Esquivel AME (2007) Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuai. Electronic Journal of Biotechnology, 11:1-12. https://doi.org/10.2225/vol11-issue1-fulltext-9

Germana MA, Lambardi M (2016) In vitro embryogenesis in higher plants. Methods Mol Biol, 1359:1–577.

Hamon P, Grover CE, Davis AP, Rakotomalala JJ, Raharimalala NE, Albert VA, Hamon S (2017) Genotyping-by-sequencing provides the first well-resolved phylogeny for coffee (*Coffea*) and insights into the evolution of caffeine content in its species: GBS coffee phylogeny and the evolution of caffeine content. Mol Phylogenet Evol 109:351–361. https://doi.org/10.1016/j.ympev.2017.02.009

Hamon P, Siljak-Yakovlev S, Srisuwan S, Robin O, Poncet V, Hamon S, De Kochko A (2009) Physical mapping of rDNA and heterochromatin in chromosomes of 16 *Coffea* species: a revised view of species differentiation. Chromosome Res 17:291–304. https://doi.org/10.1007/s10577-009-9033-2

Lashermes P, Combes MC, Robert J, Trouslot P, D'Hont A, Anthony F, Charrier A (1999) Molecular characterization and origin of the *Coffea arabica* L. genome. Mol Gen Genet 261:259–266. https://doi.org/10.1007/s004380050965

Los Santos-Briones D, Hernández-Sotomayor SM (2006) Coffee biotechnology. Brazilian Journal of Plant Physiology 18(1):217-227. https://doi.org/10.1590/S1677-04202006000100015

Maciel ALR, Pasqual M, Pereira AR, Rezende JC, Silva AB, Dutra LF (2003) Embriogênese somática indireta em explantes foliares de *Coffea arabica* L. cv. Obatã. Ciência e Agrotecnologia, 27(1)107-116. https://doi.org/10.1590/S1413-70542003000100013

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(6)699-711. https://doi.org/10.1007/s11103-007-9191-6

Mendes ANG, Carvalho GR, Botelho CE, Fazuoli LC, Silvarolla MB (2008) História das primeiras cultivares de café plantadas no Brasil. In: Carvalho CHS. Cultivares de Café. Brasília, DF: Embrapa Café.

Mishra MK, Slater A (2012) Recent advances in the genetic transformation of coffee. Biotechnology research international, 2012.

Naidu MM, Sreenath HL (1998) In vitro culture of coffee zygotic embryos for germplasm preservation. Plant Cell Tiss Organ Cult 55:227–230.

Noirot M, Poncet V, Barre P, Hamon P, Hamon S, De Kochko A (2003) Genome size variations in diploid african *Coffea* species. Ann Bot 92(5):709–714. https://doi.org/10.1093/aob/mcg183

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) Producing decaffeinated coffee plants. Nature 423:823.

Papanastasiou I, Soukouli K, Moschopoulou G, Kahia J, Kintzios S (2008) Effect of liquid pulses with 6-benzyladenine on the induction of somatic embryogenesis from coffee (*Coffea arabica* L.) callus cultures. Plant Cell Tiss Organ Cult 92:215–225. https://doi.org/10.1007/s11240-007-9326-0

Pereira AA, Oliveira ACB, Sakiyama NS (2008) Híbrido de Timor como fonte de resistência a doenças e de qualidade de bebida do cafeeiro. In: Parreiras S (ed) Manejo fitossanitário da cultura do cafeeiro. Universidade Federal de Lavras: UFLA, 13–24.

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol 2(5). https://doi.org/10.1101/cshperspect.a001446

Pinto-Maglio CAF, Da Cruz ND (1998) Pachytene chromosome morphology in *Coffea* L. II. *C. arabica* L. complement. Caryologia 51(1):19-35. https://doi.org/10.1080/00087114.1998.10589117

Quiroz-Figueroa F, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tiss Organ Cult 86:285–301. https://doi.org/10.1007/s11240-006-9139-6

Ramsey J; Ramsey TS (2014) Ecological studies of polyploidy in the 100 years following its discovery. Phil Trans R Soc B, 369:1–20. https://doi.org/10.1098/rstb.2013.0352

Renny-Byfield S; Wendel JF (2014) Doubling down on genomes: polyploidy and crop plants. Am J Bot, 101:1–15. https://doi.org/10.3732/ajb.1400119

Rijo L (1974) Observações cariológicas no cafeeiro 'Híbrido de Timor'. Portugaliae Acta Biológica, 8:157-168.

Rocha DI, Vieira LM, Tanaka FA, Silva LC, Otoni WC (2012) Somatic embryogenesis of a wild passion fruit species *Passiflora cincinnata* Masters: histocytological and histochemical evidences. Protoplasma 249:747–758.

Rocha DI, Kurczyńska E, Potocka I, Steinmacher DA e Otoni WC (2016) Histology and histochemistry of somatic embryogenesis. In: Loyola-Vargas, V.M. and Ochoa-Alejo, N. (eds.) Somatic embryogenesis: Fundamental aspects and applications. Springer, Switzerland, pp. 471–494.

Rose RJ, Mantiri FR, Kurdyukov S, Chen S-K, Wang X-D, Nolan KE, Sheahan MB (2010) Developmental biology of somatic embryogenesis. Plant Developmental Biology - Biotechnological Perspectives, 3–26. https://doi.org/10.1007/978-3-642-04670-41

Sanchez-Teyer LF, Quiroz-Figueroa FR, Loyola-Varga VM, Infante-Herrera D (2003) Culture-induced variation in plants of *Coffea arabica* cv. Caturra rojo, regenerated by direct and indirect somatic embryogenesis. Molecular Biotechnology 23:107–115.

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

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Nunes ACP, Soares TCB, Carvalho CR, Clarindo WR (2017) From chromosome doubling to DNA sequence changes: outcomes of an improved in vitro procedure developed for allotriploid "Híbrido de Timor" (*Coffea arabica* L. x *Coffea canephora* Pierre ex A. Froehner). Plant Cell Tiss Organ Cult 131:223–231. https://doi.org/10.1007/s11240-017-1278-4

Sattler MC, Carvalho CR, Clarindo WR (2016) The polyploidy and its key role in plant breeding. Planta 243(2):281-296. https://doi.org/10.1007/s00425-015-2450-x

Schöpke C, Müller LE, Kohlenbach HW (1987) Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex. Fr.). Plant Cell Tiss Organ Cult 8:243-248.

Soltis PS, Soltis DE (2009) The role of hybridization in plant speciation. Annu Rev Plant Biol 60:561–588. https://doi.org/10.1146/annurev.arplant.043008.092039

Sondahl MR, Romig WR, Bragin A (1995) Induction and selection of somaclonal variation in coffee. U.S. Patent No. 5,436,395. 25 Jul. 1995.

Staritsky G (1970) Embryoid formation in callus tissues of coffee. Plant Biol 19:509–514. https://doi.org/10.1111/j.1438-8677.1970.tb00677.x

Stebbins GL (1971) Chromosomal evolution in higher plants. Addison-Wesley, London.

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 7:847–859. https://doi.org/10.1038/nrm2020

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

Van De Peer Y; Maere S; Meyer A (2009) The evolutionary significance of ancient genome duplications. Nat Rev Genet 10:725–732.

Vanstraelen M, Benková E (2012) Hormonal interactions in the regulation of plant development. Annu Rev Cell Dev Biol 28(1):463–487. https://doi.org/10.1146/annurev-cellbio-101011-155741

Venial LR, Mendonça MAC, Amaral-Silva PM, Canal GB, Passos ABRJ, Ferreira A, Soares TCB, Clarindo WR (2020) Autotetraploid *Coffea canephora* and auto-alloctaploid *Coffea arabica* from in vitro chromosome set doubling: new germplasms for *Coffea*. Front Plant Sci 11:154. https://doi.org/10.3389/fpls.2020.00154

Yang X, Zhang X (2010) Regulation of somatic embryogenesis in higher plants. Crit Rev Plant Sci 29:36–57.

Yu Q, Guyot R, Kochko A, Byers A, Navajas-Pérez R, Langston BJ, Dubreuil-Tranchant C, Paterson AH, Poncet V, Nagai C, Ming R (2011) Micro-collinearity and genome evolution in the vicinity of an ethylene receptor gene of cultivated diploid and allotetraploid coffee species (*Coffea*). Plant J 67:305–317. https://doi.org/10.1111/j.1365- 313X.2011.04590.x

Zavattieri MA, Frederico AM, Lima M et al (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechnol 13:1–9. https://doi.org/10.2225/vol13-issue1-fulltext-4

Capítulo I: In vitro regeneration of stable allotriploid plantlets of the "Híbrido de Timor" (C*offea*): a comparative study between liquid and semisolid systems.

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Abstract

"Híbrido de Timor" (HT) ‘CIFC 4106’ is a natural allotriploid formed from the spontaneous crossing between *Coffea arabica* and *Coffea canephora*. This allotriploid has been used to elucidate morphogenic in vitro responses and to generate new individuals for germplasm banks, owing to its resistance to coffee pathogens and its semifertile condition. However, seedlings have not been efficiently regenerated from somatic embryogenesis hitherto. Therefore, this study aimed to adapt a new indirect somatic embryogenesis procedure for HT ‘CIFC 4106’, and to evaluate the genetic stability of plantlets regenerated in vitro. Leaf explants were inoculated in semisolid and liquid media for friable calli induction. Subsequently, the friable calli of the two systems were transferred to semisolid or liquid media for somatic embryo regeneration. The highest somatic embryo regeneration rate was observed in the liquid system. Semisolid system and the liquid/semisolid combination did not regenerate the somatic embryo. All recovered plantlets showed stable allotriploid karyotype. Results of this work reinforce the relevance of adjusting in vitro conditions for indirect somatic embryogenesis establishment in order to provide plantlets of the different *Coffea* germplasms. Proposed indirect somatic embryogenesis protocol in liquid system enables the propagation of HT ‘CIFC 4106’ plantlets, overcoming the seminal propagation barriers.

Keywords: Coffee; In vitro propagation; Indirect somatic embryogenesis; Plant tissue culture; Ploidy.

Introduction

Indirect somatic embryogenesis (ISE) makes use of differentiated somatic cells to recover totipotency or pluripotency, originating calli and later somatic embryos (SE) (Williams & Maheswaran 1986). The ISE pathway has been used to investigate genetic, epigenetic, physiological and morphological events that occur during the stages from cell dedifferentiation until seedling regeneration (Quiroz-Figueroa et al. 2006). Genetic, epigenetic and physiological aspects of the explant donors as well as in vitro conditions can influence the ISE establishment, rendering it a complex morphogenic pathway (Fehér 2015; Sanglard et al. 2017). Thus, procedures have been described and continuously modified to establish ISE and to accelerate the induction and proliferation stages of embryogenic cells, as well as the regeneration of SE and seedlings for different taxa, including the genus *Coffea* (van Boxtel & Berthouly 1996; Samson et al. 2006; Loyola-Vargas & Ochoa-Alejo 2016).

ISE in *Coffea* have been described mainly for *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner, due to their economic relevance (Staritsky 1970; van Boxtel & Berthouly 1996; Santana et al. 2004; Almeida et al. 2008; Ibrahim et al. 2015). Other *Coffea* species have been propagated in vitro owing to their relevance as genetic resources for breeding programs, such as *Coffea liberica* Bull ex Hiern, *Coffea dewevrei* De Wild. et Th. Dur., *Coffea eugenioides* S. Moore, and *Coffea racemosa* Lour., as well as the "Híbrido de Timor" (HT – *Coffea arabica* L. x *Coffea canephora* Pierre ex Froehner). For all *Coffea*, young leaves serve as the main explants for ISE, as they are available throughout the year and contain proliferative cells in the region of vascular exchange (van Boxtel & Berthouly 1996; Molina et al. 2002; Almeida et al. 2008).

HT ‘CIFC 4106’ is a natural allotriploid with 1C = 2.10 pg and 2n = 3x = 33 chromosomes, formed from the crossing between *C. arabica* and *C. canephora* (Clarindo et al. 2013). HT ‘CIFC 4106’ accessions are used in *Coffea* breeding programs as genetic sources of resistance to economically important diseases and pests (Romero et al. 2014; Gichimu et al. 2014). The seminal propagation of this hybrid is restricted; therefore, the development of an in vitro vegetative propagation protocol is necessary to increase the regeneration of SE and plantlets (Sattler et al. 2016). During ISE establishment, HT ‘CIFC 4106’ shows rapid and uniform morphogenic response in the induction of friable calli at 15 days, with a mean of 72.4% responsive explants in

semisolid system (Sanglard et al. 2019). However, the regeneration rate of mature cotyledonary somatic embryos (MCSE) is relatively low, with a mean of 16.2% at 180 days, similar to *C. canephora* (14.3%) and lower than *C. arabica* (60.1%) and *C. eugenioides* (30.4%; Sanglard et al. 2019). Thus, it is necessary to develop new protocols and strategies to improve the regeneration of SE in HT 'CIFC 4106'.

In vitro responses are influenced by the chemical and physical conditions of the tissue culture medium, such as the type, concentration and combination of growth regulators, inorganic salts, vitamins, carbon and energy sources, organic compounds, pH, gelling agents and photoperiod (Staritsky 1970; van Boxtel & Berthouly 1996; Papanastasiou et al. 2008). Liquid systems allow for a faster morphogenic response compared to semisolid ones owing to the increased capacity of the cultured cells to assimilate the tissue culture media (Hammerschlag 1982; Jones & Petolino 1988; van Boxtel & Berthouly 1996; Feito et al. 2001; Papanastasiou et al. 2008). In *C. arabica* and *C. canephora*, cell aggregates suspended in liquid system show increased uptake of mineral elements and growth regulators, and consequently greater metabolic activities as well as cell proliferation and SE regeneration responses (van Boxtel & Berthouly 1996; Papanastasiou et al. 2008). Several advantages have led liquid systems to gradually replace semisolid ones: reduced culture medium preparation costs; avoidance of gelling agent impurities; and increased efficiency in the acclimatization of the seedlings to the ex vitro environment (Savio et al. 2012). However, a recurrent problem in ISE, especially in liquid system, is the occurrence of somaclonal variation in plantlets regenerated in vitro, promoted by genetic changes such as euploidy, aneuploidy and DNA sequence alterations (Muzamli et al. 2016).

Nevertheless, in *Coffea* the liquid system is only used for cell aggregate proliferation and SE regeneration (van Boxtel & Berthouly 1996). This system is generally consortium or dependent on the semisolid system during the initial stage of friable calli induction. To date, no studies regarding the impact of the liquid system on the establishment of ISE in the allotriploid HT 'CIFC 4106' have been performed. Thus, the present study proposed to establish and compare ISE in HT 'CIFC 4106' in liquid and semisolid systems, and to evaluate the ploidy level stability of plantlets regenerated in vitro using cytogenetic tools and flow cytometry.

Material and methods

Biological material

Leaves of HT 'CIFC 4106' were collected from plantlets maintained under in vitro conditions at the Laboratory of Cytogenetics and Plant Tissue Culture, Universidade Federal do Espírito Santo – ES, Brazil. The collected leaves were used as explants for ISE establishment.

Friable calli induction

Six leaf explants of 2 cm^2 were inoculated in semisolid (M1) and liquid (M2) media (Table I) for friable calli induction (van Boxtel & Berthouly 1996; Sanglard et al. 2019). The culture media were sterilized in autoclave at 121°C and 1.5 atm for 20 min and exchanged monthly. Petri dishes containing 15 mL of medium M1 were kept in the dark at 25 ± 2°C, and Erlenmeyer flasks of 120 mL containing 15 mL of medium M2 were shaken at 100 rpm in the dark at the same temperature. Friable calli formation was evaluated fortnightly and the cell mass was measured after 90 days, totalizing six subcultures (S1 to S6). The experimental design was completely randomized, with twelve replicates each for the semisolid and liquid media. Variables used for statistical comparison of the semisolid and liquid systems were the mean number of responsive explants evaluated over successive subcultures (S1 to S6), and the cell mass of friable calli measured at S6. The analysis of variance (ANOVA) and polynomial regression at 5% were performed with the software R (R Core Team 2016).

For SE regeneration, 0.2 g of friable calli from each system (M1 and M2) was randomly transferred to the semisolid (M3) or liquid (M4) media (Table I; van Boxtel & Berthouly 1996; Sanglard et al. 2019). The culture media were sterilized in autoclave at 121°C and 1.5 atm for 20 min and exchanged fortnightly. Friable calli from the semisolid induction medium (M1) transferred to M3 and M4 were respectively designated SM-SM (semisolid M1–semisolid M3) and SM-LM (semisolid M1–liquid M4); friable calli obtained from liquid induction media (M2) transferred to M3 and M4 were named LM-SM (liquid M2–semisolid M3) and LM-LM (liquid M2–liquid M4), respectively. Petri dishes containing 15 mL of M3 were maintained in the dark at 25 ± 2°C. Erlenmeyer flasks of 120 mL containing 15 mL of M4 were shaken at 100 rpm in the dark at 25 ± 2°C. MCSE were counted biweekly until 17 subcultures were completed (255 days), then transferred to seedling regeneration medium (M5, Table I). The culture medium was sterilized under the same conditions described above. Test

tubes with M5 were maintained at 24°C ± 2°C under a 16:8 h (light/dark) cycle with light irradiation of 36 μmol m^{-2} s^{-1} provided by two fluorescent lamps (20 W, Osram®). The strategies adopted for the establishment of ISE in HT 'CIFC' are shown in Figure 1. The experimental design was completely randomized. The mean number of MCSE was the analyzed variable to compare the culture systems (SM-SM, SM-LM, LM-SM and LM-LM) and subcultures (S1 to S17). This comparison was accomplished through descriptive analysis and polynomial regression at 5% using the software R (R Core Team 2016).

Table 1. Composition of the culture medium used to establish ISE from leaf explants of the true allotriploid HT 'CIFC 4106'.

Compounds	**Culture medium**				
	M1	**M2**	**M3**	**M4**	**M5**
MS	2.15 g L^{-1}	2.15 g L^{-1}	4.3 g L^{-1}	4.3 g L^{-1}	4.3 g L^{-1}
Gamborg's B5 vitamins	10 mL L^{-1}	10 mL L^{-1}	10 mL L^{-1}	10 mL L^{-1}	10 mL L^{-1}
Sucrose	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}
L-cysteine	0.08 g L^{-1}	0.08 g L^{-1}	0.04 g L^{-1}	0.04 g L^{-1}	-
Malt extract	0.4 g L^{-1}	0.4 g L^{-1}	0.8 g L^{-1}	0.8 g L^{-1}	-
Casein	0.1 g L^{-1}	0.1 g L^{-1}	0.2 g L^{-1}	0.2 g L^{-1}	-
2,4-D	9.06 μM	9.06 μM	-	-	-
BAP	4.44 μM	4.44 μM	4.44 μM	4.44 μM	-
GA_3	-	-	-	-	2.89 μM
Phytagel	2.8 g L^{-1}	-	2.8 g L^{-1}	-	2.8 g L^{-1}
Activated charcoal	-	-	4.0 g L^{-1}	4.0 g L^{-1}	-
pH	5.6	5.6	5.6	5.6	5.6

M1: semisolid callus induction medium; M2: liquid callus induction medium; M3: semisolid SE regeneration medium; M4: liquid SE regeneration medium; M5: seedling regeneration medium.

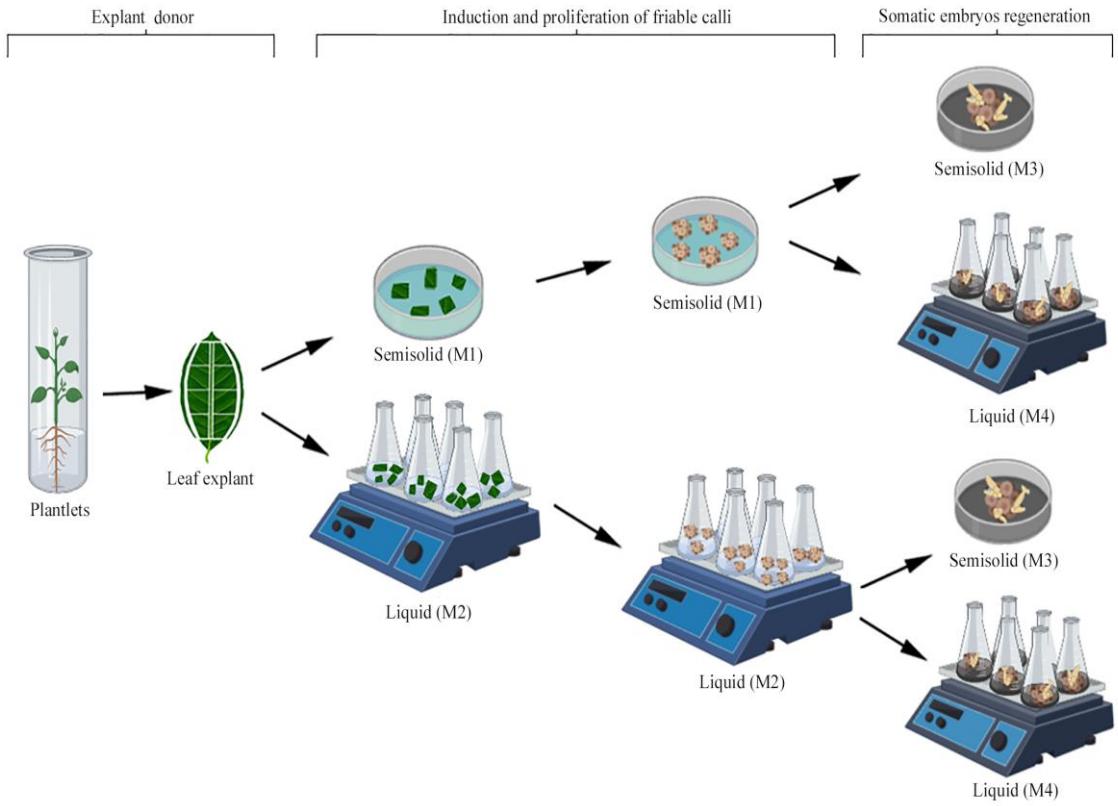


Fig. 1 – Scheme representing the strategies adopted to establish ISE in HT 'CIFC 4106'.

Chromosome number stability

The ploidy level of the regenerated plantlets was previously assessed by DNA ploidy level determination via flow cytometry. For this, nuclei suspensions were prepared from leaves of each plantlet and from the explant donor plant, according to the procedures described by Clarindo et al. (2013) and Sanglard et al. (2019). The nuclei suspensions were analyzed in a Partec PAS® cytometer (Partec® GmbH, Münster, Germany). Additionally, the chromosome number was determined from root meristems of the recovered plantlets. To achieve this, the roots were excised, individually washed in dH_2O for 15 min, treated with 3 µM amiprophos-methyl for 4 h at 30ºC, fixed in 3:1 methanol: acetic acid, and stored at -20ºC. After at least 12 h, the roots were washed in dH_2O, then macerated for 2 h at 36ºC in enzymatic pool (4% cellulase Sigma®, 0.4% hemicellulase Sigma®, 1% Macerozyme Onozuka R10 Yakult, 100% pectinase Sigma®) diluted in dH_2O in the proportion 1:8 (enzyme pool: dH_2O), washed in dH_2O, fixed in 3:1 methanol: acetic acid solution, and stored at -20ºC (Clarindo et al. 2013). Slides were prepared by cellular dissociation and air-drying techniques, followed by staining with 5% Giemsa. The metaphases were captured with a digital 12-bit CCD video

camera (Olympus®) coupled to a photomicroscope (Olympus™ BX-60) equipped with immersion objective of 100X/AN 1.4.

Results

The number of responsive explants, as determined by leaves exhibiting friable calli, was influenced by the semisolid (M1) and liquid (M2) media during the successive subcultures (Figs. 2 and 3). First responsive explants were observed at 15 days in the semisolid and liquid systems, with mean numbers of 5.75 and 1.42, respectively (Figs. 2 and 3). The mean number of responsive explants was higher in M1 than in M2 at all evaluated subcultures, presenting friable calli in all explants starting at S2 (Figs. 2 and 3). Differently, the highest mean number of responsive explants in liquid media was 4.5, seen at S5 (Figs. 2 and 3).

The cell mass of the friable calli gradually increased until S6 for both semisolid and liquid systems, but differed statistically between them. The mean value for cell mass in semisolid system was 1.21 g at 90 days (S6), compared to 0.25 g for the liquid medium. Moreover, the friable calli formed in the liquid system were detached from the leaf explant due to orbital agitation. All friable calli of both systems had yellowish appearance (Fig. 3).

The mean number of MCSE differed over time among the combinations SM-SM, LM-SM, SM-LM and LM-LM (Figs. 4 and 5). Globular, heart- and torpedo-shaped SE in HT ‘CIFC 4106’ calli were initially observed at 20 days in LM-LM. At 45 days (S3), SE were found at globular, heart, torpedo and cotyledonary stages, with a mean number of 1.29 MCSE (Figs. 4 and 5). The regeneration rate of SE in LM-LM gradually increased during the successive subcultures, with a mean number of 38.86 MCSE by 255 days (S17, Fig. 5). In turn, the first SE in SM-LM were observed at 150 days (S10), with a mean number of 0.36 MCSE (Fig. 4 and 5). The regeneration rate of SE in SM-LM increased gradually up to 255 days (S17), with a mean number of 4.64 MCSE (Figs. 4 and 5). Differently, no SE were recovered in the SM-SM and LM-SM systems (Figs. 4 and 5). Based on these results, the most suitable combination for SE regeneration of HT ‘CIFC 4106’ was LM-LM, which presented the highest mean number of MCSE in a shorter period of time (Figs. 4 and 5). Non-responsive calli in SM-SM and LM-SM were characterized as compact, exhibiting pale yellow color (Fig.

4). Contrarily, the responsive calli of the SM-LM and LM-LM were friable and exhibited dark coloration (Fig. 4).

The allotriploidy of the recovered plantlets was determined by DNA ploidy level, with all plantlets showing 1C = 2.10 pg, in agreement with the HT ‘CIFC 4106’ explant donor. In addition, the chromosome number of 2n = 3x = 33 chromosomes (Fig. 6) found for these plantlets confirmed the triploid condition of all regenerants.

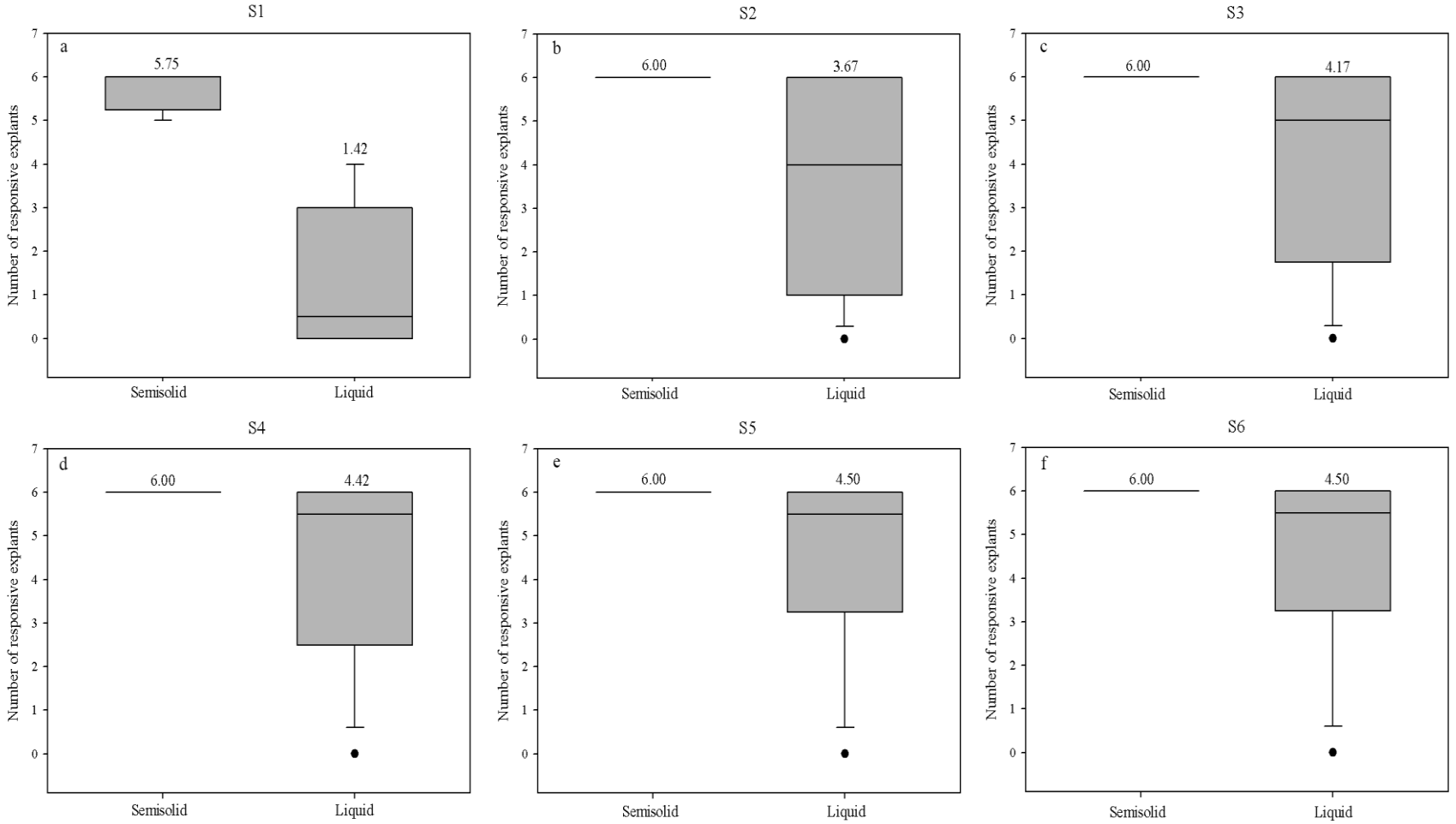
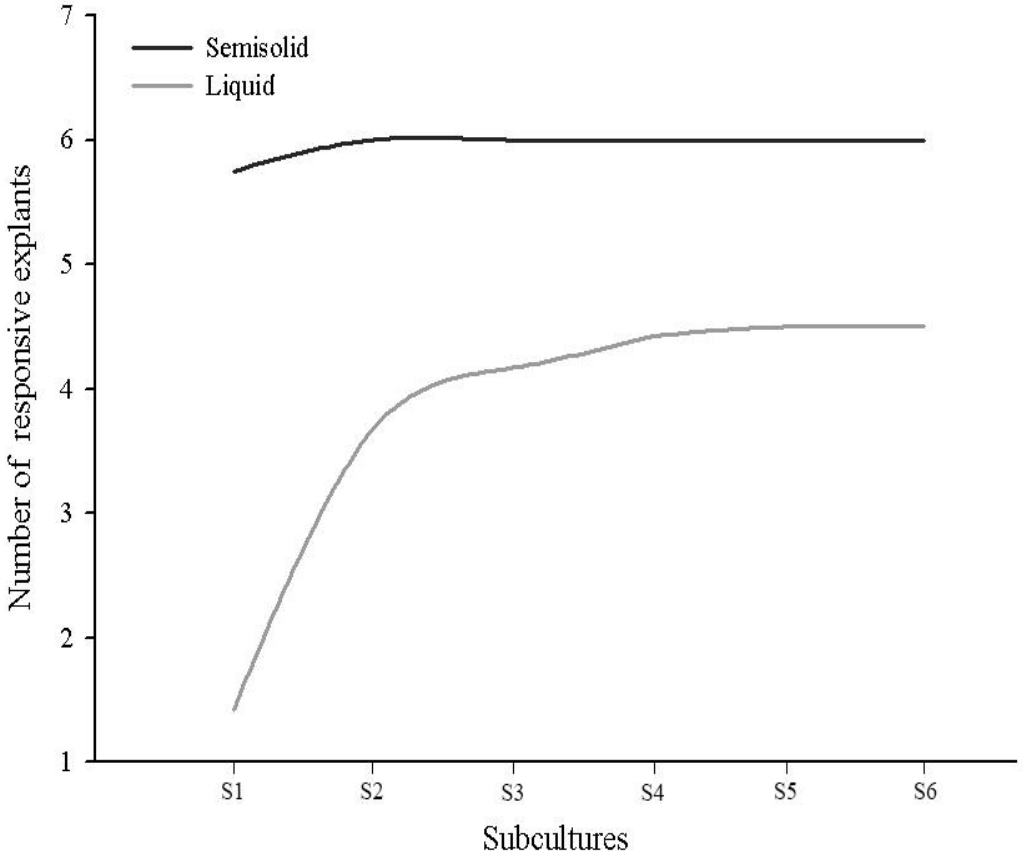
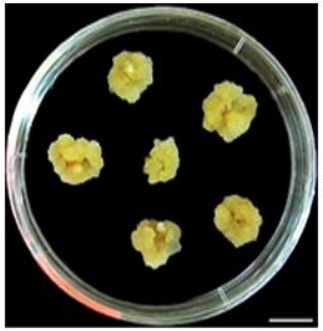
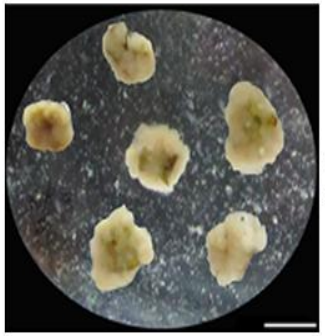


Fig. 2 – Mean number of responsive leaf explants of HT 'CIFC 4106' in semisolid and liquid media for callus induction, during the successive subcultures (S1 – S6). Box plots show that the mean number of responsive explants differed among the culture systems by the Tukey's test ($P < 0.05$). Semisolid system had the highest mean number of responsive explants compared to the liquid. Greatest variation in the number of responsive explants was observed in liquid system for all subcultures.





Callus induction in semisolid system.
Bar = 1 cm.



Callus induction in liquid system.
Bar = 0.5 cm.

Fig. 3 – Callus establishment of HT 'CIFC 4106' in M1 and M2. Graph, representing the dedifferentiation of the leaf explant cells, strutucred from the regression analysis using the mean number of responsive leaf explants in semisolid and liquid systems. Graph shows that the semisolid system had the highest mean number of responsive explants compared to the liquid system. Note that, from S2, the mean number of responsive leaf explants was maximal in semisolid system. Highest mean number of responsive leaf explants in liquid system was 4.50 from S5. The cell mass of the calli gradually increased to the S6 for both culture systems. Adjusted model was significant ($P < 0.05$) by the regression analysis for the liquid system: $Y = - 0.2307X^2 + 2.1336X - 0.1917$.

