NATÁLIA ARRUDA SANGLARD

EMBRIOGÊNESE SOMÁTICA INDIRETA E POLIPLOIDIA NO GÊNERO *Coffea*: BASE E APLICAÇÃO

Tese apresentada à Universidade Federal do Espírito Santo, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*

ALEGRE

ESPÍRITO SANTO – BRASIL

2017

Dados Internacionais de Catalogação-na-publicação (CIP)
(Biblioteca Setorial de Ciências Agrárias, Universidade Federal do Espírito Santo, ES, Brasil)
Bibliotecária: Lizzie de Almeida Chaves – CRB-6 ES-000871/O

Sanglard, Natália Arruda, 1984-
S225e Embriogênese somática indireta e poliploidia no gênero Coffea: base e aplicação / Natália Arruda Sanglard. – 2017.
112 f. : il.

Orientador: Wellington Ronildo Clarindo.
Coorientadora: Márcia Flores da Silva Ferreira.
Tese (Doutorado em Genética e Melhoramento) – Universidade Federal do Espírito Santo, Centro de Ciências Agrárias e Engenharias.

1. Plantas – Melhoramento genético. 2. Café. 3. Citogenética vegetal. 4. Tecidos vegetais – Cultura e meios de cultura. I. Clarindo, Wellington Ronildo. II. Ferreira, Márcia Flores da Silva. III. Universidade Federal do Espírito Santo. Centro de Ciências Agrárias e Engenharias. IV. Título.

CDU: 631.523

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APROVADA: 14 de julho de 2017.

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Aos meus pais Demétrio e Sonia

À minha tia Leonides Luíza (in memorian)

Ao meu irmão Demerson

Dedico

AGRADECIMENTOS

À Universidade Federal do Espírito Santo (UFES) e ao Programa de Pós-Graduação em Genética e Melhoramento (PPGGM) pela oportunidade de realização desse trabalho.

À Fundação de Amparo à Pesquisa e à Inovação do Espírito Santo (FAPES) pelo auxílio financeiro, indispensável à condução deste trabalho.

Ao meu orientador professor Wellington Ronildo Clarindo (UFES/Depto. Biologia), pela orientação constante no laboratório, sala e até mesmo em sua casa. Agradeço por ser um incentivador ao progresso da minha redação científica. Além disso, pela amizade e momentos de descontração no Karaoke.

À minha coorientadora, professora Marcia Flores da Silva Ferreira (UFES/Depto. Biologia) pelo apoio e incentivo para a realização acerca de diversos trabalhos.

Ao professor Carlos Roberto de Carvalho (UFES/Depto. Biologia), pela contribuição com as análises de citometria de fluxo.

Ao professor Adésio Ferreira (UFES/Dpto. Agronomia) pelo apoio e auxílio com as análises estatísticas.

À professora Taís Cristina Bastos Soares (UFES/Dpto. Farmácia e Nutrição), pela contribuição com as análises moleculares.

Aos pesquisadores e amigos Mariana Cansian Sattler, Paulo Marcos Amaral Silva, Stéfanie Cristina de Oliveira, Andrei Caíque Pires Nunes e Gustavo Fernandes Mariano pelas contribuições científicas para a realização desse trabalho.

À pesquisadora Eveline Teixeira Caixeta (UFV) pelo fornecimento de material biológico.

Aos coordenadores do PPGGM, professor Adésio Ferreira (UFES/Depto. Agronomia) e professora Milene Miranda Praça Fontes (UFES/Depto. Biologia), pelo zelo incansável com a manutenção das condições necessárias ao ensino de Pós-Graduação.

À secretária do PPGGM, Sabrina Lino Furtado Gonçalves, por sua organização e que sempre esteve disponível a me ajudar.

Aos professores e pesquisadores Maria Andréia Corrêa Mendonça, Elias Terra Werner, Fernanda Aparecida Ferreira Soares, Milene Miranda Praça Fontes, por terem aceitado o convite para compor a banca avaliadora deste trabalho.

Aos pesquisadores do Laboratório de Citogenética e Cultura de Tecidos/UFES, Ariane, Stéfanie, Mariana, Darley, Paulo, Lucas, Cris, Renata, Michele, Victor, Suelen, Liliana, Andrei, Alda, Anelise, Gustavo, João Paulo, Quezia, Mel, Kalia, Loren, Karen, Patrícia, Lucimara, Thiago, Fernanda, pelas informações científicas e pelas risadas.

Ao prof. Valdir Diola (UFRRJ/Dpto Genética) (*in memorian*) pelo incentivo ao doutorado, pelo exemplo de comprometimento profissional e amizade.

Aos meus maravilhosos pais Demétrio e Sonia, meu irmão Demerson e meu namorado Otaviano pelo amor e incentivo. Agradeço a Deus pelo dom da vida.

BIOGRAFIA

NATÁLIA ARRUDA SANGLARD, filha de Demétrio Antônio Sanglard e Sonia Lídia Arruda Sanglard, nasceu em Manhumirim, Minas Gerais, em 20 de dezembro de 1984.

Em novembro de 2004, iniciou o curso superior na Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, Rio de Janeiro, colando grau em julho de 2010 como Engenheira Agrônoma. No período de sua graduação, desenvolveu atividades de pesquisa como bolsista de Iniciação Científica da Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) no Departamento de Genética. Além disso, foi estagiária nos Laboratórios de Tecnologia de Sementes do Departamento de Fitotecnia (UFRRJ), Genética Molecular de Plantas do Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO/Universidade Federal de Viçosa).

Em março de 2011, iniciou o curso de Mestrado no Programa de Pós-Graduação em Fitossanidade e Biotecnologia Aplicada da UFRRJ, na área de Biotecnologia Aplicada, defendendo dissertação em fevereiro de 2013. Em junho de 2013, iniciou o curso de Pós-Graduação em nível de Doutorado, no Programa de Genética e Melhoramento da Universidade Federal do Espírito Santo (UFES), submentendo-se à defesa de tese em julho de 2017.

LISTA DE ABREVIATURAS

2,4-D ácido 2,4-diclorofenoxiacético

BAP 6-benzilaminopurina

CEF calos embriogênicos friáveis

CS constrições secundárias

ESI embriogênese somática indireta

HT "Híbrido de Timor"

SUMÁRIO

RESUMO..... xii

ABSTRACT..... xiv

1. INTRODUÇÃO GERAL..... 1

1.1. Poliploidia: considerações gerais..... 1

1.2. Poliploidia em *Coffea*..... 2

1.3. "Híbrido de Timor" (HT) e sua origem alotriploide..... 3

1.4. Idade evolutiva de *Coffea* 4

1.5. Embriogênese Somática Indireta em *Coffea*..... 5

1.6. Poliploidização in vitro: considerações gerais..... 7

2. OBJETIVOS GERAIS..... 10

3. REFERÊNCIAS..... 11

CAPÍTULO I: A further step toward understanding the indirect somatic embryogenesis response: looking for the influence of the different ploidy levels in *Coffea*..... 18

Abstract..... 19

Introduction..... 21

Materials and Methods..... 23

Biological Material..... 23

Nuclear DNA content and DNA ploidy level determination of the explant donors..... 24

ISE establishment..... 24

Statistical analysis..... 25

Chromosome number of the Coffea FEC..... 26

Results..... 29

Nuclear DNA content and DNA ploidy level determination of the explant donors..... 29

Dedifferentiation of the explant cells and FEC establishment..... 32

MCSE regeneration..... 37

Chromosome number of the Coffea FEC..... 47

Discussion..... 47

Conclusions..... 53

References..... 54

CAPÍTULO II: 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)…… 63

Abstract..... 64

Introduction..... 66

Materials and Methods..... 68

Plant Material..... 68

FEC induction..... 68

ISE chromosome doubling..... 69

Somatic embryogenesis (SE) and plantlet regeneration..... 69

Hexaploid screening following ploidy level assessment..... 72

Evaluation of genetic stability by SSR markers..... 72

Statistical analysis..... 73

Results..... 74

In vitro plant recovery from FEC..... 74

Hexaploid screening following ploidy level assessment..... 74

Evaluation of genetic stability by SSR markers..... 77

Discussion..... 81

Conclusions..... 85

References..... 87

4. CONSIDERAÇÕES FINAIS..... 96

RESUMO

Sanglard, Natália Arruda, D. Sc., Universidade Federal do Espírito Santo, julho de 2017. **Embriogênese somática indireta e poliploidia no gênero *Coffea*: base e aplicação.** Orientador: Wellington Ronildo Clarindo. Coorientadora: Marcia Flores da Silva Ferreira.

A embriogênese somática indireta (ESI) é uma das aplicações da cultura de tecidos que permite a manutenção, a propagação e a geração de novos germoplasmas. Assim, a partir do estabelecimento da ESI em quatro *Coffea* com diferentes níveis de ploidia (diploides *Coffea canephora* Pierre ex Froehner, *Coffea eugenioides* Moore, alotriploide "Híbrido de Timor" – HT, alotetraploide *Coffea arabica* L.), os objetivos do presente estudo foram: (a) verificar a influência das características cariotípicas (número de cromossomos, nível de ploidia e conteúdo de DNA nuclear) na ESI; e (b) duplicar o número de cromossomos do *Coffea* alotriploide HT 'CIFC 4106'. Sob as mesmas condições in vitro, os quatro *Coffea* diferiram entre si durante todas as etapas da ESI. Os alopoliploides apresentaram maior número médio de calos embriogênicos friáveis (CEF), em tempo relativamente curto, e exibiram visualmente proliferação celular mais pronunciada. CEF de *C. arabica* resultaram em maior número médio de embriões somáticos cotiledonares maduros, seguido de HT e *C. eugenioides* com mesmo número médio, e *C. canephora* com o menor. A partir dos dados de ESI, análise do cariótipo e mensuramento do valor 2C nuclear dos diferentes germoplasmas, as respostas de ESI em *Coffea* foram relacionadas com o nível de ploidia. Com a ESI estabelecida, a duplicação cromossômica do alotriploide HT 'CIFC 4106', o anortoploide com 2n = 3x = 33 cromossomos, foi conduzida associando esse sistema in vitro com o tratamento envolvendo a colchicina. Um total de 65 plântulas foram regeneradas a partir dos CEF

tratados com colchicina (0,5; 1,5 ou 2,5 mM) por 96 horas. Independentemente da concentração de colchicina empregada, 49,3% de hexaploides (2n = 6x = 66 cromossomos, 2C = 4,20 pg) foram obtidos. Além disso, a estratégia estabelecida (ESI/colchicina) resolveu os principais gargalos da duplicação cromossômica in vitro: baixa taxa de poliploides, alto número de mixoploides e alta taxa de mortalidade. Esse estudo gerou novos dados acerca das bases do entendimento da ESI e contribuiu com uma nova estratégia de duplicação cromossômica. Além disso, um novo germoplasma de *Coffea*, os hexaploides do HT 'CIFC 4106', foi gerado.

ABSTRACT

Sanglard, Natália Arruda, D. Sc., Universidade Federal do Espírito Santo, july de 2017. **Indirect somatic embryogenesis and polyploidy in the genus *Coffea*: base and application.** Adviser: Wellington Ronildo Clarindo. Co-advisers: Marcia Flores Ferreira da Silva Ferreira.

Indirect somatic embryogenesis (ISE) is one of the tissue culture applications that allow the maintenance, propagation and generation of new germplasms. Thus, from the establishment of ISE in four *Coffea* with different ploidy levels (diploids *Coffea canephora* Pierre ex Froehner and *Coffea eugenioides* Moore; allotriploid "Híbrido de Timor" – HT, and allotetraploid *Coffea arabica* L.), the aims of the present study were: (a) to verify the relation between karyotype features (chromosome number, ploidy level and nuclear DNA content) and ISE; and (b) doubling the chromosome number of the allotriploid HT 'CIFC 4106'. Under the same in vitro conditions, the four *Coffea* varieties differed from each other during all steps of the ISE. The allopolyploids provided the highest mean number of friable embryogenic calli (FEC), in relatively short time, and visually exhibiting more pronounced cell proliferation. FEC of *C. arabica* resulted in the highest mean number of mature cotyledonary somatic embryos, followed by HT and *C. eugenioides* with the same mean value, and *C. canephora* with the lowest amount. Regarding the ISE data, chromosome number, ploidy level and nuclear 2C value, in vitro responses in *Coffea* were related to ploidy level. From established ISE, chromosome doubling of the allotriploid HT 'CIFC 4106', an anorthoploid with 2n = 3x = 33 chromosomes, was conducted by associating this in vitro system with colchicine treatment. A total of 65 plantlets were regenerated from the FEC treated with colchicine (0.5, 1.5 or 2.5 mM) for 96 h. Independently of the applied

colchicine concentration, a rate of 49.3% of hexaploids (2n = 6x = 66 chromosomes, 2C = 4.20 pg) was obtained. In addition, the established strategy (ISE/colchicine) solved the main bottlenecks of in vitro chromosome doubling: low rate of polyploids, high number of mixoploids and high mortality. This study provided new data on the basics of ISE, understanding and contributing with a new strategy of chromosome doubling. In addition, a new *Coffea* germplasm, the HT 'CIFC 4106' hexaploids, was generated.

1. INTRODUÇÃO GERAL

1.1. Poliploidia: considerações gerais

A poliploidia é um dos processos evolutivos mais importantes em plantas, sendo que 100% das angiospermas são poliploides (Soltis et al. 2014). Dados de análises genômicas indicam que o processo de poliploidia ocorreu pelo menos uma vez ao longo da história evolutiva desse grupo (Jiao et al. 2011). Além disso, tem sido sugerido que 15% dos eventos de especiação nas angiospermas envolveram a poliploidia, sendo, então, a poliploidização o principal mecanismo de especiação em plantas (Wood et al. 2009).

Para entender a poliploidia é preciso conhecer alguns conceitos fundamentais em citogenética, onde as contribuições do botânico George Ledyard Stebbins são fundamentais. O imenso impacto do seu trabalho sobre a poliploidia ao longo de mais de 60 anos foi revisado por Soltis et al. (2014) e Spoelhof et al. (2017).

A poliploidia é definida como a presença de mais de dois conjuntos cromossômicos, sendo os poliploides classificados em auto ou alopoliploides conforme o mecanismo de formação. Autopoliploides são originados a partir da fusão de células reprodutivas não reduzidas de uma mesma espécie (poliploidização sexual), ou a partir da duplicação genômica em células somáticas, por um processo de endomitose ou endorreduplicação (poliploidização assexual). Já os alopoliploides são formados pelo cruzamento entre espécies diferentes (Ramsey and Schemske 1998), podendo envolver ou não a fusão de células reprodutivas não reduzidas (Mallet 2007).

Os alopoliploides podem ainda ser subdivididos em segmental e verdadeiro. Alopoliploides segmentais são provenientes de espécies distintas que, erm virtude de homologias cariotípicas entre os progenitores, apresentam univalentes, bivalentes e/ou

multivalentes durante a meiose. Alopoliploides verdadeiros também são formados a partir do cruzamento de espécies distintas, porém possuem comportamento disômico/Mendeliano na meiose (Sattler et al. 2016a).

Outras definições importantes em poliploides estão relacionadas com o tempo após a sua formação, sendo denotados os termos neo, meso e paleopoliploides na ordem de origem mais recente, intermediária ou mais antiga. Entretanto, esses termos têm sido utilizados de diferentes maneiras, como, por exemplo, a partir da interpretação de diferenciação citológica ou pareamento cromossômico (Weiss-Schneeweiss et al. 2013). Paleopoliploides são poliploides que surgiram há milhões de anos, passaram por um processo de diploidização e assumiram uma condição diploide (Blanc e Wolfe 2004; Van de Peer et al. 2017), enquanto os neopoliploides são aqueles que não passaram por esse processo. Outros autores referem paleopoliploides como os poliploides que apresentam pelo menos um dos seus progenitores desconhecidos ou os ancestrais foram extintos, enquanto os neopoliploides são aqueles poliploides cujos progenitores são conhecidos e as espécies parentais ainda existem na natureza (Guerra 2008).

A poliploidia pode ser inferida quando números cromossômicos entre espécies seguem uma série euploide, como, por exemplo: 2n = 22 (2x = diploide – *Coffea canephora* Pierre ex Froehner, *Coffea eugenioides* Moore), 33 (3x = triploide – "Híbrido de Timor" – HT), 44 (4x = tetraploide – *Coffea arabica* L.).

1.2. Poliploidia em *Coffea*

Dados citogenéticos e moleculares sugerem que todas as espécies do gênero *Coffea* foram originadas a partir de um único ancestral, o qual possuía um número básico de x = 11 cromossomos (Mahé et al. 2007). Todas as espécies são diploides (2x

= 22), exceto *C. arabica*, considerada a única alotetraploide do gênero, (4x = 44) (Clarindo e Carvalho 2009).

Por ser a única espécie tetraploide do gênero, *C. arabica* tem sido foco de estudos evolutivos, com o intuito de elucidar os aspectos da origem e evolução de seu genoma. Essa espécie é considerada um alotetraploide verdadeiro (*C. canephora* x *C. eugenioides*, Lashermes et al. 1999; Yu et al. 2011; Hamon et al. 2015), com 2C = 2,62 picogramas (pg) e 2n = 4x = 44 cromossomos (Clarindo e Carvalho 2009). Seus potenciais progenitores exibem mesmo número cromossômico (2n = 2x = 22 cromossomos, Clarindo e Carvalho 2009) e tamanho de genoma nuclear similar, sendo reportado para *C. eugenioides* 2C = 1,36 pg (Noirot et al. 2003) e para *C. canephora* 2C = 1,41 pg (Clarindo e Carvalho 2009).

Outras características cariotípicas reportadas para esses *Coffea* são número de constrições secundárias (CS) e número de loci rDNA 18S e rDNA 5S. O número de sítios de rDNA é representativo da complexidade do cariótipo de *Coffea*. *C. eugenioides*, um dos progenitores de *C. arabica*, apresenta um sítio de rDNA 5S e duas CS correspondentes aos dois sítios de rDNA 18S (Hamon et al. 2009). *C. canephora*, outro potencial progenitor de *C. arabica*, possui dois sitios de rDNA 5S e uma constrição secundária equivalente ao sítio de rDNA 18S (Clarindo e Carvalho 2006; Hamon et al. 2009). *C. arabica* exibe dois sítios de rDNA 5S e três CS correspondentes aos três sítios de rDNA 18S (Pinto-Maglio e Da Cruz 1998; Hamon et al. 2009).

1.3. “Híbrido de Timor” (HT) e sua origem alotriploide

O HT é um híbrido interespecífico oriundo de um cruzamento natural entre *C. arabica* (2n = 4x = 44 cromossomos) e *C. canephora* (2n = 2x = 22). A suposta primeira planta de HT foi encontrada em 1927, em uma população de *C. arabica*

‘Typica’, estabelecida entre 1917/18 na Ilha de Timor (Bettencourt 1973). HT foi introduzido por propagação vegetativa no Centro de Investigação das Ferrugens do Cafeeiro (CIFC), em Portugal, sob o código de registro ‘CIFC 4106’ (Pereira et al. 2008). Desde então, o HT tem sido de grande valor no que diz respeito ao melhoramento genético do cafeeiro, uma vez que apresenta resistência à ferrugem *(Hemileia vastatrix* Berk. et Br.), além de outras patologias (Capucho et al. 2009).

A partir de dados obtidos usando técnicas citogenéticas e de citometria de fluxo, Clarindo et al. (2013) e Sattler et al. (2016) sugeriram que o acesso HT ‘CIFC 4106’ é um alotriploide verdadeiro (*C. arabica* x *C. canephora*), com 3x = 33 cromossomos e 1C = 2,10 pg. Em virtude do HT ‘CIFC 4106’ exibir número ímpar de conjuntos cromossômicos (anortoploide), ocorrem anormalidades na meiose (Ramsey and Schemske, 1998), resultando em aneuploidias. Assim, o baixo número de sementes viáveis reportadas para esse híbrido (Pereira et al. 2008) está associado à sua condição alotriploide (Clarindo et al. 2013; Sattler et al. 2016b).

1.4. Idade evolutiva de *Coffea*

Além das diferenças cariotípicas (número de cromossomos, nível de ploidia e conteúdo de DNA nuclear) entre *C. arabica*, seus potenciais progenitores (*C. eugenioides* e *C. canephora*) e o HT (*C. arabica* x *C. canephora*), esses *Coffea* apresentam idade evolutiva distinta. *C. arabica* tem origem estimada em ~0,665 milhões de anos atrás e seus progenitores se divergiram a ~4,2 milhões de anos atrás (Yu et al. 2011). HT ‘CIFC 4106’ foi originado a ~100 anos atrás, na Ilha do Timor (Bettencourt 1973). Assim, HT é um alopoliploide mais recente que *C. arabica.* Comparativamente, HT pode ser considerado um neopoliploide em relação a *C. arabica*, uma espécie paleopoliploide.

Nos últimos 150 anos, poucos gêneros (*Cardamine* L., *Spartina* Schreb., *Senecio* L., *Tragopogon* L. e *Mimulus* L.) têm sido reconhecidos por apresentarem espécies alopoliploides naturais (Mandáková et al. 2013). Visto *C. arabica* e HT, a alopoliploidia é um processo evolutivo recorrente no gênero *Coffea*. Neste sentido, assim como *C. arabica*, o HT pode ser apontado como potencial modelo para estudos de poliploidia em *Coffea*. A ampliação do número de organismos alopoliploides contribui para a compreensão dos mecanismos envolvidos na sua origem e manutenção, bem como nas alterações genéticas e epigenéticas que ocorrem após um evento de hibridação.

1.5. Embriogênese somática indireta em *Coffea*

A embriogênese somática indireta (ESI), na qual embriões somáticos são originados de um calo (Williams e Maheswaran 1986), tem sido relatada em *Coffea* desde os anos 70 (Staritsky 1970), especialmente como uma estratégia de propagação massal. As espécies de *Coffea* foco para o estabelecimento da ESI são *C. canephora* e *C. arabica* em virtude da relevância agronômica. Para estas espécies, a ESI tem sido estabelecida a partir de explantes foliares e distintas condições químicas da cultura de tecidos, principalmente o tipo, a concentração e a combinação dos reguladores de crescimento usados em cada etapa. Além disso, diferentes condições físicas, tais como fotoperíodo e escuro, são utilizadas conforme a etapa do estabelecimento in vitro.

Os protocolos são frequentemente baseados em meios suplementados com auxinas [ácido 2,4-diclorofenoxiacético (2,4-D), ácido indol-3-acético (AIA), ácido indol butírico (AIB) – van Boxtel e Berthouly (1996), e / ou ácido naftalenoacético (ANA) – Almeida et al. (2008)] e citocininas [N6-benziladenina (BA), cinetina, 2-isopenteniladenina (2-iP), 6-benzilaminopurina (BAP) – van Boxtel e Berthouly (1996) e Samson et al. (2006), ou thidiazuron (TDZ) – Ibrahim et al. (2015)]. Apesar do uso de

reguladores de crescimento distintos, a combinação de 2,4-D / BAP é mais comum para a desdiferenciação celular em meio de indução de calos embriogênicos friáveis (CEF) (van Boxtel e Berthouly 1996), caracterizada como a primeira etapa da ESI em *Coffea*. Na segunda etapa, CEF são transferidos para meio de regeneração de embriões somáticos. Este meio geralmente é suplementado com uma citocinina para alcançar a competência embriogênica. Finalmente, o embrião somático é germinado e as plântulas desenvolvidas em meio de regeneração contendo ou não regulador de crescimento.