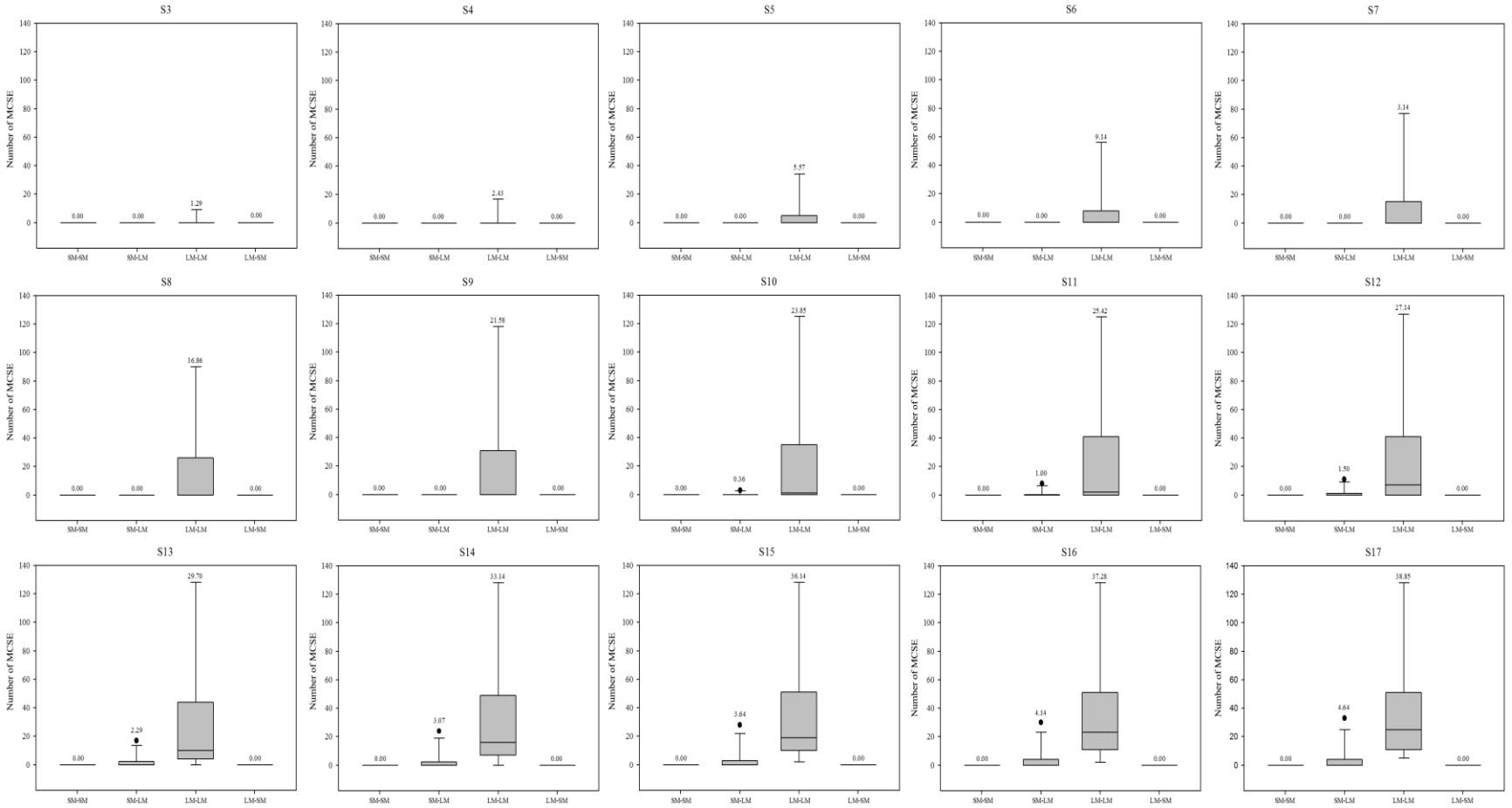


Fig. 4 – Mean number of MCSE (45 - 255 days) regenerated from HT 'CIFC 4106' calli. Box plots show that the mean number of MCSE differed between SM-SM, SM-LM, LM-LM and LM-SM between S3 and S17 (45 and 255 days). The highest mean number MCSE was observed in LM-LM with 38.85 MCSE in S17 (255 days). Although SM-LM also regenerated SE, this combination took longer to be established in vitro and had a lower mean number MCSE. The combination SM-SM and LM-SM did not regenerate SE. Means followed by different letters differ significantly by Tukey's test ($P < 0.05$).

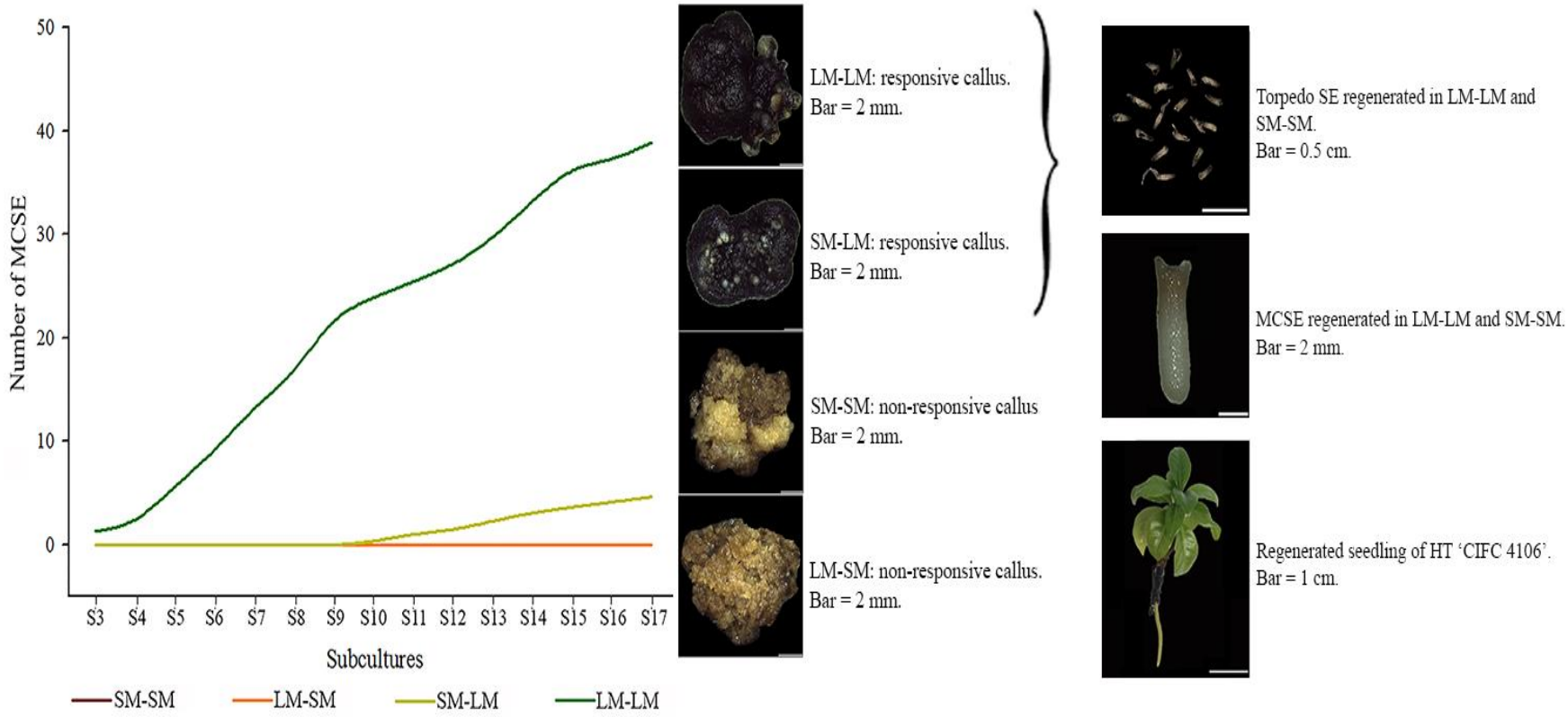


Fig. 5 – Distinct in vitro responses HT 'CIFC 4106' calli obtained from SM-SM, SM-LM, LM-LM and LM-SM. The graph evidences the higher mean number of MCSE for the calli cultivated in the LM-LM, during all the evaluated subcultures (S3 - S17). Calli cultured in the SM-LM also presented embryogenic responses, however the regeneration rate of MCSE was reduced. Calli cultured in SM-SM and LM-SM no showed embryogenic responses. It is observed the acquisition of competence and cellular determination, tissue differentiation and SE regeneration in FC of HT 'CIFC 4106' cultivated in LM-LM and SM-LM. The adjusted model was significant ($P < 0.05$) by the regression analysis for the LM-LM system: $Y = - 0.0003X^2 + 0.279X - 12.43$.

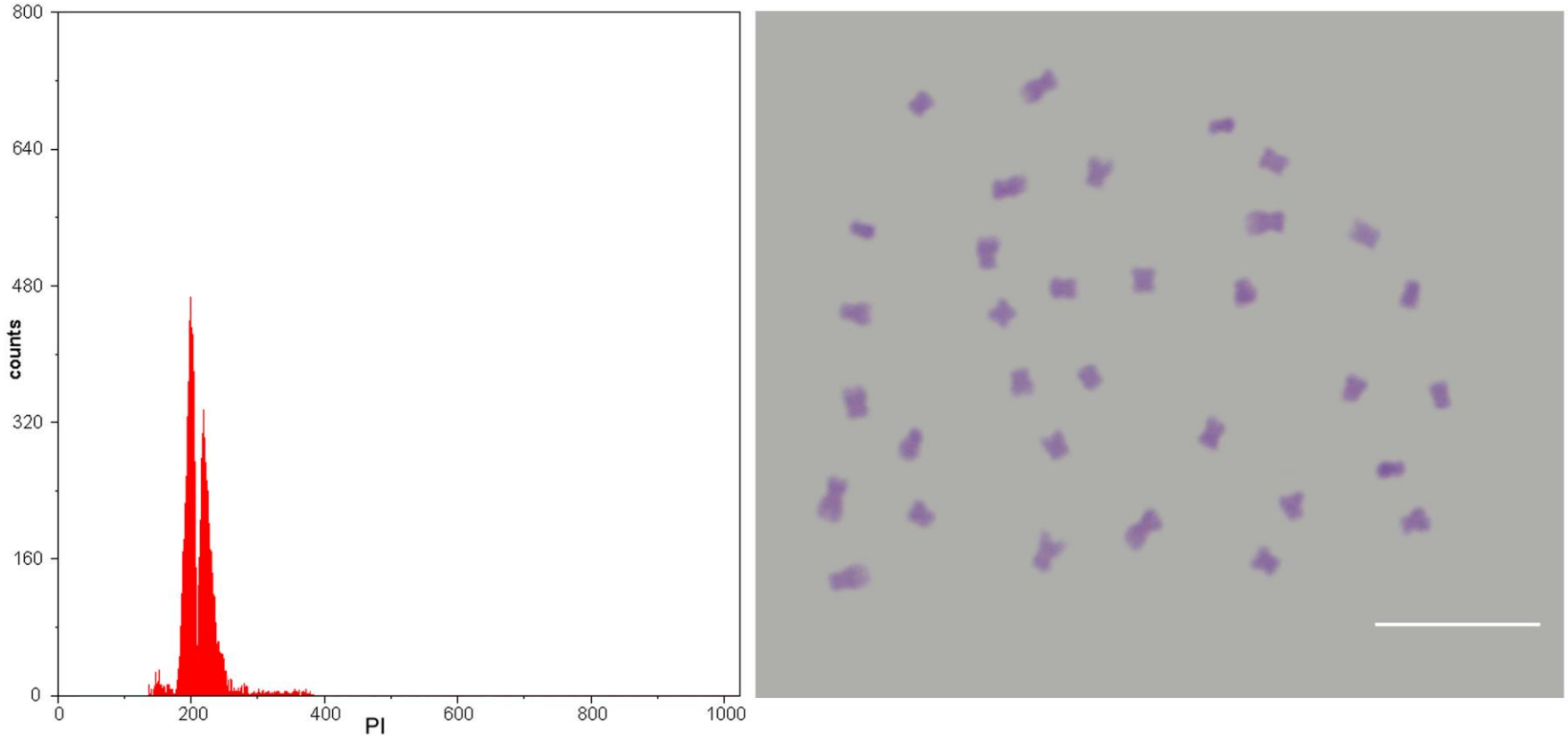


Fig. 6 – Ploidy confirmation and chromosome number in HT 'CIFC 4106' plantlets regenerated in vitro in LM-LM and SM-LM system. Representative histogram shows the G_0/G_1 peaks of the intact nuclei of *Solanum lycopersicum L.* in channel 200 and HT 'CIFC 4106' in channel 210. Karyotype with 2n = 3x = 33 HT 'CIFC 4106' chromosomes, obtained from roots of plantlets in vitro, confirming the maintenance of the number of chromosomes. Bar = 5 µm.

Discussion

In this work, the liquid system proved to be a viable, reproducible and efficient alternative for the establishment of the complete ISE pathway in HT 'CIFC 4106'. This result reinforces the importance of in vitro conditions for ISE in *Coffea*. In addition, the present study demonstrated a protocol for the relatively large-scale propagation of HT 'CIFC 4106', regenerating a mean number of 38.85 MCSE per friable callus. This mean value is higher than previous data for HT 'CIFC 4106': 1.51 MCSE per leaf explant via direct somatic embryogenesis (Sattler et al. 2016) and 6.06 MCSE per friable callus via ISE (Sanglard et al. 2019), both conducted in semisolid system.

The in vitro morphogenic response varied significantly between the liquid and semisolid systems. Moreover, the calli, presenting pale yellow and friable appearance, were formed in the relatively short time of two months in both culture systems; this result was already expected, as the time of callogenesis establishment in *Coffea* has been reported to vary from two months (for *C. canephora*, *C. arabica* and HT 'CIFC 4106'; van Boxtel & Berthouly 1996; Samson et al. 2006; Sanglard et al. 2017, 2019) to six months (for *C. canephora* and *C. eugenioides*; Sanglard et al. 2019). In addition, the cell mass of the friable calli increased over time, particularly in the semisolid system, evincing cell proliferation. This result corroborates previous studies evaluating friable calli multiplication in *C. arabica* which observed the highest induction of friable calli in semisolid system (Albarran et al. 2005; Rezende et al. 2012). In contrast, Teixeira et al. (2004) found no significant difference in the multiplication of friable calli of *C. arabica* in liquid and semisolid systems.

The SM-SM, SM-LM, LM-LM and LM-SM combinations influenced the SE regeneration across successive subcultures. Both LM-LM and SM-LM exhibited embryogenic responses from friable calli of HT 'CIFC 4106'. However, the highest short-term SE regeneration was observed in LM-LM, where friable calli originated and remained in liquid system. It is likely that the LM-LM system promoted an increased assimilation capacity due to the greater accessibility of the cultured cells to the compounds of the tissue culture medium (Hammerschlag 1982; Jones and Petolino 1988; Ziv 1995; van Boxtel & Berthouly 1996; Made et al. 2001; Papanastasiou et al. 2008). For instance, cytokinins are more effective in liquid system because they are not conjugated to the gelling agent, which results in more pronounced SE regeneration (Papanastasiou et al. 2008). In addition, the osmotic potential may also have influenced

the in vitro response in HT ‘CIFC 4106’, since the liquid system had lower osmotic potential compared to the semisolid system and regenerated the highest mean number of MCSE. According to Jeannin et al. (1995), osmotic pressure below 400 mOsm kg^{-1} H_2O is associated with organogenesis, while osmotic pressure above this threshold is associated with somatic embryogenesis. However, no difference was observed in the nature of the morphogenic pathway between culture systems in the present study, since all in vitro plantlets were regenerated via ISE.

SM-SM and LM-SM combinations were not effective for ISE establishment in HT ‘CIFC 4106’, as indicated by the lack of embryogenic response. This can be a consequence of reduced contact area of the friable calli with the culture medium. In addition, the semisolid condition promoted by gelling agents probably reduces the assimilation capacity (Gatica-Arias et al. 2007; Papanastasiou et al. 2008). Nutrient availability in the semisolid system is limited by the osmotic potential of the culture media (Papanastasiou et al. 2008). The low rate and/or non-response of SE regeneration in semisolid system was also reported by Sanglard et al. (2019) when comparing in vitro responses of *Coffea* among the diploids *C. canephora* and *C. eugenioides*, the allotriploid HT ‘CIFC 4106’ and the true allotetraploid *C. arabica*.

The 2C nuclear DNA level of plantlets regenerated in vitro via ISE in LM-LM and SM-LM was identical to that of the explant donor plant. Cytogenetic analysis confirmed that these plantlets remained allotriploid, showing the same number of 2n = 3x = 33 chromosomes ascertained in the explant donor plants. Therefore, the strategies for regeneration of HT ‘CIFC 4106’ SE in liquid system were reproducible, efficient and safe, since the 2C nuclear DNA content and ploidy level remained unchanged and preserved. This result is in agreement with Sattler et al. (2016), who evaluated ploidy level stability in HT ‘CIFC 4106’ plantlets regenerated in vitro via direct somatic embryogenesis and showed that allotriploidy was conserved in all regenerated plantlets. According to Ducos et al. (2003), no somaclonal variants were detected in *C. canephora* trees derived from the production of SE in liquid media, showing that in vitro propagation is safe and maintains genetic fidelity.

Conclusions

The present approach reported, for the first time, an ISE procedure in liquid system suitable for mass propagation of HT ‘CIFC 4106’. This hybrid was chosen in

view of the difficulties related to the seminal propagation of a triploid hybrid, and owing to the relevance of this allotriploid for *Coffea* breeding programs. Based on the present results, this study represents a breakthrough in *Coffea* tissue culture, as plantlets regenerated in vitro will be introduced into the field for maintenance and conservation of the germplasm bank. All plantlets recovered in vitro exhibited stable allotriploid karyotype, with preserved chromosomal number and nuclear DNA content. Moreover, the results of this work emphasize the adjustment of in vitro conditions for the establishment of ISE in *Coffea*.

Acknowledgements

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References

Albarran J, Bertrand B, Lartaud M, Etienne H (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. Plant Cell, Tiss and Organ Cult 81:27–36. https://doi.org/10.1007/s11240-004-2618-8

Almeida JAS, Silvarolla MB, Fazuoli LC, Stancato GC (2008) Embriogênese somática em genótipos de *Coffea arabica* L. Coffee Science 3:143–151.

Clarindo WR, Carvalho CR, Caixeta ET, Koehler AD (2013) Following the track of "Híbrido de Timor" origin by cytogenetic and flow cytometry approaches. Genet Resour Crop Evol 60:2253–2259. https://doi.org/10.1007/s10722-013-9990-3

Ducos JP, Alenton R, Reano JF, Kanchanomai C, Kanchanomai A, Pétiard V (2003) Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. Euphytica 131:215–223.

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

Feito I, Gonzalez A, Centeno ML, Fernandez B, Rodriguez A (2001) Transport and distribution of benzyladenine in *Actinidia deliciosa* explants cultured in liquid and solid media. Plant Physiol Biochem 39:909–916. https://doi.org/10.1016/S0981-9428(01)01309-2

Gatica-Arias AM, Arrieta-Espinoza G, Esquivel AME (2007) Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuai. Electronic Journal of Biotechnology 11:1–12. https://doi.org/10.2225/vol11-issue1-fulltext-9

Gichimu BM, Gicheru EK, Mamati GE, Nyende AB (2014) Variation and association of cup quality attributes and resistance to Coffee Berry Disease in *Coffea arabica* L. composite cultivar, Ruiru 11. Afr J Hortic Sci 7:22–35.

Hammerschlag F (1982) Factors affecting establishment and growth of peach shoots in vitro. HortScience 17:85–86.

Ibrahim MSD, Hartati RRS, Rubiyo R, Purwito A, Sudarsono S (2015) The induction of primary and secondary somatic embryo to support arabica coffee propagation. Journal of Tropical Crop Science 2:1–13. https://doi.org/10.29244/jtcs.2.3.6-13

Jeannin G, Bronner R, Hahne G (1995) Somatic embryogenesis and organogenesis induced on the immature zygotic embryo of sunflower (*Helianthus annuus* L.) cultivated in vitro: role of the sugar. Plant Cell Reports 15:200–204.

Jones AM, Petolino JF (1988) Effects of support médium on embryo and plant production from cultured anthers of soft-red winter wheat (*Triticum aestivum* L.). Plant Cell Tiss Organ Cult 12:253–261. https://doi.org/10.1007/BF00034366

Loyola-Vargas VM, Ochoa-Alejo N (2016) “Somatic embryogenesis. An overview,” in somatic embryogenesis: fundamental aspects and applications. Springer, New York, 1:1–10, 2016. https://doi.org/10.1007/978-3-319-33705-0

Molina DM, Aponte ME, Cortina H, Moreno G (2002) The effect of genotype and explant age on somatic embryogenesis of coffee. Plant Cell Tiss Org Cult 71:117–123. https://doi.org/10.1023/A:1019965621041

Muzamli A, Mujib A, Tonk D, Zafar N (2017) Plant regeneration through somatic embryogenesis and genome size analysis of *Coriandrum sativum* L. Protoplasma 254:343–352. https://doi.org/10.1007/s00709-016-0954-2

Otto FJ (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewiez Z, Crissman HA, Robinson JP (eds) Methods in cell biology, vol. 33. Academic Press, San Diego, pp 105–110.

Papanastasiou I, Soukouli K, Moschopoulou G, Kahia J, Kintzios S (2008) Effect of liquid pulses with 6-benzyladenine on the induction of somatic embryogenesis from coffee (*Coffea arabica* L.) callus cultures. Plant Cell Tiss Organ Cult 92:215–225. https://doi.org/10.1007/s11240-007-9326-0

Quiroz-Figueroa F, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tiss Organ Cult 86:285–301. https://doi.org/10.1007/s11240-006-9139-6

Rezende JC, Carvalho CHS, Ramia ACS, Pasqual M, Teixeira JB (2012) Multiplication of embryogenic calli in *Coffea arabica* L. Acta Scientiaru 34:93–98. https://doi.org/10.4025/actasciagron.v34i1.11230

Romero G, Vásquez LM, Lashermes P, Herrera JC (2014) Identification of a major QTL for adult plant resistance to coffee leaf rust (*Hemileia vastatrix*) in the natural Timor hybrid (*Coffea arabica* x *C. canephora*). Plant Breeding 133:121–129. https://doi.org/10.1111/pbr.12127

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 Tiss Organ 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 RC, Clarindo WR (2019) Indirect somatic embryogenesis in *Coffea* with different ploidy levels: a revisiting and updating study. Plant Cell Tiss and Organ Cult 136:255–267. https://doi.org/10.1007/s11240-018-1511-9

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Nunes ACP, Soares TCB, Carvalho CR, Clarindo WR (2017) From chromosome doubling to DNA sequence changes: outcomes of an improved in vitro procedure developed for allotriploid "Híbrido de Timor" (*Coffea arabica* L. x *Coffea canephora* Pierre ex A. Froehner). Plant Cell Tiss Organ Cult 131:223–231. https://doi.org/10.1007/s11240-017-1278-4

Santana N, González ME, Valcárcel M, Canto-Flick A, Hernandez MM, Fuentes-Cerda CF J, Barahona F, Mijangos-Cortés J, Loyola-Vargas VM (2004) Somatic embryogenesis: a valuable alternative for propagating selected robusta coffee (*Coffea canephora*) clones. In vitro Cell Dev Bio-Plant 40:95–101. https://doi.org/10.1079/IVP2003486

Sattler MC, Carvalho CR, Clarindo WR (2016) Regeneration of allotriploid *Coffea* plants from tissue culture: resolving the propagation problems promoted by irregular meiosis. Cytologia 81:125–132. https://doi.org/10.1508/cytologia.81.125

Savio LEB, Astarita LV, Santarém ER (2012) Secondary metabolism in micropropagated *Hypericum perforatum* L. grown in non-aerated liquid medium. Plant Cell Tiss Organ Cult 108:465–472. https://doi.org/10.1007/s11240-011-0058-9

Staritsky G (1970) Embryoid formation in callus tissues of coffee. Plant Biol 19:509–514. https://doi.org/10.1111/j.1438-8677.1970.tb00677.x

Teixeira JB, Junqueira CS, Pereira AJPC, Mello RIS, Silva APD, Mundim DA (2004) Multiplicação clonal de café (*Coffea arabica* L.) via embryogenesis somática. Brasília: Embrapa Recursos Genéticos e Biotecnologia, documentos, 121.

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

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57:443–462. https://doi.org/10.193/oxfordjournals.aob.a087127

Ziv M (1995) The control of bioreactor environment for plant propagation in liquid culture. Acta Horticulturae 393:25–38. https://doi.org/10.17660/ActaHortic.1995.393.3

Capítulo II: Ploidy level, epigenetic and in vitro environment influence the indirect somatic embryogenesis of the new synthetic autoallohexaploid *Coffea*.

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Abstract

Autoallohexaploid plantlets of “Híbrido de Timor” (HT, *Coffea*) were recently regenerated by chromosome set doubling of the allotriploid HT. Besides the in vitro propagation and chromosome doubling set, indirect somatic embryogenesis allows us to investigate the short-term consequences of the polyploidy. Here, we aim to establish and compare the indirect somatic embryogenesis in natural allotriploid and syntehtyic autoallohexaploid HT, evaluating the role of the in vitro environment, ploidy level and global level of 5-methylcytosine on somatic embryo regeneration and karyotype stability. Our results show that the autoallohexaploid needed more time for induction and proliferation of friable calli, showing lower numbers of responsive explants and a higher level of 5-methylcytosine. The autoallohexaploid also showed a higher 5-methylcytosine level during the somatic embryo regeneration, but both hybrids exhibited the same mean value of mature cotyledonary somatic embryos for friable callus. Regarding the in vitro environment, the activated charcoal at 8 and 16 g L^{-1} increased global level of 5-methylcytosine and the mean number of abnormal somatic embryo in autoallohexaploid. Therefore, 5-methylcytosine variations in autoallohexaploid HT calli correspond to an adaptive response to in vitro conditions. Based on these results, the autoalohexaploid exhibited a lower in vitro response compared to its allotriploid ancestor, despite having at least twice as many copies of in vitro response genes. Therefore, our data show that the in vitro environment, ploidy level and global 5-methylcytosine level influence the *Coffea* indirect somatic embryogenesis, evidencing the outcome of the short-term induced polyploidy.

Keywords: Coffee; Epigenetic; In vitro propagation; Plant tiussue culture; Polyploidy.

Introduction

Polyploidy is a natural and common event in angiosperms that plays an important role in the origin and evolution of wild and cultivated plants (Yang et al. 2010; Sattler et al. 2016a). Since the discovery of antitubulin compounds, mainly colchicine, induced polyploidy has been widely applied to understand the short-, middle- and long-term effects of the polyploidization on the “omics” (Wang and Bodovitz 2010; Soltis et al. 2016; Alix et al. 2017; Münzbergová 2017). The newly formed polyploids undergo a rapid restructuring of the genome, epigenome, transcriptome, metabolome and other "-omes", which results in morphological, physiological and reproductive differences in relation to their ancestors (Otto 2007; Yang et al. 2010; Sattler et al. 2016a). Natural and synthetic chromosome set doubling is often explored in plant breeding programs, since polyploidy may be related to an increase of the hybrid´s vigor, to adaptations to new environmental conditions and to changes in vegetative and/or reproductive organs (“Gigas effect”), resulting in higher rates of production compared to their ancestors (Hancock and Overton 1960; Van de Peer et al. 2009; Boller et al. 2012; Renny-Byfield and Wendel 2014; Sattler et al. 2016a). However, natural or synthetic polyploids are not always more productive and vigorous than their progenitors, since the modifications induced by polyploidization in the genome, epigenome, transcriptome and other "-omes" can also be neutral or even deleterious (Renny-Byfield and Wendel 2014).

Recently, our research group induced the hexaploidy (2C = 4.20 pg and 2n = 6x = 66 chromosomes) from the allotriploid “Híbrido de Timor” 'CIFC 4106' (HT, 1C = 2.10 pg and 2n = 3x = 33 chromosomes, Clarindo et al. 2013) through the treatment of friable calli with colchicine (Sanglard et al. 2017). The allotriploid HT originated from the natural interspecific crossing between *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner. The first individuals were found about 100 years ago on the Timor Island (Bettencourt 1973; Capucho et al. 2009; Setotaw et al. 2010). This hybrid has been used in plant tissue culture to provide information on differences in morphogenetic responses in vitro between *Coffea* species with distinct ploidy level (Sanglard et al. 2019). As reported by the authors, the origin and time of formation (evolutionary history) of diploid *Coffea* (*C. canephora* and *Coffea eugenioides* S. Moore) and polyploids (allotetraploid *C. arabica* and allotriploid HT) probably influence the in vitro response due to the extensive genetic and epigenetic changes that have already occurred

or are occurring in the genome through changes from chromosome rearrangements, DNA sequence and epigenome. Thus, the in vitro establishment of the autoallohexaploid HT is necessary to provide relevant information on the behavior of synthetic polyploids in different environments in vitro and to investigate karyotype stability of regenerated plantlets via indirect somatic embryogenesis (ISE). In addition, it will increase the number of individuals and make plant material available for comparative genomic and phenotypic studies in an attempt to understand the effects of chromosome set doubling.

In vitro morphogenic responses are influenced by the in vitro conditions (van Boxtel and Berthouly 1996) and by genetic, epigenetic and physiological characteristics of the explant donors. Epigenetic variations are promoted by chemical changes in the DNA and chromatin status. In in vitro cultivated plant cells, the epigenetic changes correspond to an adaptive response to tissue culture conditions, in which (López et al. 2010; Wang et al. 2012; Wang and Wang 2012; Nic-Can et al. 2015). According to Fehér (2015), cytosine methylation is involved in the ISE pathway during the stages of dedifferentiation and cellular redifferentiation, since the gene expression can be altered by DNA methylation in the locus-specific modulation, such as *wusche* (*wus*), *agamous-like 15* (*agl15*), *somatic embryogenesis receptor kinase* (*serk*), *baby boom 1* (*bbm1*), *leafy cotyledon 1 and 2* (*lec1 and lec2*). In *C. canephora* cicatricial calli obtained by direct somatic embryogenesis, cytosine hypermethylation has been associated with the increase, conversion and maturation of somatic embryos (SE) (Nic-Can et al. 2013). Considering that the increase in the chromosomal number leads to epigenomic changes (Soltis et al. 2016; Alix et al. 2017), the quantification of the global levels of DNA methylation should be performed to investigate and compare the autoallohexaploid HT relative to its allotriploid HT ancestor during the stages of callus induction and proliferation and SE regeneration.

According to Sanglard et al. (2019), the in vitro conditions should be adjusted for the allotriploid HT in order to increase the rate of SE regeneration, suggesting that higher concentrations of activated charcoal should be tested. Activated charcoal provides faster embryogenic response and, consequently, increases the number of SE (Pan e van Staden 1998; Thomas 2008). This effect occurs because the activated charcoal has a surface area with well-distributed pores capable of adsorbing undesirable substances from the tissue culture medium, such as phenolic compounds and exogenous residues of plant growth regulators (Johansson et al. 1982; Pan e van Staden 1998;

Thomas 2008). In addition, activated charcoal promotes the inactivation of polyphenoloxidase and peroxidase, thus increasing the survival rate of regenerated plantlets (Thomas 2008).

The aims of this study were to: (a) establish the ISE in the natural allotriploid and the synthetic autoallohexaploid HT, (b) verify and compare whether the ploidy level and the global level of methylated cytosine influence the in vitro response, (c) evaluate the effect of the activated charcoal on SE regeneration in the synthetic hexaploid HT, and (d) evaluate the karyotype stability of in vitro regenerated plantlets in the natural allotriploid and the synthetic hexaploid HT via ISE. It is noteworthy that the allotriploid HT represented an experimental control to compare the ISE morphogenic response with the synthetic autoallohexaploid HT, and to evidence and undestand the influence of the ploidy level in the in vitro responses.

Material and methods

Biological material

Leaves of the allotriploid HT 'CIFC 4106' (1C = 2.10 pg and 2n = 3x = 33 chromosomes) and the synthetic autoallohexaploid HT 'CIFC 4106' (2C = 4.20 pg and 2n = 6x = 66 chromosomes) were collected from plantlets maintained in in vitro conditions. These plantlets had been maintained in a growth room under a light/dark regime of 16/8 h with 36 μmol m^{-2} s^{-1} of light radiation and at 25 ± 2°C. The culture medium consisted of 4.3 g L^{-1} basal MS salts (Murashige and Skoog 1962), 10 mL L^{-1} B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel and pH = 5.6 (Sanglard et al. 2019). Collected leaves were used as source of explant for the ISE establishment, nuclear DNA content measurement and DNA ploidy level determination.

ISE establishment

For callus induction, the tissue culture medium consisted of 2.15 g L^{-1} ½MS basal salts (Murashige and Skoog 1962), 10 mL L^{-1} B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, 0.08 g L^{-1} L-cysteine, 0.4 g L^{-1} malt extract, 0.1 g L^{-1} hydrolised casein, 9.06 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μM 6-benzylaminopurine (BAP) and 2.8 g L^{-1} Phytagel, pH = 5.6 (van Boxtel and Berthouly 1996; Sanglard et al.

2019). Five leaf explants of the allotriploid or the autoallohexaploid HT were inoculated in each Petri dish, totaling 15 Petri dishes for each HT. After each three months, the explants were transfered, with or without callus, to a new callus induction medium. Petri dishes were kept in the dark at 25 ± 2°C. Callus formation was evaluated biweekly until 150 days. The analysis of variance (ANOVA) was performed and the mean values were compared by the Tukey's test ($P < 0.05$) to compare the mean number of responsive explants of the allotriploid and the autoallohexaploid until 150 days. Then, a regression analysis ($P < 0.05$) was applied using the R software (R Core Team 2016).

Friable calli were transferred to the SE regeneration medium, which consisted of 4.3 g L^{-1} basal MS salts (Murashige and Skoog 1962), 10 mL L^{-1} B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, 0.04 g L^{-1} L-cysteine, 0.8 g L^{-1} malt extract, 0.2 g L^{-1} hydrolised casein, 4.44 μM BAP, 2.8 g L^{-1} Phytagel, and 2 g L^{-1} activated charcoal, pH = 5.6 (Sanglard et al. 2019). One callus was inoculated in each Petri dish and kept in the dark at 25 ± 2°C, totaling 112 dishes for the allotriploid and for the autoallohexaploid. After each three months, the calli were transfered to a new SE regeneration medium. Mean number of normal mature cotyledonary somatic embryos (MCSE) and abnormal SE were evaluated monthly until 330 days. Using the software R (R Core Team 2016), in vitro SE regeneration between allotriploid and autoallohexaploid was described and compared with regard to the number of normal MCSE and abnormal SE. From the results, calli of the synthetic autoallohexaploid HT were also inoculated in the SE regeneration medium supplemented with 4, 8 or 16 g L^{-1} activated charcoal. The Petri dishes were maintained under the same in vitro conditions, totaling 28 dishes for each concentration of activated charcoal. The number of recovered MCSE from these media was also compared using the software R (R Core Team 2016).

Normal MCSE were transferred to the seedling regeneration medium that consisted of 4.3 g L^{-1} basal MS salts (Murashige and Skoog 1962), 10 mL L^{-1} B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel and pH = 5.6 (Sanglard et al. 2019). In each test tube, one MCSE was inoculated. Subsequently, the test tubes were maintained in a growth room under a light/dark regime of 16/8 h with 36 μmol m^{-2} s^{-1} of light radiation supplied by two fluorescent lamps (20 W, Osram®), at 25 ± 2°C.

Global level of 5-methylcytosine (5-mC%)

Genomic DNA was extracted separately from samples of the friable calli of the allotriploid and the autoallohexaploid HT collected after 150 days in the callus induction medium and after 330 days in the SE regeneration medium. Genomic DNA extraction was performed as described by Doyle and Doyle (1990). DNA concentration, quality and purity were estimated using the NanoDrop equipment (ThermoScientific® 2000c). Subsequently, the quantification of genomic DNA was adjusted to an amount of 30 μg of DNA in 100 μL of autoclaved water type 1. Acid hydrolysis of the genomic DNA was accomplished according to the methodology proposed by Chen et al. (2013). Analysis by High Performance Liquid Chromatography (HPLC) was performed using Prominence HPLC equipment (Shimadzu, Japan). The level of 5-mC% present in the genomic DNA samples was expressed as a percentage of the cytosine level, calculated by the equation proposed by Chen et al. (2013). In order to compare the mean 5-mC%, the data were submitted to analysis of variance (ANOVA) and the mean values were compared by the Tukey test ($P < 0.05$) using the R software (Core Team 2016).

Autoallohexaploidy confirmation

To determine the nuclear 2C value and DNA ploidy level of the SE and plantlets of the regenerated allotriploid and hexaploid HT, a flow cytometry procedure with external and pseudo-internal standardization was applied. The SE and the leaves of the plantlets were separately placed in distilled water and cut into 2 cm^2 fragments. These materials were chopped (Galbraith et al. 1983) in nuclei extraction buffer (Otto 1990), and the nuclear suspensions were filtered and stained with propidium iodide (Clarindo et al. 2013). After 20 min in the dark, the nuclear suspensions were analyzed with a Partec-PAS® flow cytometer (Partec® Gmbh, Munster, Germany) and the DNA ploidy level was determined. Next, a joint analysis with the pseudo-internal standard allotriploid and the autoallohexaploid was performed to determine the nuclear 2C value. G_0/G_1 nuclei peak of the allotriploid HT was the reference for nuclear 2C value measurement of the autoallohexaploid.

To determine the 2n chromosome number, metaphases were obtained from in vitro root meristems of the allotriploid and the autoallohexaploid HT plantlets. The root meristems were treated with 3 μM amiphrophos-methyl, washed in dH_2O, fixed and enzymatically macerated. The slides were then prepared with cell dissociation and air-drying techniques. Afterwards, the slides were placed on a hot plate at 50°C for 5 min,

stained with 5% Giemsa (Merck®) for 5 min, rinsed with dH_2O and air-dried (Clarindo et al. 2013). The images of the chromosomes were captured using a 100x objective and a CCD camera (Nikon EvolutionTM) accopled to a Nikon 80i microscope (Nikon, Japan)**.**

Results

ISE establishment

The allotriploid and the autoallohexaploid HT exhibited distinct mean numbers of responsive explants (leaf fragment with callus) over time (Fig. 1). The first allotriploid HT responsive explants were observed at 15 days in the callus induction medium, with a mean number of 4.00 responsive explants (Fig. 1). Differently, the first leaf responsive explants of the autoallohexaploid HT were observed at 90 days, presenting a mean number of 0.33 (Fig. 1). Progressively during this in vitro step, the mean number of responsive explants was higher in allotriploid HT (Fig. 2). Callus formation increased over time for both allopolyploids until the 45^{th} day of culture for the allotriploid and until the 135^{th} day for the autoallohexaploid, at a mean final number of 5.00 and 2.53 responsive explants, respectively (Fig. 2). Callus mass for both allopolyploids gradually increased up to 150 days in the induction medium. Besides, all calli showed a pale-yellow and friable appearance (Fig. 2).

In SE regeneration medium, the MCSE number at 330 days was identical for the calli of the allotriploid and the autoallohexaploid HT maintained in 2 g L^{-1} activated charcoal. However, the time for MCSE regeneration was distinct between the allotriploid and the autoallohexaploid calli. First MCSE were observed in allotriploid HT calli at 60 days, exhibiting a mean number of 0.10 MCSE per callus. For the autoallohexaploid HT calli, the first regenerated MCSE were observed at 270 days, presenting a mean number of 0.34 MCSE per callus (Fig. 3). Remarkably, the MCSE number was influenced by the activated charcoal for the autoallohexaploid HT. MCSE regeneration increased over time, presenting a mean number of 1.39, 0.30, 1.22 and 0.60 MCSE per callus at the 330 days for culture medium supplemented with 2, 4, 8 or 16 g L^{-1} activated charcoal, respectively (Fig. 3).

Over time, abnormal SE were also regenerated from the autoallohexaploid HT calli maintained at different activated charcoal concentrations. These abnormal SE were

identified due to their abnormalities in the apical-basal pattern, such as deformation and absence of the root apical meristem, hypocotyl, shoot apical meristem, and/or cotyledons. The first abnormal SE were observed at 120 days in the autoallohexaploid HT calli in regeneration medium supplemented with 8 and 16 g L^{-1} activated charcoal, with a mean of 0.45 abnormal SE per callus (Fig. 4). In SE regeneration medium supplemented with 2 and 4 g L^{-1} activated charcoal, the first abnormal SE were observed at 270 days with a mean number of 0.10 and 0.45 per callus, respectively (Fig. 4). Abnormal SE regeneration from the autoallohexaploid HT increased over time, with a mean number of 0.89, 1.00, 4.00 and 2.81 abnormal SE per callus at 330 days in culture medium supplemented with 2, 4, 8 and 16 g L^{-1} of activated charcoal, respectively (Fig. 4). It is noteworthy that no regeneration of abnormal SE was observed in the allotriploid HT calli cultivated in culture medium supplemented with 2 g L^{-1} activated charcoal (Fig. 4).

In general, the different concentrations of activated charcoal influenced the SE regeneration in the autoallohexaploid HT. The highest concentrations of 8 and 16 g L^{-1} activated charcoal provided the regeneration of normal and abnormal SE of autoallohexaploid HT in a relatively short time. At lower concentrations of activated charcoal (2 and 4 g L^{-1}), however, the SE recovery time was longer and had a lower mean number of abnormal SE of the autoallohexaploid HT (Figs. 3 and 4). Culture medium supplemented with 2 g L^{-1} of activated charcoal resulted in a higher mean number of SE in the autoallohexaploid HT, since it regenerated the highest mean number of MCSE and the lowest mean number of abnormal SE.

Global level of 5-mC%

After 150 days in the callus induction and proliferation steps, the autoallohexaploid HT had the highest mean level of 5-mC% equivalent to 29.85%, while the one for the allotriploid was 13.50%. The 5-mC% mean level increased for the two HT allopolyploids during the SE regeneration step (Fig. 5). The allotriploid HT calli cultured in 2 g L^{-1} activated charcoal had a mean 5-mC% level of 14.78% (Fig. 5). The mean level of 5-mC% in autoallohexaploid HT calli was influenced in SE regeneration medium due to different concentrations of activated charcoal. The highest mean level of 5-mC% was observed in calli of those autoallohexaploid HT cultured with 16 g L^{-1} activated charcoal, with 43.37%. The second highest mean level of 5-

mC% was observed in friable calli of those autoallohexaploid HT cultured with 8 g L^{-1} activated charcoal, with 36.52%. Meanwhile, the autoallohexaploid HT calli cultured with 2 and 4 g L^{-1} activated charcoal had mean 5-mC% levels of 31.12% and 31.79%, respectively, and did not differ statistically (Fig. 5).

Autoallohexaploidy confirmation

Autoallohexaploidy was confirmed for all normal and abnormal SE regenerated from the autoallohexaploid HT calli in tissue culture medium supplemented with 2, 4, 8 and 16 g L^{-1} activated charcoal (Fig. 5). Normal and abnormal SE exhibited the same DNA ploidy level and nuclear DNA content when compared to the explant donor plantlet (Fig. 5). However, some regenerated abnormal SE with 8 and 16 g L^{-1} activated charcoal did not provide intact, isolated and stoichiometrically stained nuclei, and thus it was not possible to verify the G_0/G_1 nuclei peaks (Fig. 6).

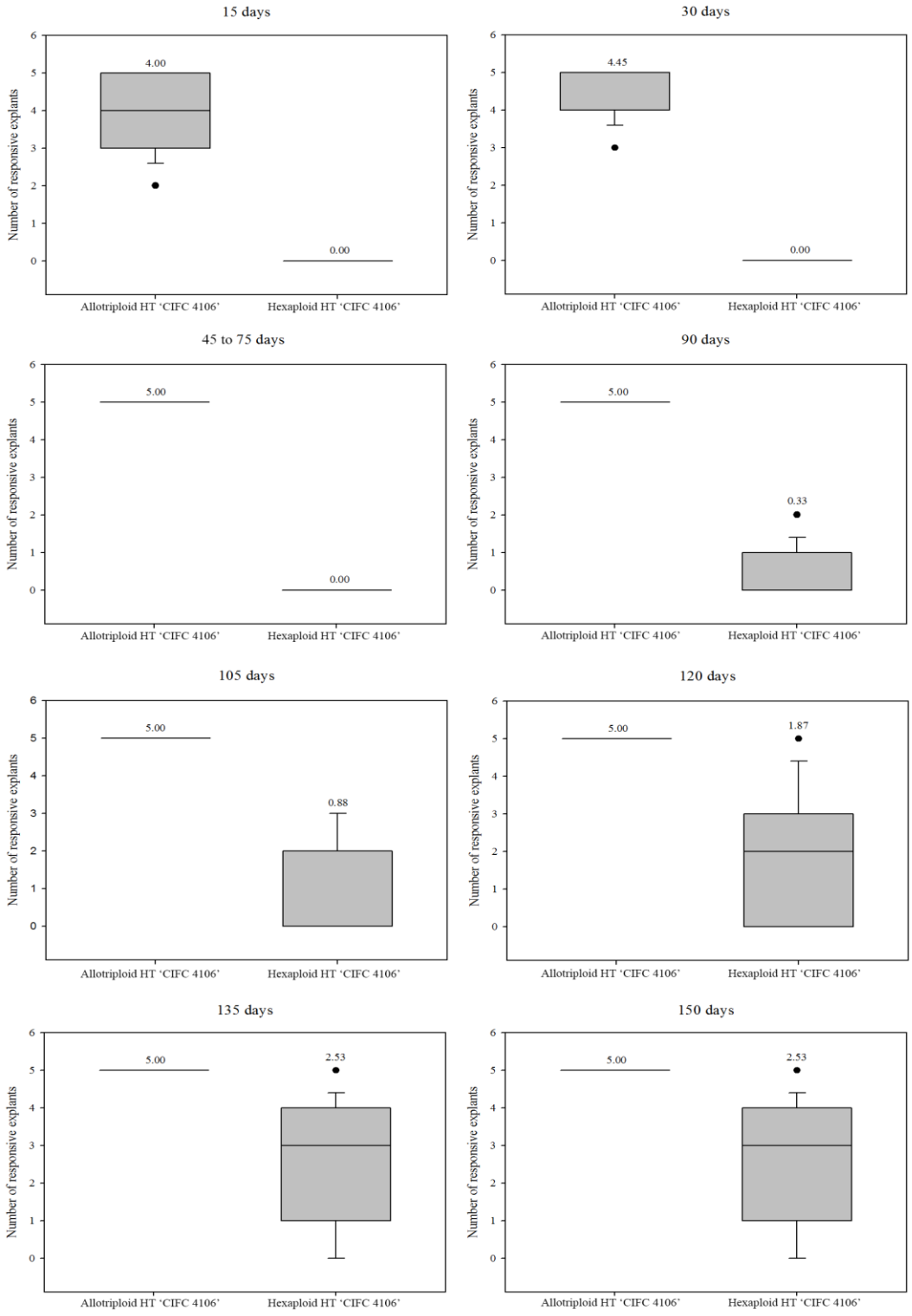

Fig. 1 – Mean number of responsive leaf explants of the allotriploid and hexaploid HT 'CIFC 4106' in callus induction medium during the 150 days. Box plots show that the mean number of responsive explants was higher in allotriploid than hexaploid HT 'CIFC 4106' by the Tukey's test ($P < 0.05$). The number of responsive explants was not varied between the 45 to 75 days.

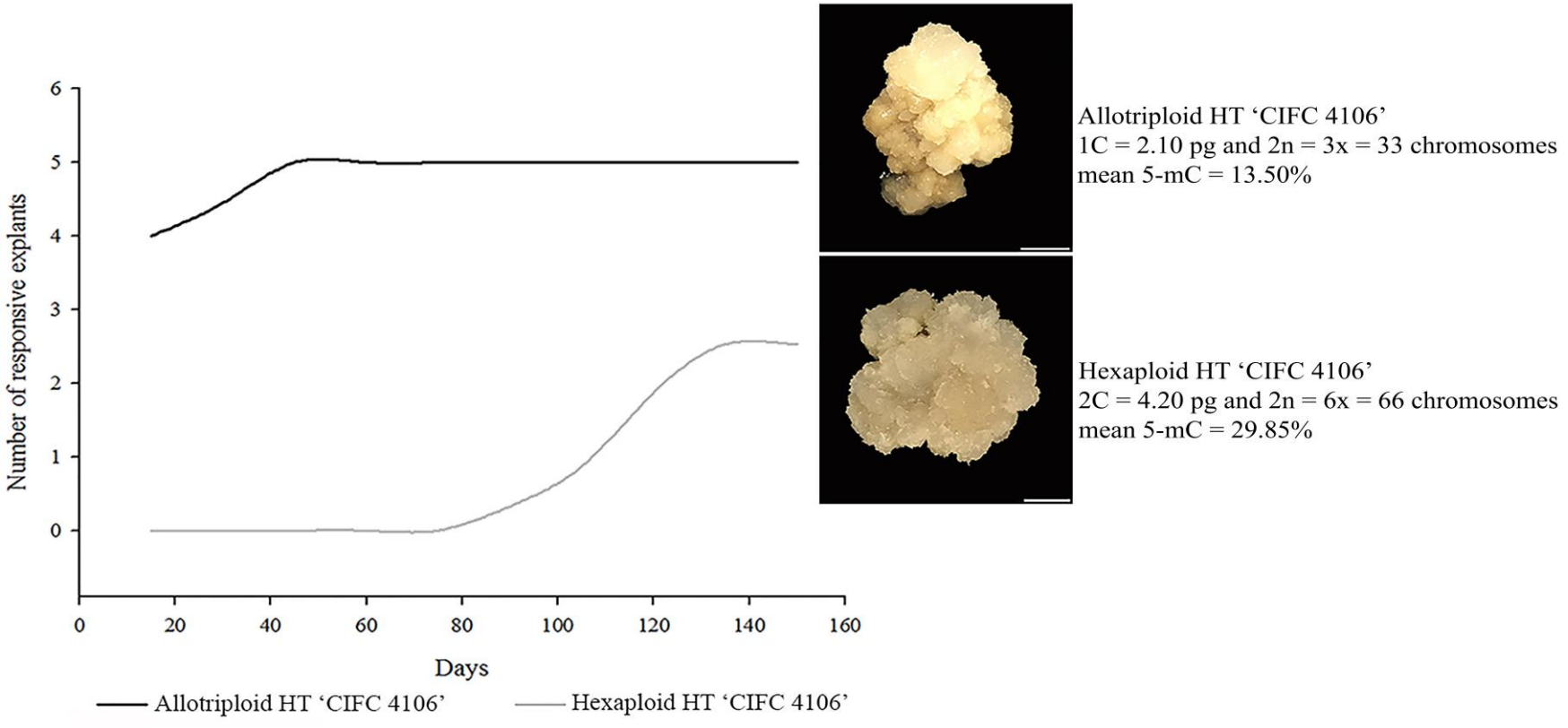

Fig. 2 – Mean number of responsive explants of the allotriploid and hexaploid HT 'CIFC 4106' over time in the callus induction step. Graph shows that the allotriploid HT 'CIFC 4106' had a higher mean number of responsive explants and took less time for callus formation. Meanwhile, the first callus of the hexaploid HT 'CIFC 4106' was observed at 90 days. Callus formation increased over time, being that all calli exhibited a pale yellow color and friable appearance. Adjusted model was significant ($P < 0.05$) by the regression analysis for the hexploid HT 'CIFC 4106': $Y = 0.0469X^2 - 0.1842X$. Bar = 2 mm.

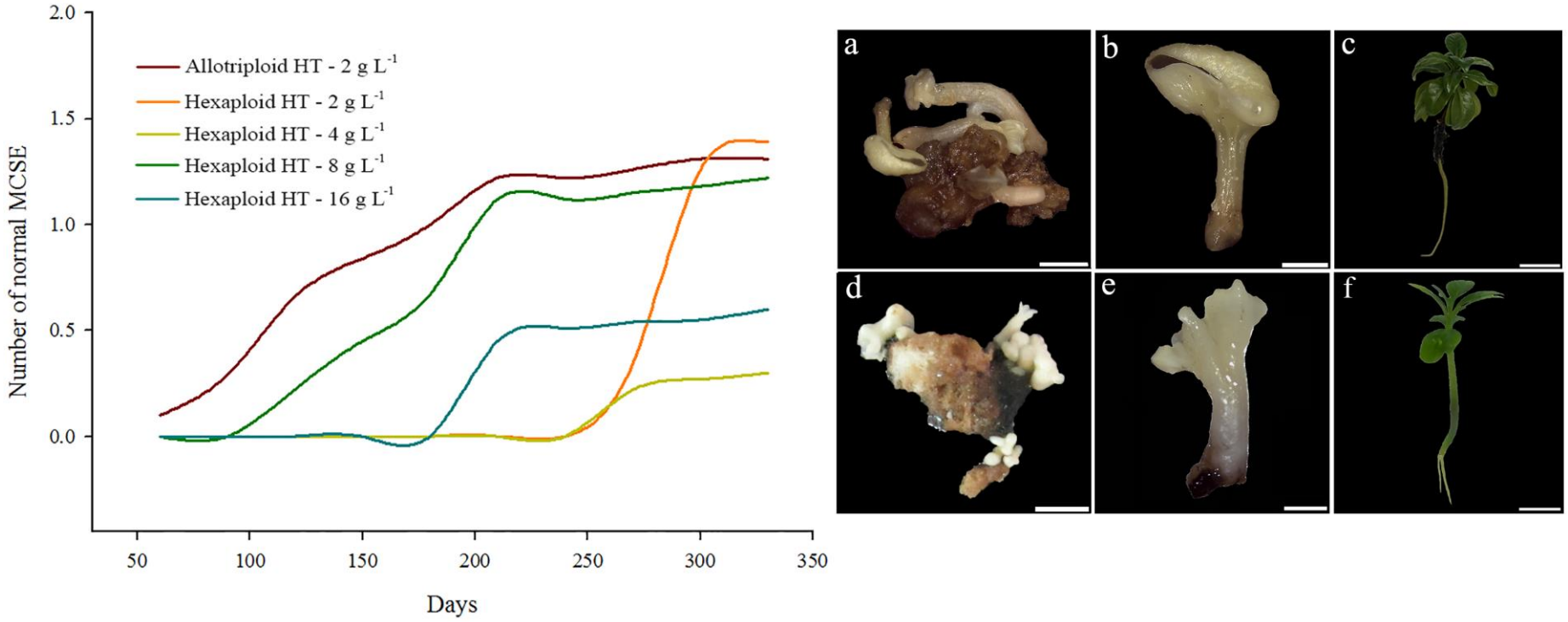

Fig. 3 – Mean number of normal regenerated MCSE of the allotriploid and the hexaploid HT 'CIFC 4106' over time in the SE regeneration medium supplemented with disticnt concentrations of activated charcoal. The first MCSE were observed in allotriploid HT 'CIFC 4106' at 60 days, and the mean number of MCSE for the two allopolyploids increased during the 330 days. (a) Responsive callus of the allotriploid and (d) the hexaploid HT 'CIFC 4106'. Bar = 2 mm. (b) MCSE of the allotriploid and (e) the hexaploid HT 'CIFC 4106'. Bar = 2 mm. (c) Plantlet regenerated from the allotriploid (f) the hexaploid HT 'CIFC 4106' MCSE. Bar = 1 cm.

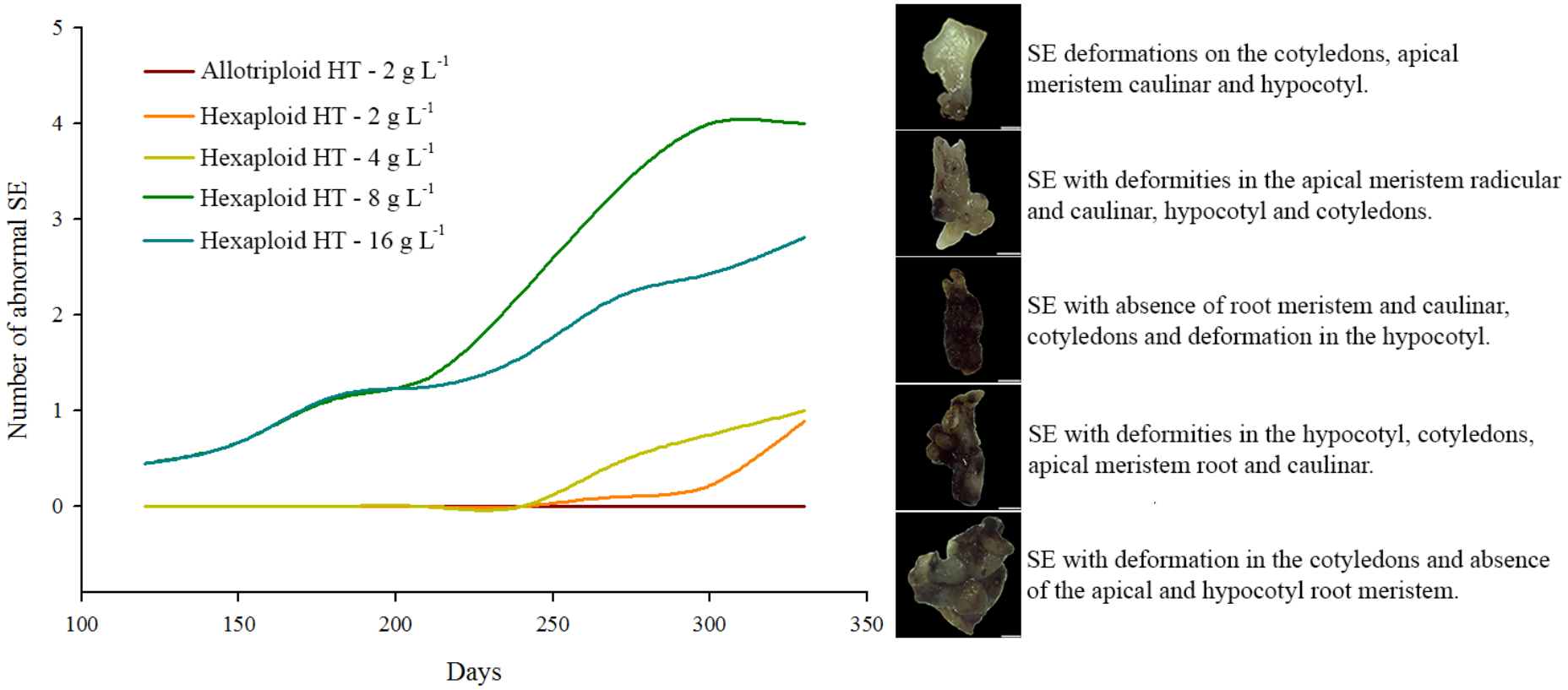

Fig. 4 – Mean number of regenerated abnormal SE of the allotriploid and hexaploid HT 'CIFC 4106' over time in tissue culture medium supplemented with different concentrations of activated charcoal. The graph shows the highest mean number of abnormal SE regenerated in tissue culture medium containing 8 g L^{-1} activated charcoal, and the lowest mean number in the calli maintained in 2 g L^{-1}. No abnormal SE was observed in allotriploid HT 'CIFC 4106' calli. Bar = 2 mm.

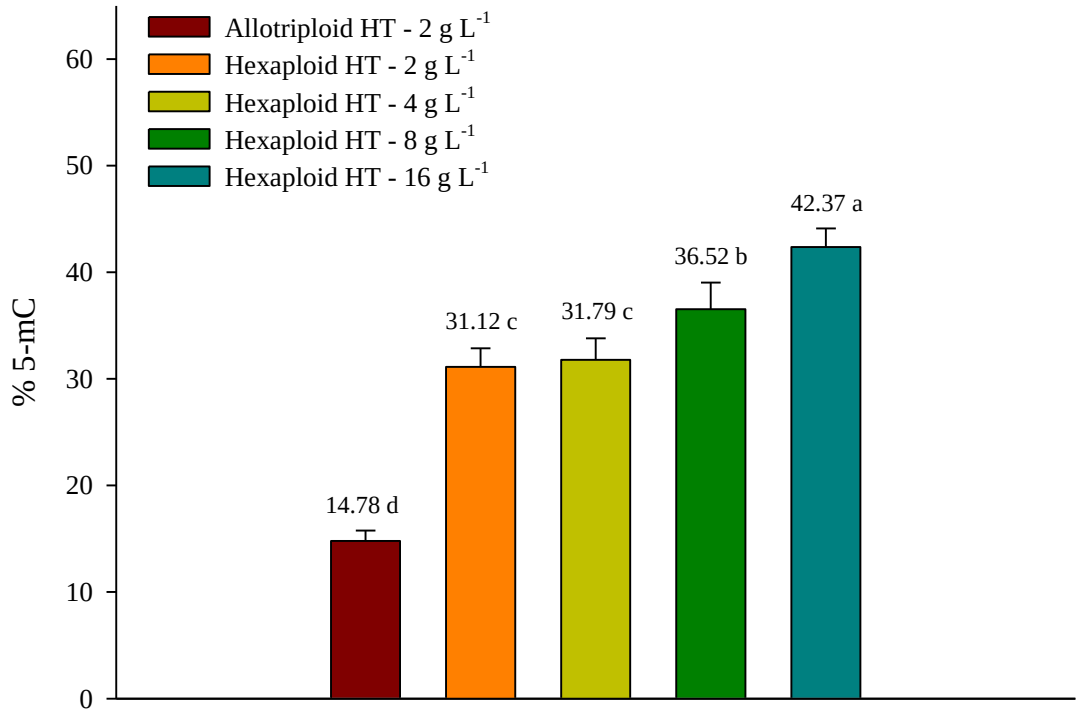

Fig. 5 – Mean level of 5-mC% of the allotriploid HT ‘CIFC 4106’ calli cultured in SE regeneration medium containing 2 g L^{-1} activated charcoal, and the hexaploid HT ‘CIFC 4106’ calli cultured in 2, 4, 8 or 16 g L^{-1} activated charcoal. Activated charcoal concentrations influenced the mean 5-mC% level in hexaploid HT 'CIFC 4106'. The variations of 5-mC% in callus of the hexaploid HT ‘CIFC 4106’ represent an adaptive response to in vitro conditions. The lowest mean level of 5-mC% in hexaploid HT ‘CIFC 4106’ was observed in calli cultured with 2 g L^{-1} and 4 g L^{-1} activated charcoal, and the highest mean level of 5-mC% in calli cultured with 16 g L^{-1} activated charcoal. The mean level of 5-mC% was lower in the calli samples of the allotriploide HT ‘CIFC 4106’ in relation to the hexaploid calli of all tissue culture medium.

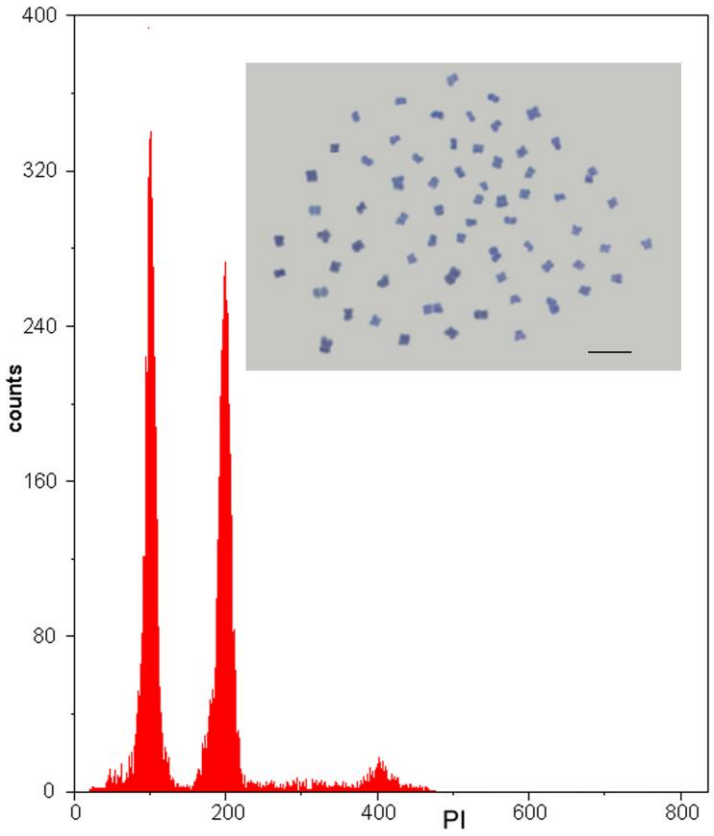

Fig. 6 – Confirmation of hexaploidy and genomic stability in hexaploid HT 'CIFC 4106'. Representative histogram exhibiting G_0/G_1 peaks of intact nuclei of allotriploid (channel 100, 1C = 2.10 pg) and hexaploid HT ‘CIFC 4106’ (channel 200, 2C = 4.20 pg) regenerated in vitro. Karyotype with 2n = 6x = 66 chromossomes of hexaploid HT 'CIFC 4106' obtained from seedling roots in vitro, confirming the maintenance of the number of chromosomes. Bar = 5 µm.

Discussion

ISE was established for the autoallohexaploid HT based on a reproducible procedure proposed by van Boxtel and Berthouly (1996) and modified by Sanglard et al. (2019), involving the callus induction and proliferation, followed by SE and plantlet regeneration. Basically, the changes were the addition of 4, 8 and 16 g L^{-1} activated charcoal in the SE regeneration medium and the absence of gibberellic acid in the plantlet recovery medium. This tissue culture procedure allowed us to verify the effect of the autoallohexaploidy in the HT and to compare the ISE responses with its ancestor, the allotriploid HT. The ISE establishment in the autoallohexaploid HT required more

time and had a lower mean number of responsive explants when compared to the allotriploid HT. Therefore, our data showed differences between the allotriploid and the autoallohexaploid HT during the ISE, evidencing that the ploidy level influences the in vitro response.

The autoallohexaploidy, with a nuclear genome size of 2C = 4.20 pg and a chromosome number of 2n = 6x = 66, influenced the in vitro morphogenic response of the HT. The autoallohexaploid HT showed a slower morphogenic response and a lower mean number of responsive explants compared to the allotriploid HT. Karyotype divergences (i.e. chromosome number, ploidy level and nuclear 2C value) have been indicated as factors that interfere in the in vitro morphogenic pathway in *Coffea* (Sanglard et al. 2019). The autoallohexaploid HT has six chromosome sets, $CCC^aC^aE^aE^a$, originating from the chromosome set doubling of the allotriploid HT that has three chromosome sets CC^aE^a, which themselves originate from a interspecific cross between *C. canephora* (CC) and *C. arabica* ($C^aC^aE^aE^a$, Sanglard et al. 2017). One of the allotriploid HT ancestors, the true allotetraploid *C. arabica* has four $C^aC^aE^aE^a$ chromosome set originating from an interspecific cross between *C. canephora* (CC) and *C. eugenioides* (EE), followed by a natural polyploidization event (Lashermes et al. 1999, 2010; Cenci et al. 2012; Hamon et al. 2015). According to Sanglard et al. (2019), the four *Coffea* with different ploidy levels presented distinct ISE responses: the allotriploid HT (CC^aE^a) had a higher mean number of responsive explants in a shorter period of time (from 3.62 to 15 days to 4.90 to 90 days), followed by the allotetraploid *C. arabica* ($C^aC^aE^aE^a$, 2.17 to 15 days at 2.37 to 90 days), while the diploids *C. canephora* (CC) and *C. eugenioides* (EE) had a lower mean number of responsive explants (1.07 and 1.04 to 90 days, respectively). According to the evolutionary history, it is believed that the genome of *C. arabica* ($C^aC^aE^aE^a$) has a greater contribution or influence on the in vitro response in the autoallohexaploid HT ($CCC^aC^aE^aE^a$), since the mean number of responsive explants was similar.

The autoallohexaploid HT exhibited a lower response of friable callus induction compared to its ancestor, despite having twice the in vitro response-related genes due to its autopolyploid condition. After a polyploidization event, genetic changes occur including structural chromosome rearrangements, aneuploidy, mutations in the DNA sequence, duplicate genes loss, gene conversion, and epigenetic variations (Osborn et al. 2003; Soltis et al. 2004). Such genetic and epigenetic alterations trigger immediate and long-term disturbances in the genome, epigenome, transcriptome and other "-omes",

experiencing a variety of collective phenomena that are termed "genomic shock" (McClintock 1984). Thus, the autoallohexaploid HT did not meet the initial expectation of having a better in vitro response than its allotriploid HT progenitor.

During the SE regeneration step, both the allotriploid and the autoallohexaploid HT exhibited a slow response and a low regeneration rate of MCSE. However, the time for MCSE regeneration was faster in the allotriploid HT than in the autoallohexaploid HT. The relatively slow response and the low rate of MCSE regeneration of the allotriploid HT were reported by Sanglard et al. (2019) when comparing in vitro responses in *Coffea* among the diploids *C. canephora* (CC) and *C. eugenioides* (EE), the allotriploid HT (CC^aE^a) and the true allotetraploid *C. arabica* ($C^aC^aE^aE^a$). According to the authors, the allotetraploid *C. arabica* ($C^aC^aE^aE^a$) had a higher mean number of MCSE in a shorter period of time (from 1.71 to 60 days to 9.94 to 180 days), while the diploids *C. eugenioides* (EE) and *C. canephora* (CC) and the allotriploid (CC^aE^a) presented lower mean MCSE and did not differ significantly (2.04, 0.29 and 1.00 to 180 days, respectively). Interestingly, in the second part of ISE (SE regeneration), the autoallohexaploid HT ($CCC^aC^aE^aE^a$) presented a morphogenic response similar to the diploids *C. canephora* (CC) and *C. eugenioides* (EE) and to the allotriploid HT (CC^aE^a). Therefore, based on our results and those of Sanglard et al. (2019), karyotypic divergences (chromosome number, ploidy level and nuclear 2C value), origin (allopolyploid and autoalopolyploid) and time of formation of polyploids (~0.665 Mya for allotetraploid *C. arabica*, ~100 years for the allotriploid HT and 2 years for the autoallohexaploid HT) influence the establishment of ISE in *Coffea*.