A maioria dos estudos de ESI em *Coffea* têm sido conduzidos a partir de uma única espécie (*C. arabica* - Sondahl e Sharp 1977; Cid et al. 2004; Almeida et al. 2008; Ibrahim et al. 2015 ou *C. canephora* – Santana et al. 2004), sendo poucos os relatos de trabalhos que concomitantemente envolveram mais espécies sob as mesmas condições in vitro: *C. canephora*, *Coffea liberica* Bull ex Hiern. e *C. arabica* – Staritsky 1970; *C. canephora*, *C. arabica*, 'Arabusta' (*C. arabica* x *C. canephora*) e 'Congusta' (*C. congiensis* Fr. x *C. canephora* – van Boxtel e Berthouly 1996; *C. canep*hora, *Coffea heterocalyx* Stoffelen, *C.* sp. Moloundou, *C. arabica* – Samson et al. 2006). Dentre esses, van Boxtel e Berthouly (1996) tem sido amplamente utilizado como base para o procedimento de ESI em *Coffea*. Os autores consideraram os explantes foliares de *C. canephora* (diploide), 'Arabusta' (alopoliploide) e 'Congusta' (homoploide) como os mais responsivos para formação de CEF em relação a *C. arabica* (alotetraploide). O tempo para a regeneração de plântulas variou de 7 a 8 meses para *C. canephora* e 'Arabusta' e 9 a 10 meses para *C. arabica*. Usando dois meios modificados de van Boxtel e Berthouly (1996), Samson et al. (2006) observaram que o tempo de desenvolvimento de embriões somáticos para os diploides *C. canephora* e *Coffea heterocalyx* e o alotetraploide *C. arabica* foi reduzido em até 3 meses.

A variação na resposta in vitro pode estar relacionada às diferenças genotípicas entre plantas doadoras de explantes (van Boxtel e Berthouly 1996; Cid et al. 2004). Além disso, a resposta da ESI em *Coffea* pode estar relacionada ao estado fisiológico do explante (Santana et al. 2004). Considerando a variação na resposta de ESI relatada para *Coffea* (van Boxtel e Berthouly 1996; Samson et al. 2006) e as divergências em relação ao cariótipo (número de cromossomos, nível de ploidia e conteúdo de DNA nuclear), este gênero se torna um potencial táxon a ser investigado quanto às relações entre características do cariótipo e respostas in vitro.

1.6. Poliploidização in vitro: considerações gerais

A ESI representa uma das bases da propagação in vitro, sendo, assim, pré-requisito para o desenvolvimento de outras estratégias na cultura de tecidos, tais como a obtenção de poliploides sintéticos. Os primeiros experimentos de poliploidização in vitro, também denominada duplicação cromossômica, datam de 1966 em ensaios com *Nicotiana tabacum* L. (Murashige e Nakano 1966). A partir da década de 90, a duplicação cromossômica in vitro tem sido um método estabelecido na cultura de tecidos vegetais, sendo realizada com a perspectiva de dobrar o número de cromossomos total do cariótipo. Os poliploides sintéticos obtidos podem apresentar alterações genômicas, morfológicas e fisiológicas (Dhooghe et al. 2011). Além disso, a duplicação do número de cromossomos pode restaurar a fertilidade dos homoploides (Song et al. 1997) e anortoploides (Faleiro et al. 2016).

Uma visão geral sobre a duplicação cromossômica in vitro foi apresentada por Dhooghe et al. (2011), sumarizando dezenas de trabalhos que empregaram diversos compostos antimitóticos, tais como colchicina (36), orizalina (20), trifuralina (6), etalfluralina (1), amiprofos-metil (1) e 2,6-dinitroalinina (1). Dentre esses compostos, a

colchicina é o amplamente utilizado. A colchicina é um alcaloide com capacidade de ligar-se aos dímeros de tubulina e formar um complexo colchicina/tubulina, impedindo, dessa forma, a polimerização dos microtúbulos e a formação do fuso mitótico ou meiótico (Panda et al. 1995).

Assim como os compostos antimitóticos, existem muitos tipos de explantes que podem ser empregados para a indução da duplicação cromossômica, tais como calos, suspensões celulares, segmentos nodais, brotos axilares, embriões somáticos, ápices caulinares, embriões zigóticos, sementes e plântulas (Dhooghe et al. 2011). Diante dessa variedade de materiais biológicos passíveis de indução da duplicação cromossômica, os autores têm testado a melhor concentração do agente antimitótico, assim como o tempo de exposição. Doses excessivamente elevadas são letais e baixas concentrações podem não promover a duplicação cromossômica (Allum et al. 2007). Além disso, características fisiológicas de cada espécie e até mesmo entre explantes de um mesmo indivíduo devem ser consideradas para padronizar um método de indução da duplicação cromossômica.

Dentre os métodos que distinguem indivíduos gerados a partir da duplicação cromossômica, o mais utilizado é a citometria de fluxo. Este método possibilita a seleção de poliploides putativos em um curto período de tempo (Doležel et al. 2007). No entanto, a contagem cromossômica é o único método que inequivocamente confirma o nível de ploidia (Doležel et al. 2007).

As diversas estratégias empregadas para indução da duplicação cromossômica têm gerado baixa taxa de poliploides e alta taxa de mixoploides. Em virtude desses obstáculos, um ensaio de duplicação cromossômica a partir da ESI de HT 'CIFC 4106' (anortoploide, 2n = 3x = 33 cromossomos) é relevante, em virtude deste híbrido

apresentar natureza alotriploide e sistema embriogênico estabelecido por nosso grupo de pesquisa.

OBJETIVOS GERAIS

A partir do estabelecimento da ESI em *Coffea* diploides (*C. canephora* e *C. eugenioides*), alotriploide (HT 'CIFC 4106') e alotetraploide (*C. arabica*), os objetivos foram:

a) verificar a influência das características cariotípicas (número de cromossomos, nível de ploidia e conteúdo de DNA nuclear) na resposta in vitro;

b) duplicar o número de cromossomos do alotriploide do HT ‘CIFC 4106’.

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CAPÍTULO I

Title: Title: A further step toward understanding the indirect somatic embryogenesis response: looking for the influence of the different ploidy levels in *Coffea*.

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Abstract

Indirect somatic embryogenesis (ISE) is required for propagation and mass multiplication of elite plant lines. It is also a prerequisite for the development of applications that may provide new germplasms. Genetic, epigenetic and/or physiological features of the explant donor plants are appointed as barriers for ISE establishment, hindering the wide use of this morphogenic pathway. Despite the identification and/or expression analysis of genes during ISE, no approach to establish the karyotype aspects has been performed so far. Therefore, the present study aimed to investigate the relationship between karyotype features and the ISE response in diploid (*C. canephora* and *C. eugenioides*), true allotriploid ("Híbrido de Timor" – HT) and true allotetraploid (*C. arabica*) *Coffea*. Under the same in vitro conditions, the four *Coffea* differed from each other in all steps of the ISE response. Leaf explants of the true allopolyploids (HT and *C. arabica*) yielded the highest mean number of friable embryogenic calli (FEC), in relative low time and visually exhibiting cell proliferation more pronounced than in the diploids. FEC of the allotetraploid *C. arabica* presented the highest mean number of mature cotyledonary somatic embryos (MCSE), which were also recovered faster in this species. However, surprisingly the mean number of MCSE in the allotriploid HT was the same as in the diploid *C. eugenioides*, and these *Coffea* presented high mean number of MCSE in relation to *C. canephora*. Associating the ISE to the karyotypes of the four *Coffea*, it can be suggested that the ISE responses in *Coffea* are probably related to the ploidy level. Based on this initial study, other approaches should be performed considering the ploidy level and other karyotype features, aiming to better understand the ISE response.

Keywords: plant tissue culture; somatic embryogenesis; chromosome number; ploidy level, nuclear 2C value.

Abbreviations

2,4-D 2,4-dichlorophenoxyacetic acid

BAP 6-benzylaminopurine

FEC Friable embryogenic calli

GA_3 Gibberellic acid

HT "Híbrido de Timor"

ISE Indirect somatic embryogenesis

MCSE Mature cotyledonary somatic embryo

SE Somatic embryos

Introduction

Indirect somatic embryogenesis (ISE), an approach in which somatic embryos (SE) originate from calli (Williams and Maheswaran 1986), represents the basis for the development of tissue culture applications, for instance: in vitro propagation (Staritsky 1970; van Boxtel and Berthouly 1996; Samson et al. 2006), germplasm conservation (Etienne et al. 2002), androgenesis (Herrera et al. 2002), genetic transformation (Gatica-Arias et al. 2008), production of secondary metabolites (Karuppusamy 2009), and chromosome doubling (Murashige and Nakano 1966; Sanglard et al. 2017). Experimental conditions to achieve an ISE response, from cell dedifferentiation to plantlet regeneration, are specific to the genetic, epigenetic and/or physiological features of the explant (Fehér et al. 2015). For this reason, ISE has been established mainly in phylogenetically close taxa, such as within the genus *Coffea* (Staritsky 1970; van Boxtel and Berthouly 1996; Samson et al. 2006).

ISE in *Coffea* emerged in the 1970s (Staritsky 1970) as a strategy to propagate the species with agronomic relevance, such as *Coffea canephora* Pierre ex Froehner and *Coffea arabica* L. For this genus (van Boxtel and Berthouly 1996; Samson et al. 2006; Almeida et al. 2008; Ibrahim et al. 2015), in vitro conditions have differed in relation to physical (photoperiod – light/dark ratio) and chemical aspects (mainly concentration of macro- and micronutrients, as well as type, combination and concentration of growth regulators) for induction of friable embryogenic calli (FEC) from leaf explants, and for regeneration of SE and plantlets.

Similarly to chemical and physical tissue culture conditions, genetic features also influence the ISE responses (Fehér et al. 2015). Substantial differences between in vitro responses (i.e. time and rate) associated with FEC induction and/or SE and plantlet

recovery have been reported for the diploid *C. canephora*, the true allotetraploid *C. arabica* (Staritsky 1970; van Boxtel and Berthouly 1996; Samson et al. 2006), the diploids *Coffea heterocalyx* Stoffelen and *Coffea* sp. Moloundou (Samson et al. 2006), the allopolyploid 'Arabusta' (*C. arabica* x *C. canephora*), and the homoploid 'Congusta' (*C. congensis* Fr. x *C. canephora*) (van Boxtel and Berthouly 1996). van Boxtel and Berthouly (1996), who proposed the tissue culture procedure most applied in *Coffea*, considered the leaf explants of the diploid *C. canephora*, allopolyploid 'Arabusta' and homoploid 'Congusta' to be more responsive toward FEC formation than the true allotetraploid *C. arabica*. The time for plantlet regeneration also varied among these *Coffea*, comprising 7–8 months for *C. canephora* and 'Arabusta' and 9–10 for *C. arabica*.

Thus, there are differences among the ISE responses obtained in *Coffea* (van Boxtel and Berthouly 1996; Samson el al. 2006) with pronounced karyotype attributes. *C. canephora*, differentiated ~4.2 mya (million years ago) (Yu et al. 2011), presents 2C = 1.41 picograms (pg), 2n = 2x = 22 chromosomes, two sites of 5S rDNA (Hamon et al. 2009), and one chromosome (6) with secondary constriction (SC) (Clarindo and Carvalho 2006) corresponding to one 45S rDNA site (Hamon et al. 2009). *C. arabica*, a true allotetraploid (*C. canephora* x *Coffea eugenioides* Moore; Lashermes et al. 1999; Yu et al. 2011; Hamon et al. 2015), originated ~0.665 mya (Yu et al. 2011), possesses 2C = 2.62 pg, 2n = 4x = 44 chromosomes (Clarindo and Carvalho 2009), two sites of 5S rDNA (Hamon et al. 2009), and three chromosomes (14, 20, 21) with SC (Pinto-Maglio and Da Cruz 1998) relative to three 45S rDNA sites (Hamon et al. 2009).

Considering all the above, the present work aimed to elucidate whether a relationship exists between the karyotype features and the ISE response in *Coffea*. For this purpose, *C. eugenioides*, as a possible progenitor of *C. arabica* (Lashermes et al.

1999; Hamon et al. 2015), was one of the species included in the analyses. *C. eugenioides* presents 2C = 1.36 pg (Noirot et al. 2003), 2n = 2x = 22 chromosomes, one 5S rDNA site, and two 45S rDNA sites (Hamon et al. 2009). However, since no in vitro tissue culture study has been conducted in this species so far, at first ISE should be established for *C. eugenioides* in order to compare its morphogenic responses with those of other *Coffea*.

"Híbrido de Timor" (HT) (*C. arabica* x *C. canephora*) is a polyploid *Coffea* relevant to clarify the matter at hand, owing to its ancestry and evolutionary origin. This natural allopolyploid originated ~100 years ago on the Timor Island (Bettencourt 1973). HT 'CIFC 4106', the probable first HT plant, is an allotriploid with 1C = 2.10 pg and 2n = 3x = 33 chromosomes (Clarindo et al. 2013), and has been recently propagated via direct somatic embryogenesis (Sattler et al. 2016) and ISE (Sanglard et al. 2017).

Considering the karyotype features of the four above-mentioned *Coffea*, this study aimed to verify the relation of these aspects to the ISE. In this sense, the following objectives were fulfilled for all studied *Coffea*: (a) establishment of the ISE; (b) confirmation of the chromosome number, ploidy level, and nuclear DNA content; (c) comparison of the four *Coffea* in relation to the ISE response; and (d) establishment of a relationship between karyotype and ISE response.

Materials and methods

Biological material

Leaves were collected from three individuals of *C. canephora*, *C. eugenioides*, HT 'CIFC 4106' and *C. arabica* (explant donors). *C. eugenioides* and *C. arabica*

'Catuaí Vermelho' had been grown in greenhouse of the *Coffea* germplasm bank of the Universidade Federal de Viçosa (UFV – Minas Gerais, Brazil, 20°45'S, 42°52'W) under controlled phytosanitary and environmental conditions. *C. canephora* and HT 'CIFC 4106' had been propagated in vitro (Universidade Federal do Espírito Santo – UFES, Espírito Santo, Brazil) in medium consisting of MS (Murashige and Skoog 1962) salts, 10 mL L^{-1} Gamborg's B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, and 7.0 g L^{-1} Agar (Sigma®) (Sattler et al. 2016). Collected leaves were used as explant sources for ISE, as well as for nuclear DNA content measurement and DNA ploidy level determination.

Nuclear DNA content and DNA ploidy level determination of the explant donors

Leaf fragments of each *Coffea* explant donor plant 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). The nuclei suspensions were processed, stained (Otto 1990; Clarindo and Carvalho 2009; Clarindo et al. 2013) and analyzed in a Partec PAS® flow cytometer (Partec® GmbH, Muenster, Germany). The 2C value was measured considering the G_0/G_1 nuclei peak of the *Coffea* samples and *S. lycopersicum*. From the mean 2C value, the DNA ploidy level was also confirmed for all *Coffea*.

ISE establishment

The leaves of *C. eugenioides* and *C. arabica* explant donor plants, cultivated in greenhouse, were disinfected prior to inoculation (Clarindo et al. 2012). Leaf explants (~1 cm^2) of the four *Coffea* were excised, and five fragments were inoculated in Petri dish containing FEC induction medium (M1, Table 1). In accordance with the availability of leaves from each *Coffea*, 51 Petri dishes (repetitions) were accomplished

for *C. eugenioides*, 30 for *C. canephora*, 29 for HT 'CIFC 4106', and 102 for *C. arabica*. The Petri dishes were maintained in the dark at 25 ± 2°C.

After 90 days, only the explants showing FEC were individually transferred to Petri dishes containing SE regeneration medium (M2, Table 1). A total of 23 Petri dishes (repetitions) were prepared for *C. eugenioides*, 21 for *C. canephora,* 98 for HT 'CIFC 4106', and 143 for *C. arabica*. The culture was maintained in the dark at 25 ± 2°C for 180 days. Subsequently, each mature cotyledonary somatic embryo (MCSE) was transferred to plantlet recovery medium (M3, Table 1) for SE germination and seedling development. The tubes were maintained at 24°C ± 2°C, under a 16/8 h (light/dark) regimen with 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram®).

Statistical analysis

ISE responses of *C. canephora, C. eugenioides*, HT 'CIFC 4106' and *C. arabica* were compared with regard to two steps: (a) dedifferentiation of explant cells and FEC establishment, and (b) MCSE regeneration (competence acquisition, FEC cell determination and differentiation, and MCSE regeneration). For the first ISE step (a), statistical analysis was performed using the number of responsive explants, which were defined by FEC presence at 15, 30, 45, 60, 75 and 90 days. For the second ISE step (b), the number of regenerated MCSE was compared at 30, 60, 90, 120, 150 and 180 days, first considering all FEC, then only the FEC presenting SE.

The numbers of FEC or MCSE per Petri dish were compared by analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$), and depicted as box-plot graphics. Subsequently, regression analysis ($p < 0.05$) was performed from quantitative

data: mean number of responsive leaf explants and mean number of MCSE. All analyses were accomplished using the software R (R Core Team 2016).

Chromosome number of the Coffea FEC

For each *Coffea*, 0.5 g of a random sample of 90-day FEC was collected and transferred to Erlenmeyer flasks containing FEC proliferation medium (M1, Table 1) without Phytagel. At least five flasks were prepared for each *Coffea*, and maintained on shaker at 100 rpm and 24°C ± 2°C. Cell aggregate suspensions were subcultured every 15 days. After 60 days, the suspensions were treated with 4 μM of amiprophos methyl for 8 h, fixed (3:1 methanol:acetic acid) and enzymatically macerated with pectinase solution (1:20 pectinase:dH_2O) for 1 h 40 min at 34°C (Clarindo and Carvalho 2009). Cell dissociation and air-drying techniques were applied for slide preparation (Carvalho et al. 2007). After staining with 5% Giemsa solution, mitotic images were captured with a Media Cybernetics® Camera Evolution™ charge-coupled device video camera, mounted on a Nikon 80i microscope (Nikon, Japan).

Table 1. Composition of the tissue culture media used for ISE establishment from leave explants of diploid *C. canephora* and *C. eugenioides*, true allotetraploid *C. arabica*, and true allotriploid HT 'CIFC 4106'.

Compounds	Culture media		
	M1	**M2**	**M3**
MS (Sigma®)	2.15 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}
Sucrose (Sigma®)	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}
L-cysteine (Sigma®)	0.08 g L^{-1}	0.04 g L^{-1}	-
Malt extract (Sigma®)	0.4 g L^{-1}	0.8 g L^{-1}	-
Casein (Sigma®)	0.1 g L^{-1}	0.2 g L^{-1}	-
2,4-D (Sigma®)	9.06 µM	-	-
BAP (Sigma®)	4.44 µM	4.44 µM	-
GA_3 (Sigma®)	-	-	2.89 µM
Phytagel (Sigma®)	2.8 g L^{-1}	2.8 g L^{-1}	2.8 g L^{-1}
Activated charcoal (Isofar®)	-	2.0 g L^{-1}	-
pH	5.6	5.6	5.6

M1: FEC induction medium; M2: SE regeneration medium; M3: plantlet recovering medium; MS: Murashige e Skoog (1962); Gamborg et al. (1968), 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzilaminopurina; GA_3: gibberellic acid. All media were autoclaved for 20 min at 121°C and, then, poured out into Petri dishes (M1 or M2), Erlenmeyers (liquid M1), tubes (M3). Differently of van Boxtel and Berthouly (1996), the ISE procedure only involved three sequential steps in semi-solid media. In the first step (leave cell dedifferentiation), the main adjustments for FEC induction

medium were the concentration of 2,4-D (9.06 µM), BAP (4.44 µM), casein hydrolysate (0.10 g L^{-1}), malt extract (0.40 g L^{-1}), sucrose (30 g L^{-1}) and L-cysteine (0.08 g L^{-1}). For the second step (FEC cell competence acquisition, determination, and differentiation), the SE regeneration medium differed in relation to the concentration of BAP (4.44 µM), casein hydrolysate (0.10 g L^{-1}) and sucrose (30 g L^{-1}), and the addition of active charcoal (2.00 g L^{-1}). The last step was accomplished from plantlet recovering medium, in which the growth regulators indole-3-acetic acid and BAP were replaced by GA_3. Physical conditions were identical to the often used for vitro propagation in *Coffea* by ISE.

Results

Nuclear DNA content and DNA ploidy level determination of the explant donors

The mean nuclear 2C DNA content value of the explant donors was identical to previous data: 2C = 1.38 ± 0.060 pg for *C. eugenioides* (Fig. 1a), 2C = 1.41 ± 0.012 pg for *C. canephora* (Fig. 1b), 2C = 2.62 ± 0.043 pg for *C. arabica* (Fig. 1c), and 1C = 2.10 ± 0.003 pg for HT 'CIFC 4106' (Fig. 1d). Thus far, our research group had expressed the nuclear genome size of HT 'CIFC 4106' in 2C (Clarindo et al. 2013; Sattler et al. 2016). However, since the karyotype characterization and karyogram assembly were not performed, chromosome pairs were not identified for this allotriploid. Therefore, 1C = 2.10 pg was assumed for HT 'CIFC 4106' (Fig. 1d). Based on the mean nuclear 2C value, the DNA ploidy level of the explant donors was found to be diploid for *C. eugenioides* and *C. canephora*, triploid for HT 'CIFC 4106', and tetraploid for *C. arabica*.

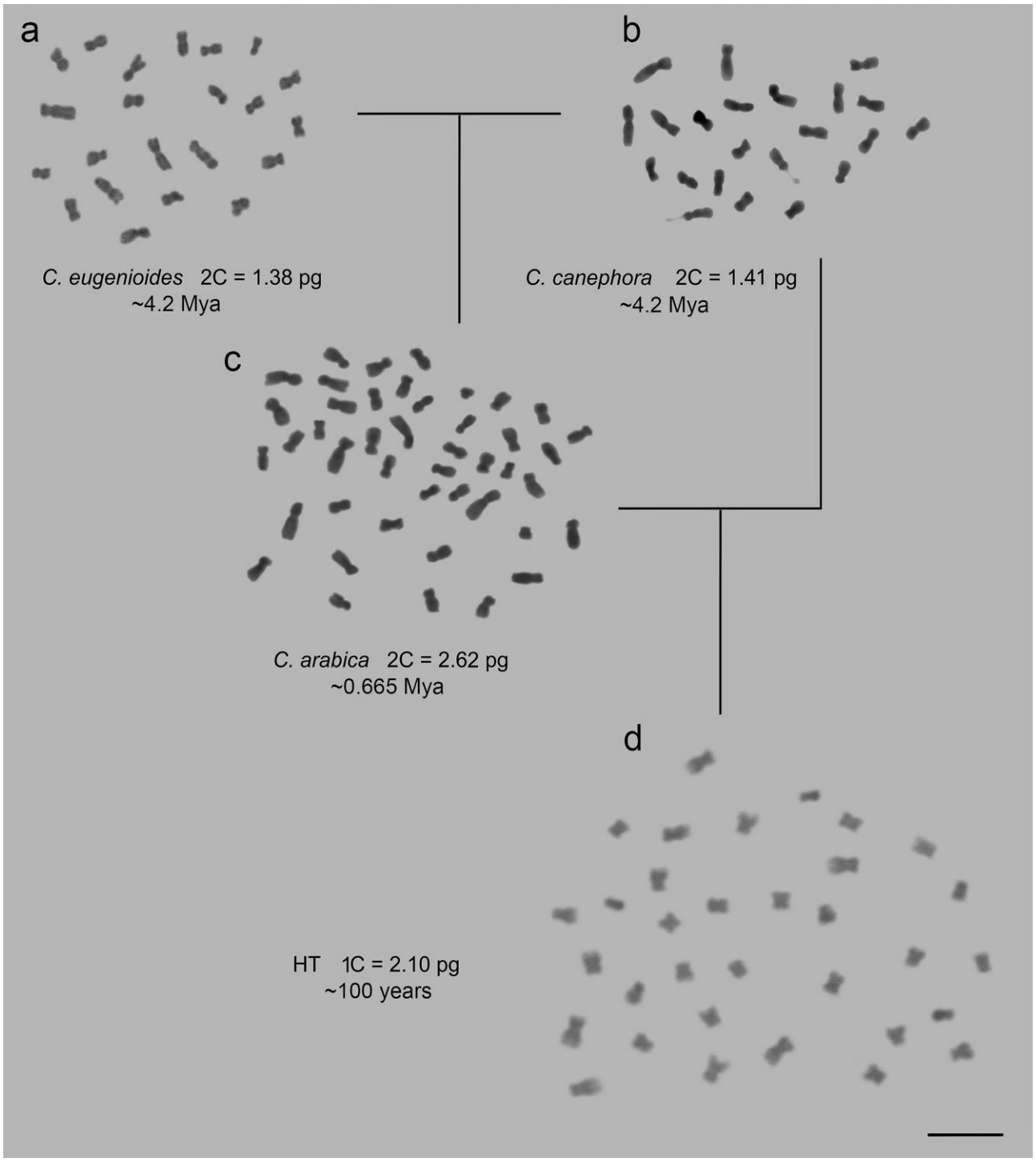
a
b
C. eugenioides 2C = 1.38 pg
~4.2 Mya
C. canephora 2C = 1.41 pg
~4.2 Mya
c
C. arabica 2C = 2.62 pg
~0.665 Mya
d
HT 1C = 2.10 pg
~100 years

Fig. 1 Karyotypes, 2C DNA content values and evolutionary ages of the four *Coffea*. a) *C. eugenioides* (EE) – 2n = 2x = 22 chromosomes, 2C = 1.38 ± 0.060 pg. b) *C. canephora* (CC) – 2n = 2x = 22 chromosomes, 2C = 1.41 ± 0.012 pg. The two species (a, b) are diploids originated around 4.2 mya (Yu et al. 2011). (c) *C. arabica* ($C^aC^aE^aE^a$) – 2n = 4x = 44 chromosomes, 2C = 2.62 ± 0.043 pg. This species originated ~0.665 mya (Yu et al. 2011). d) HT 'CIFC 4106' (CC^aE^a), allotriploid karyotype with 2n = 3x = 33 chromosomes, 1C = 2.10 ± 0.003 pg. This *Coffea* arose from a backcrossing between *C. arabica* ($C^aC^aE^aE^a$) and its possible progenitor *C. canephora* (CC). HT emerged around 100 years ago (Bettencourt 1973). Due to its allotriploid origin, the nuclear genome size for HT was shown in 1C.