The allotriploid and the autoallohexaploid HT exhibited distinct levels of global DNA methylation as well as varying methylation levels during ISE. The autoalohexaploid HT showed higher levels of global methylation during all ISE, probably due to the polyploid condition. After the whole genome duplication, epigenetic changes occurred including the methylation of the cytosine (Sattler et al. 2016). In addition, during the induction and proliferation, the friable callus presented lower values of 5-mC%, while higher levels of 5-mC% were observed during the SE regeneration stage. Therefore, changes in DNA methylation levels during the ISE are related to cells acquiring competence, determination and differentiation. According to Karim et al. (2016), DNA hypomethylation is associated with suppression of SE conversion and maturation, and the hypermethylation is associated with SE regeneration. DNA methylation is a reversible epigenetic mechanism that promotes dedifferentiation and

redifferentiation of plant cells (Féher 2015; Peng and Zhang 2009). Therefore, our data show that the increase in global methylation levels during the ISE plays a fundamental role in the in vitro morphogenic response of newly formed polyploid plants.

Activated charcoal, the only difference between the media used for SE recovery, influenced the mean levels of 5-mC% and the MCSE regeneration of the autoallohexaploid HT. 8 and 16 g L^{-1} of activated charcoal resulted in a faster embryogenic response and provided a higher mean number of abnormal SE, indicating at least the phytotoxic effect. In addition, these concentrations of activated charcoal increased the mean levels of 5-mC% and, consequently, caused DNA hypermethylation. The 5-mC% variations observed in the callus cells correspond to an adaptive response to in vitro conditions. The probable cause of the rapid embryogenic response and the high rate of regeneration of the abnormal SE, caused by the addition of activated charcoal, is due to epigenetic factors. Elevated levels of 5-mC% in autoallohexaploid HT friable calli may have repressed or silenced some genes that are involved in cell differentiation, possibly adding a methyl group to the DNA promoter and causing the formation of abnormal SE. According to Berdasco et al. (2008), hypermethylation of the DNA caused repression of the genes *mitogen-activated protein kinase 12 (mapk12), glutathione S-transferase U10 (gstu10), beta-xylosidase 1 (bxl1), transparent testa glabra 1 (ttg1), glutathione S-transferase F5 (gstf5), su(var)3-9 homolog8 (suvh8), fimbrin* and *carotenoid cleavage dioxygenase 7 (ccd7)* from in vitro response in undifferentiated *Arabidopsis thaliana* cells. For the MCSE recovery, it is estimated that 3500 genes must be expressed, at least 40 are involved in the establishment of the apical-basal embryonic axis. Thus, the cells differentiate and specialize to form the different organs of the SE (von Arnold et al. 2002).

Abnormal SE via direct somatic embryogenesis of the allotriploid HT were also observed by Sattler et al. (2016b). The authors reported that 6% of the regenerated SE of the allotriploid HT exhibited limited and abnormal development, providing unusual plantlets characterized by the occurrence of cotyledons fusion, absence of apical meristem, hypocotyl thickening and callus formation. Although the formation of abnormal SE of the allotriploid HT was not observed in the present work, the regenerated abnormal SE of autoallohexaploid HT showed deformations similar to those observed by Sattler et al (2016b).

In addition to the epigenetic variations, the formation of abnormal SE may be associated with genetic alterations. However, our cytogenetic and flow cytometry data

show that changes in the in vitro environment did not cause euploidy or aneuploidy in autoallohexaploid HT. Abnormal SE and regenerated autoallohexaploid plantlets presented mean values of 2C DNA (2C = 4.20) and a ploidy level identical to the autoallohexaploid explant donor plantlets. The cytogenetic analysis confirmed that the regenerated plantlets remained hexaploids, with 2n = 6x = 66 chromosomes. The regenerated allotriploid HT plantlets also exhibited 2C DNA mean values (1C = 2.10 pg) and a chromosome number (2n = 3x = 33) equivalent to the allotriploid HT explant donor plant, as well as the same ploidy level reported by Clarindo et al. (2013) for the original allotriploid HT plants. Therefore, our data showed that the protocol of ISE did not promote chromosomal alterations, resulting in the stability of the nuclear genome of regenerated plantlets of allotriploid and autoallohexaploid.

Conclusions

Besides the propagation of the new synthetic autoallohexaploid HT, we here report the consequences of short-term induced polyploidy in the ISE. The newly formed polyploids of HT exhibit a worse response in vitro and higher methylated cytosine levels than its allotriploid progenitor. In addition, as a response to the tissue culture conditions, changes in DNA methylation occurred during ISE promoting the competence, determination and differentiation of the plant cells. In conclusion, the indirect somatic embryogenesis of the polyploid *Coffea* was influenced by ploidy level, epigenetic and in vitro environmental.

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References

Alix K, Gérard P.R, Schwarzacher T, Heslop-Harrison JS (2017) Polyploidy and interspecific hybridization: partners for adaptation, speciation and evolution in plants. Annals of botany 120(2):183-194. https://doi.org/10.1093/aob/mcx079

Berdasco M, Alcázar R, García-Ortiz MV, Ballestar E, Fernández AF, RoldánArjona T, Tiburcio AF, Altabella T, Buisine N, Quesneville H, Baudry A, Lepiniec L, Alaminos M, Rodríguez R, Lloyd A, Colot V, Bender J, Canal MJ, Esteller M, Fraga MF (2008) Promoter DNA hypermethylation and gene repression in undifferentiated *Arabidopsis* cells. PLoS ONE 3(10):e3306. http://dx.doi.org/10.1371/journal.pone.0003306

Bettencourt AJ (1973) Considerações gerais sobre o "Híbrido de Timor". Campinas, Brasil: Instituto Agronômico de Campinas, Cicular 23.

Boller B, Schubiger FX, Kölliker (2012) Red clover. In: Boller B, Posselt UK, Veronesi F (eds) Fodder crops and amenity grasses. Springer-Verlag, New York, pp 439–455.

Capucho AS, Caixeta ET, Zambolim EM, Zambolim L (2009) Herança da resistência de Híbrido de Timor UFV 443-03 à ferrugem-do-cafeeiro. Pesq Agropec Bras 44:276-282.

Cenci A, Combes MC, Lashermes P (2012) Genome evolution in diploid and tetraploid *Coffea* species as revealed by comparative analysis of orthologous genome segments. Plant Mol Biol 78:135-145. http://dx.doi.org/10.1007/s11103-011-9852-3

Chen Q, Tao S, Bi X, Xu X, Wang L, Li X (2013) Research of total levels on DNA methylation in plant based on HPLC analysis. American Journal of Molecular Biology 3:98-101. https://doi.org/10.4236/ajmb.2013.32013

Clarindo WR, Carvalho CR, Caixeta ET, Koehler AD (2013) Following the track of "Híbrido de Timor" origin by cytogenetic and flow cytometry approaches. Genet Resour Crop Evol 60:2253-2259. https://doi:10.1007/s10722-013-9990-3

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13-15.

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

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049-1051. https://doi.org/10.1126/science.220.4601.1049

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50:151-158. https://doi.org/10.1016/0014-4827(68)90403-5

Hamon P, Hamon S, Razafinarivo NJ, Guyot R, Siljak-Yakovlev S, Couturon E, Crouzillat D, Rigoreau M, Akaffou S, Rakotomalala JJ, de Kochko A (2015) *Coffea* genome organization and evolution. In: Coffee in health and disease prevention. Academic, San Diego, pp 29-37. https://doi.org/10.1016/B978-0-12-409517-5.00004-8

Hancock NI, Overton JR (1960) Behavior and adaptation of Balbo and Tetra Petkus rye. University of Tennessee Agricultural Experiment Station. Bulletin 307.

Jarillo JA, Piñeiro M, Cubas P, Martínez-Zapater JM (2009) Chromatin remodeling in plant development. Int J Dev Biol 53:1581-1596. https://doi.org/10.1387/ijdb.072460jj

Johansson L, Andersson B, Eriksson T (1982) Improvement of anther culture technique: activated charcoal bound in agar medium in combination with liquid medium and elevated CO_2 concentration. Physiol Plantarum 54:24-30. https://doi.org/10.1111/j.1399-3054.1982.tb00571.x

Karim R, Nuruzzaman M, Khalid N, Harikrishna JA (2016) Importance of DNA and histone methylation in in vitro plant propagation for crop improvement: a review. Ann Appl Biol 169:1-16. https://doi.org/10.1111/aab.12280

Lashermes P, Combes MC, Ribas A, Cenci A, Mahé L, Etienne H (2010) Genetic and physical mapping of the SH3 region that confers resistance to leaf rust in coffee

tree (*Coffea arabica* L.). Tree Genet Genomes 6:973-980. http://dx.doi.org/10.1007/s11295-010-0306-x

Lashermes P, Combes MC, Robert J, Trouslot P, D'Hont A, Anthony F, Charrier A (1999) Molecular characterization and origin of the *Coffea arabica* L. genome. Mol Gen Genet 261:259-266. http://dx.doi.org/10.1007/s004380050965

López CMR, Wetten AC, Wilkinson MJ (2010) Progressive erosion of genetic and epigenetic variation in callus-derived cocoa (*Theobroma cacao*) plants. New Phytol 186:856-868

Münzbergová Z (2017) Colchicine application significantly affects plant performance in the second generation of synthetic polyploids and its effects vary between populations. Annals of botany 120(2), 329-339. https://doi.org/10.1093/aob/mcx070

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

Nic-Can GI, Galaz-Ávalos RM, De-la-Peña C, Alcazar-Magaña A, Wrobel K, Loyola-Vargas VM (2015) Somatic embryogenesis: identified factors that lead to embryogenic repression. A case of species of the same genus. PLoS One 10(6):e0126414. https://doi.org/10.1371/journal.pone.0126414

Nic-Can GI, Lopez-Torres A, Barredo-Pool F, Wrobel K, LoyolaVargas VM, Rojas-Herrera R, De-la-Pena C (2013) New insights into somatic embryogenesis: LEAFY COTYLEDON1, BABY BOOM1 and WUSCHEL-RELATED HOMEOBOX4 are epigenetically regulated in *Coffea canephora*. PLoS ONE 8:e72160. https://doi.org/10.1371/journal.pone.0072160

Osborn TC, Pires JC, Birchler JA et al (2003) Understanding mechanisms of novel gene expression in polyploids. Trends Genet 19:141–147. https://doi:10.1016/S0168-9525(03)00015-5

Otto FJ (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewiez Z, Crissman HA, Robinson JP (eds) Methods in cell biology, vol. 33. Academic Press, San Diego, pp 105–110

Otto SP (2007) The evolutionary consequences of polyploidy. Cell 131:452-462. https://doi.org/10.1016/j.cell.2007.10.022

Pan MJ, van Staden J (1998) The use of charcoal in in vitro culture: a review. Plant Growth Regul 26:155-163. https://doi.org/10.1023/A:1006119015972

Peng H, Zhang J (2009) Plant genomic DNA methylation in response to stresses: potential applications and challenges in plant breeding. Progress in Natural Science 19:1037-1045. https://doi.org/10.1016/j.pnsc.2008.10.014

Renny-Byfield S, Wendel JF (2014) Doubling down on genomes: polyploidy and crop plants. Am J Bot 101:1–15. https://doi.org/10.3732/ajb.1400119

Reyes JC (2006) Chromatin modifiers that control plant development. Curr Opin Plant Biol 9:21-27. https://doi.org/10.1016/j. pbi.2005.11.010

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

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

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Nunes ACP, Soares TCB, Carvalho CR, Clarindo WR (2017) From chromosome doubling to DNA sequence changes: outcomes of an improved in vitro procedure developed for allotriploid “Híbrido de Timor” (*Coffea arabica* L. x *Coffea canephora* Pierre ex A. Froehner). Plant Cell Tiss Organ Cult 131:223-231. https://doi.org/10.1007/s11240-017-1278-4

Sattler MC, Carvalho CR, Clarindo WR (2016a) The polyploidy and its key role in plant breeding. Planta 243:281-296. https://doi.org/10.1007/s00425-015-2450-x

Sattler MC, Carvalho CR, Clarindo WR (2016b) Regeneration of allotriploid *Coffea* plants from tissue culture: resolving the propagation problems promoted by irregular meiosis. Cytologia 81:125–132. https://doi.org/10.1508/cytologia.81.125

Setotaw TA, Caixeta ET, Pena GF, Zambolim EM, Pereira AA, Sakiyama NS (2010) Breeding potential and genetic diversity of "Híbrido do Timor" coffee evaluated by molecular markers. Crop Breed Appl Biotechnol 10:298-304. https://doi.org/10.1590/S1984-70332010000400003

Slotkin K, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. Nat Rev Genet 8:272–285. http://dx.doi.org/10.1038/nrg2072

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since Plant speciation. New Phytol 161:173–191. https://doi.org/10.1046/j.1469-8137.2003.00948.x

Soltis DE, Visger CJ, Marchant DB, Soltis PS (2016) Polyploidy: pitfalls and paths to a paradigm. American Journal of Botany 103:1146-1166. https://doi.org/10.3732/ajb.1500501

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnology Advances 26:618-63. https://doi.org/10.1016/j.biotechadv.2008.08.003

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

Van de Peer Y, Maere S, Meyer A (2009) The evolutionary significance of ancient genome duplications. Nat Rev Genet 10:725–732. https://doi.org/10.1038/nrg2600

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filanova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tissue and Organ Culture, 69:233-49.

Wang D, Bodovitz S (2010) Single cell analysis: the new frontier in 'omics'. Trends in Biotechnology 28:281-290. https://doi.org/10.1016/j.tibtech.2010.03.002

Wang Q-M, Wang L (2012) An evolutionary view of plant tissue culture: somaclonal variation and selection. Plant Cell Rep 31:1535-1547. https://doi.org/10.1007/s00299-012-1281-5

Wang Q-M, Wang Y-Z, Sun L-L, Gao F-Z, Sun W, He J, Gao X, Wang L (2012) Direct and indirect organogenesis of *Clivia miniata* and assessment of DNA methylation changes in various regenerated plantlets. Plant Cell Rep. https://doi.org/10.1007/s00299- 012-1248-6

Xu L, Huang H (2014) Genetic and epigenetic controls of plant regeneration. Curr Top Dev Biol 108:1-33. https://doi.org/10.1016/B978-0-12-391498-9.00009-7

Yang X, Ye C-Y, Cheng Z-M, Tschaplinski TJ, Wullschleger SD, Yin W, Xia X, Tuskan G. A. (2010). Genomic aspects of research involving polyploid plants. Plant Cell, Tissue and Organ Culture 104(3):387-397. https://doi.org/10.1007/s11240-010-9826-1

Capítulo III: Global cytosine methylation is associated with in vitro regeneration in *Coffea arabica* lines.

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Abstract

Coffea arabica has been established in vitro to propagate superior genotypes. However, different *C. arabica* genotypes present different responses in vitro. Epigenetic modifications have been pointed out as one of the factors that influence these distinct responses. Here, we aim to evaluate and compare the global cytosine methylation levels of three *C. arabica* lines ('Catuaí Vermelho', 'Caturra' and 'Oeiras') during indirect somatic embryogenesis, and to verify the ploidy level fidelity of the regenerated plantlets. Leaf explants were inoculated in callus induction and proliferation medium. After 90 days, the calli were transferred to regeneration medium, and the mature cotyledonary somatic embryos were transferred to the seedling regeneration medium. 'Oeiras' exhibited the highest number of responsive leaf explants (4.30), followed by 'Caturra' (4.00) and 'Catuaí Vermelho' (2.32). Cytosine methylation levels increased over time in the friable calli of 'Catuaí Vermelho' (20.73% at 60 days and 30.79% at 90 days) and 'Caturra' (38.70% at 60 days and 53.40% at 90 days), remaining constant in 'Oeiras' (34.34% at 60 days and 33.51% at 90 days). 'Oeiras' did not regenerate somatic embryos, while 'Catuaí Vermelho' exhibited the highest number at all evaluated times. Embryogenic calli of 'Catuaí Vermelho' and 'Caturra' showed different values of methylated cytosine, with a mean value of 54.09% and 43.35%, respectively. Therefore, a global increase in methylated cytosine is necessary for the regeneration of somatic embryos in *C. arabica*. In addition, all regenerated plantlets exhibited mean values of 2C DNA (2C = 2.62 pg) and remained tetraploid, identical to the explant donor plant.

Keywords: Coffee; Epigenetic; Indirect somatic embryogenesis.

Introduction

The coffee tree belongs to the genus *Coffea*, which includes approximately 103 species. Among these species, *Coffea arabica* L. has the greatest economic importance representing about 61% of the world's coffee production (ICO Report 2018). Coffee production is onerous due to the long culture cycle and because it is subject to adverse biotic and abiotic factors, limiting its productive potential (Santana-Buzzy et al. 2007; Loyola-Vargas et al. 2016). Coffee breeding programs have sought to develop and select more productive genotypes, resistant to biotic and abiotic stress, and adapted to the soil conditions of marginal areas and with less environmental impact (Santana-Buzzy et al. 2007). As the coffee plants are perennial and the conventional breeding requires 30 to 35 years to select superior genotypes, corresponding to six or seven cycles of self-pollination, the use and improvement of other strategies is fundamental (Loyola-Vargas et al. 2016).

In this context, plant biotechnology coupled with conventional breeding has contributed to genetic breeding in an attempt to create and develop superior genotypes using more sophisticated and rapid techniques to meet farmers' expectations (Los Santos-Briones and Hernández-Sotomayor 2006; Campos et al. 2017). The plant tissue culture applied to coffee breeding is an important biotechnological tool, since through in vitro culture it is possible to: (a) propagate superior genotypes on a large scale (van Boxtel and Berthouly 1996); (b) maintain, conserve and exchange germplasm (Naidu and Sreenath 1998; Sanglard et al. 2019); (c) obtain homozygous plantlets from another culture (Silva et al. 2011); (d) induce polyploidization (Sanglard et al. 2017) and somaclonal variations (Sondahl et al. 1995; Sanchez-Teyer et al. 2003); (e) regenerate transgenic cells or tissues (Ogita et al. 2003 and 2004; Da Silva and Menéndez-Yuffá 2003; Mishra and Slater 2012); and (f) overcome incompatibility barriers through protoplast fusion (Schöpke et al. 1987; Adams and Zarowitz 1994). However, for the plant tissue culture techniques to be applied to coffee breeding as described above, a prerequisite is that *Coffea* varieties, hybrids and species are established in vitro.

Indirect somatic embryogenesis (ISE) is a morphogenetic pathway that consists of the cultivation of plant tissue segments in sterile and specific culture medium, in the originating callus and, later, in the somatic embryos (SE) and plantlets. The capacity of the plant cell to organize and give rise to a new plant identical to the explant donor plant is based on the concept of cellular totipotency, postulated by Haberlandt in 1902

(Williams and Maheswaran 1986). Cellular totipotency involves complex mechanisms of gene expression reprograming, cellular division and metabolism and cellular development reprogramming (Fehér et al. 2003). The genetic (Sanglard et al. 2019), epigenetic (Nic-Can et al. 2015; Fehér 2015) and physiological and/or morphological (Santana et al. 2004; Fehér 2015) aspects of the explant donors, as well as the conditions in vitro (van Boxtel and Berthouly 1996; Bychappa et al. 2019), influence ISE.

In addition to the characteristics inherent to explant donors and the in vitro environment, the epigenome also influences ISE (LoSchiavo et al. 1989; Nic-Can et al. 2013; Amaral-Silva et al. 2020). Epigenetic variations are defined as covalent modifications that occur in chromatin, allowing cells to maintain distinct and different characteristics, although they contain the same genetic material (Us-Camas et al. 2014; Amaral-Silva et al. 2020). Variations in cytosine methylation levels have been reported in plant cells grown in vitro as a mechanism that controls the morphogenetic processes, since it regulates the expression of genes that are involved in somatic embryogenesis such as *wus* (*wuschel*), *lec 1* and *2* (*leafy cotyledon 1* and *2*), *fus3* (*fusca3*), *cuc1* (*cup-shaped cotyledon 1*) and *wind1* (*wound-induced dedifferentiation 1*), *serk* (*somatic embryogenesis receptor like kinase*), *bbm1* (*baby boom 1*) and *agl15* (*agamous-like 15*) (Fehér 2015; Karim et al. 2016; Kadokura et al. 2018). According to Nic-Can et al. (2013), the cytosine hypermethylation has been associated with the increase, conversion and maturation of SE in *C. canephora* by direct somatic embryogenesis.

In vitro propagation of *Coffea* via ISE has been associated with genetic instability resulting from somaclonal variation (Sanchez-Teyer et al. 2003; Landey et al. 2015; Etienne et al. 2016; Bychappa et al. 2019). The somaclonal variation is associated to genomic (nucleus, mitochondria and plastid) and/or epigenomic changes, which can result in phenotypic variations of the regenerated plantlets in relation to the plant explant donors – called genetic fidelity loss (de Oliveira et al. 2019). These variations usually present cytological changes, such as chromosome number changes (aneuploidy or polyploidy), chromosomal rearrangements (deletions, duplications, inversions, and translocations), and/or modifications in DNA sequences (Phillips et al. 1994; Bairu et al. 2011). Somaclonal variations can be verified and evaluated by flow cytometry (Sattler et al. 2016, Sanglard et al. 2017, Konar et al. 2018), cytogenetics (Sattler et al 2016, Sanglard et al. 2017), molecular markers (Konar et al. 2018; Bychappa et al. 2019), DNA methylation (Grzybkowska et al. 2018; Kadokura et al. 2018), histone

modifications (De-la-Pena et al. 2015) and microRNAs (Szyrajew et al. 2017), allowing detailed analyses of the genome and epigenome at different levels.

In this study, we used three lines of *C. arabica*, 'Catuaí Vermelho', 'Caturra' and 'Oeiras', which originated from genetic recombination, mass selection and pedigree, respectively, to understand the genotypic influence on the morphogenic response in vitro and also to understand the role of global cytosine methylation. Thus, the present study aimed to (a) establish, compare and evaluate the ISE in *C. arabica* 'Catuaí Vermelho', 'Caturra' and 'Oeiras' under the same in vitro condition, (b) measure, compare and relate the global levels of methylated cytosine in these *C. arabica* lines during the callus induction/proliferation and the SE regeneration, and (c) evaluate ploidy level instability of the regenerated plantlets by chromosome counting and DNA ploidy level.

Material and methods

ISE establishment

Completely expanded leaves of *C. arabica* 'Caturra', 'Oeiras' and 'Catuaí Vermelho' were collected from orthotropic nodes of plants that have been maintained in the germplasm bank of the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil, 20° 45'S, 42° 52'W, under adequate phytosanitary and environmental conditions. The collected leaves were used as explant source for ISE.

The collected leaves were washed with liquid detergent and rinsed with running water for 2 h, and then disinfected in a laminar flow chamber by immersion in 70% ethanol for 20 s, and 1.5% sodium hypochlorite solution for 20 min (Clarindo et al. 2012). Posteriorly, leave explants of 1 – 2 cm^2 were excised, and five fragments were inoculated in Petri dishes containing callus induction medium (M1, Table 1). The callus formation was evaluated biweekly until 90 days, and after, the calli were individually transferred to SE regeneration medium (M2, Table 1, van Boxtel and Berthouly 1996; Sanglard et al. 2019). The Petri dishes were kept in the dark at 25 ± 2°C. Mature cotyledon somatic embryos (MCSE) regeneration was evaluated monthly up to 180 days. The MCSE were counted and transferred to the seedling recovery medium (M3, Table 1, Sanglard et al. 2019). In each test tube, one MCSE was inoculated. Subsequently, the test tubes were maintained in a growth room under a light/dark

regime of 16/8 h with 36 μmol m^{-2} s^{-1} of light radiation supplied by two fluorescent lamps (20 W, Osram®) at 25 ± 2°C.

Global cytosine methylation (5-mC%)

Friable callus samples from 'Catuaí Vermelho', 'Caturra' and 'Oeiras' were separately collected after 60 and 90 days in callus induction and proliferation medium. Embryogenic callus samples (callus showing an embryogenic response) from 'Catuaí Vermelho' and 'Caturra' were also separately collected after MCSE formation in SE regeneration medium. Then, the collected samples were macerated separately in the MagNALyser (Roche®, Germany) for 60 s at 7,000 rpm. Genomic DNA was extracted according to Doyle and Doyle (1990) with the addition of 7.5 M ammonium acetate and excluding the nocturnal period for DNA precipitation (Sanglard et al. 2017). DNA concentration, quality and purity were determined using the NanoDrop spectrophotometer (Thermo Scientific® 2000c) and DNA integrity was assessed by 0.8% agarose gel electrophoresis.

For the measurement of the 5-mC%, 30 μg of genomic DNA were diluted in 100 μL of dH_2O. Then, 50 μL of 70% (v/v) perchloric acid were added to the diluted DNA, and then hydrolyzed for 1 h in a 100°C water bath. The pH of the hydrolysates was adjusted between 3 – 5 with KOH (1 M) (Demeulemeester et al. 1999; Chen et al. 2013). Cytosine and 5-methylcytosine standard stock solutions were prepared by weighing 0.44 mg cytosine and 0.55 g 5-methylcytosine and dissolving it in 0.1% perchloric acid. Posteriorly, concentrations of cytosine and 5-methylcytosine stock solution were 4×10^2 μmol L^{-1} and 20 μmol L^{-1}, respectively (Chen et al. 2013). 5-methylcytosine was analyzed according to Chen et al. (2013) by High Performance Liquid Chromatography (Shimadzu® HPLC, model LC-20AT), which is equipped with a photodiode array detector (SPD–M20A) using a silica-based reverse phase column C_{18} (VP– 150 × 4.6 mm, 5 μm). The sample separations were carried out under a specific mobile phase of potassium dihydrogen phosphate (50 mmol L^{-1}, pH 5.8) at a flow rate of 0.5 mL min^{-1}, in which each component of the sample interacts in a different way with the sorbent material, generating different speeds and leading to separation as they run through the column. Cytosine and its methylated derivate detection were performed at a wavelength of 285 nm. The 5-mC% was calculated by 5-mC% = [5-methylcytosine

/ (cytosine + 5-methylcytosine)] x 10, comparing the values with cytosine and 5-methylcytosine standards.

Ploidy level stability

Leaf fragments (2 cm^2) from in vitro regenerated plantlets of *C. arabica* 'Catuaí Vermelho' and 'Caturra' and of the internal standard *Solanum lycopersicum* L. (2C = 2.00 pg, Praça-Fontes et al. 2011) were co-chopped in nuclei extraction buffer (Otto 1990; Praça-Fontes et al. 2011). The suspensions were processed, stained (Otto 1990; Sanglard et al. 2019) and analyzed on a flow cytometer (Partec® GmbH, Muenster, Germany). The 2C value was measured considering the G_0/G_1 nuclei peak of the 'Catuaí Vermelho' or 'Caturra' and of *S. lycopersicum*. In addition, the root meristem of the regenerated plantlets of 'Catuaí Vermelho' and 'Caturra' were collected, washed, fixed and enzymatically macerated. The slides were prepared using cell dissociation and air drying techniques (Sanglard et al. 2019). After staining with 5% Giemsa, mitotic images were captured using a 100x objective and a CCD camera (Nikon Evolution™) connected to a Nikon 80i microscope (Nikon, Japan). From the mean 2C value and chromosome number, the DNA ploidy level was confirmed for the regenerated 'Catuaí Vermelho' and 'Caturra' plantlets.

Statistical analysis

ISE responses of *C. arabica* 'Catuaí Vermelho', 'Caturra' and 'Oeiras' were compared during callus induction/proliferation and during SE regeneration stages. In the callus induction and proliferation stage, statistical analysis was performed using the mean number of responsive explants, which were defined by the presence of calli at 15, 30, 45, 60, 75 and 90 days, totaling 45 repetitions for 'Caturra', 60 for Oeiras' and 102 for 'Catuaí Vermelho'. To compare the mean number of responsive explants of 'Catuaí Vermelho', 'Caturra' and 'Oeiras' over time, an analysis of variance (ANOVA) was applied and the mean values were compared by Tukey's test ($P < 0.05$). During the SE regeneration stage, an ANOVA was also performed for 'Catuaí Vermelho' and 'Caturra', using the mean number of MCSE regenerated at 30, 60, 90, 120, 150 and 180 days, totaling 116 repetitions for 'Caturra' and 144 for 'Catuaí Vermelho'. The mean values

were compared by the *F* test ($P < 0.05$) and displayed with box-plot graphs. Then, a regression analysis was performed at 5% of the probability level ($P < 0.05$).

The 5-mC% was measured during the callus induction/proliferation and the SE regeneration stages. The ANOVA was applied to compare the 5-mC% of the 'Catuaí Vermelho', 'Caturra' and 'Oeiras' friable calli collected at 60 (8 repetitions for 'Catuaí Vermelho', 9 for 'Caturra' and 20 for 'Oeiras') and 90 days (6 repetitions for 'Catuaí Vermelho' and 'Caturra' and 20 for 'Oeiras') in callus induction and proliferation medium. Mean values were compared by Tukey's test ($P < 0.05$). To compare the 5-mC% in 'Catuaí Vermelho' (7 repetitions) and 'Caturra' (3 repetitions) friable calli in SE regeneration medium, an ANOVA was applied and mean values were compared with the *F* test ($P < 0.05$). All analyzes were performed using the software R (R Core Team 2018).

Results

C. arabica 'Catuaí Vermelho', 'Caturra' and 'Oeiras' presented distinct values of responsive explants (callus in the leaf fragment) over time. The first responsive explants were observed at 15 days in callus induction and proliferation medium for all lines of *C. arabica*. 'Oeiras' exhibited the highest mean number of responsive explants (4.30), followed by 'Caturra' (4.00) and 'Catuaí Vermelho' (2.32) (Fig. 1). There was no significant difference among the mean number of responsive explants over time of the lines. Callus proliferation gradually increased at 30 days, becoming visually stable at 90 days. The friable callus from all *C. arabica* lines showed pale-yellow coloration (Fig. 1).

'Catuaí Vermelho', 'Caturra' and 'Oeiras' friable calli presented distinct mean values of 5-mC% at 60 and 90 days. 5-mC% levels gradually increased over time for 'Catuaí Vermelho' (20.73% at 60 days and 30.79% at 90 days, Fig. 2a) and 'Caturra' (38.70% at 60 days and 53.40% at 90 days, Fig. 2a). While for 'Oeiras' the friable calli showed the same mean values equivalent to 34.34% at 60 days and 33.51% at 90 days, not differing statistically (Fig. 2a). When comparing *C. arabica* lines at 60 and 90 days, it is observed that the 'Oeiras' and 'Caturra' friable calli exhibited 5-mC% mean values equivalent to 34.34% and 35.35% at 60 days statistically differing in relation to 'Catuaí Vermelho', which exhibited an mean value of 20.73% (Fig. 2b). At 90 days, the

'Caturra' friable calli showed mean values of 5-mC% equivalent to 50.40% statistically distinct compared to 'Oeiras' and 'Catuaí Vermelho', which presented equal mean values equivalent to 33.51% and 30.80%, respectively (Fig. 2b).

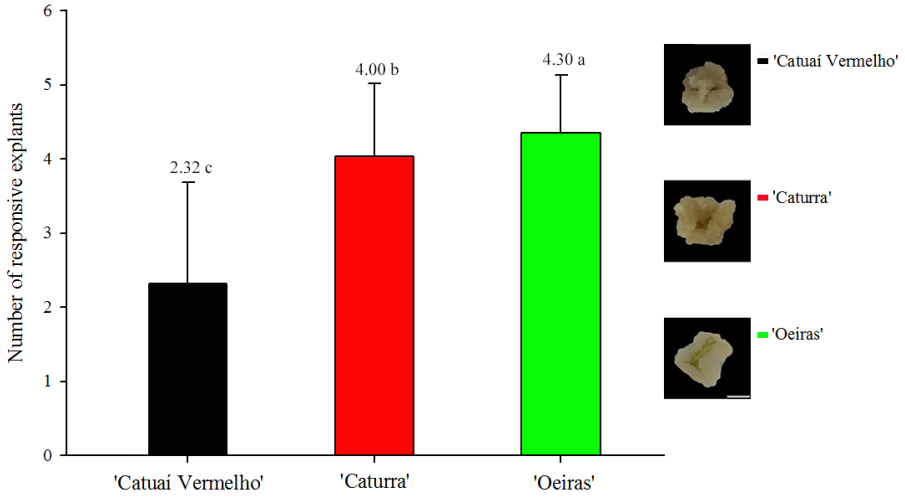

Fig. 1 – Mean number of responsive explants of 'Catuaí Vermelho', 'Caturra' and 'Oeiras' in callus induction and proliferation medium. Note that *C. arabica* lines exhibited distinct values of responsive explants. The highest mean number of responsive explants was observed in 'Oeiras', followed by 'Caturra' and 'Catuaí Vermelho'. Bar = 2 mm.

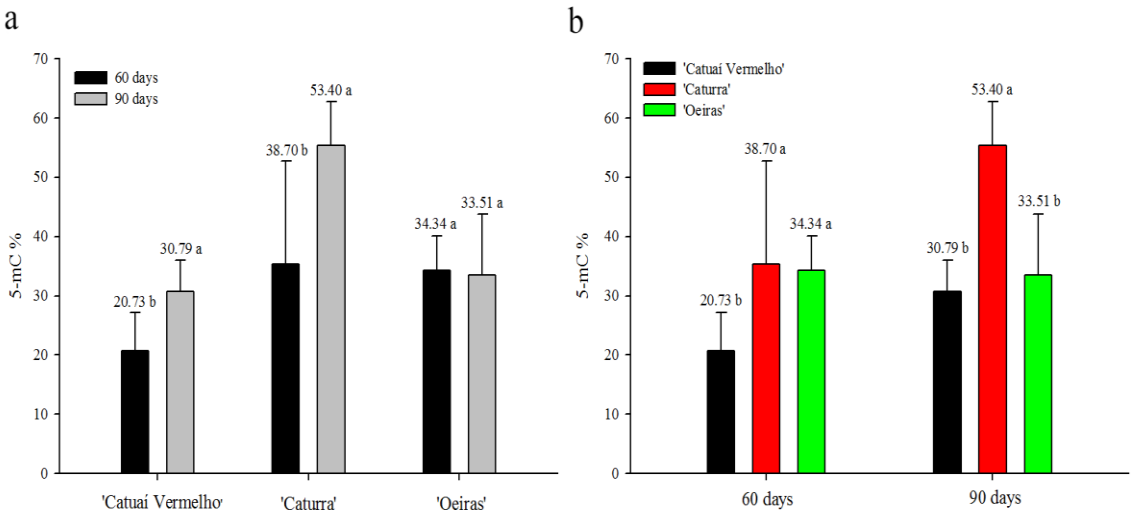

Fig. 2 – Graphics show the 5-mC% level measured for 'Catuaí Vermelho', 'Caturra' and 'Oeiras' friable calli at 60 and 90 days. (a) Comparison of the global 5-mC% level at 60

and 90 days among each *C. arabica* line. (b) Comparison of the global 5-mC% level between all *C. arabica* lines at 60 and 90 days.