Dedifferentiation of the explant cells and FEC establishment

The number of responsive explants differed among the *Coffea* over time (Fig. 2a–f). The amount of FEC (responsive leaf fragment explants) increased over time until the 60th day for all *Coffea* (Figs. 2a–d, 3). HT 'CIFC 4106' exhibited the highest mean number of FEC (from 3.62 at 15 days to 4.90 at 90 days), followed by *C. arabica* (2.17 at 15 days to 2.37 at 90 days), *C. canephora* (0.26 to 1.07) and *C. eugenioides* (0.08 to 1.04) (Fig. 2a–f). Thus, the polyploids (HT and *C. arabica*) had a higher mean number of responsive explants than the diploids (*C. canephora* and *C. eugenioides*). Differently from the polyploids, the diploid *Coffea* did not differ from each other over time (Fig. 2a–f). At 15–30 days, only the polyploids showed at least one responsive explant (Fig. 2a–b), and FEC were generated in larger quantities and in less time in these *Coffea*. The number of responsive explants (i.e. leaves exhibiting FEC) was constant for all *Coffea* after 60 days, and already after 15 days for *C. arabica* (Fig. 3). Furthermore, at 90 days all *Coffea* displayed explants which did not generate calli (Fig. 2f), except for HT 'CIFC 4106', which at 45 days showed all explants to be responsive (Fig. 2c–f). Considering each *Coffea*, variation in mean FEC number was observed for *C. arabica*, *C. eugenioides* and *C. canephora* (Fig. 2).

In addition to quantitative data, the cellular mass of the FEC gradually increased until the 90th day of culture for all *Coffea*. However, cell proliferation in the FEC was more pronounced in the polyploids, followed by the diploids (Figs. 3, 4a).

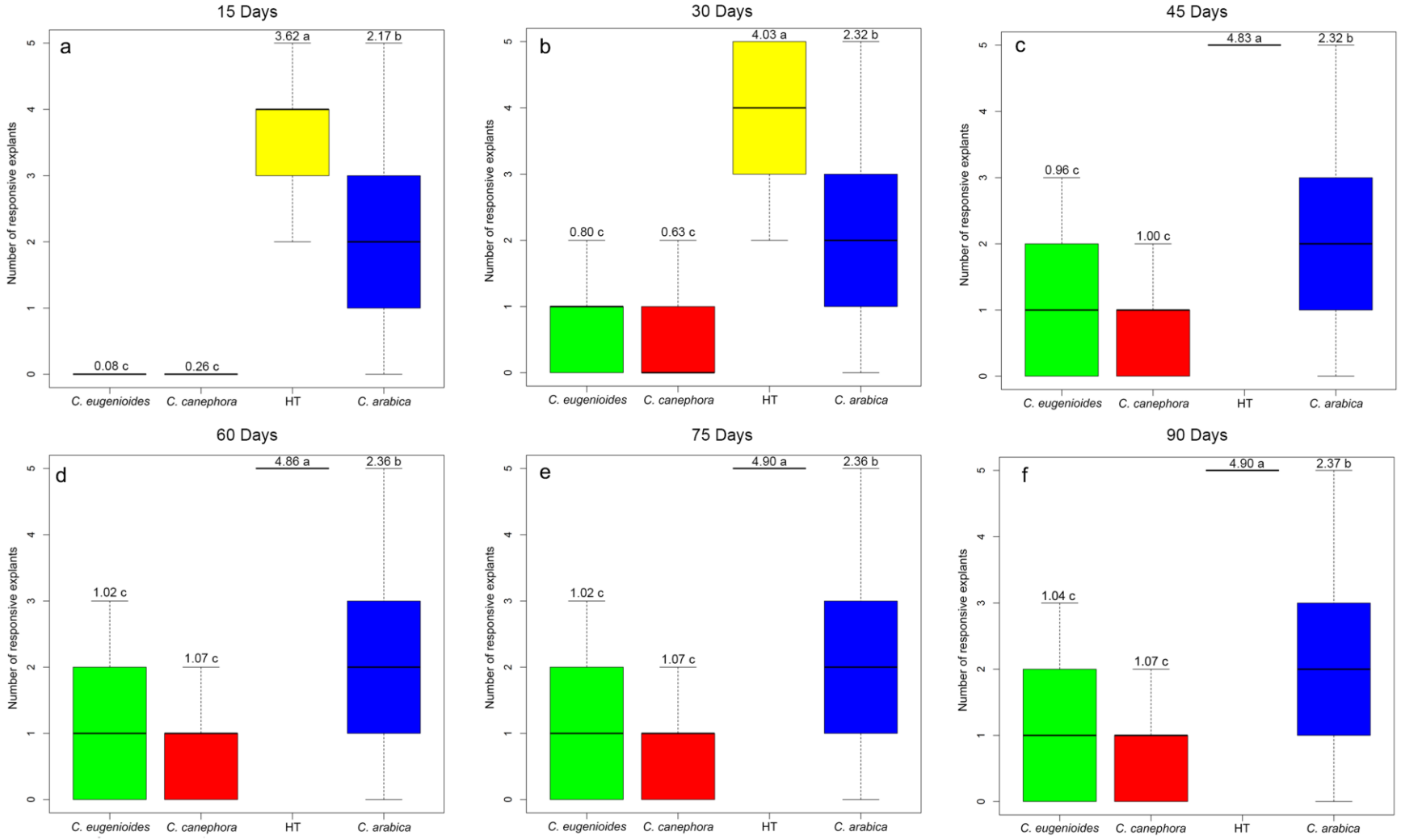
15 Days
a
3.62 a
2.17 b
0.08 c
0.26 c
30 Days
b
4.03 a
2.32 b
0.80 c
0.63 c
45 Days
c
4.83 a
2.32 b
0.96 c
1.00 c
60 Days
d
4.86 a
2.36 b
1.02 c
1.07 c
75 Days
e
4.90 a
2.36 b
1.02 c
1.07 c
90 Days
f
4.90 a
2.37 b
1.04 c
1.07 c
Number of responsive explants
C. eugenioides
C. canephora
HT
C. arabica

Fig. 2 Mean number of responsive leaf explants from *C. eugenioides, C. canephora*, HT 'CIFC 4106' and *C. arabica* over time (15–90 days) in FEC induction medium (M1, Table 1) – dedifferentiation step of the explant cells (Fig. 4a). The box plots show that the mean number of responsive explants differed among the *Coffea* (a–f), with the highest number being observed for HT 'CIFC 4106' (2n = 3x = 33 chromosomes, 1C = 2.10 pg), followed by *C. arabica* (2n = 4x = 44, 2C = 2.62 pg), *C. eugenioides* (2n = 2x = 22, 2C = 1.38 pg) and *C. canephora* (2n = 2x = 22, 2C = 1.41 pg) (Fig. 1). Notice the variation in responsive explant number in each *Coffea*, particularly in *C. arabica* and *C. eugenioides*. The mean number of FEC followed by the same letter are not different by Tukey's test ($p < 0.05$).

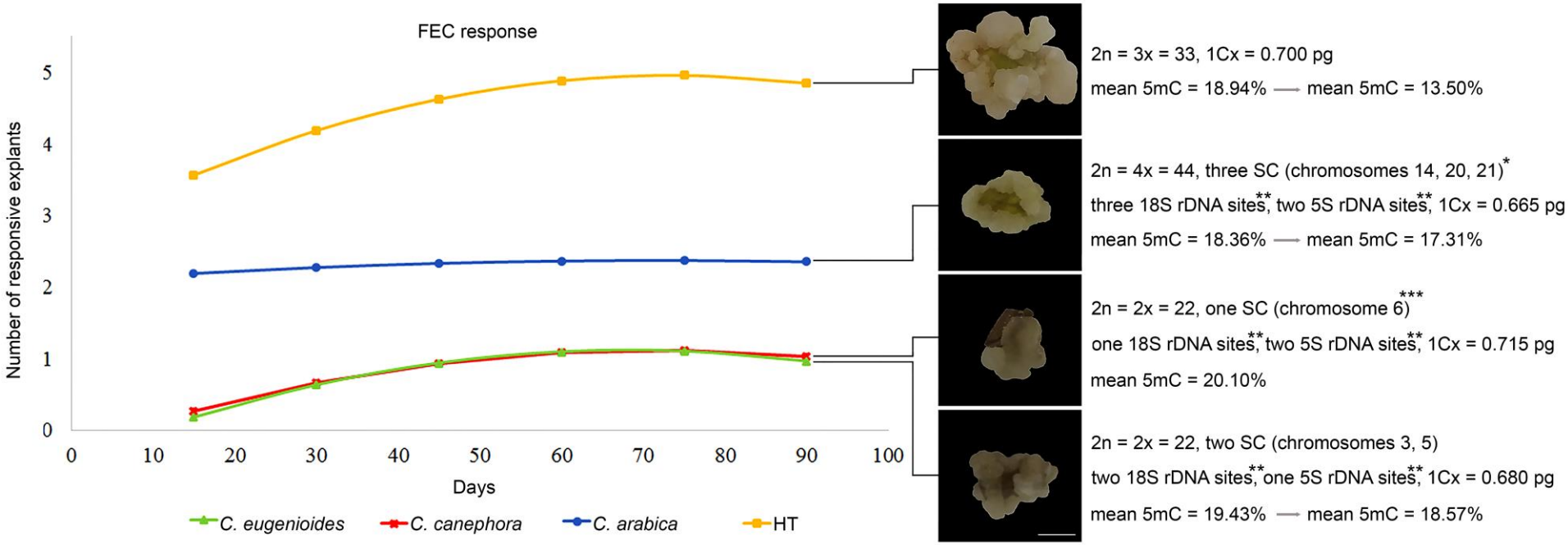
FEC response
Number of responsive explants
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
Days
C. eugenioides
C. canephora
C. arabica
HT
2n = 3x = 33, 1Cx = 0.700 pg
mean 5mC = 18.94% → mean 5mC = 13.50%
2n = 4x = 44, three SC (chromosomes 14, 20, 21)*
three 18S rDNA sites**, two 5S rDNA sites**, 1Cx = 0.665 pg
mean 5mC = 18.36% → mean 5mC = 17.31%
2n = 2x = 22, one SC (chromosome 6)***
one 18S rDNA sites**, two 5S rDNA sites**, 1Cx = 0.715 pg
mean 5mC = 20.10%
2n = 2x = 22, two SC (chromosomes 3, 5)
two 18S rDNA sites**, one 5S rDNA sites**, 1Cx = 0.680 pg
mean 5mC = 19.43% → mean 5mC = 18.57%

Fig. 3 FEC establishment in *C. eugenioides*, *C. canephora*, HT 'CIFC 4106' and *C. arabica*. Graphic representing dedifferentiation of the leaf explant cells, obtained from regression analysis using the mean number of responsive leaf explants of the four *Coffea*. The graphic shows that the allopolyploids (yellow – HT 'CIFC 4106'; blue – *C. arabica*) had higher mean number of responsive explants than the diploids (red – *C. canephora*; green – *C. eugenioides*). Observe that, from the 60th day onwards, the mean number of responsive leaf explants was constant in each *Coffea*, and already from the 15th day in *C. arabica*. Furthermore, the response presented by each *Coffea* also differed in relation to the time and cellular proliferation of the FEC (right). In addition to chromosome number and 2C/1Cx nuclear value, the four *Coffea* also exhibit other karyotype divergences, such as the number of SC and rDNA sites. Fitted quadratic models were significant ($p < 0.05$) by regression analysis for all *Coffea*: *C. eugenioides* – $Y = - 0.4236 + 0.04513X - 0.0004X^2$; *C. canephora* – $Y = - 0.2367 + 0.03772X - 0.0003X^2$; HT 'CIFC 4106' – $Y = 2.7724 + 0.05967X - 0.0005X^2$; and *C. arabica* – $Y = 2.0774 + 0.00838X - 0.0001X^2$. *Pinto-Maglio and Da Cruz 1998; **Hamon et al. 2009; ***Clarindo and Carvalho 2006.

MCSE regeneration

First signs of globular SE recovery occurred at 21, 27, 52 and 90 days for *C. arabica*, HT 'CIFC 4106', *C. eugenioides* and *C. canephora*, respectively. During the 180 days, SE reached different development stages (globular, heart, torpedo and cotyledonary), characterizing the *Coffea* ISE as asynchronous. Only the MCSE (Fig. 4b) were recorded to compare the studied *Coffea* (Figs. 5, 6, 7), owing to their clear identification and isolated embryo stage for plantlet recovery. MCSE emerged after 30 days in *C. arabica*, 51 days in HT 'CIFC 4106', 97 days in *C. eugenioides*, and 145 days in *C. canephora*.

The mean number of MCSE per FEC differed among the *Coffea* from 60 to 180 days (Fig. 4b–f). At 180 days, 60.1% of the FEC in *C. arabica* exhibited MCSE, as did 30.4% in *C. eugenioides*, 16.2% in HT 'CIFC 4106', and 14.3% in *C. canephora*. This embryogenic response increased over time, with mean number of MCSE varying from 1.71 to 9.94 for *C. arabica*, 0 to 2.04 for *C. eugenioides*, 0.10 to 1.0 for HT 'CIFC 4106', and 0 to 0.29 for *C. canephora* at 60 and 180 days, respectively (Fig. 5b–f). *C. arabica* showed the highest mean number of MCSE, differing over time from the other *Coffea*. *C. eugenioides, C. canephora* and HT 'CIFC 4106' presented the same mean number of MCSE until 90 days (Fig. 5b–c), being that this embryo stage was firstly found in the FEC of the allotriploid HT 'CIFC 4106'. After 180 days, these three *Coffea* showed the same mean number of MCSE (Fig. 5f).

Considering only the FEC that generated MCSE, the mean number varied from 3.18 to 18.45 for *C. arabica*, 0.62 to 6.06 for HT 'CIFC 4106', 0 to 6.71 for *C. eugenioides*, and 0 to 2.0 for *C. canephora* at 60 and 180 days, respectively (Fig. 6b–f). From 60 days onwards, *C. arabica* presented the highest mean number of MCSE, while the other *Coffea* were identical until 150 days (Figs. 6b–e, 7). After 180 days, *C.*

canephora presented the lowest mean number of MCSE (Figs. 6f, 7). Regarding each *Coffea*, variation in MCSE number was also found (Figs. 5, 6).

The ISE morphogenic pathway was established in relatively short time, varying from 150 days in *C. arabica*, 180 days in HT 'CIFC 4106', 270 days in *C. eugenioides*, to 300 days in *C. canephora* (Fig. 4).

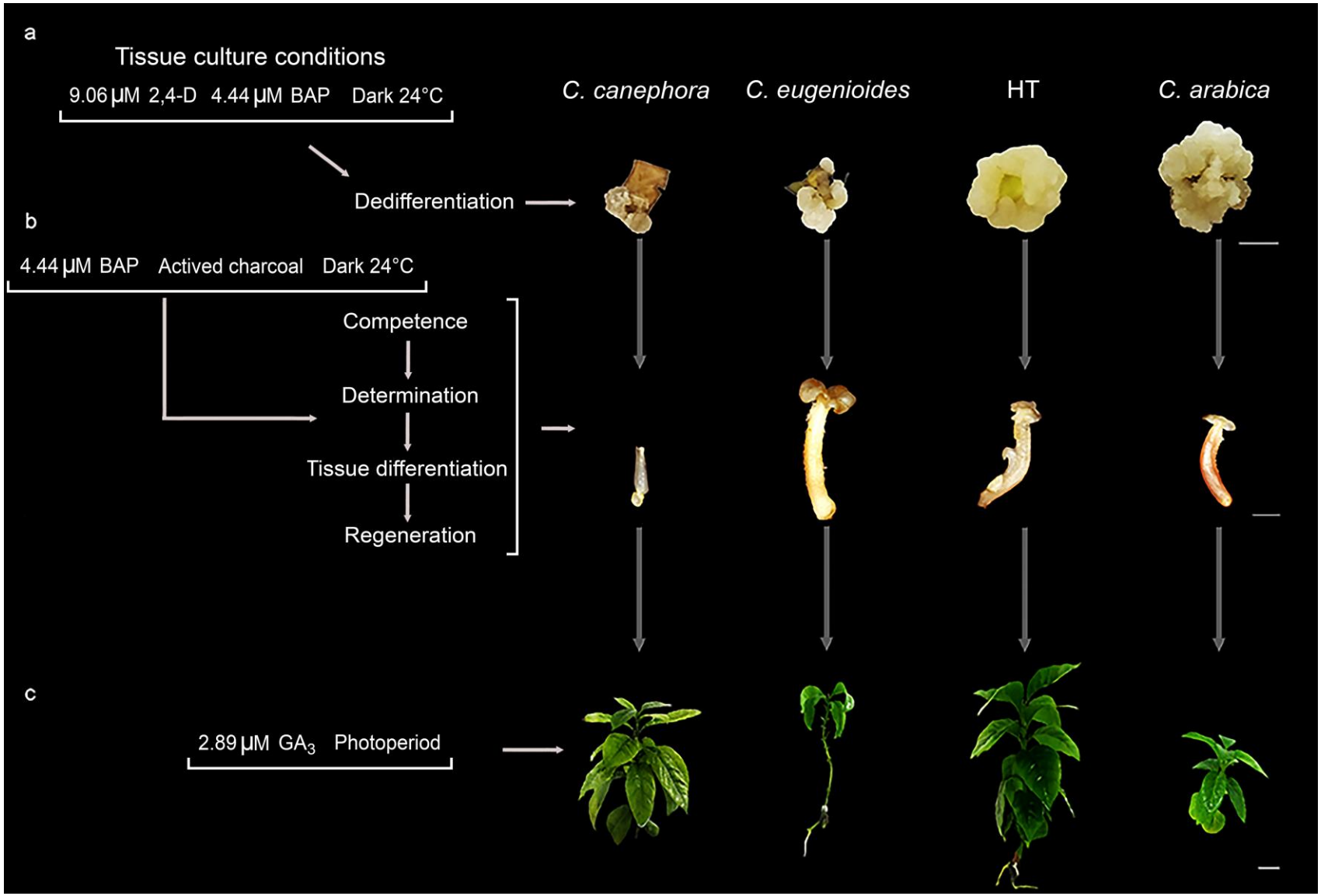
a
Tissue culture conditions
9.06 µM 2,4-D 4.44 µM BAP Dark 24°C
Dedifferentiation
C. canephora
C. eugenioides
HT
C. arabica
b
4.44 µM BAP Actived charcoal Dark 24°C
Competence
Determination
Tissue differentiation
Regeneration
c
2.89 µM GA3 Photoperiod

Fig. 4 ISE achievement in *C. canephora*, *C. eugenioides*, HT 'CIFC 4106' and *C. arabica* (left to right, respectively). Above the brackets, the main chemical and physical in vitro conditions for FEC induction (a, M1 – Table 1), SE regeneration (b, M2 – Table 1) and plantlet recovery (c, M3 – Table 1) are shown. (a) Dedifferentiation of leaf cells in these *Coffea* was promoted in medium supplemented with 2,4-D and BAP, in the dark. Besides differences in mean number of responsive explants, also the cell proliferation in the FEC was more pronounced in the allopolyploids (HT 'CIFC 4106' and *C. arabica*) than in the diploids (*C. eugenioides* and *C. canephora*). Bar = 1 cm. (b) Acquisition of cell competence and determination, tissue differentiation and SE regeneration in FEC occurred in medium supplemented with BAP and active charcoal, in the dark. Representative SE were obtained for all *Coffea*: immature cotyledonary SE of *C. canephora* and mature cotyledonary SE of *C. eugenioides*, HT 'CIFC 4106' and *C. arabica* after 180 days in medium M2 (Table 1). Note a globular SE in the apical root meristem of the *C. canephora* SE, evidencing the occurrence of secondary somatic embryogenesis. Bar = 2 mm. c) MCSE germination and seedling development was promoted in medium supplemented with GA_3, under photoperiod. The ISE morphogenic pathway was established in 150 days for *C. arabica*, 180 days for HT 'CIFC 4106', 270 days for *C. eugenioides*, and 300 days for *C. canephora*. Bar = 1 cm.

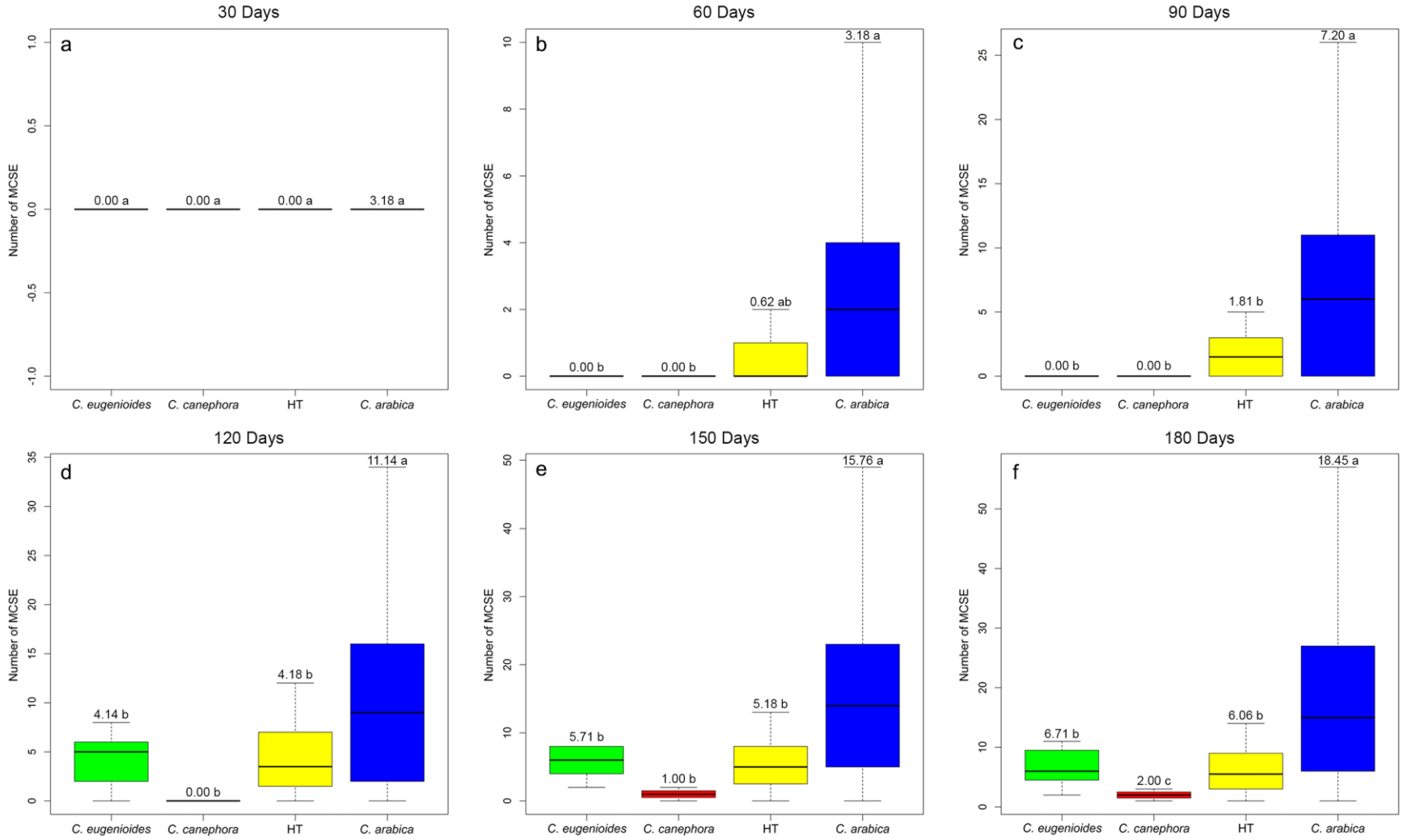
30 Days
a
Number of MCSE
1.0
0.5
0.0
-0.5
-1.0
0.00 a
0.00 a
0.00 a
3.18 a
C. eugenioides
C. canephora
HT
C. arabica
60 Days
b
Number of MCSE
10
8
6
4
2
0
3.18 a
0.62 ab
0.00 b
0.00 b
C. eugenioides
C. canephora
HT
C. arabica
90 Days
c
Number of MCSE
25
20
15
10
5
0
7.20 a
1.81 b
0.00 b
0.00 b
C. eugenioides
C. canephora
HT
C. arabica
120 Days
d
Number of MCSE
35
30
25
20
15
10
5
0
11.14 a
4.18 b
4.14 b
0.00 b
C. eugenioides
C. canephora
HT
C. arabica
150 Days
e
Number of MCSE
50
40
30
20
10
0
15.76 a
5.18 b
5.71 b
1.00 b
C. eugenioides
C. canephora
HT
C. arabica
180 Days
f
Number of MCSE
50
40
30
20
10
0
18.45 a
6.06 b
6.71 b
2.00 c
C. eugenioides
C. canephora
HT
C. arabica