During SE regeneration, *C. arabica* lines exhibited distinct MCSE mean values over time (Figs. 3 and 4). The 'Catuaí Vermelho' friable calli presented the first MCSE at 30 days with a mean value of 0.03 MCSE, while the first MCSE of 'Caturra' were observed at 60 days with a mean value of 0.02 (Fig. 3). After, the mean number of MCSE gradually increased for 'Caturra' and 'Catuaí Vermelho' (Figs. 3 and 4). However, 'Catuaí Vermelho' exhibited the highest mean number of MCSE at all time, differing statistically from 'Caturra' after 60 days (Fig. 3). The final mean number of MCSE regenerated at 180 days was 0.24 and 12.25 for 'Caturra' and 'Catuaí Vermelho', respectively (Fig. 3). No SE was recovered up to 180 days for 'Oeiras'.

During the SE regeneration, the responsive calli of 'Catuaí Vermelho' and 'Caturra' presented different mean values of 5-mC%. The highest level of 5-mC% was observed in responsive calli of 'Caturra' with 54.09%, while the responsive calli of 'Catuaí Vermelho' exhibited mean value of 43.35% (Fig. 4). 'Catuaí Vermelho' had a 12.56% (30.79 to 43.35%) increase of 5-mC%, while 'Caturra' had a 0.69% increase (53.40 to 54.09%) (Fig. 4). In general, the first ISE moment of induction and proliferation of friable calli was characterized by lower values of 5-mC% (Fig. 2). Meanwhile, the second ISE moment involving SE regeneration was marked by high values of 5-mC% (Fig. 4).

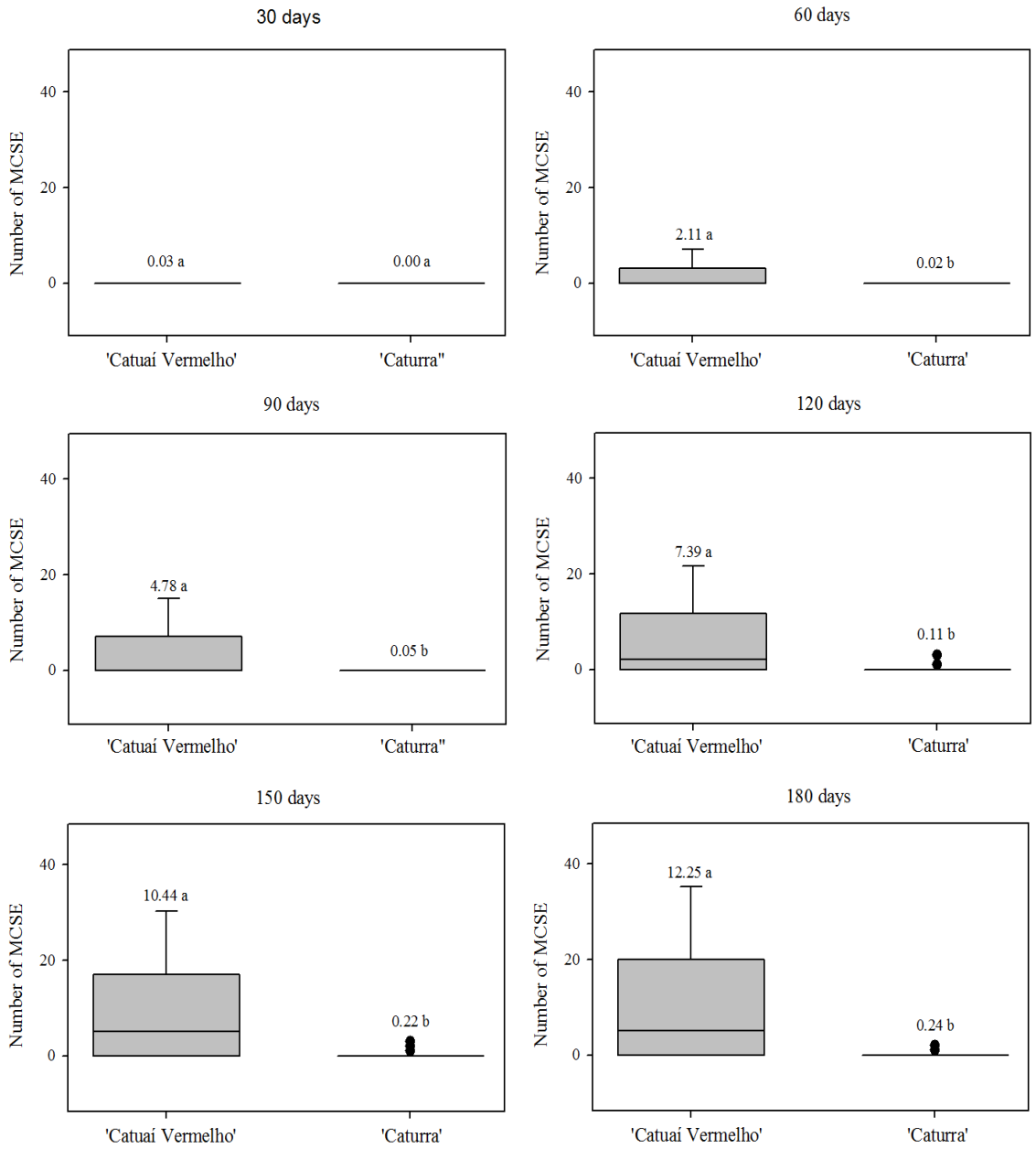

Fig. 3 – MCSE mean number for 'Catuaí Vermelho' and 'Caturra' in SE regeneration medium during 180 days. Box plots show that 'Catuaí Vermelho' and 'Caturra' exhibited distinct MCSE mean numbers. The highest mean number of MCSE was observed in 'Catuaí Vermelho', differing from 'Caturra' at 60 days.

ISE was established for 'Catuaí Vermelho' and 'Caturra' (Fig. 5). The ES exhibited distinct stages of development (globular, heart, torpedo and cotyledonary), presenting an asynchronous response during ISE. SE regeneration continued for over 180 days for 'Catuaí Vermelho' and 'Caturra', with seedling recovery potential over several months (Fig. 5). The embryogenic response of 'Catuaí Vermelho' was classified

as higher in relation to the other lines, regenerating a mean number of 12.25 MCSE per callus. ISE was not established for 'Oeiras', as SE regeneration was not observed for 180 days. Karyotypic variations (number of chromosomes 2n, ploidy levels and nuclear values of 2C) were not observed between explant donors and in vitro regenerated plantlets (Fig. 6).

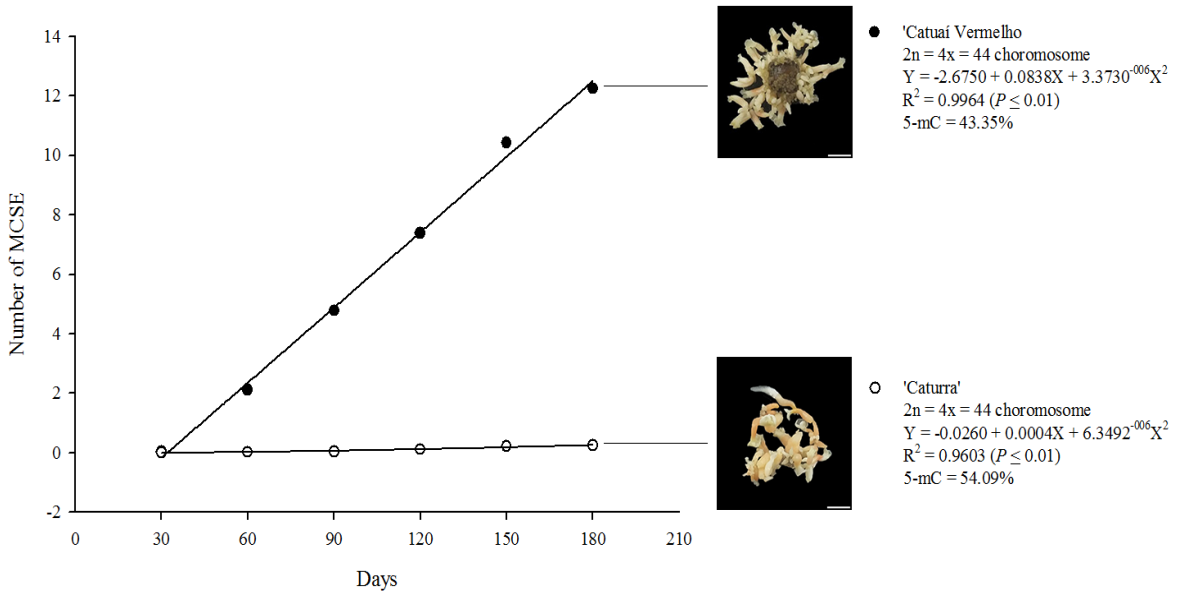

Fig. 4 – Mean number of MCSE regenerated from 'Catuaí Vermelho' and 'Caturra' over time. 'Catuaí Vermelho' exhibited the highest mean number of MCSE compared to 'Caturra'. Regression analysis was significant for 'Catuaí Vermelho' and 'Caturra' ($P < 0.01$). The responsive calli presented different mean values of 5-mC% with 43.35% and 54.09% for 'Catuaí Vermelho' and 'Caturra', respectively. The regenerated SE exhibited different stages of globular, heart, torpedo and cotyledonary SE. Bar = 3 mm.

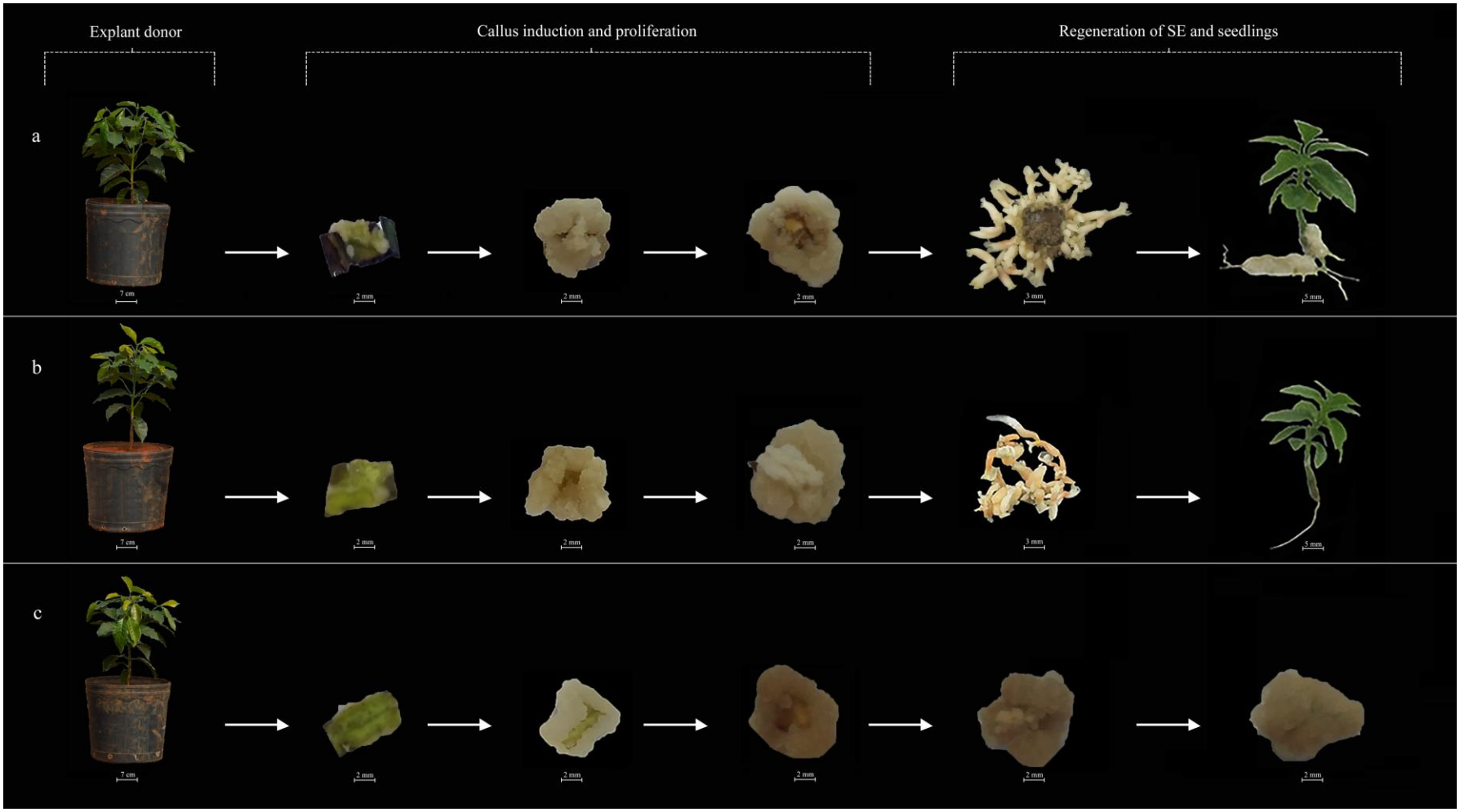

Fig. 5 – Response of ISE for C. arabica lines. ISE was established for 'Catuaí Vermelho' (a) and 'Caturra' (b), involving the induction and proliferation stages of callus, followed by SE and plantlets regeneration. ISE was not established for 'Oeiras' (c) as it did not regenerate SE and plantlets.

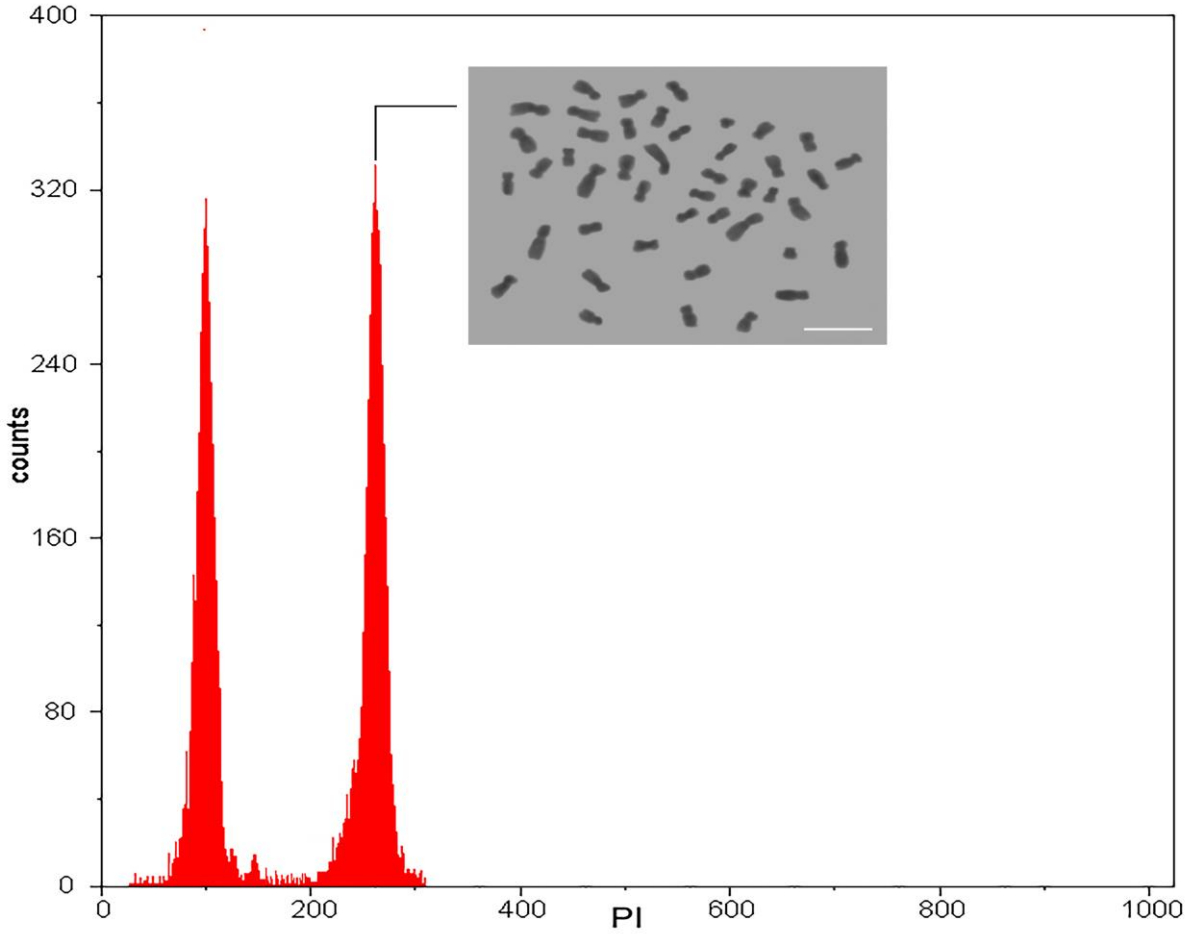

Fig. 6 – Ploidy confirmation and chromosomal number of *C. arabica* plantlets regenerated in vitro. Representative histogram showing G_0/G_1 peaks of intact nuclei of *Solanum lycopersicum* L. in channel 100 and *C. arabica* in channel 262. Karyotype with 2n = 4x = 44 *C. arabica* chromosomes, obtained from plantlets roots in vitro, confirming maintaining the number of chromosomes. Bar = 5 µm.

Discussion

In vitro procedures were developed under the same conditions to compare and evaluate *C. arabica* lines 'Catuai Vermelho', 'Caturra' and 'Oeiras' in relation to ISE response. In addition, it was possible to determine, compare and associate global levels of 5-mC% in the *C. arabica* lines during ISE responses and to evaluate the karyotype stability of in vitro regenerated plantlets. The three tested *C. arabica* lines differed in their in vitro response (mean number of responsive leaf explants, mean number of MCSE and response time) and in their global 5-mC% levels. ISE was established for 'Catuaí Vermelho' and 'Caturra' based on an improved and reproducible procedure proposed by van Boxtel and Berthouly (1996) and Sanglard et al. (2019), involving the callus induction and proliferation, followed by the SE and plantlets regeneration. However, the ISE procedure (in vitro conditions) was not effective for 'Oeiras', since there were no SE recovered, the development remained in the callus phase. For ISE establishment, differentiated plant cells must return to their undifferentiated state and regain totipotency, giving rise to calli and later competence acquisition to regenerate SE (Williams and Maheswaran 1986). According to Namasivayam et al. (2007), the process of acquiring embryogenic competence by somatic cells should involve the reprogramming of gene expression patterns, as well as changes in physiology and morphology. These changes reflect dedifferentiation, activation of cell division, and a change in cell fate through down-regulation of some genes operating in differentiated cells and up-regulation of genes needed for transition (reviewed by Fehér 2015). However, Campos et al. (2017) suggested an alternative hypothesis about totipotency, in which meristematic cells are able to differentiate into SE without going through the dedifferentiation. These meristematic cells maintain their totipotency throughout the development of the plant and, under the right stimulus, multiply and differentiate to form a new SE.

During the callus induction and proliferation, 'Oeiras' leaf explants were the most responsive, providing friable calli in the relatively short time of one month, followed by 'Caturra' and 'Catuai Vermelho' (Fig. 1). In the second ISE step (acquisition of competence, determination and differentiation of friable callus cells and regeneration of SE), a higher mean number of MCSE was observed for 'Catuaí Vermelho' compared to 'Caturra', which presented the highest mean number of responsive explants (Figs. 1, 3 and 4). Therefore, our data show differences between all three *C. arabica* lines,

suggesting that the in vitro response is dependent and influenced by the genotype. The 'Caturra' lines originated from a natural mutation of the 'Bourbon' line; the breeding method was mass selection based on the phenotype without progeny testing (Mendes et al. 2008). The 'Oeiras' line was developed by the pedigree method from the hybrid ‘CIFC HW 26/5’, which was obtained from a crossing between 'Caturra Vermelho' (CIFC 19/1) and “Híbrido de Timor” ‘CIFC 832/1’ (Mendes et al. 2008). The 'Catuaí Vermelho' originated from a recombination of the 'Caturra Amarelo' and 'Mundo Novo' lines (Mendes et al. 2008). Therefore, all *C. arabica* lines here under study originated and were selected by different breeding methods and have a restricted but different genetic base, which may have influenced the in vitro response. Corroborating, other studies also showed that in vitro response in *C. arabica* is genotype dependent and influenced by the in vitro environment (Bieysse et al. 1993; van Boxtel and Berthouly 1996; Samson et al. 2006). Bieysse et al. (1993), evaluating the in vitro ISE responses of the commercial *C. arabica* ‘Caturra Rojo’ and the wild type *C. arabica* of the Ethiopian (ET 25.1, ET 20.1, ET 1.1, ET 12.4, ET 12.5, KF 2.1 and KF 6.3), observed that only KF 2.1, ET 25.1, ET 12.5 and ET 1 regenerated SE and plantlets.

Our data show that the in vitro tissue culture condition alters the methylation patterns, as global levels of 5-mC% increased gradually over time in 'Catuaí Vermelho' friable calli (20.73% at 60 days and 30.79% at 90 days) and 'Caturra' (38.70% at 60 days and 53.40% at 90 days). Variations in global 5-mC% levels were also observed between *C. arabica* lines during the SE regeneration. The increase in the global level of 5-mC% observed in in vitro established *C. arabica* lines is associated with a chromatin remodeling from a eucromatic to a heterochromatic state. Cytosine methylation is a conserved epigenetic modification that plays an important role in chromatin remodeling (Zhang et al. 2018) and transcriptional control of gene expression, influencing the somatic embryogenesis establishment (Nic-Can et al. 2013; Pasternak and Dudits 2019). In addition, cytosine methylation is associated with controlling genome stability by suppressing transcription of mobile DNA elements in plants (Kidwell and Lisch 2007). Cytosine methylation can be passed down through several generations or induced by in vitro environmental stimuli: stress, culture medium, growth regulators and culture system (Zhang et al. 2018). According to Chen and Li (2004), the level of cytosine methylation in plants varies from 6 to 30% in somatic cells. This high degree of cytosine methylation in plants is attributed to the fact that, in plants, methylation can be present in three CG islands, CHG and CHH, where H can be any deoxynucleotide,

being more common in CG islands, characteristic of transposons, mainly due to the high presence of these elements in plant genomes.

The mechanisms of gene regulation have been the subject of studies on several plant species of agronomic interest, in order to investigate and elucidate morphogenetic events in vitro (Zhang et al. 2018; Pasternak and Dudits 2019; de Oliveira et al. 2019). According to Nic-Can et al. (2013), epigenetic changes in somatic embryogenic tissues of *Coffea canephora* are controlled by histone modifications and 5-mC%. As observed in this study, an increase of 5-mC% was necessary for the regeneration and maturation of the SE in *C. arabica* 'Caturra' and 'Catuaí Vermelho'. The increase of 5-mC% observed in these *C. arabica* lines during the ISE may have silenced a large number of genes that are not involved in the process of cell induction and dedifferentiation, acquisition of competence and SE formation. This result corroborates with Nic-Can et al. (2013) and de Oliveira et al. (2019) when they investigated the structural, physiological and molecular events that occur during embryonic development.

The occurrence of somaclonal variation is undesirable in plant tissue culture, as a high level of somaclonal variation can ruin a possible genotype. According to Landey et al. (2013), low rates of somaclonal variation were identified in *C. arabica* genotypes using molecular markers. Corroborating, Etienne et al. (2016) noted that 99% of *C. arabica* trees regenerated in vitro are in compliance with the explant donor plant and that phenotypic variants are induced by aneuploidy, showing that this somaclonal variation occurs during in vitro propagation. However, genetic somaclonal variations (euploidy and aneuploidy) were not observed in the present study between in vitro regenerated plantlets in relation to leaf explant donors. Flow cytometry analysis showed that the regenerated plantlets had mean 2C DNA values (2C = 2.62 pg) and ploidy level identical to those of the explant donor plant. In addition, the cytogenetic approach confirmed that regenerated plantlets of 'Catuaí Vermelho' and 'Caturra' remain tetraploid, with 2n = 4x = 44 chromosomes. Therefore, our data showed that the ISE protocol is reproducible, viable and safe, since it did not promote chromosomal alterations.

Conclusions

'Catuaí Vermelho' was the *C. arabica* line that exhibited the best in vitro response to the proposed in vitro conditions, regenerating the highest mean number of

SE. Cytosine methylation is a dynamic and variable mechanism and the increase in methylation is necessary for the regeneration and maturation of SE in *C. arabica* lines, since this increase is associated with a chromatin remodeling from the euchromatic to the heterochromatic state. In vitro regenerated plantlets were identical to explant donor plants and therefore no karyotype and phenotypic variations were identified. The culture medium has been shown to be efficient, reproducible and viable as it maintains genetic fidelity and can be applied when multiplying the superior *Coffea* genotypes.

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References

Adams TL, Zarowitz MA (1994). Stably transformed coffee plant cells and plantlets. U.S. Patent No. 5,334,529, 2 Aug. 1994.

Amaral-Silva PM, Clarindo WR, Guilhen JHS, De Jesus Passos ABR, Sanglard NA, Ferreira A (2020) Global 5-methylcytosine and physiological changes are triggers of indirect somatic embryogenesis in *Coffea canephora*. Protoplasma 1-13. https://doi.org/10.1007/s00709-020-01551-8

Bairu MW, Aremu AO, Van Staden J (2011) Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation 63:147–173. https://doi.org/10.1007/s10725-010-9554-x

Bieysse D, Gofflot A, Michaux-Ferrière N (1993) Effect of experimental conditions and genotypic variability on somatic embryogenesis in *Coffea arabica*. Canadian Journal of Botany 71(11):1496-1502. https://doi.org/10.1139/b93-181

Bychappa M, Mishra MK, Jingade P, Huded AKC (2019) Genomic alterations in coding region of tissue culture plants of *Coffea arabica* obtained through

somatic embryogenesis revealed by molecular markers. Plant Cell Tiss Organ Cult 139:91–103. https://doi.org/10.1007/s11240-019-01666-8

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

Carvalho CR, Clarindo WR, Almeida PM (2007) Plant cytogenetics: still looking for the perfect mitotic chromosomes. Nucl 50:453–462.

Chen Q, Tao S, Bi X, Xu X, Wang L, Li X (2013) Research of total levels on DNA methylation in plant based on HPLC analysis. Am J Mol Biol 3(2):98–101. https://doi.org/10.4236/ajmb.2013.32013

Chen T, Li E (2004) Structure and function of eukaryotic DNA methyltransferases. Current Topics in Developmental Biology 60:55–89. https://doi.org/10.1016/S0070-2153(04)60003-2

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

Da Silva RF, Menéndez-Yuffá A (2003) Transient gene expression in secondary somatic embryos from coffee tissues electroporated with the genes gus and bar. Electronic Journal of Biotechnology 6(1):11-12.

De Oliveira KC, Guimarães PS, Bazioli JM, Martinati JC, Santos MM, Padilha L, Guerreiro-Filho O, Maluf MP (2019) Effects of somatic embryogenesis on gene expression of cloned coffe heterozygous hybrids. Acta Physiol Plant 41:118. https://doi.org/10.1007/s11738-019-2917-7

De-la-Peña C, Nic-Can GI, Galaz-Ávalos RM, Avilez-Montalvo R, Loyola-Vargas VM (2015) The role of chromatin modifications in somatic embryogenesis in plants. Front Plant Sci. https://doi.org/10.3389/fpls.2015.00635

Demeulemeester MAC, Stallen NV, Droft PMP (1999) Degree of DNA methylation to chicory (*Cichorium intybus* L.): influence of plant age and vernalization. Plant Science 142:101-108. https://doi.org/10.1016/S0168-9452(99)00010-2

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15.

Etienne H, Bertrand B, Dechamp E, Maurel P, Georget F, Guyot R, Breitler JC (2016) Are Genetic and Epigenetic Instabilities of Plant Embryogenic Cells a Fatality? The Experience of Coffee Somatic Embryogenesis. Human Genetics & Embryology 06(01). https://doi.org/10.4172/2161-0436.1000136

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

Fehér A, Pasternak T, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss Organ Cult 74:201–228.

Fraga HPF, Vieira LN, Caprestano CA, Steinmacher DA, Micke GA, Spudeit DA, Pescador R, Guerra MP (2012) 5-Azacytidine combined with 2,4-D improves somatic embryogenesis of *Acca sellowiana* (O. Berg) Burret by means of changes in global DNA methylation levels. Plant Cell Rep 31:2165–2176. https://doi.org/10.1007/s00299-012-1327-8

Grzybkowska D, Morończyk J, Wójcikowska B, Gaj MD (2018) Azacitidine (5-AzaC)-treatment and mutations in DNA methylase genes affect embryogenic response and expression of the genes that are involved in somatic embryogenesis in *Arabidopsis*. Plant Growth Regulation 85:243–256. https://doi.org/10.1007/s10725-018-0389-1

ICO (2018) International Coffee Organization. http://www.ico.org/Market-Report-16-17-e.asp. Available at: http://www.ico.org/documents/cy2017-18/cmr-0618-e.pdf

Kadokura S, Sugimoto K, Tarr P, Suzuki T, Matsunaga S (2018) Characterization of somatic embryogenesis initiated from the Arabidopsis shoot apex.

Developmental Biology 442:13–2714. https://doi.org/10.1016/j.ydbio.2018.04.023

Karim R, Nuruzzaman M, Khalid N, Harikrishna JA (2016) Importance of DNA and histone methylation in in vitro plant propagation for crop improvement: a review. Ann Appl Biol 169:1–16. https://doi.org/10.1111/aab.12280

Kidwell MG, Lisch DR (2007) Perspective: transposable elements, parasitic DNA, and genome evolution. Evolution 55:1–24. https://doi.org/10.1111/j.0014-3820.2001.tb01268.x

Konar S, Karmakar J, Ray A, Adhikari S, Bandyopadhyay TK (2018) Regeneration of plantlets through somatic embryogenesis from root derived calli of *Hibiscus sabdariffa* L. (Roselle) and assessment of genetic stability by flow cytometry and ISSR analysis. Plos One 13(8):e0202324. https://doi.org/10.1371/journal.pone.0202324

Landey BR, 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(2):e56372. https://doi.org/10.1371/journal.pone.0056372

Landey RB, Cenci A, Guyot R (2015) Assessment of genetic and epigenetic changes during cell culture ageing and relations with somaclonal variation in *Coffea arabica*. Plant Cell Tiss Organ Cult 122:517–531. https://doi.org/10.1007/s11240-015-0772-9

Los Santos-Briones D, Hernández-Sotomayor SM (2006) Coffee biotechnology. Brazilian Journal of Plant Physiology 18(1):217-227. https://doi.org/10.1590/S1677-04202006000100015

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and

hypomethylating drugs. Theor Appl Genet 77:325–331. https://doi.org/10.1007/BF00305823

Loyola-vargas VM, Avilez-Montalvo JR, Avilés-Montalvo RN, Marquez-Lopez R, Galaz-Avalos R, Mellado-Mojica E (2016) "Somatic embryogenesis in *Coffea* spp," in Somatic embryogenesis: fundamental aspects and applications, eds V. M. Loyola-vargas and N. Ochoa-Alejo (Cham: Springer International Publishing), 297–318.

Mendes ANG, Carvalho GR, Botelho CE, Fazuoli LC, Silvarolla MB (2008) História das primeiras cultivares de café plantadas no Brasil. In: Carvalho CHS. Cultivares de Café. Brasília, DF: Embrapa Café.

Mishra MK, Slater A (2012) Recent advances in the genetic transformation of coffee. Biotechnology research international, 2012.

Naidu MM, Sreenath HL (1998) In vitro culture of coffee zygotic embryos for germplasm preservation. Plant Cell Tiss Organ Cult 55:227–230.

Namasivayam P (2007) Acquisition of embryogenic competence during somatic embryogenesis. Plant Cell Tiss Organ Cult 90:1–8. https://doi.org/10.1007/s11240-007-9249-9

Nic-Can GI, Lopez-Torres A, Barredo-Pool F, Wrobel K, Loyola-Vargas VM, Rojas-Herrera R, De-la-Pena C (2013) New insights into somatic embryogenesis: LEAFY COTYLEDON1, BABY BOOM1 and WUSCHEL-RELATED HOMEOBOX4 are epigenetically regulated in *Coffea canephora*. Plos One 8:e72160. https://doi.org/10.1371/journal.pone.0072160

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) Producing decaffeinated coffee plants. Nature 423:823.

Ogita S, Uefuji Hi, Morimoto M, Sano H (2004) Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. Plant Molecular Biology 54:931–94.

Otto FJ (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewiez Z, Crissman HA, Robinson JP Methods in cell biology v. 33. Academic Press, San Diego, pp 105–110.

Pasternak T, Dudits D (2019) Epigenetic clues to better undestanding of the assexual embryogenesis in planta and in vitro. Front Plant Sci 10:778. https://doi.org/10.3389/fpls.2019.00778

Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. PNAS 91(12):5222–5226. https://doi.org/10.1073/pnas.91.12.5222

Praça-Fontes MM, Carvalho CR, Clarindo WR (2011) C-value reassessment of plant standards: an image cytometry approach. Plant Cell Rep 30:2303–2312. https://doi.org/10.1007/s00299-011-1135-6

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

Sanchez-Teyer LF, Quiroz-Figueroa FR, Loyola-Varga VM, Infante-Herrera D (2003) Culture-induced variation in plants of *Coffea arabica* cv. Caturra rojo, regenerated by direct and indirect somatic embryogenesis. Molecular Biotechnology 23:107–115.

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

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Nunes ACP, Soares TCB, Carvalho C R, Clarindo WR (2017) From chromosome doubling to DNA sequence changes: outcomes of an improved in vitro procedure developed for allotriploid Híbrido de Timor (*Coffea arabica* L. × *Coffea canephora* Pierre ex A. Froehner). Plant Cell Tiss Org 131:223–231. https://doi.org/10.1007/s11240-017-1278-4

Santana N, González ME, Valcárcel M, Canto-Flick A, Hernandez MM, Fuentes-Cerda CFJ, Barahona F, Mijangos-Cortés J, Loyola-Vargas VM (2004) Somatic embryogenesis: a valuable alternative for propagating selected robusta coffee (*Coffea canephora*) clones. In vitro Cell Dev Bio-Plant 40:95–101. https://doi.org/10.1079/IVP2003486

Santana-Buzzy N, Rojas-Herrera R, Galaz-Ávalos RM, Ku-Cauich JR, Mijangos-Cortés J, Gutiérrez-Pacheco LC, Canto A, Quiroz-Figueroa F, Loyola-Vargas VM (2007) Advances in coffee tissue culture and its practical applications. In vitro Cell Dev Bio-Plant, 43:507–520. https://doi.org/10.1007/s11627-007-9074-1

Sattler MC, Carvalho CR, Clarindo WR (2016) Regeneration of allotriploid *Coffea* plants from tissue culture: resolving the propagation problems promoted by irregular meiosis. Cytologia 81:125–132. https://doi.org/10.1508/cytologia.81.125

Schöpke C, Müller LE, Kohlenbach HW (1987) Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex. Fr.). Plant Cell Tiss Organ Cult 8:243-248.

Silva AS, Magno J, Luz Q, Rodrigues TM, Alves C (2011) Callus induction and embryo regeneration in *Coffea arabica* L. anthers by silver nitrate and ethylene. Rev. Ciênc Agron 42:921–929. https://doi.org/10.1590/S1806-66902011000400014

Sondahl MR, Romig WR, Bragin A (1995) Induction and selection of somaclonal variation in coffee. U.S. Patent No. 5,436,395. 25 Jul. 1995.

Szyrajew K, Bielewicz D, Dolata J, Wójcik AM, Nowak K, Szczygieł-Sommer A, Szweykowska-Kulinska Z, Jarmolowski A, Gaj MD (2017) MicroRNAs are intensively regulated during induction of somatic embryogenesis in *Arabidopsis*. Front Plant Sci 8:18. https://doi.org/10.3389/fpls.2017.00018

Us-Camas R, Rivera-Solís G, Duarte-Aké F, De-la-Pena C (2014) In vitro culture: an epigenetic challenge for plants. Plant Cell Tiss Organ Culture 118(2):187-201. https://doi.org/10.1007/s11240-014-0482-8

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

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57:443–462. https://doi.org/10.1093/oxfordjour-nals.aob.a087127

Zhang H, Lang Z, Zhu J-K (2018) Dynamics and function of DNA methylation in plants. Nature Reviews Molecular Cell Biology 19:489–506. https://doi.org/10.1038/s41580-018-0016-z

Capítulo IV – Nuclear DNA methylation pattern and 2,4-dichlorophenoxyacetic genotoxicity indirect somatic embryogenesis in *Coffea arabica* and *Coffea canephora*

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Abstract

Indirect somatic embryogenesis is a morphogenic pathway in which somatic cells form callus and, later, somatic embryos. 2,4-dichlorophenoxyacetic (2,4-D) is a synthetic auxin widely used in indirect somatic embryogenesis, influencing the development of the somatic embryos. During somatic embryogenesis, variations in DNA methylation patterns have been reported as fundamental for the acquisition of embryogenic competence. From indirect somatic embryogenesis in *Coffea arabica* and *Coffea canephora*, we aimed to evaluate the global methylated cytosine and the 2,4-D effect, as well as its genotoxic effect. Leaf explants were inoculated in media with different 2,4-D concentrations. After 90 days, the calli were transferred to regeneration medium of somatic embryos, and the number of normal and abnormal somatic embryos was counted monthly. The increase of the of 2,4-D concentration increased the number of responsive explants in *C. arabica* and *C. canephora*. At concentrations of 9.06, 36.24 and 54.36 μM 2,4-D, *C. arabica* presented the highest values of responsive explants, differing from *C. canephora*. The regeneration of normal and abnormal somatic embryos increased in relation to the time and 2,4-D concentrations. Global methylated cytosine levels varied at different stages of indirect somatic embryogenesis in both *Coffea*. In addition, 2,4-D concentration was correlated with global methylation levels. The abnormal somatic embryos of *C. arabica* and *C. canephora* exhibited nuclei with DNA damage, probably induced by 2,4-D. Therefore, we concluded that this synthetic auxin promotes genotoxic and phytotoxic damages in *Coffea* somatic embryos. In addition, variations in global cytosine methylation levels are an adaptive response to environmental conditions in vitro, mainly to the growth regulator - 2,4-D.

Keywords: Coffee; Epigenetic; Plant tissue culture; Comet Assay.

Introduction

Coffee is an important tropical product that has been highlighted due to it is the source of income for many developing countries. Brazil is the world's largest producer of coffee beans, with a contribution of 30% of the international market, an amount equivalent to the sum of the production of the other five largest producing countries. The genus *Coffea* comprises about 103 species (Davis et al. 2006), with *Coffea arabica* and *Coffea canephora* being the most economically important. The first is an autogamous allotetraploid species with 2n = 4x = 44 chromosomes and 2C = 2.62 pg, while the second is a allogamous diploid species with 2n = 2x = 22 chromosomes and 2C = 1.41 pg. The first in vitro approaches involving these species by somatic embryogenesis were carried out by Staritsky (1970) for *C. canephora* and by Söndahl and Sharp (1977) for *C. arabica*, aiming at the mass propagation.

Indirect somatic embryogenesis (ISE) is a morphogenic pathway that involves the callus formation and, later, of somatic embryos (SE) regeneration, in a sterile and appropriate in vitro conditions, from the inoculation of tissues from plant explant donors – based on the Totipotency Theory (Maximova et al. 2002; Fehér et al. 2003). Although totipotency is a characteristic of plant cells, not all plant cells express it (Germana and Lambardi 2016). According to Queiroz-Figueroa et al. (2006), the ability of a given tissue to generate SE is a characteristic restricted to a limited fraction of the cells. For differentiation, cells need to acquire competence and become determined to follow a new morphogenic pathway (Leite et al. 2019). A new hypothesis has been proposed by Campos et al. (2017), in which meristematic cells are able to differentiate into SE without going through the process of dedifferentiation. From in vitro approaches, it is possible to elucidate the biological issues involved in the dedifferentiation, proliferation and regeneration of SE and plantlets, allowing a detailed analysis of the genome and epigenome at different levels, as well as their interaction (Amaral-Silva et al. 2020).

The in vitro environment influences the ISE, since the morphogenetic response is determined by the action of environmental stimuli through the composition of the culture medium, phytoregulators, photoperiod and pH (Fehér et al. 2003; Zavattieri et al. 2010; Vanstraelen and Benková 2012). 2,4-dichlorophenoxyacetic (2,4-D) growth regulator is widely used in ISE in *Coffea* (van Boxtel and Berthouly 1996; Sanglard et al. 2017 and 2019), as it plays a crucial role in the process of cell dedifferentiation and SE multiplication (Lloyd et al. 1980; Pasternak 2002; Raghavan 2004; Vondráková et

al. 2011). This growth regulator is related to the cell division processes at the S input and the G_2-M transition (Teale et al. 2006), and also to the cell expansion processes (Perrot-Rechenmann 2010). However, the high exposure of the explant to 2,4-D results in the formation of abnormal somatic embryos (ASE), due to the interruption of the genetic and physiological processes of the cells, since they cause a rapid efflux of protons, enzymatic activation, transcription and translation of proteins and synthesis of polysaccharides, resulting in loss of cell wall stability, depletion of reserves and inactivation of cell repair mechanisms (Cruz et al. 1990; Stuart and McCall 1992; Tokuji and Masuda 1996; Gaj 2004; Pescador et al. 2008; Vondráková et al. 2011). The main abnormalities exhibited in SE are fusion of two or more embryos, absence of apical and root meristems, translucent embryos, multiple cotyledons and loss of bipolarity (reviewed by Garcia et al. 2019).

Although 2,4-D causes cytological, epigenetic and genetic changes in cells, such as aneuploidy or polyploidy, DNA methylation, chromosomal rearrangements, changes in DNA sequences and/or loss of nuclear DNA and promotes somaclonal variations (LoSchiavo et al. 1989; Phillips et al. 1994; Leljak-Levanić et al. 2004; Bairu et al. 2011; Fraga et al. 2012; Fehér 2015), this growth regulator is the most used in most embryogenic systems that require the initial presence of auxins in the pre-incubation or induction stages (van Boxtel and Berthouly 1996; Sanglard et al. 2017 and 2019; Bychappa et al. 2019; Amaral-Silva et al. 2020). In *Coffea*, the use of this growth regulator causes genomic and epigenomic changes in plants regenerated in vitro, leading to loss of genetic fidelity and the formation of ASE, which may be undesirable in plant propagation programs (Landey et al. 2013 and 2015; Etienne et al. 2016; Bychappa et al. 2019). For example, Etienne et al. (2016) observed phenotypic variants in *C. arabica* trees regenerated in vitro induced by aneuploidy. Bychappa et al. (2019) analyzed that *C. arabica* plants regenerated in vitro did not show genetic fidelity with their mother plant, since the differential fragments amplified by SRAP primers in the regenerated plants revealed the occurrence of somaclonal variation in the coding region with functional attribution to zinc finger protein. Landey et al. (2013) revealed through molecular analyzes that genetic and epigenetic changes are particularly limited during somatic embryogenesis in *C. arabica*, and that the main modification in most phenotypic variants is aneuploidy, indicating that mitotic changes play a major role in somaclonal variation in *Coffea*. Later, Landey et al. (2015) when identifying the mechanisms associated with somaclonal variation, showed that the methylation

polymorphism in *C. arabica* plantlets regenerated in vitro was low and varied between 0.087 and 0.149% and that aneuploidy (monosomy) was systematically found in phenotypic variants. In addition, the authors revealed that the allopolyploid structure of *C. arabica* allowed aneuploid cells to survive and regenerate viable plants.

Epigenetics is a regulatory mechanism sensitive to the environment that influences the development of the organism and the process of its inheritance (Yakovlev et al. 2011; Bräutigam et al. 2013; Wang et al. 2020). During somatic embryogenesis, DNA methylation, histones and miRNAs are susceptible to epigenetic changes, since it regulates/controls morphogenetic processes in vitro (Nic-Can et al. 2013; Fehér 2015; Griffiths et al. 2015; Amaral-Silva et al. 2020; Wang et al. 2020). These epigenetic variations are transient and can be constantly altered through DNA methylation, acetylation, phosphorylation, methylation and ubiquitination of histones (Becker and Weigel 2012; Bräutigam et al. 2013; Fehér 2015; Wang et al. 2020). However, to promote and stimulate cell proliferation and the formation of SE and plantlets, genetic and epigenetic factors need to be in coordination (Smulders and de Klerk 2011; Miguel and Marum 2011; Leite et al. 2019). When genetic and epigenetic factors are not in coordination, the main problem of somatic embryogenesis occurs, which is the large-scale production of abnormal embryos (Márquez-López et al. 2017; Garcia et al. 2019). As embryo abnormalities tend to be associated with physiological disorders and/or somaclonal variations where point mutations or epigenetic changes can influence the development of the embryo and, consequently, the resulting plant morphology (Bobadilla Landey et al. 2015; Márquez-López et al. 2017; Garcia et al. 2019), it is essential to quantify the levels of global methylation during the entire process of indirect somatic embryogenesis and to evaluate the genotoxic effect of 2,4-D in the regeneration of SE in *Coffea*. In addition, it is important to relate 2,4-D concentrations to methylation levels, given that this growth regulator promotes genetic and epigenetic changes. The detection of genetic changes and the genotoxic effect of 2,4-D in ASE can be verified by means of comet assays that allow the detection of very small changes - not yet fully determined - cell by cell in the DNA structure, providing a more detailed analysis of the genome (Koppen et al. 1999). To date, the comet assay has not been used with this approach in plant tissue culture.

Thus, we aimed in *C. arabica* and *C. canephora* to: (a) quantify the levels of global methylated cytosine during ISE, (b) determine the best concentration of 2,4-D in the induction and proliferation of friable callus, well as its genotoxic effect in the

regeneration of SE, and (c) relate the concentrations of 2,4-D whint levels of global methylation and genetic changes.

Material and methods

Biological material

C. arabica and *C. canephora* plantlets kept in vitro (Universidade Federal do Espírito Santo, Espírito Santo, Brazil) were used as explant donor. These plantlets have been cultivated in medium consisting of 4.3 g L^{-1} basal MS (Murashige and Skoog, 1962) salts, 10 mL L^{-1} B5 vitamins, 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel and pH = 5.6 (Sanglard et al. 2019). Leaves were excised for ISE, nuclear DNA content measurement by flow cytometry, ploidy level determination (Sanglard et al. 2019) and global methylated cytosine (Amaral-Silva et al. 2020).

Callogenesis

Five leaf fragments (2 cm^2) of each *C. arabica* and *C. canephora* explant donor plant were individually inoculated in Petri dishes containing 15 mL friable callus induction medium constituted with 2.15 g L^{-1} ½ MS basal, 10 mL L^{-1} B5 vitamins, 30 g L^{-1} sucrose, 0.08 g L^{-1} L-cysteine, 0.4 g L^{-1} malt extract, 0.1 g L^{-1} hydrolyzed casein, 4.44 μM 6-benzylaminopurine (BAP), 2.8 g L^{-1} Phytagel, pH = 5.6, and supplemented with 9.06, 36.24, 54.36 and 72.48 μM of 2,4-D. Ten Petri dishes for each 2,4-D concentration were prepared for *C. arabica* and *C. canephora*. The culture media were sterilized at 121°C and 1.5 atm for 20 min. Petri dishes were maintained in the dark at 25 ± 2°C for 90 days. Friable callus formation was evaluated biweekly and after 90 days some samples were collected to extract genomic DNA and determine the level of global methylation.

Embryogenesis and plantlet regeneration

Friable callus of *C. arabica* and *C. canephora* were individually inoculated in Petri dishes containing 15 mL of SE regeneration medium consisting of 4.3 g L^{-1} basal MS, 10 mL L^{-1} B5 vitamins, 30 g L^{-1} sucrose, 0.04 g L^{-1} L-cysteine, 0.8 g L^{-1} malt extract, 0.2 g L^{-1} hydrolyzed casein, 4.44 μM BAP, 2.8 g L^{-1} Phytagel, and 4 g L^{-1} activated charcoal, pH = 5.6 (Sanglard et al. 2019), totaling 60 repetitions for *C. arabica*

and 44 for *C. canephora*. The culture media were autoclaved at 121°C and 1.5 atm for 20 min and changed every three months. Petri dishes were kept in the dark at 25 ± 2°C for 240 days. The regeneration of normal mature cotyledon somatic embryo (MCSE) and ASE was monthly evaluated. Samples of embryogenic callus, MCSE and ASE were collected to extract genomic DNA and determine the level of global methylation. In addition, the MCSE and ASE samples were used for the comet assay.

The MCSE were individually transferred to a test tube containing plantlet regeneration medium consisting of 4.3 g L^{-1} basal MS, 10 mL L^{-1} B5 vitamins, 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel and pH = 5.6 (Sanglard et al. 2019). Culture media were autoclaved at 121°C and 1.5 atm for 20 min. Test tubes were maintained at 24°C ± 2°C under a 16:8 h (light/dark) cycle with light irradiation of 36 μmol m^{-2} s^{-1} provided by two fluorescent lamps (20 W, Osram®).

Global methylated cytosine

The collected samples (leaf from the donors of explants, friable callus, embryogenic callus, MCSE and ASE) were macerated separately in the MagNALyser (Roche®, Germany) for 60 s at 7,000 rpm. Genomic DNA was extracted according to Doyle and Doyle (1990), with modifications, adding 7.5 M ammonium acetate and excluding night precipitation. The concentration, purity and integrity of the DNA was determined by electrophoresis on 0.8% agarose gel and by a NanoDrop spectrophotometer (Thermo Scientific® 2000c). 15 μg of genomic DNA was diluted in 50 μL of dH_2O and then 25 μL of 70% (v/v) perchloric acid was added and the samples were hydrolyzed for 1 h in a dry bath at 100°C. The pH of the hydrolysates was adjusted between 3 and 5 with KOH 1.0 mol L^{-1} solution (Demeulemeester et al. 1999; Chen et al. 2013). In this step, the formation of a precipitate, white solid, of potassium perchlorate ($KClO_4$) occurred. Subsequently, the hydrolysates and precipitates were centrifuged at 10,000 rpm for 5 min. The supernatant (hydrolysates) was collected and transferred to a new microtube. The microtube containing the precipitate was washed twice with 200 μL of dH_2O and centrifuged for 10,000 rpm for 5 min. Then, the supernatant was transferred to the microtube containing the hydrolysate. The samples were placed in SpeedVac. After solvent evaporation, the samples were stored in the refrigerator. To determine the global methylation level, the lyophilized samples were suspended in 100 μL of dH_2O and analyzed in high performance liquid chromatography

(HPLC Shimadzu®, model LC-20AT) equipped with a photodiode matrix detector (SPD - M20A) using a column of reverse phase C_{18} based on silica (4.6 × 250 mm, 5 μm), according to Demeulemeester et al. (1999).

From 9.06, 36.24, 54.36 and 72.48 μM of 2,4-D, the global methylation cytosine was measured for: a) 20 samples of friable callus of *C. arabica* and *C. canephora*, totaling 5 repetitions for each treatment; b) 12 samples of embryogenic callus of *C. arabica* and *C. canephora*, totaling 3 replicates for each treatment; c) 12 ASE samples from *C. arabica*, totaling 3 replicates for each treatment; d) 3 samples of *C. canephora* ASE from 72.48 μM 2,4-D because in other concentrations there was not enough biological material; e) 3 MCSE samples from *C. arabica* and *C. canephora*; and f) 3 samples of explant donors from *C. arabica* and *C. canephora*.

Comet assay

To perform the Comet assay, the slides were prepared one day before, immersed in normal 1% agarose at 60°C, dried and stored in 4°C. The MCSE and the ASE of *C. arabica* and *C. canephora* were individually collected and chopped with a razor blade in 300 μL of nuclear isolation solution (400 mM Tris-HCl pH 7.5, 20% polyethylene glycol – PEG). The resulted suspension was filtered through a 30 μm nylon filter and kept in the dark for 5 min. Subsequently, 40 μL of the nuclear suspension and 60 μL of 1% low-melting agarose at 39°C was mixed and placed onto the slide. The slides were covered with a coverslip (24 x 50 mm) and incubated at 4°C for 15 min, then at -22°C for 1 min. The coverslips were removed and the slides incubated in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris) for 15 min. Then, the slides were transferred to a previously cooled electrophoresis through containing alkaline electrophoresis buffer (250 mM Tris, 10 mM NaOH and 1 mM EDTA, pH 12), where they were incubated for 5 min for DNA unfolding and then electrophoresed at 18 V for 15 min. After electrophoresis, the slides were incubated in neutralization buffer (400 mM Tris, pH 7.5) for 15 min and then stained with 100 μL of acridine orange 50 mM for 15 min, and washed in cold dH_2O. The slides were analyzed under a 20x objective accoupled in a fluorescence microscope Olympus BX-60 (Olympus, Tóquio, Japão) and nucleoids images were captured with a Photometrics CoolSNAP Pro cf (Roper Scientific, Tucson, AZ). 300 nucleoids per slide were evaluated, totaling 4 repetitions for the MCSE of *C.*

arabica and *C. canephora* and 4 repetitions for the ASE of *C. arabica* and *C. canephora* regenerated from friable callus in 9.06, 36.24, 54.36 and 72.48 μM of 2,4-D.

Statistical analysis

ISE responses of *C. arabica* and *C. canephora* were compared during the stages of callus formation and of SE regeneration. During the callogenesis, the number of responsive explants of *C. arabica* and *C. canephora* were evaluated at 15, 30, 45, 60, 75 and 90 days for the 9.06, 36.24, 54.36 and 72.48 μM of 2,4-D,. The mean values were compared from analysis of variance (ANOVA) and Tukey's test ($P < 0.05$). Then, a regression analysis ($P < 0.05$) was applied using the R software (Core Team 2016). The number of regenerated MCSE and ASE were evaluated at 15, 30, 45, 60, 75 and 90 days, applying ANOVA. The data did not present a normal distribution and the mean values were compared using the Bonferroni test ($P < 0.05$). Then, a regression analysis ($P < 0.05$) was applied using the R software (Core Team 2016).

To understand the influence of global methylation cytosine during the ISE in *C. arabica* and *C. canephora*, the global 5-mC% level was measured in donor explant plants, friable callus, embryogenic callus, ASE and MCSE. ANOVA was applied to compare the global methylation cytosine level of friable callus, embryogenic callus, ASE and MCSE of *C. arabica* and *C. canephora* that were originated from the induction medium supplemented with 9.06, 36.24, 54,36 and 72.48 μM 2,4-D. The mean values were compared using the Tukey's test ($P < 0.05$). A new ANOVA was applied disregarding the factor 2,4-D to assess and compare the levels of global methylation in all stages of the ISE (explant donor, friable callus, embryogenic callus, ASE and MCSE), since there was no material vegetable available in all concentrations of 2,4-D during the different stages of ISE and also to give an overview of the patterns of global methylation throughout the process of ISE. The mean values were compared using the Tukey's test ($P < 0.05$).

The influence of the 2,4-D on the ASE regeneration of *C. arabica* and *C. canephora* was verified from the Comet Assay performed with MCSE (control) and ASE regenerated from friable callus originated in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 μM 2,4-D. The number of nucleoids with DNA damage was counted and, later, ANOVA was applied. The mean values were compared using the Tukey's test ($P < 0.05$).

Results

Callogenesis

C. arabica and *C. canephora* exhibited a distinct mean number of responsive explants (leaf fragment with callus) over time influenced by the friable callus induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 μM 2,4-D (Figs. 1 and 2). The first responsive explants of *C. arabica* and *C. canephora* were observed at 30 days for all media of callus induction and proliferation. *C. arabica* exhibited the highest mean number of responsive explants in the media supplemented with 9.06, 36.24 and 54.36 μM of 2,4-D at all times evaluated, differing of *C. canephora* (Fig. 3). At the concentration of 72.48 μM, the mean number of responsive explants of *C. arabica* and *C. canephora* were statistically equal (Fig. 3).

When analyzing the *Coffea* species individually, it is observed that the 2,4-D concentrations influenced the mean number of responsive explants in *C. arabica* at 30 days (Fig. 4). 36.24 μM of 2,4-D exhibited the highest mean number of responsive explant and differed from 9.06 and 72.48 μM (Fig. 4). After 30 days, the number of responsive explants of *C. arabica* was statistically equal (Fig. 4). When analyzing *C. canephora*, it is observed that the concentrations of 2,4-D influenced the mean number of responsive explants at all times evaluated (Fig. 3). 72.48 μM 2,4-D exhibited the highest mean number of responsive explant at all times evaluated, differing from the 36.24 and 54.36 μM, and these greater than the 9.06 μM concentration (Fig. 4). The callus proliferation of *C. arabica* and *C. canephora* gradually increased at 60 days, becoming visually stable at 90 days. In addition, all callus showed a pale yellow and friable appearance (Fig. 1).

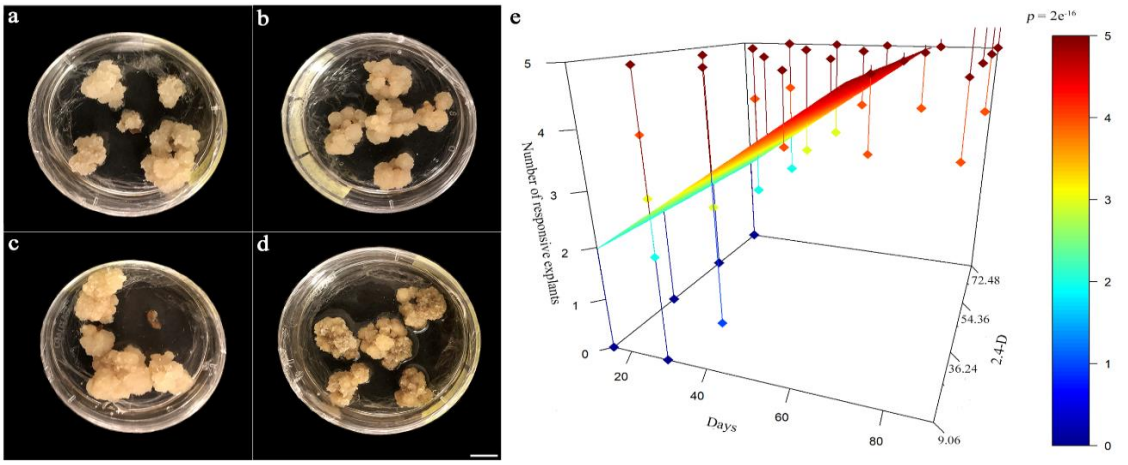

Fig. 1 – Response surface showing the influence of time (in days) and 2,4-D concentration (in µM) on the mean number of responsive explants of *C. arabica*. (e) The graph shows that the mean number of responsive explants increased in relation to the exposure time and the concentration of 2,4-D, as can be seen in the figure opposite. Friable callus of *C. arabica* originated in induction medium supplemented with 9.06 (a), 36.24 (b), 54.36 (c) and 72.48 (d) µM of 2,4-D. The adjusted quadratic model was significant ($P < 0.05$) by regression analysis: Z = 0.56 + 0.052X -0.1X2+ 0.54Y. Bar = 1 cm.

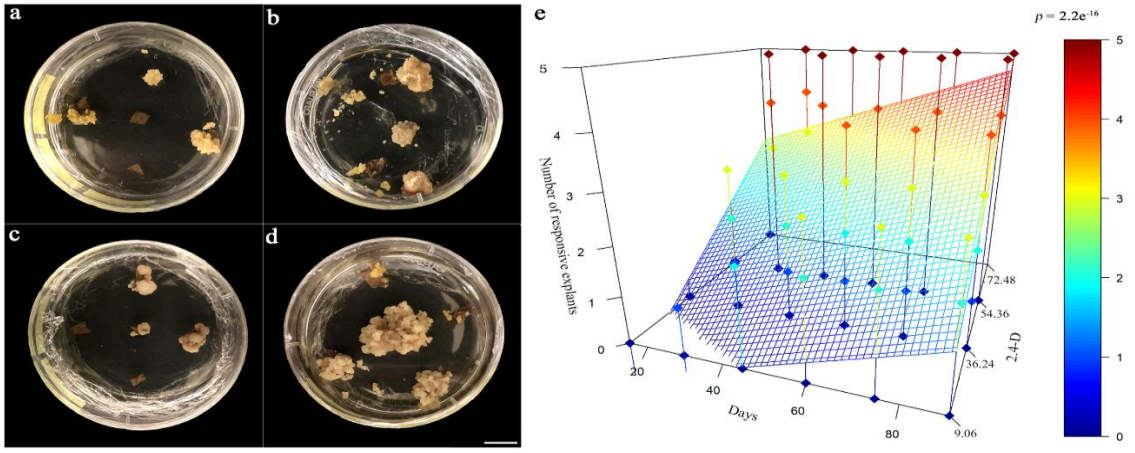

Fig. 2 – Response surface showing the influence of time (in days) and 2,4-D concentration (in µM) on the mean number of responsive explants of *C. canephora*. (e) The graph shows that the mean number of responsive explants increased in relation to the exposure time and the concentration of 2,4-D, as can be seen in the figure opposite. Friable callus of *C. canephora* originated in induction medium supplemented with 9.06 (a), 36.24 (b), 54.36 (c) and 72.48 (d) µM of 2,4-D. The adjusted quadratic model was significant ($P < 0.05$) by regression analysis: Z = -1.15 + 0.026X + 0.25X2-0.11Y. Bar = 1 cm.

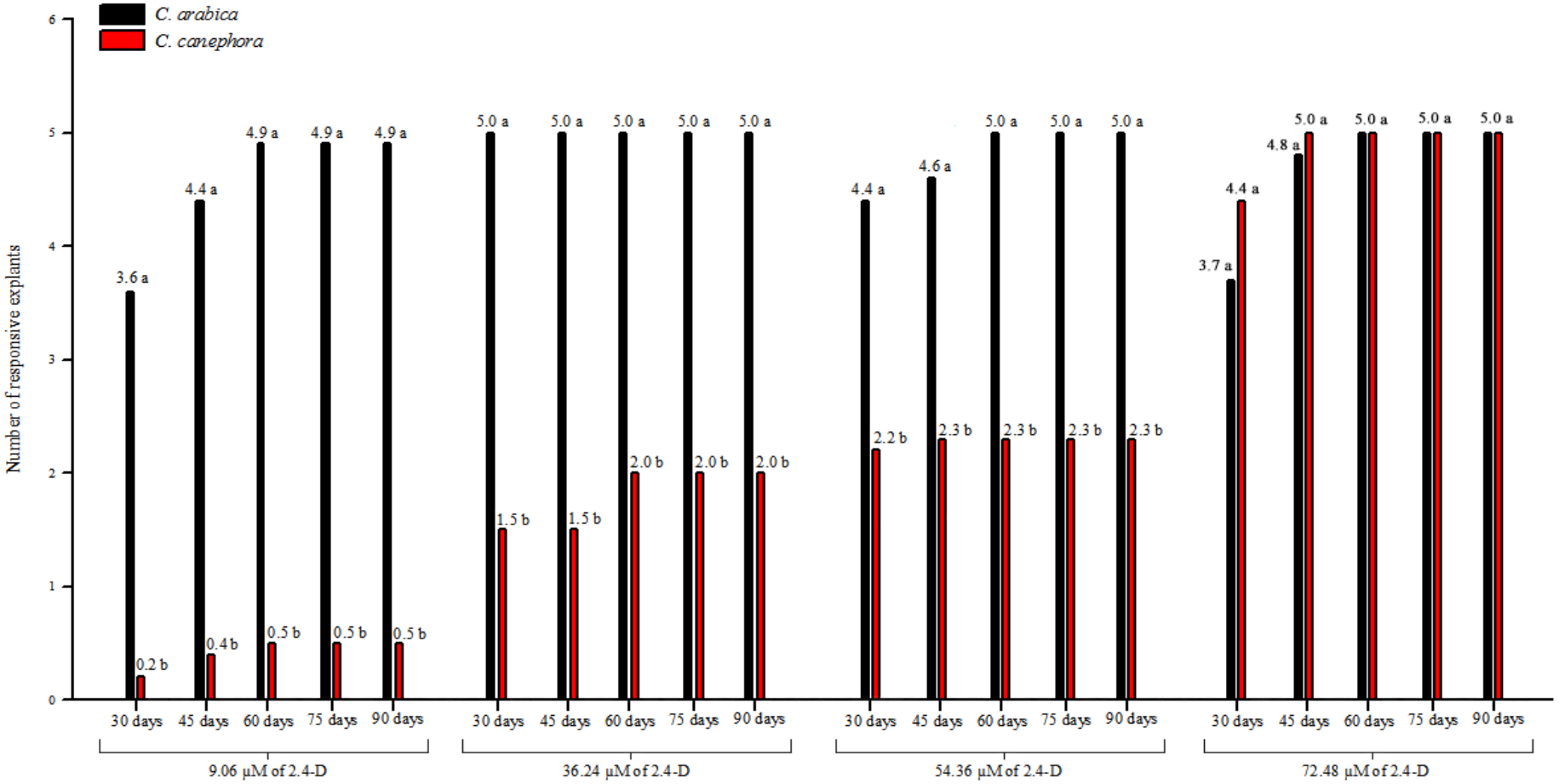

Fig. 3 – Comparison of the mean number of responsive explants of *C. arabica* and *C. canephora* in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D at 30, 45, 60, 75 and 90 days. The graph shows that the mean number of responsive explants was higher in *C. arabica* than in *C. canephora* at all times evaluated at concentrations of 9.06, 36.24 and 54.36 µM 2,4-D by the test Tukey's ($P < 0.05$). At the concentration of 72.48 µM, there was no significant difference.

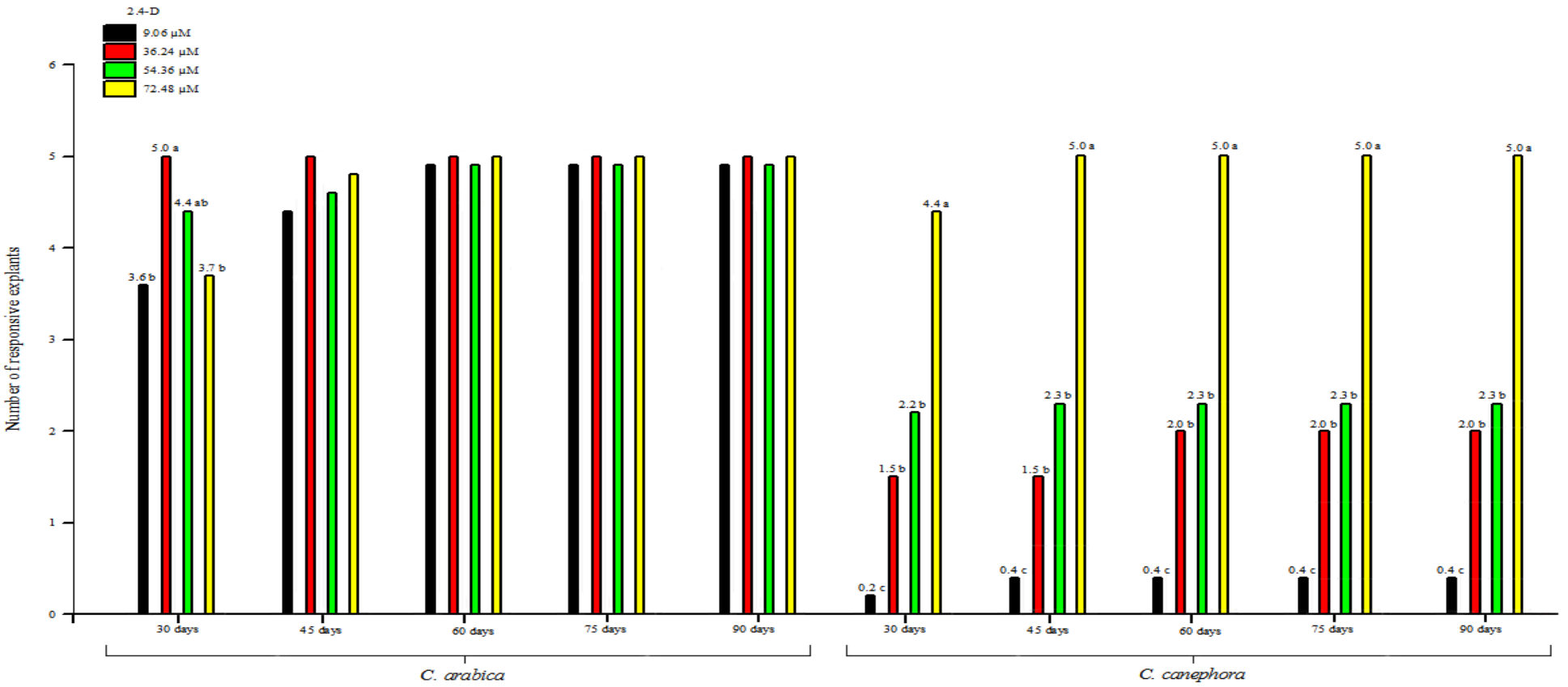

Fig. 4 – Comparison of the mean number of responsive explants in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D in *C. arabica* and *C. canephora* at 30, 45, 60, 75 and 90 days. In *C. arabica*, at 30 days the median number of responsive explants was higher at the concentration of 26.24 µM 2,4-D and differed from the concentration of 9.06 µM.. After 30 days, 2,4-D concentrations were statistically equal. In *C. canephora*, 2,4-D concentrations influenced the induction of responsive explants at all times evaluated. The highest mean number of responsive explants was observed at a concentration of 72.48 µM 2.4-D.