Fig. 5 Mean number of MCSE (30–180 days) for *C. eugenioides*, *C. canephora*, HT 'CIFC 4106' and *C. arabica* considering all FEC in SE regeneration medium (M2, Table 1) – step of competence acquisition, FEC cell determination and differentiation, and MCSE regeneration (Fig. 4b). The box plots show that the mean number of MCSE differed among the *Coffea* between 60 and 180 days (b–f), with the highest number of FEC exhibiting MCSE being observed for *C. arabica* (2n = 4x = 44 chromosomes, 2C = 2.62 pg). In addition, MCSE were recovered faster in the FEC of this species (b). At 180 days, *C. eugenioides*, *C. canephora* and HT 'CIFC 4106' showed the same mean number of MCSE. Mean MCSE numbers followed by the same letter do not differ by Tukey's test ($p < 0.05$).

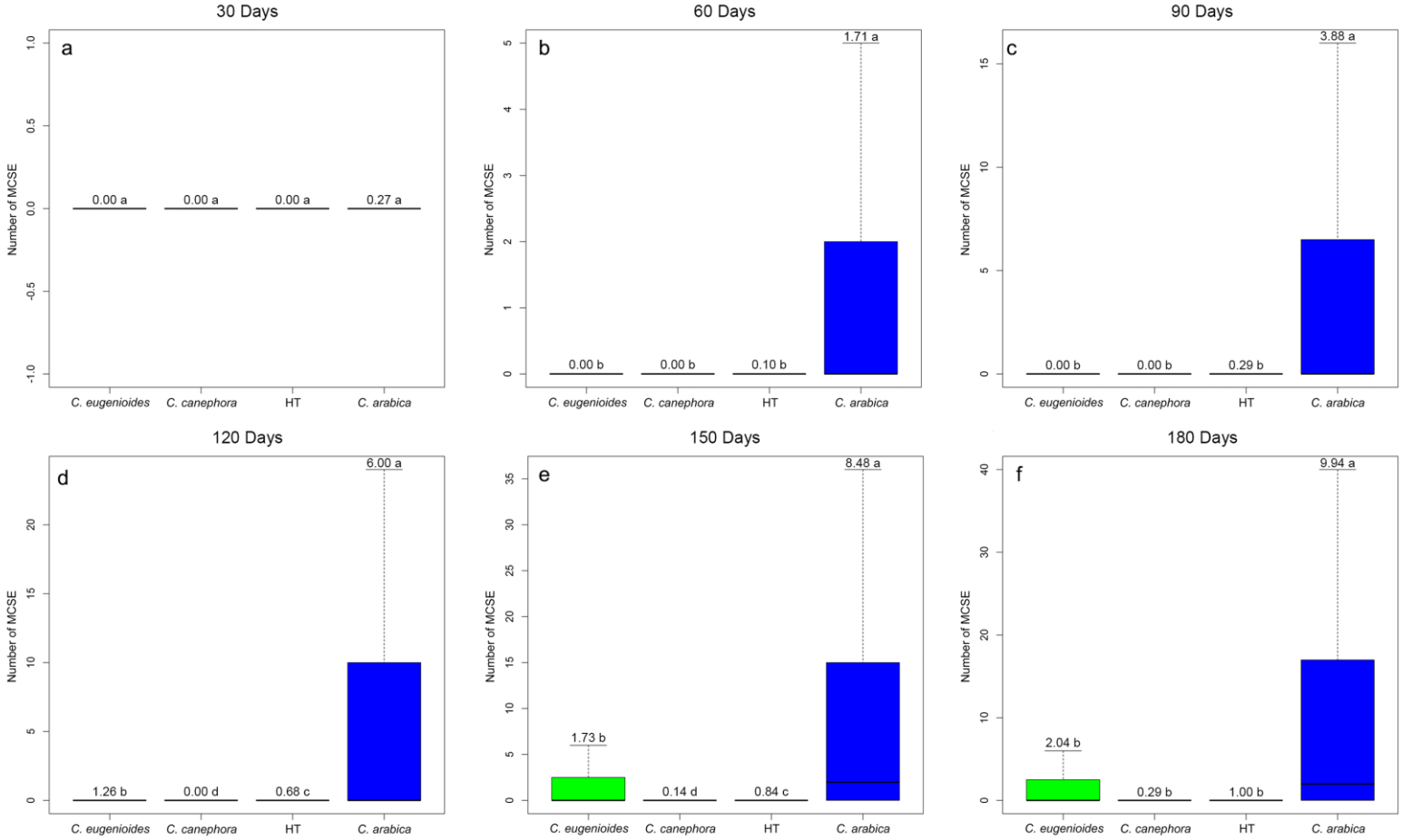
30 Days
a
Number of MCSE
0.00 a
0.00 a
0.00 a
0.27 a
C. eugenioides
C. canephora
HT
C. arabica
60 Days
b
1.71 a
0.00 b
0.00 b
0.10 b
90 Days
c
3.88 a
0.00 b
0.00 b
0.29 b
120 Days
d
6.00 a
1.26 b
0.00 d
0.68 c
150 Days
e
8.48 a
1.73 b
0.14 d
0.84 c
180 Days
f
9.94 a
2.04 b
0.29 b
1.00 b

Fig. 6 Box plots representing the mean number of MCSE for the responsive FEC of the four studied *Coffea*. Because physiological and epigenetic aspects may impede competence acquisition, and consequently determination and differentiation, the mean number of MCSE in the *Coffea* was compared considering only the responsive FEC. As shown in Fig. 5, the highest number of MCSE was observed for *C. arabica* between 30 – 180 days. In contrast, at 180 days the FEC of *C. canephora* presented the lowest mean number of MCSE in relation to *C. eugenioides* and HT 'CIFC 4106'. Observe the variation in MCSE number mainly for *C. arabica* and *C. eugenioides*, as shown in Figures 2 and 5. Mean MCSE numbers followed by the same letter do not differ by Tukey's test ($p < 0.05$).

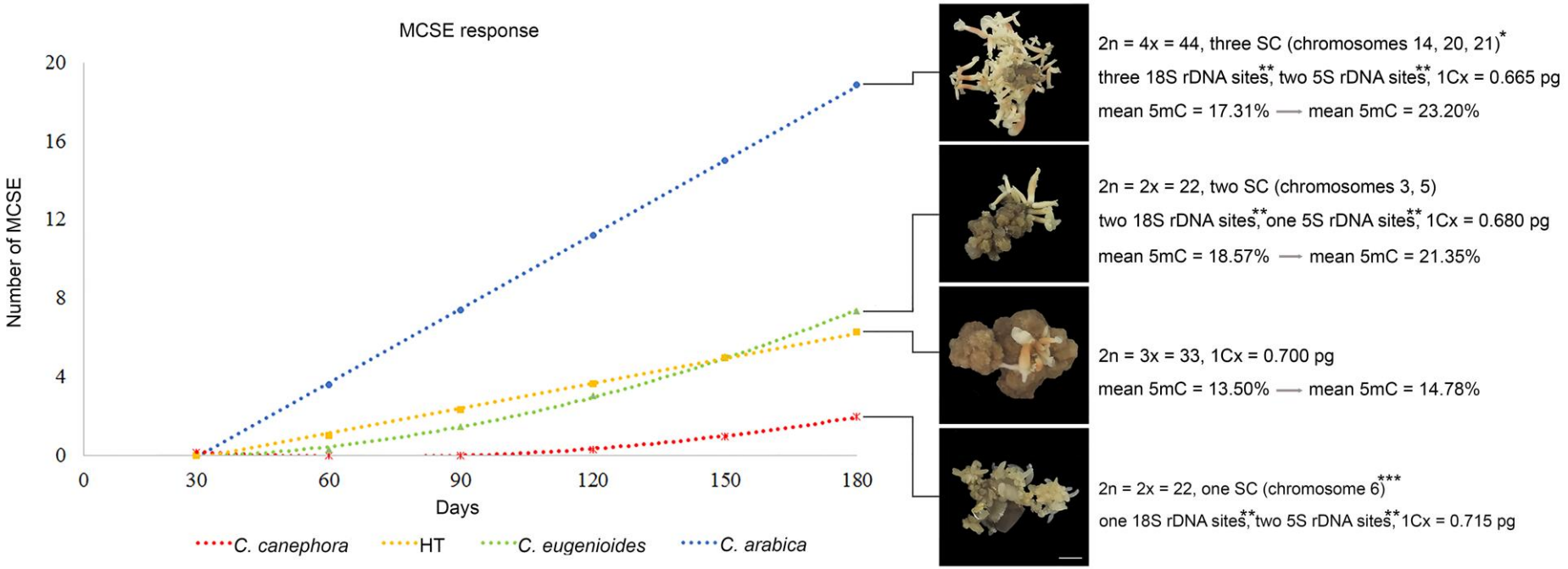
MCSE response
Number of MCSE
20
16
12
8
4
0
0
30
60
90
120
150
180
Days
C. canephora
HT
C. eugenioides
C. arabica
2n = 4x = 44, three SC (chromosomes 14, 20, 21)*
three 18S rDNA sites**, two 5S rDNA sites**, 1Cx = 0.665 pg
mean 5mC = 17.31% → mean 5mC = 23.20%
2n = 2x = 22, two SC (chromosomes 3, 5)
two 18S rDNA sites**, one 5S rDNA sites**, 1Cx = 0.680 pg
mean 5mC = 18.57% → mean 5mC = 21.35%
2n = 3x = 33, 1Cx = 0.700 pg
mean 5mC = 13.50% → mean 5mC = 14.78%
2n = 2x = 22, one SC (chromosome 6)***
one 18S rDNA sites**, two 5S rDNA sites**, 1Cx = 0.715 pg

Fig. 7 In vitro response as shown by SE recovery in *C. canephora*, HT 'CIFC 4106', *C. eugenioides* and *C. arabica*. Graphic obtained from regression analysis using the mean number of MCSE (Fig. 6) of the four *Coffea*. The graphic shows the highest mean number of MCSE for *C. arabica* (blue) during the entire period of SE conversion and maturation. *C. eugenioides* (green) and HT 'CIFC 4106' (yellow) exhibited the same mean number of MCSE at 30–180 days. After 180 days, *C. canephora* FEC showed the lowest mean number of MCSE. Besides the chromosome number and 2C/1Cx nuclear value, these *Coffea* also differed with regard to other cytogenetic features, such as the number of SC and 45S rDNA and 5S rDNA sites. Fitted models were significant ($p < 0.05$) by regression analysis: *C. eugenioides* – $Y = - 0.8428 + 0.00522X + 0.0002X^2$; *C. canephora* – $Y = 0.7 - 0.02512X + 0.0001X^2$; HT 'CIFC 4106' – $Y = - 1.6583 + 0.0441X$; and *C. arabica* – $Y = - 4.0693 + 0.1273X$. *Pinto- Maglio and Da Cruz 1998; **Hamon et al. 2009; ***Clarindo and Carvalho 2006.