Embryogenesis and plantlet regeneration

During the SE regeneration stage, *C. arabica* and *C. canephora* exhibited different MCSE mean values and, in addition, ASE regeneration was observed. The origin of the friable callus (9.06, 36.24, 54.36 or 72.48 μM 2,4-D) and the time (30, 60, 90, 120, 150, 180, 210 and 240 days) influenced the regeneration of MCSE and ASE from *C. arabica* and *C. canephora* (Figs. 5 and 6). The first MCSE and ASE were observed at 60 days for *C. arabica* with a mean of 0.05 MCSE and 0.03 ASE per callus, and at 90 days for *C. canephora* with a mean of 0.05 MCSE and 0.07 ASE per callus (Figs. 5 and 6). The regeneration of MCSE and ASE for both *Coffea* species increased over time, according to the concentrations of 2,4-D to which the friable callus was originated and exposed (Figs. 5 and 6).

C. arabica exhibited the highest mean number of MCSE and differed from *C. canephora* at concentrations of 9.06 and 72.48 μM 2,4-D (Fig. 7a). MCSE regeneration increased over time for all 2,4-D concentrations, but only at the concentration of 72.48 μM, a significant difference was observed (Fig. 7b). The greatest regeneration of MCSE was observed at 210 days in *C. arabica* and subsequently remained constant (Fig. 8a). In *C. canephora*, MCSE regeneration increased progressively up to 240 days (Fig. 8a). *C. arabica* regenerated more MCSE than *C. canephora* up to 150 days. Subsequently, *C. canephora* regenerated more MCSE than *C. arabica* and differed at 240 days (Fig. 8b).

Friable callus of *C. arabica* that originated in the culture medium with 72.48 μM 2,4-D exhibited mean values of 10.7, 16.7, 24, 44.1 and 47.5 ASE per callus in 120 , 150, 180, 210 and 240 days, respectively, differing from C. canephora, which presented an mean value of 1.5, 6, 12.1, 18.6 and 19.9 ASE per callus (fig. 9).

Friable callus of *C. arabica* in concentration of 72.48 μM 2,4-D exhibited mean values of 10.7, 16.7, 24, 44.1 and 47.5 ASE per callus at 120, 150, 180, 210 and 240 days, respectively, differing from *C. canephora*, which showed an mean value of 1.5, 6, 12.1, 18.6 and 19.9 ASE per callus (Fig. 9). However, concentration of 9.06 μM 2,4-D, the friable callus of *C. arabica* regenerated more ASE than *C. canephora* at 180, 210 and 240 days, showing an mean value of 8.4, 17.9 and 17.9 ASE per callus, respectively (Fig. 9). The regeneration of ASE in *C. arabica* increased with time for all concentrations of 2,4-D, but only at concentrations of 9.06 and 72.48 μM, significant differences were observed. In these concentrations, ASE regeneration increased up to

210 days and showed mean values of 17.9 and 45.5 ASE per callus (Fig. 10). In *C. canephora*, ASE regeneration increased over time and differed in the concentration of 72.48 μM 2,4-D (Fig. 10).

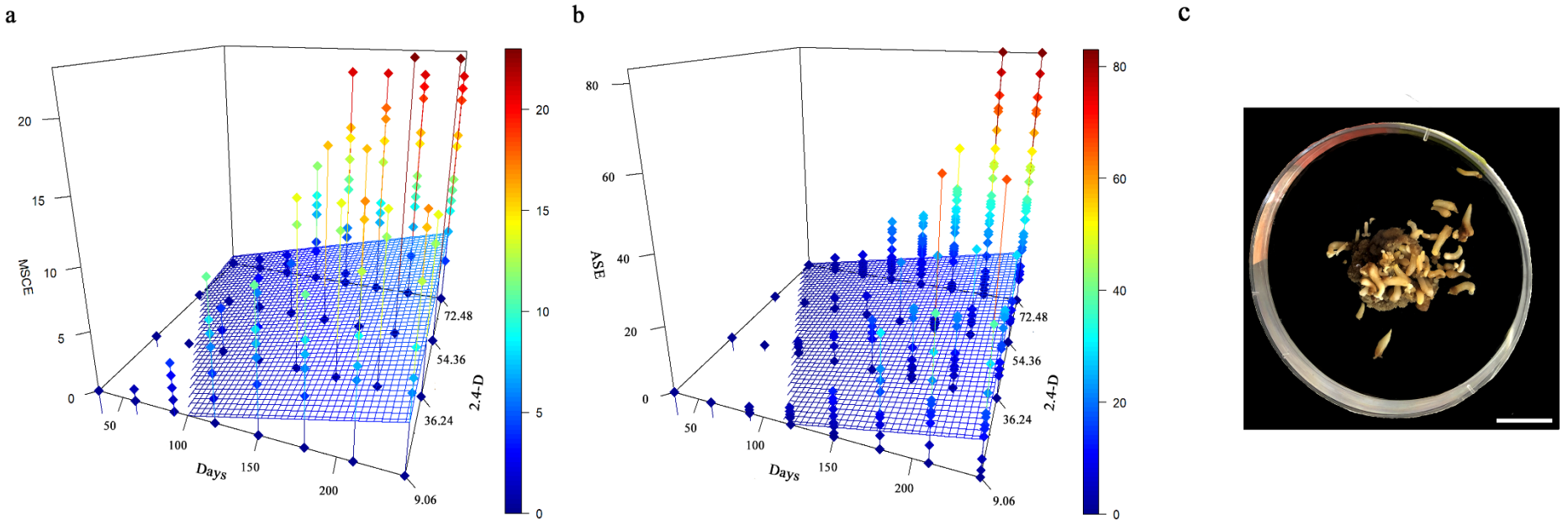

Fig. 5 – Response surface showing the influence of time (in days) and the origin of friable callus exposed to 9.06, 36.24, 54.36 and 72.48 µM de 2,4-D on SE regeneration in *C. arabica*. Mean number of MCSE (a) and ASE (b) increased over time according to the 2,4-D concentrations to which the friable callus were exposed (9.06, 36.24, 54.36 and 72.48 µM). (c) Embryogenic callus of *C. arabica* with the presence of MCSE and ASE. The adjusted quadratic model was significant ($P < 0.05$) by regression analysis: (a) $Z = 1.99 + 0.53X + 3.76X^2 – 8.21Y$; and (b) $Z = 2.46 + 0.99X + 6.03X^2 – 13.009Y$. Bar = 1 cm.

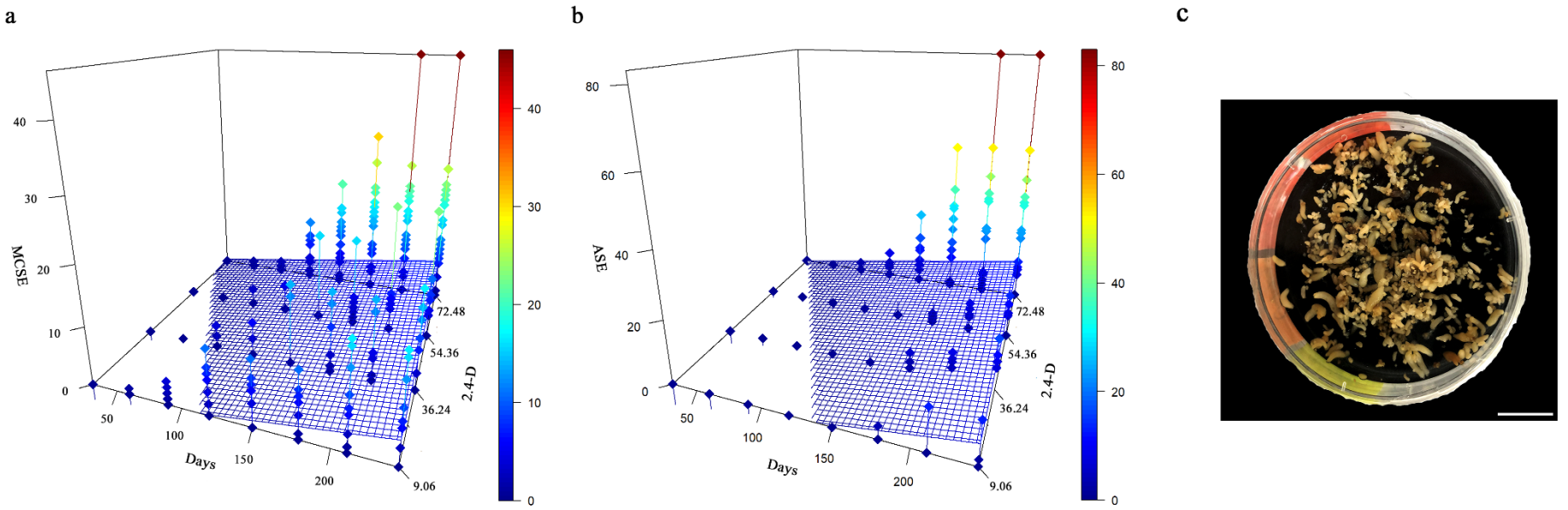

Fig. 6 – Response surface showing the influence of time (in days) and the origin of friable callus exposed to 9.06, 36.24, 54.36 and 72.48 µM de 2,4-D on SE regeneration in *C. canephora*. Mean number of MCSE (a) and ASE (b) increased over time according to the 2,4-D concentrations to which the friable callus were exposed (9.06, 36.24, 54.36 and 72.48 µM). (c) Embryogenic callus of *C. canephora* with the presence of MCSE and ASE. The adjusted quadratic model was significant ($P < 0.05$) by regression analysis: (a); $Z = 2.46 + 0.99X + 6.03X^2 – 13.009Y$ and (b) $Z = – 4.4 + 0.85X – 0.026X^2 + 0.802Y$. Bar = 1 cm.

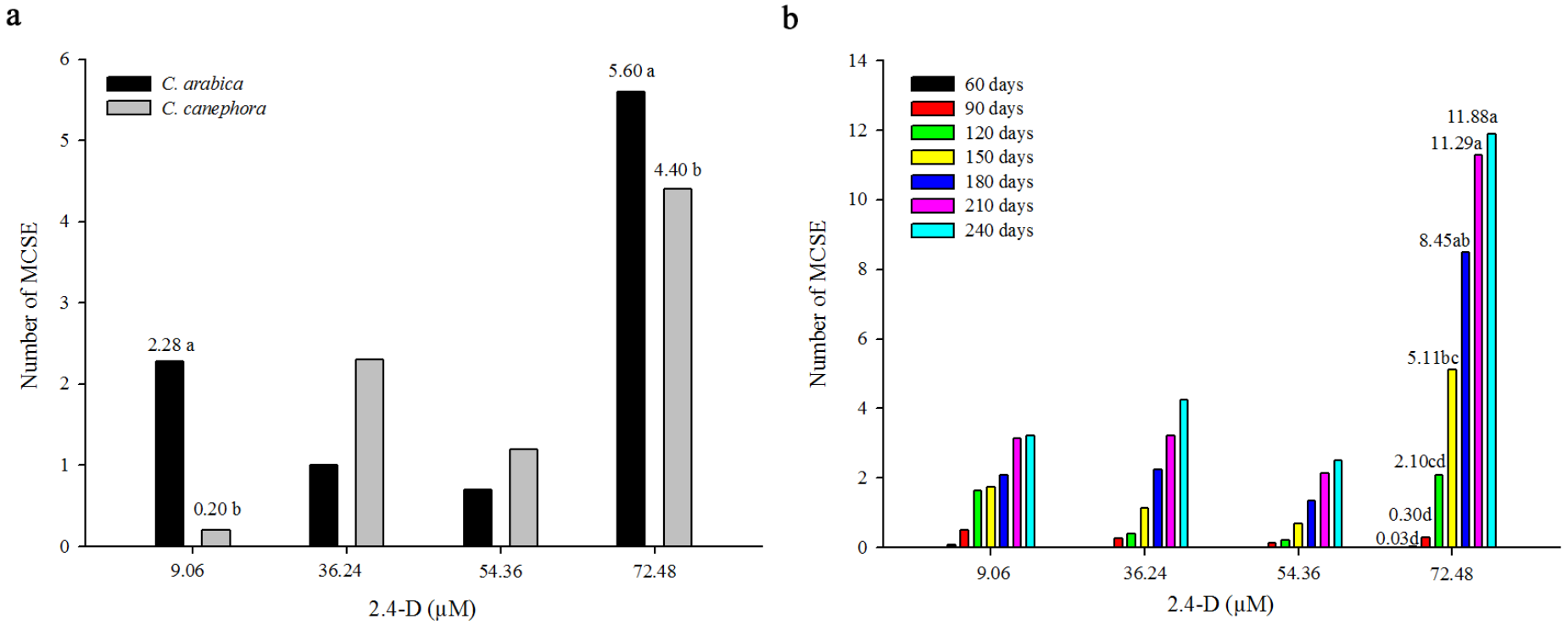

Fig. 7 – (a) Graph shows that the MCSE regeneration of *C. arabica* and *C. canephora* was influenced by the origin of the friable callus supplemented with 9.06, 36.24, 54.36 and 72.48 µM of 2,4-D. At concentrations of 9.06 and 72.48 µM 2,4-D, *C. arabica* exhibited a greater number of MCSE and differed from *C. canephora*. (b) Graph shows that MCSE regeneration over time (60, 90, 120, 150, 180, 210 and 240 days) was influenced by the origin of the friable callus (9.06, 36.24, 54.36 and 72.48 µM 2,4-D). The number of MCSE has increased over time. However, it was only at the concentration of 72.48 µM that time significantly influenced in mean number of MCSE. *Means followed by the same lowercase letter did not differ statistically by the Bonferroni test ($P < 0.05$).

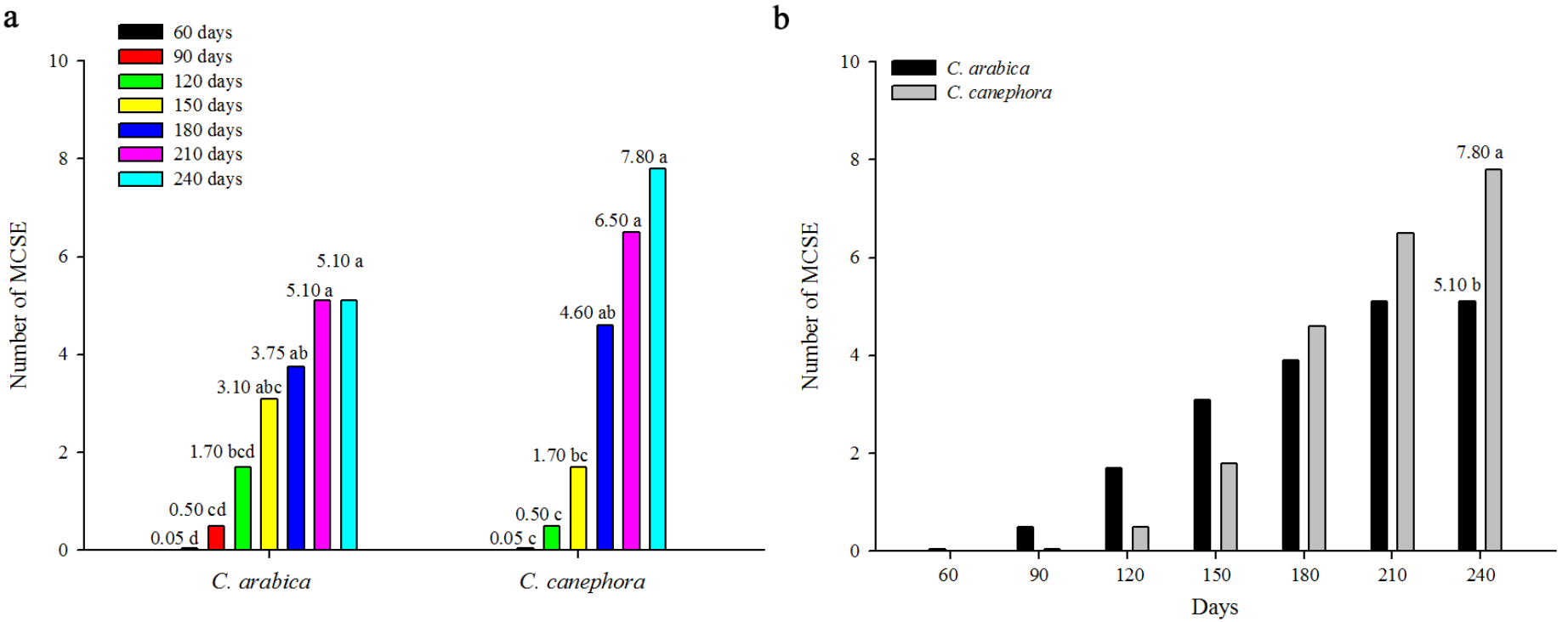

Fig. 8 – Graphs show that the number of MCSE of *C. arabica* and *C. canephora* increased over time (60, 90, 120, 150, 180, 210 and 240 days) and differed statistically. (a) The greatest number of MCSE of *C. arabica* was observed at 210 days, after that day it remained constant. In *C. canephora*, the largest number of MCSE was observed at 240 days. (b) *C. canephora* exhibited the highest mean number of MCSE at 240 days, differing from *C. arabica*. *Means followed by the same lowercase letter did not differ statistically by the Bonferroni test ($P < 0.05$).

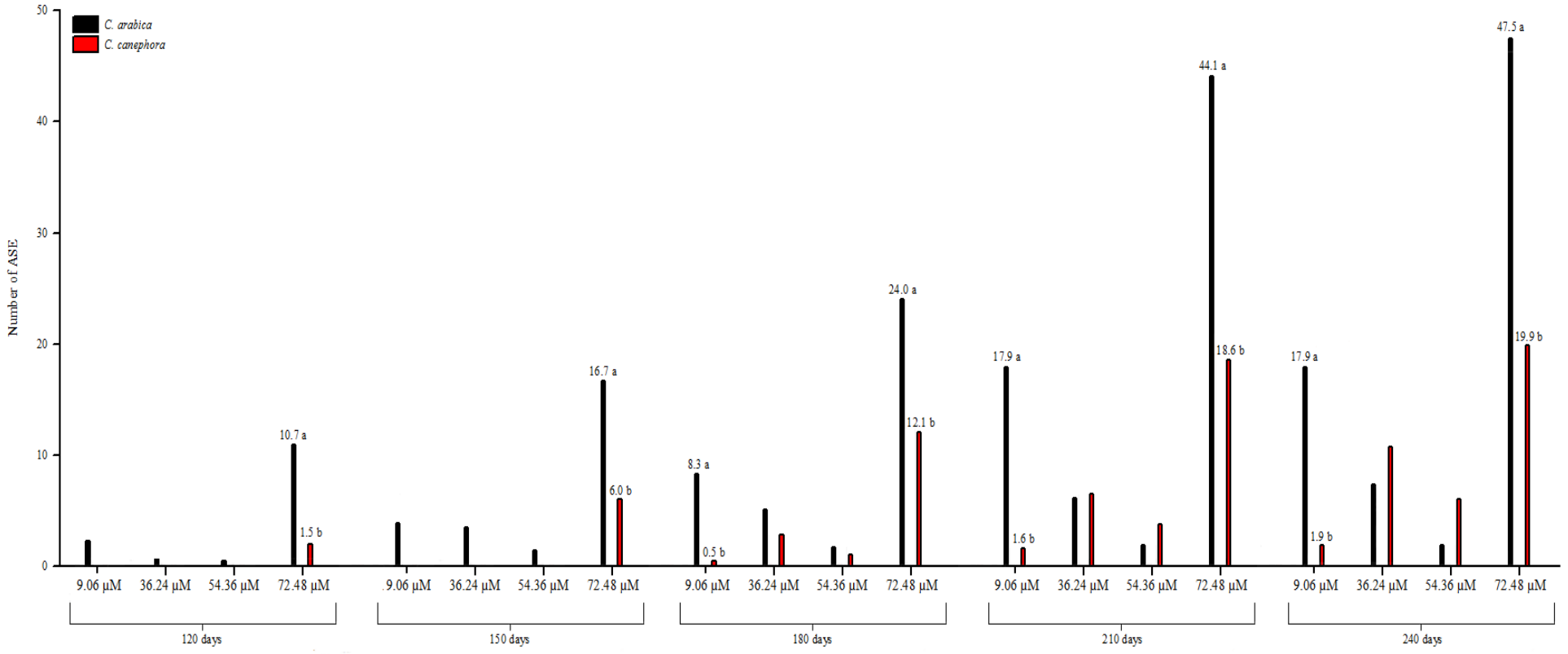

Fig. 9 – Comparison of the mean number of ASE regenerated from friable callus originated in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM of 2,4-D in *C. arabica* and *C. canephora* at 120, 180, 210 and 240 days. *Means followed by the same lowercase letter did not differ statistically by the Bonferroni test ($P < 0.05$).

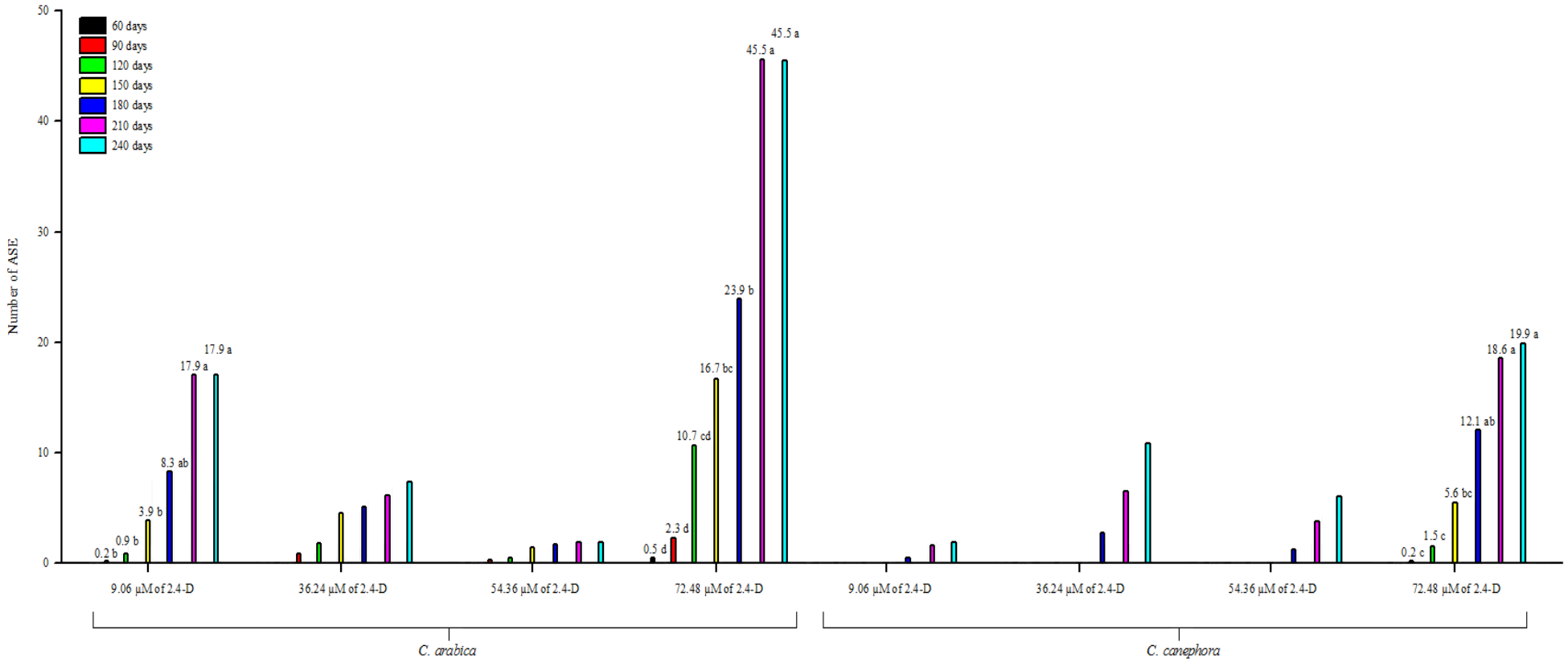

Fig. 10 – Graph shows that the ASE regeneration of *C. arabica* and *C. canephora* over time (60, 90, 120, 150, 180, 210 and 240 days) was influenced by the origin of the friable callus (9.06, 36.24, 54.36 and 72.48 µM 2,4-D). *Means followed by the same lowercase letter did not differ statistically by the Bonferroni test ($P < 0.05$).

Global DNA methylation

The friable callus of *C. arabica* and *C. canephora* originated in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 μM of 2,4-D showed different values of global methylation at 90 days (Fig. 29). In the concentration of 36.24 μM 2,4-D, the friable callus of *C. arabica* exhibited the highest levels of global methylation, with a mean of 21.80% and differed from the friable callus induced in culture medium with 72.48 μM 2,4-D, which showed an mean value of 11.50% (Fig. 11a). In *C. canephora*, the friable callus induced in a culture medium with 72.48 μM 2,4-D exhibited the highest levels of global methylation, with an mean of 24.20%, differing from the friable callus induced with 36.24 and 54.36 μM 2,4-D, with a mean of 12.80 and 14.60%, respectively (Fig. 11a). The levels of global methylation of friable callus differed between *Coffea* species. The levels of global methylation of friable callus differed between Coffea species. The friable callus of *C. arabica* induced with 36.24 μM 2,4-D exhibited higher levels of global methylation, with a mean of 21.80%, and differed from *C. canephora*, which had a mean value of 12.80% (Fig. 11b). At the concentration of 72.48 μM 2,4-D, the friable callus of *C. canephora* presented the highest level of global methylation, with a mean of 24.20%, differing from the friable callus of *C. arabica*, which presented mean values of 11.50% (Fig. 11b).

The origin of the friable callus did not interfere in the global methylation level of the embryogenic callus of *C. arabica* and *C. canephora*, and therefore the interaction was not significant. However, it was observed that *Coffea* species exhibited distinct values of global methylation. The embryogenic callus of *C. canephora* exhibited mean values of 44% of global methylation, while the embryogenic callus of *C. arabica* exhibited mean values of 26.80% (Fig. 12a).

The origin of the friable callus (9.06, 36.24, 54.36 and 72.48 μM of 2,4-D) interfered with the global methylation levels of MCSE and ASE of *C. arabica*. The global methylation increased in the ASE according to the concentrations of 2,4-D (Fig. 12b). MCSE and ASE regenerated with 9.06 μM 2,4-D exhibited a lower level of methylation, with a mean of 8.9 and 11.3%, and differed from the regenerated ASE with 54.36 and 72.48 μM 2,4-D, with a mean of 18.4 and 24.4%, respectively (Fig. 12b). In addition, the ASE regenerated with 36.24 μM of 2,4-D exhibited a lower level of global methylation, differing from the ASE regenerated with 72.48 μM of 2,4-D (Fig. 12b). Not possible to determine the gobal methylation level in the ASE of *C. canephora*

regenerated from friable callus with 9.06, 36.24 and 54.36 μM of 2,4-D, since the biological material was not sufficient.

The levels of global methylation varied in the different stages and responses of the ISE (explant donor, friable callus, embryogenic callus, ASE and MCSE) in *C. arabica* and *C. canephora* (Fig. 13). The embryogenic callus of *C. arabica* exhibited higher levels of global methylation, with a mean of 26.8%, and differed from MCSE, friable callus and ASE, which presented mean values of 8.9, 16.6 and 24.4%, respectively (Fig. 13a). In addition, *C. arabica* explant donors exhibited higher methylation values, with a mean of 20.6%, differing from MCSE (Fig. 13a). The embryogenic callus of *C. canephora* showed a mean value of 44% methylation and differed from the MCSE, explant donor and friable callus, which presented mean values of 11, 16.4 and 18.1%, respectively (Fig. 13b). In addition, ASE exhibited higher methylation values, with a mean of 22%, differing from MCSE (Fig. 13b). When comparing *C. arabica* and *C. canephora* during each moment of the ISE, it is observed that there was a significant difference only between the embryogenic callus. *C. canephora* exhibited a mean value of 44% of global methylation, while *C. arabica* exhibited a mean value of 26.8%.

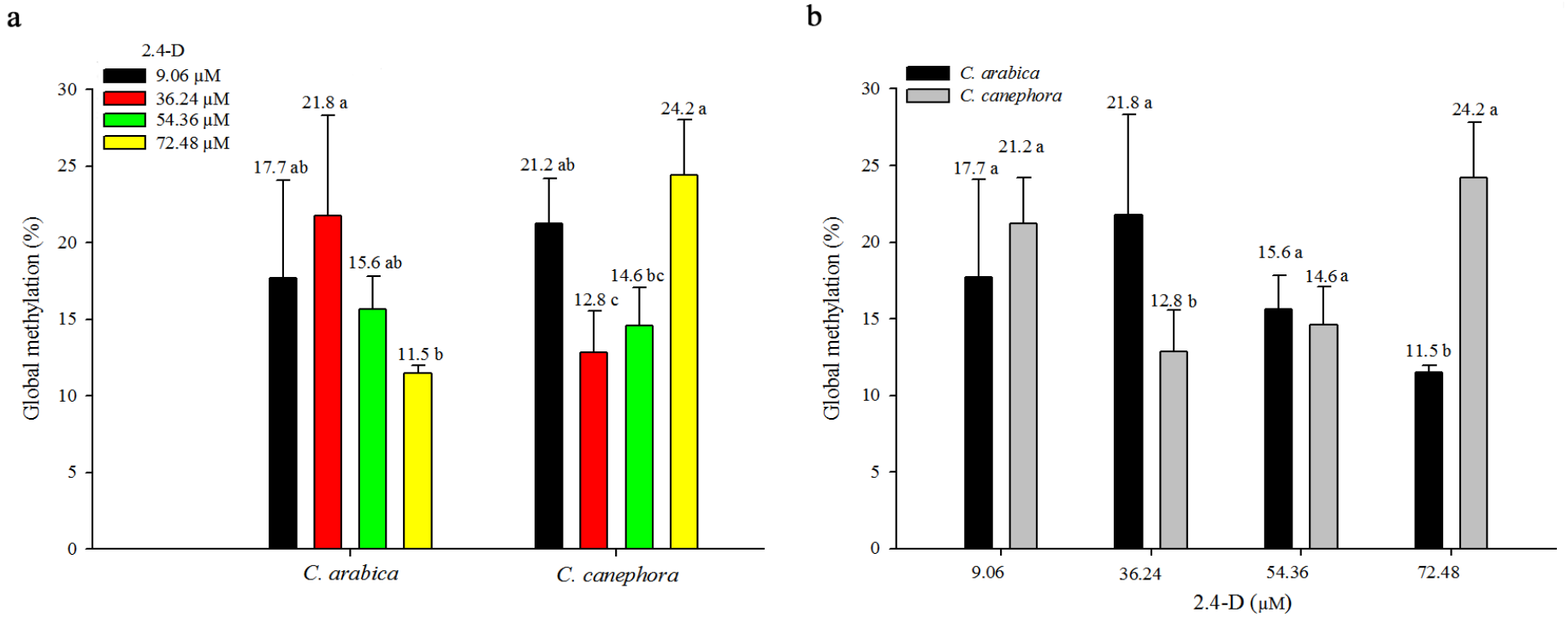

Fig. 11 – Graphs show the global methylation level of the friable callus of *C. arabica* and *C. canephora* originated from the induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D. (a) Comparison of the global DNA methylation level of friable callus originated in induction medium with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D in *C. arabica* and *C. canephora*. (b) Comparison of the global DNA methylation level of friable callus originated in induction medium with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D between *C. arabica* and *C. canephora*. *Means followed by the same lowercase letter did not differ statistically by the Tukey's test ($P < 0.05$).

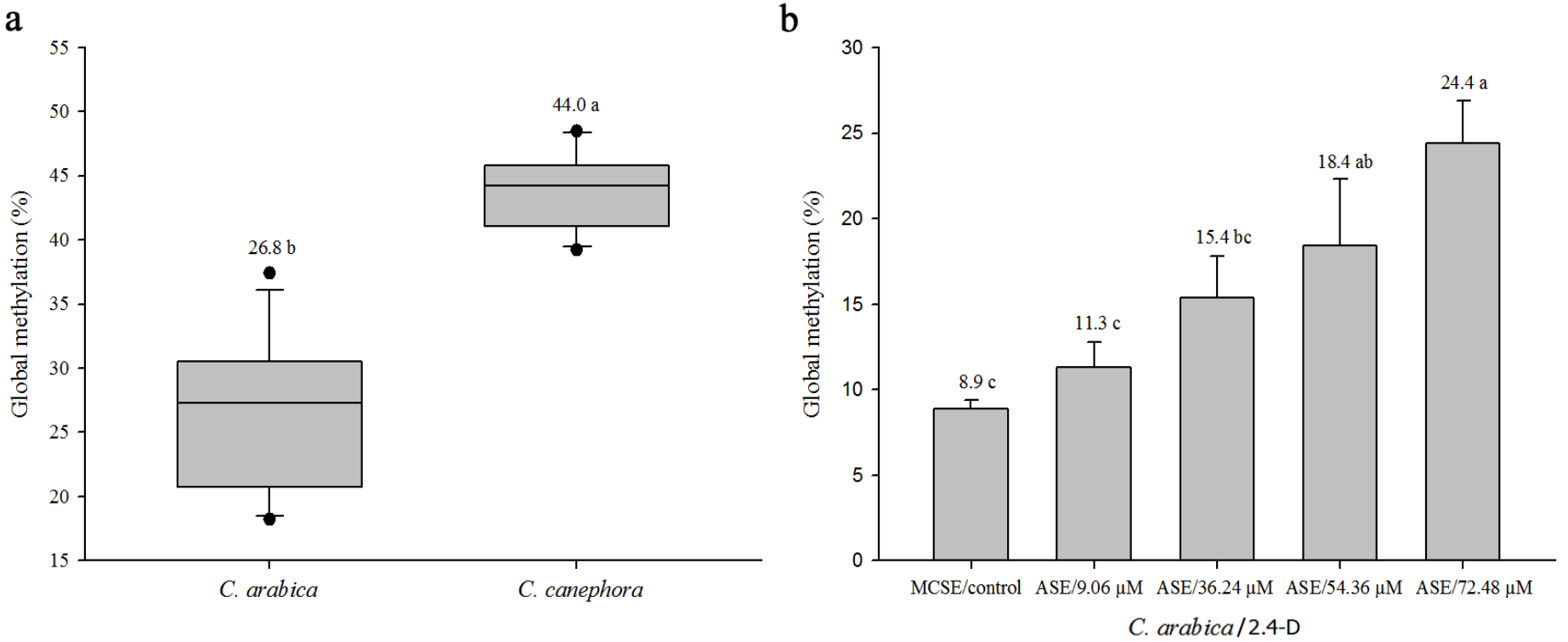

Fig. 12 – (a) Levels of global DNA methylation in embryogenic callus of *C. arabica* and *C. canephora*. The embryogenic callus showed different values of global methylation and differed statistically. *C. canephora* exhibited a mean value of 44% and *C. arabica* 26.8%. Origin of the friable callus (9.06, 36.24, 54.36 and 72.48 µM of 2,4-D) did not interfere in the levels of global methylation of embryogenic callus. (b) Levels of global DNA methylation in MCSE and ASE of *C. arabica* regenerated from friable callus originated in induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM of 2,4-D. *Means followed by the same lowercase letter did not differ statistically by the Tukey's test ($P < 0.05$).

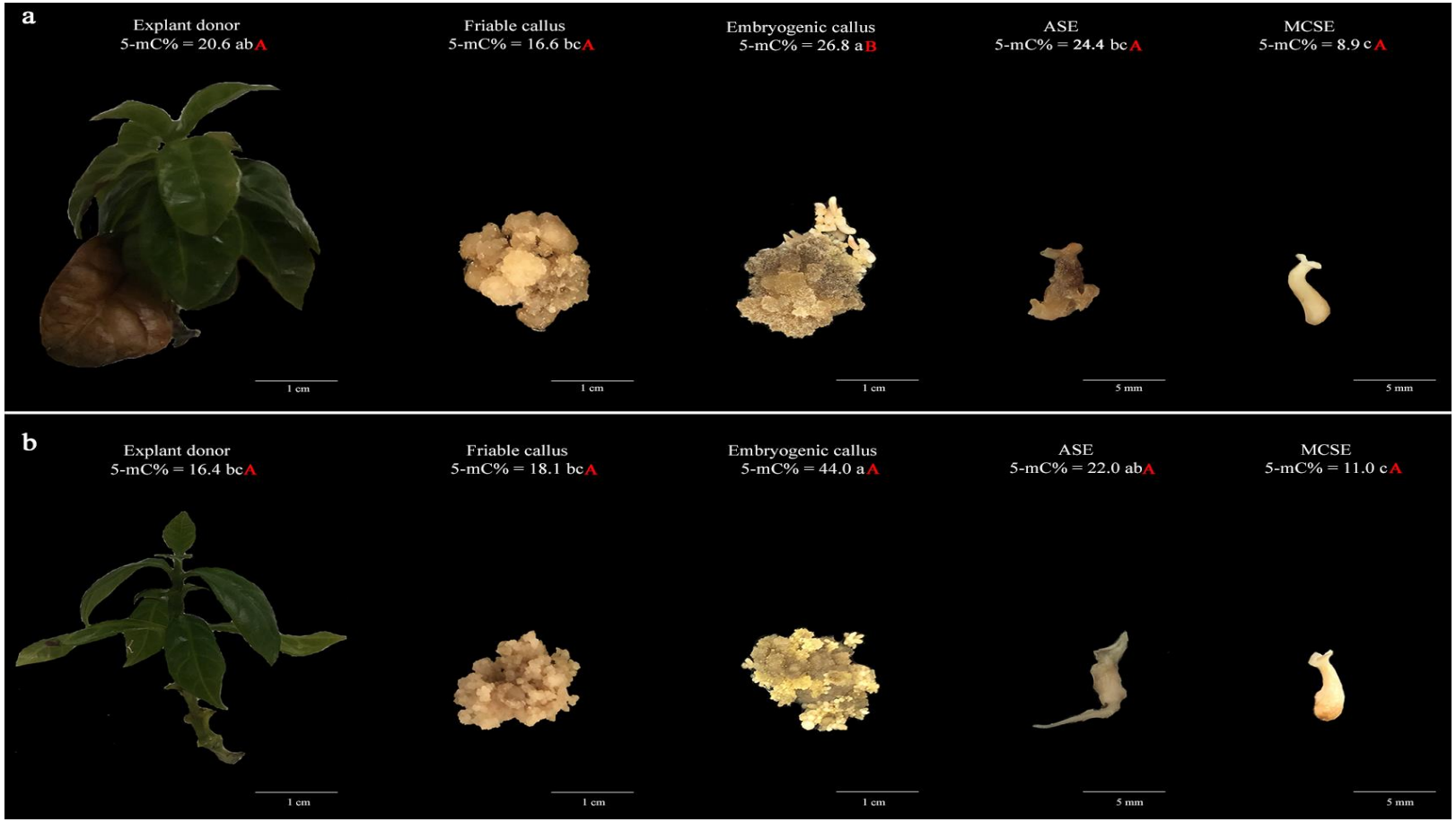

Fig. 13 – Determination of global DNA methylation levels during the establishment of ISE in *C. arabica* and *C. canephora*. (a) Comparison of the global methylation levels in *C. arabica* with the different stages and response of ISE (explant donor, friable callus, embryogenic callus, ASE and MCSE). (b) Comparison of the global methylation levels in *C. canephora* with the different stages and response of ISE (explant donor, friable callus, embryogenic callus, ASE and MCSE). (axb) Comparison of the global levels of methylation between the species *C. arabica* and *C. canephora* with the different stages and response of the ISE. *Means followed by the same lowercase letter, in the horizontal do not differ statistically, by the Tukey's test ($P < 0.05$). *Means followed by the same capital letter, in the vertical do not differ statistically, by the Tukey's test ($P < 0.05$).

Comet assay

MCSE and the ASE of *C. arabica* and *C. canephora* exhibited nucleus with DNA damage due to the genotoxic action of 2,4-D (Fig. 14). In *C. arabica*, MCSE exhibited a mean number of 9.8 nuclei with DNA damage, followed by ASE regenerated with 9.09 μM 2,4-D, with a mean of 49.8 and, later, by ASE regenerated with 36.24, 54.36 and 72.48 μM of 2,4-D, with a mean of 94, 98.2, 104 and 104.3 nuclei with DNA damage, respectively (Fig. 14a). In *C. canephora*, the lowest mean number of nuclei with DNA damage was observed in MCSE that differed from ASE (Fig. 14a). ASE of *C. canephora* exhibited higher mean values of nuclei with DNA damage in all concentrations of 2,4-D and differed from *C. arabica* (Fig. 14b) . While the MCSE of *C. arabica* and *C. canephora* were statistically equal (Fig. 14b).

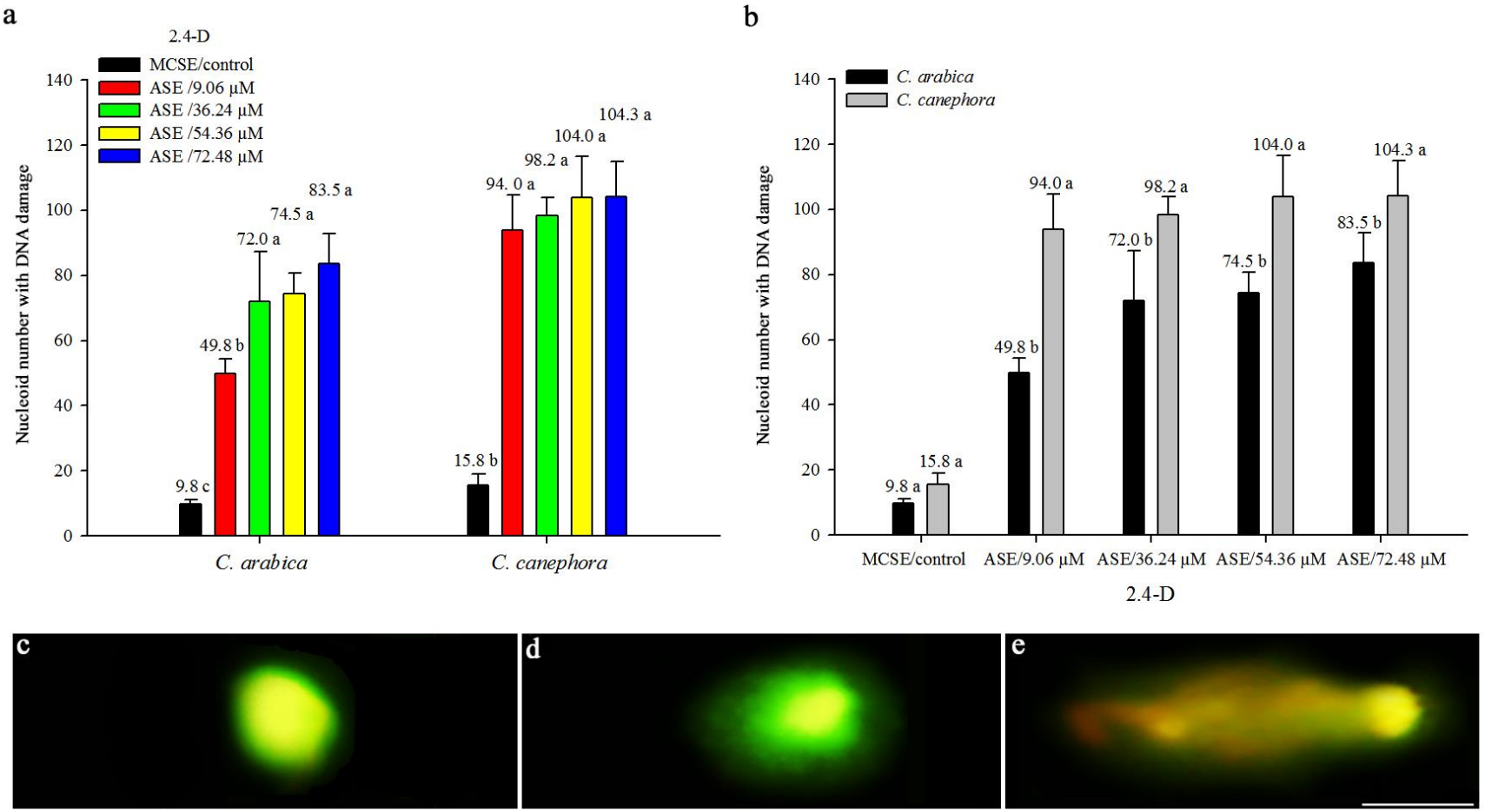

Fig. 14 – Graphs show the mean number of nuclei with DNA damage in MCSE (control) and ASE of *C. arabica* and *C. canephora* that were regenerated from friable callus originated in an induction medium supplemented with 9.06, 36.24, 54.36 and 72.48 µM 2,4-D. (a) Comparison of the mean number of nuclei with DNA damage in MCSE and ASE of *C. arabica* and *C. canephora* in function of 2,4-D concentrations. (b) Comparison of the mean number of nucleoids with DNA damage between MCSE and ASE of *C. arabica* and *C. canephora*.(c) Nucleus without DNA damage. (d) Nucleus with moderate DNA damage. (e) Nucleus with severe DNA damage. Bar = 5 µm.

Discussion

The ISE was established for *C. arabica* and *C. canephora* based on a procedure proposed by Sanglard et al. (2019), involving the stages of callus induction and proliferation (callogenesis), followed by regeneration SE (embryogenesis) and plantlets. Basically, the modifications were the addition of 36.24, 54.36 and 72.48 μM 2,4-D in the callus induction and proliferation medium, and also the absence of gibberellic acid in the plantlets recovery medium. This tissue culture procedure allowed to verify the immediate effect of 2,4-D in the induction and proliferation of friable callus of *C. arabica* and *C. canephora*, as well as its remaining effect during the SE regeneration stage, in embryogenic callus, MCSE and ASE. In addition, the in vitro procedure also made it possible to: to compare *Coffea* species based on the in vitro response (mean number of responsive explants, mean number of MCSE, ASE and response time); determine and compare the levels of global methylation of donors of explants, friable callus, embryogenic callus, MCSE and ASE; and associate the level of global methylation with ISE and concentrations of 2,4-D.

The addition of 2,4-D in the medium of induction and proliferation of friable callus influenced the rate and time of obtaining responsive explants of *C. arabica* and *C. canephora*, as well as the levels of global methylation. Furthermore, it showed that in *C. arabica* the lowest concentration of 2,4-D is sufficient to reach 100% responsive explants, while *C. canephora* requires the highest concentration of 2,4-D to reach 100% responsive explants. These different 2,4-D needs to induce ISE can be attributed to genetic and epigenetic factors. Once *C. arabica* (true allotetraploid, 2C = 2.62 pg and 2n = 4x = 44 chromosomes) contains twice the chromosome set of *C. canephora* (diploid, 2C = 1.41 pg 2n = 2x = 22 chromosomes) and probably, has more genes that encode the proteins involved in auxin metabolism (Pérez-Pascual et al. 2018; Saptari and Susila 2019), and also because the friable callus exhibited distinct global methylation values that are important factors affecting ISE (Fehér 2015). Variations in global DNA methylation levels have already been reported in somatic embryogenesis and are associated with plant growth regulators and the in vitro environment (Miguel and Marum 2011; Yang et al. 2013).

2,4-D is a synthetic substance analogous to the auxin class and it is widely used in plant tissue culture to induce ISE (van Boxtel and Berthouly 1996; Ardiyani and Sulistyani 2017; Bartos et al. 2018; Sanglard et al. 2019). This substance is responsible

for triggering the cellular transition processes from the somatic to embryogenic state, and this process is complex and includes de-differentiation, reactivation and cell division, in addition to metabolic and developmental reprogramming, conferring competence to the cells present in the explants (Fehér 2015). However, the excess of 2,4-D can cause extreme epigenetic and genetic changes in cells, such as methylation and DNA mutations, compromising ISE, since there must be a balance between auxin and cytokinin (LoSchiavo et al. 1989; Leljak-Levanić et al. 2004; Fraga et al. 2012; Fehér 2015). Variations in global methylation levels were observed in the present study due to the addition of 2,4-D. In friable callus of *C. arabica*, for example, the highest concentration of 2,4-D showed the lowest level of global methylation, whereas in the friable callus of *C. canephora*, the highest concentration of 2,4-D resulted in the highest level methylation. Therefore, it is observed that methylation is a complex and variable mechanism, and that the addition of 2,4-D will not always result in an increase in global methylation, since each species behaves differently. For example, in cell suspension cultures of *Daucus carota* L., LoSchiavo et al. (1989) showed a positive correlation between the mean values of global methylation and the concentrations of 2,4-D. In addition, the authors showed the strong and reversible effect of these exogenous auxins on global methylation and, consequently, on cell proliferation.

During the SE regeneration stage it was observed that embryogenic callus exhibited higher levels of global methylation than friable callus and that this increase was not caused by the addition of 2,4-D. In addition, *Coffea* species showed different values of global methylation. The increase in global methylation levels in *C. arabica* and *C. canephora* is associated with a remodeling of chromatin from the euchromatic to the heterochromatic state. Similar results have been reported by Fraga et al. (2012) when analyzing the levels of global methylation during the ISE in *Acca sellowiana*, showed that in the regeneration stage of SE there is an global increase in those of methylation. However, contrasting results have been reported by Chakrabarty et al. (2003), who, when assessing cytosine methylation patterns during ISE in *Eleutherococcus senticosus*, revealed that embryogenic callus have a lower level of global methylation compared to non-embryogenic callus. But in general, as reviewed by Karim et al. (2015), generally global hypermethylation of DNA is associated with somatic embryogenesis, while hypomethylation is associated with suppression of somatic embryogenesis.

The origin of the friable callus (9.06, 36.24, 54.36 and 72.48 μM of 2,4-D) influenced the regeneration and the levels of global methylation in SE of *C. arabica* and *C. canephora*. The highest concentrations of 2,4-D resulted in the highest mean number of MCSE and ASE. In addition, in *C. arabica*, the addition of 2,4-D increased the levels of global methylation in ASE. Studies already prove that auxins, mainly 2,4-D, are necessary for the induction of ISE and multiplication of SE in the search to potentiate/optimize the regeneration of plantlets (Lloyd et al. 1980; Pasternak 2002; Raghavan 2004; Vondráková et al. 2011), but they must be removed in the stages of expression, development and maturation, since the presence of 2,4-D hinders the development of the embryo and its subsequent conversion into a plant (Pasternak 2002; Zavattieri et al. 2010).

Although 2,4-D was eliminated from the SE regeneration medium in the present study, it was observed that the friable callus presented excess 2,4-D and that this accumulation inside the cells causing ASE regeneration. The main abnormalities exhibited in the SE of *C. arabia* and *C. canephora* were the fusion of two or more embryos, lack of apical and radical meristems, translucent embryos, multiple cotyledons and loss of bipolarity. The formation of ASE has already been reported in different species such as *Medicago truncatula* (Tvorogova et al. 2015), *Theobroma cacao* (Chanatásig 2004), *Vinca herbacea* (2008) and *Melia azedarach* (Vila et al. 2010), and abnormalities were similar to that described in this work. According to Guerra et al. (1999), the pattern of development of a SE is characterized by the differentiation of a bipolar structure consisting of stem and root apex, passing through the stages of pre-embryonic and embryonic development, globular, cordiform, torpedo and cotyledonary in eudicotiledonea.

Our data show that a positive correlation was observed with the increase of 2,4-D concentrations with the levels of global methylation in ASE regeneration. Probably, the addition of 2,4-D increased the levels of global methylation and, consequently, silenced the genes involved in embryonic development *met1* (*DNA methyltransferase 1*), *cmt 2* and *3* (*chromomethylase 2* and *3*), *drm 1* and *2* (*domain rearranged methyltransferase 1* and *2*), *kyp* (*kryptonite, histone H3 lysine 9 methyltransferase*), *jmj14* (*jumonji 14, histone H3 lysine 4 demethylase*), *wu*s (*wuschel*), *lec 1* and *2* (*leafy cotyledon 1* and *2*), *fus3* (*fusca3*), *tir1* (*transport inhibitor response 1*), *cuc1* (*cup-shaped cotyledon 1*) and *wind1* (*wound-induced dedifferentiation 1*), *serk* (*somatic embryogenesis receptor like kinase*), *bbm1* (*baby boom 1*) and *agl15* (*agamous-like 15*),

that control in vitro induced morphogenic processes (Karim et al. 2016), causing physiological disturbances and malformations. According to Garcia et al. (2019), the abnormalities of the SE are associated with physiological disorders and/or somaclonal variations, where mutations or epigenetic changes influence the embryonic development and, consequently, the morphology of the resulting plants.

To assess and understand the abnormalities present in the SE of *C. arabica* and *C. canephora*, in addition to quantifying the levels of global methylation, comet assays were carried out to check the DNA degradation caused by the in vitro environment, mainly by adding 2,4-D. Our data show that *C. canephora* is more sensitive to 2,4-D and *C. arabica* has a certain tolerance in the concentration of 9.06 μM since the ASE exhibited lower mean values of nucleoids with DNA damage. Therefore, it is observed that each species of *Coffea* reacts differently to the environment in vitro, and that the genetic characteristics, as well as the evolution of the species, influence the response in vitro (Sanglard et al. 2019). *C. arabica* probably showed a certain tolerance to 2,4-D because it is a recent polyploid from an evolutionary point of view and its genome is still unstable. *C. canephora* is a diploid, progenitor of *C. arabica* and its genome is more stable and, therefore, for the preservation of the genetic content, cells with abnormalities, regardless of their degree of severity, present in ASE entered the apopitotic path more quickly.

In general, our data show that the condition of tissue culture in vitro alters DNA methylation/demethylation patterns in *C. arabica* and *C. canephora*, since global methylation levels decrease in friable callus and increase in embryogenic callus and, later, demethylation occurs in MCSE and ASE. In addition, the explant donor's inherent predisposition can also affect DNA methylation and consequently the in vitro response. Similar results were reported by De-la-Pena et al. (2012), showing that the conditions in vitro alter the methylation patterns in *Agave angustifolia* and *Agave fourcroydes*, and that this change depends on the species studied and the environmental conditions. Therefore, it is observed that to establish the somatic embryogenesis requires complex cellular, biochemical and molecular processes. Epigenetics has a fundamental role in somatic embryogenesis, since it is a mechanism of genetic regulation that influences in vitro morphogenetic processes.

Conclusion

The addition of 2,4-D to the culture medium stimulates the induction and proliferation of friable calluses in *C. arabica* and *C. canephora* and consequently increases the regeneration of SE. However, high concentrations of 2,4-D promote morphological and epigenetic changes and furthermore cause genetic disorders in cells, compromising the development of SE. *C. canephora* is more sensitive to the action of 2,4-D and, therefore, requires greater attention and care with in vitro conditions. As observed in this study, DNA methylation is a susceptible, dynamic and variable mechanism to the in vitro environment. For the establishment of ISE to occur, epigenetic variations are necessary during the induction and proliferation of friable corns and SE regeneration in *Coffea*.

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References

Ardiyani F, Sulistyani P (2017) "Morphological variation of somatic embryos of *Coffea arabica* L. during dome sub-culture periods." Pelita Perkebunan 33(2):97-108. https://doi.org/10.22302/iccri.jur.pelitaperkebunan.v33i2.263

Bartos PMC, Gomes HT, Amaral LV, Teixeira JB, Scherwinski-Pereira JE (2018) Biochemical events during somatic embryogenesis in *Coffea arabica* L. 3 Biotech 8(4):209. https://doi.org/10.1007/s13205-018-1238-7

Becker C, Weigel D (2012) Epigenetic variation: origin and transgenerational inheritance. Curr Opin Plant Biol 15(5):562–567. https://doi.org/10.1016/j.pbi.2012.08.004

Bräutigam K, Vining KJ, Lafon-Placette C, Fossdal CG, Mirouze M, Marcos JG, Fluch S, Fraga MF, Guevara MÁ, Abarca D, Johnsen Ø, Maury S, Strauss SH,

Campbell MM, Rohde A, Díaz-Sala C, Cervera MT (2013) Epigenetic regulation of adaptive responses of forest tree species to the environment. Ecol Evol 3(2):399–415. https://doi.org/10.1002/ece3.461

Chakrabarty D, Yu KW, Paek KY (2003) Detection of DNA methylation changes during somatic embryogenesis of siberian ginseng (*Eleuterococcus senticosus*). Plant Sci 165(1):61–68. https://doi.org/10.1016/S0168-9452(03)00127-4

Chanatásig CI (2004) Inducción de la embriogénesis somática en clones superiores de cacao (*Theobroma cacao* L.), con resistencia a enfermedades fungosas. CATIE 1–86. Retrieved from http://orton.catie.ac.cr/repdoc/A0275e/A0275e.pdf

Chen Q, Tao S, Bi X, Xu X, Wang L, Li X (2013) Research of total levels on DNA methylation in plant based on HPLC analysis. American Journal of Molecular Biology 3:98-101. https://doi.org/10.4236/ajmb.2013.32013

Cruz GS, Canhoto JM, Abreu MAV (1990) Somatic embryogenesis and plant regeneration from zygotic embryos of *Feijoa sellowiana* Berg. Plant Sci 66:263–270.

De-la-Pena C, Nic-Can G, Ojeda G, Herrera-Herrera J, Lopez-Torres A, Wrobel K, Robert-Diaz M (2012) *KNOX1* is expressed and epigenetically regulated during in vitro conditions in *Agave* spp. BMC Plant Biol 12(1):203. http://www.biomedcentral.com/1471-2229/12/203

Demeulemeester MAC, Stallen NV, Droft PMP (1999) Degree of DNA methylation to chicory (*Cichorium intybus* L.): influence of plant age and vernalization. Plant Science 142:101-108. https://doi.org/10.1016/S0168-9452(99)00010-2

Fehér A (2015) Somatic embryogenesis stress-induced remodeling of plant cell fate. Biochim et Biophys Acta (BBA)-Gene Regul Mech 1849:385–402. https://doi.org/10.1016/j.bbagrm.2014.07.005

Fehér A, Pasternak T, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228.

Fraga HPF, Vieira LN, Caprestano CA, Steinmacher DA, Micke GA, Spudeit DA, Pescador R, Guerra MP (2012) 5-Azacytidine combined with 2,4-D improves somatic embryogenesis of *Acca sellowiana* (O. Berg) Burret by means of changes in global DNA methylation levels. Plant Cell Rep 31:2165–2176. https://doi.org/10.1007/s00299-012-1327-8

Gaj M (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul 43:27–47.

Garcia C, Almeida A-AF, Costa M, Britto D, Valle R, Royaert S, Marelli J-P (2019) Abnormalities in somatic embryogenesis caused by 2,4-D: an overview. Plant Cell, Tissue and Organ Culture 137:193–212. https://doi.org/10.1007/s11240-019-01569-8

Garcia C, Corrêa F, Findley S et al (2016) Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L. Afr J Biotechnol 15:1936–1951. https://doi.org/10.5897/AJB2016.15513

Germana MA, Lambardi M (2016) In vitro embryogenesis in higher plants. Methods Mol Biol 1359:1–577.

Guerra MP, Torres AC, Teixeira JB (1999) Embriogênese somática e sementes sintéticas. In: Culturas de tecidos e transformação genética em plantas. Brasília: Embrapa-CBAB 2:533–568.

Hashemloian BD, Ataei-Azimi A, Majd A, Ebrahimzadeh H (2008) Abnormal plantlets regeneration through direct somatic embryogenesis on immature seeds of *Vinca herbacea* Waldst. and Kit. Afr J Biotechnol 7:1679–1683.

Leite CT, Ferreira DAT, Vieira AT, Praça-Fontes MM, Ferreira A, Carvalho CR, Clarindo WR (2019) In vitro responses in Passiflora species with different chromosome numbers, ploidy levels and nuclear 2C values: revisiting and providing new insights. Plant Cell, Tissue and Organ Culture 136(3):549–560. https://doi.org/10.1007/s11240-018-01536-9

Leljak-Levanić D, Bauer N, Mihaljević S, Jelaska S (2004) Changes in DNA methylation during somatic embryogenesis in *Cucurbita* pepo L. Plant Cell Rep 23:120–127. https://doi.org/10.1007/s00299-004-0819-6

Lloyd CW, Lowe SB, Peace GW (1980) The mode of action of 2,4-D in counter-acting the elongation of carrot cells grown in culture. J Cell Sci 45:257–268.

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–331. https://doi.org/10.1007/BF00305823

Maximova SN, Alemanno L, Young A et al (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. Vitro Cell Dev Biol-Plant 38:252–259. https://doi.org/10.1079/IVP2001257

Miguel C, Marum L (2011) An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond. J Exp Bot 62(11):3713–3725. https://doi.org/10.1093/jxb/err155

Nic-Can GI, Lopez-Torres A, Barredo-Pool F, Wrobel K, Loyola-Vargas VM, Rojas-Herrera R, De-la-Pena C (2013) New insights into somatic embryogenesis: LEAFY COTYLEDON1, BABY BOOM1 and WUSCHEL-RELATED HOMEOBOX4 are epigenetically regulated in *Coffea canephora*. Plos One 8:e72160. https://doi.org/10.1371/journal.pone.0072160

Pasternak TP (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol 129:1807–1819. https://doi.org/10.1104/pp.000810

Pérez-Pascual D, Jiménez-Guillen D, Villanueva-Alonzo H, Souza-Perera R, Godoy-Hernández G, Zúñiga-Aguilar JJ (2018) Ectopic expression of the *Coffea canephora* SERK1 homolog-induced differential transcription of genes involved in auxin metabolism and in the developmental control of embryogenesis. Physiologia plantarum 163(4):530-551. https://doi.org/10.1111/ppl.12709

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol 2(5). https://doi.org/10.1101/cshperspect.a001446

Pescador R, Kerbauy GB, Viviani D, Kraus JE (2008) Anomalous somatic embryos in Acca sellowiana (O. Berg) Burret (Myrtaceae). Braz J Bot 31:155–164.

Queiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell, Tissue and Organ Culture 86(3):285-301. https://doi.org/10.1007/s11240-006-9139-6

Raghavan V (2004) Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of Arabidopsis: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. Am J Bot 91:1743–1756. https://doi.org/10.3732/ajb.91.11.1743

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

Sanglard NA, Amaral-Silva PM, Sattler MC, Oliveira SC, Nunes ACP, Soares TCB, Carvalho C R, Clarindo WR (2017) From chromosome doubling to DNA sequence changes: outcomes of an improved in vitro procedure developed for allotriploid Híbrido de Timor (*Coffea arabica* L. × *Coffea canephora* Pierre ex A. Froehner). Plant Cell Tiss Org 131:223–231. https://doi.org/10.1007/s11240-017-1278-4

Saptari RT, Susila H (2019) Data mining study of hormone biosynthesis gene expression reveals new aspects of somatic embryogenesis regulation. In Vitro Cellular & Developmental Biology Plant 55(2):139-152. https://doi.org/10.1007/s11627-018-9947-5

Söndahl MR, Sharp WR (1977) High frequency induction of somatic embryos in cultured leaf expiants of *Coffea arabica* L. Zeitschrift für Pflanzenphysiologie 81(5):395-408. https://doi.org/10.1016/S0044-328X(77)80175-X

Springer NM (2013) Epigenetics and crop improvement. Trends Genet 29(4):241–247.

Stuart DA, McCall CM (1992) Induction of somatic embryogenesis using side chain and ring modified forms of phenoxy acid growth regulators. Plant Physiol 99:111–118. https://doi.org/10.1104/pp.99.1.111

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 7:847–859. https://doi.org/10.1038/nrm2020

Tokuji Y, Masuda H (1996) Duration of treatment of carrot hypocotyl explants with 2,4-Dichlorophenoxyacetic acid for direct somatic embryogenesis. Biosci Biotechnol Biochem 60:891–892. https://doi.org/10.1271/bbb.60.891

Tvorogova VE, Lebedeva MA, Lutova LA (2015) Expression of WOX and PIN genes during somatic and zygotic embryogenesis in *Medicago truncatula*. Russ J Genet 51:1189–1198. https://doi.org/10.1134/S1022795415120121

Us-Camas R, Rivera-Solís G, Duarte-Aké F, De-la-Pena C (2014) In vitro culture: an epigenetic challenge for plants. Plant Cell, Tissue and Organ Culture 118(2):187-201. https://doi.org/10.1007/s11240-014-0482-8

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

Vanstraelen M, Benková E (2012) Hormonal interactions in the regulation of plant development. Annu Rev Cell Dev Biol 28(1):463–487. https://doi.org/10.1146/annurev-cellbio-101011-155741

Vila S, Gonzalez A, Rey H, Mroginski L (2010) Effect of morphological heterogeneity of somatic embryos of *Melia azedarach* on conversion into plants. Biocell 34:7–13.

Vondráková Z, Eliášová K, Fischerová L, Vágner M (2011) The role of auxins in somatic embryogenesis of *Abies alba*. Open Life Sci 6:587–596. https://doi.org/10.2478/s11535-011-0035-7

Yakovlev IA, Asante DKA, Fossdal CG, Junttila O, Johnsen O (2011) Differential gene expression related to an epigenetic memory affecting climatic adaptation in Norway spruce. Plant Sci 180(1):132–139.

Zavattieri MA, Frederico AM, Lima M et al (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechnol 13:1–9. https://doi.org/10.2225/vol13-issue1-fulltext-4

CONSIDERAÇÕES FINAIS

As abordagens in vitro permitiram, pela primeira vez, a regeneração de plântulas em larga escala do acesso alotriploide HT 'CIFC 4106' em sistema líquido. Esse híbrido é relevante para programas de melhoramento por ser fonte de genes de resistência. O protocolo de embriogênese somática indireta em sistema líquido é seguro, eficaz e reprodutível, uma vez que todas as plântulas regeneradas apresentaram cariótipo estável como alotriploides, preservando a fidelidade genética. Além disso, por ser restrito, esse protocolo foi eficaz e contornou os problemas relacionados à propagação seminal em HT 'CIFC 4106'.

Com o estabelecimento da embriogênese somática indireta em HT 'CIFC4106' alotriploide verdadeiro e autoalohexaploide sintético, as abordagens in vitro permitiram analisar a consequência da poliploidia induzida a curto prazo e, além disso, possibilitaram a propagação de novos autoalohexaploides sintéticos. Desse modo, foi possível observar que a origem, o tempo de formação dos poliploides e o nível de ploidia influenciam a resposta in vitro. A adição de carvão ativado em altas concentrações no meio de regeneração altera os padrões de metilação global das células vegetais e, consequentemente, resulta em embriões somáticos anormais para o HT 'CIFC4106' autoalohexaploide sintético.

Desde os primeiros estudos de embriogênese somática indireta em *Coffea*, abordou-se que as respostas in vitro são influenciadas por aspectos genéticos, epigenéticos e pelo ambiente in vitro, sendo o genótipo mencionado como um fator determinante. No presente estudo, observou-se que as linhagens de *C. arabica* apresentam respostas distintas in vitro. 'Catuaí Vermelho' exibe a melhor resposta à condição de cultura de tecidos proposta, mostrando que o aumento da metilação global está associada com a regeneração e maturação de embriões somáticos. Como observado, a metilação do DNA é um mecanismo dinâmico e variável que interfere no estabelecimento de embriogênese somática indireta em linhagens de *C. arabica*. O meio de cultura foi eficaz, reprodutível e viável, pois manteve a fidelidade genética, regenerando plântulas idênticas às plantas doadoras de explantes e, portanto, nenhuma variação do cariótipo foi identificada.

O regulador de crescimento ácido 2,4-diclorofenoxiacético desempenha um papel crucial na embriogênese somática indireta, uma vez que esta auxina sintética

promove indução e proliferação de calos friáveis em *C. arabica* e *C. canephora*, aumentando a regeneração de embriões somáticos. A adição de ácido 2,4-diclorofenoxiacético promove mudanças nos padrões de metilação do DNA, mostrando que a metilação do DNA é um mecanismo suscetível, dinâmico e variável ao ambiente in vitro. Variações epigenéticas ocorrem para o estabelecimento de embriogênese somática indireta em *Coffea*. Altas concentrações de ácido 2,4-diclorofenoxiacético no meio de indução têm ação fitotóxica, uma vez que causa alterações morfológicas, epigenéticas e genéticas nos embriões somáticos de *Coffea*.