Chromosome number of the Coffea FEC

The chromosome number established in the FEC was identical to that reported for each *Coffea*: *C. eugenioides* and *C. canephora* showed 2n = 2x = 22 chromosomes (Fig. 1a, b), *C. arabica* had 2n = 4x = 44 (Fig. 1c), and HT 'CIFC 4106' presented 2n = 3x = 33 (Fig. 1d). These data confirmed the euploid series based on the chromosome number x = 11. All metaphases showed constant chromosome number for each *Coffea*, indicating that no numerical variation occurred during the in vitro tissue culture. Thus, chromosome number evidences the stability of the karyotype, indicating that no somaclonal variation related to euploidy and/or aneuploidy has occurred.

Based on chromosome number and nuclear genome size, the 2C/1C value was converted to 1Cx (1C DNA content for the basic chromosome set x =11). In this way, the mean 1Cx value of *C. eugenioides* was equivalent to 0.680 pg, with 1Cx = 0.715 pg for *C. canephora*, 1Cx = 0.665 pg for *C. arabica*, and 1Cx = 0.700 pg for HT 'CIFC 4106'.

Discussion

Differently from other studies also involving *Coffea* with distinct ploidy level (Staritsky 1970; van Boxtel and Berthouly 1996; Samson et al. 2006), the tissue culture procedure was effective for ISE establishment in *C. canephora*, *C. eugenioides*, HT 'CIFC 4106' and *C. arabica* under the same in vitro conditions. This experimental design allowed verifying whether the karyotype features can be related to ISE responses in these *Coffea*.

Briefly, the karyotype of the four *Coffea* analyzed here differed with regard to chromosome number, resulting in distinct ploidy level (based on x = 11 chromosomes)

and nuclear 2C values (Figs. 1, 3, 7). The number of SC, 18S and 5S rDNA sites (Figs. 3, 7) is also distinct among these *Coffea*. The *C. eugenioides* karyotype exhibits two SC (chromosomes 3 and 5, data not shown), whereas one is found in *C. canephora* (chromosome 6; Clarindo and Carvalho 2006; Figs. 3, 7) and three in *C. arabica* (chromosomes 14, 20 and 21; Pinto-Maglio and Da Cruz 1998; Figs. 3, 7). Further, the number of 18S rDNA sites (Hamon et al. 2009) corroborates the number of SC. The karyotypes of *C. canephora* and *C. arabica* show two 5S rDNA sites, while *C. eugenioides* has one (Hamon et al. 2009).

Based on the quantitative (mean number of responsive leaf explants, mean number of MCSE, and response time) and qualitative variables (FEC cell mass), the four *Coffea* differed in relation to ISE response. In the first ISE step (dedifferentiation of explant cells and FEC establishment), leaf explants of the true allopolyploids (allotriploid HT 'CIFC 4106' and allotetraploid *C. arabica*) were the most responsive, providing FEC in relatively low time (Figs. 2, 3) and visually exhibiting more pronounced cell proliferation (Figs. 4a, 6). Other studies concomitantly analyzing *Coffea* with different ploidy levels also reported differences in relation to FEC responses. Leaf explants of the diploid *C. canephora*, the allopolyploid 'Arabusta', and the homoploid 'Congusta' were more responsive than the allotetraploid *C. arabica* (van Boxtel and Berthouly 1996). Independently of the FEC induction strategy (two steps in semisolid medium and one step in liquid medium, van Boxtel and Berthouly 1996; or a single step in semisolid medium, present study), differences were observed in FEC responses for *Coffea* with different ploidy levels.

In the second ISE step (competence acquisition, FEC cell determination and differentiation, and MCSE regeneration), a higher mean number of MCSE was observed for the true allotetraploid *C. arabica*, which were also recovered faster in the FEC of

this species (Figs. 6, 7). Since the first ISE study (Staritsky 1970), differences in relation to SE formation have been noted in *Coffea* with different ploidy levels (van Boxtel and Berthouly 1996; Samson et al. 2006). Hence, van Boxtel and Berthouly (1996) highlighted the importance of considering the genetic background of selected *Coffea*. In that sense, since differences in relation to SE formation have been pointed out but not debated, the present study verified the relationship between karyotype features (chromosome number, ploidy level and nuclear DNA content) and ISE response. Therefore, the experimental design of the present study constitutes a further step towards understanding the ISE response in *Coffea*.

Associating the ISE to the karyotype and nuclear 2C value, and considering that the 1Cx value is close among the four *Coffea* (1Cx = 0.680 pg in *C. eugenioides*, 1Cx = 0.715 pg in *C. canephora*, 1Cx = 0.665 pg in *C. arabica*, and 1Cx = 0.700 pg in HT 'CIFC 4106'; Figs. 3, 7), a relationship between those karyotype features and the ISE responses may exist. Nuclear genome size and karyotype divergences have been pointed out as factors that directly influence the gene regulation involved in morphogenesis, interfering with the in vitro response (Chandler et al. 2008; Irikova et al. 2012; Xu and Huang 2014). In a euploid series (monoploid, diploid, triploid and tetraploid) of *Zea mays* L., the most analyzed genes exhibited a relative dosage effect in multiples of the basic chromosome complement, x = 10. The expression level rose proportionally to the ploidy level, increasing from monoploid up to tetraploid (Guo et al. 1996).

The high mean number of responsive leaf explants in relatively low time (Figs. 2, 3) and the FEC cell proliferation were more pronounced in HT 'CIFC 4106' (2n = 3x = 33 chromosomes) compared with the other three *Coffea* (Figs. 3, 4a). However, surprisingly the mean number of MCSE in HT 'CIFC 4106' was the same as in *C. eugenioides* (2n = 2x = 22) and lower than in *C. arabica* (2n = 4x = 44) (Fig. 6f).

Possibly, HT ‘CIFC 4106’ FEC cells exhibited a high level of endogenous auxins, such as indole-3-acetic acid, which maintain the cell undifferentiated (Fehér et al. 2003). This physiological aspect, which may be related to the allotriploidy of HT ‘CIFC 4106’, prevents the regeneration, conversion and maturation of SE under the tissue culture conditions established here (M2, Table 1). Therefore, for future ISE in the allotriploid HT ‘CIFC 4106’, the tissue culture medium should be adjusted to consider the ploidy level, so as to increase the mean number of MCSE. Further suggestions are to decrease the 2,4-D level in the FEC induction medium (M1, Table 1), and/or increase the concentration of active charcoal or sucrose, and/or add abscisic acid (ABA) to the SE regeneration medium (M2, Table 1).

At 180 days, FEC of *C. eugenioides* presented a higher mean number of MCSE in relation to *C. canephora* (Fig. 6f). Thus, quantitative divergence regarding ISE response was verified for *Coffea* with similar nuclear genome sizes and same chromosome number (2n = 2x = 22). However, the karyotype of these diploid species presented differences, such as the number of rDNA sites: *C. eugenioides* presents one 5S rDNA site and two 18S rDNA sites, while *C. canephora* possesses two sites of 5S rDNA and one of 18S rDNA (Hamon et al. 2009). 5S and 18S rDNA sites are equally important for the formation of the ribosome subunits (Neves et al. 2005) and, consequently, for the biosynthesis of polypeptides. Therefore, the ploidy level is not the only karyotype feature determining the notable divergences in ISE responses in *Coffea*: also the number of rDNA sites is representative of the karyotype complexity (Figs. 3, 7).

The ISE establishment for *C. canephora*, *C. eugenioides*, HT ‘CIFC 4106’ and *C. arabica* under the same in vitro conditions was fundamental, since in vitro response is influenced by the in vitro environment and the species’ genotype (Fehér et al. 2003).

Some authors have assumed a species-specific response for *Coffea* (Molina et al. 2002; Samson et al. 2006) based on the ISE response rates among hybrids, species and lines of the same species. Figure 4 is a guideline that summarizes the ISE procedure, showing the chemical and physical conditions of each step (FEC induction, SE regeneration and plantlet recovery). This guideline represents a substantial advancement for the conduction of future ISE studies in *Coffea*.

For the first time, SE and plantlets were regenerated for *C. eugenioides* via ISE. All steps of the ISE procedure (dedifferentiation, Figs. 2, 3, 4a; SE conversion and maturation, Figs. 4b, 5–7; and seedling development, Fig. 4c), in particular the chemical and physical in vitro conditions, were adapted from van Boxtel and Berthouly (1996). The procedure proposed by these authors has represented the basis for the in vitro propagation of *C. canephora* and *C. arabica* lines, and has been improved in our ISE routine (Sattler et al. 2016; Sanglard et al. 2017).

Considering the ISE responses and the more adequate in vitro procedures summarized in Table 1, certain in vitro conditions should be compiled for *Coffea*. FEC formation and cell proliferation in *Coffea* leaf explants (Figs. 2, 3, 4a) confirmed the role of the growth regulators 2,4-D and BAP in cell dedifferentiation. Thus, the use and balance of these compounds was crucial for callus induction, a precondition for ISE in *Coffea*. The essential role of 2,4-D in dedifferentiation has been demonstrated via a stress-related mechanism (Fehér et al. 2015). Such stress is promoted by an increased amount of oxidative compounds, such as hydrogen peroxide, produced in plant cells exposed to this growth regulator (Pfeiffer and Hoftberger 2001). Another advantage of the synthetic 2,4-D is associated to its chemical structure (Gaspar et al.1996), which is stable under in vitro conditions and not recognized by intracellular enzymes (Gaspar et al. 1996; Karami and Saidi 2010). Similarly to 2,4-D, the cytokinin BAP, a derivative of

a purine base not cleaved by cytokinin oxidase, affects the plant growth and development, such as cell division, shoot initiation and growth, and apical dominance (Kieber and Schaller 2014). Despite being fundamental for the *Coffea* ISE (Table 1), the role of BAP, as of other cytokinins, should be further investigated in tissue culture studies.

SE formation in *Coffea* is promoted after removal of the exogenous auxin from the culture medium, eliminating the main chemical component that maintains plant cells in a totipotent condition (Rose et al. 2010; Nic-Can and Loyola-Vargas 2016). Accordingly, activated charcoal has been added to plant tissue culture due to its capacity of adsorbing residues of exogenous 2,4-D (Pan and van Staden 1998), which has been demonstrated by its inhibitory effect on direct somatic embryogenesis in *C. canephora* (Hatanaka et al. 1991). In the present study, for the FEC providing MCSE in all *Coffea*, the 2,4-D excess was adsorbed by active charcoal, enabling to speed up the determination and differentiation process.

Coffea MCSE inoculation in medium supplemented with GA_3 provided seedlings at 30 days. This compound is metabolized by gibberellin 3-oxidase, resulting in GA_3 conversion to its bioactive forms. Hence, GA_3 plays a direct role in bioactive gibberellin levels (Mitchum et al. 2006), promoting SE germination.

Variation in the mean number of responsive leaf explants (Fig. 2) and MCSE (Figs. 5, 6) was observed in each *Coffea*, particularly *C. arabica* and *C. eugenioides* (Figs. 2f, 5f, 6f). The larger variation in these *Coffea* was probably conditioned by propagation of the explant donors in greenhouse. In contrast, *C. canephora* and HT 'CIFC 4106' had been propagated in a more homogeneous physical and chemical environment, namely in vitro. Variation in the ISE response has been reported in *C. arabica* (van Boxtel and Berthouly 1996; Cid et al. 2004) and *C. canephora* (van Boxtel

and Berthouly 1996), being suggested to result from genotypic differences among explant donor plants. In addition, differences in ISE response among *Coffea* species can be related to the physiological status of the explant, such as the endogenous level of growth regulators (Santana et al. 2004). Nevertheless, it should be noted that genetic factors (such as the karyotype features analyzed in this study) and epigenetic features (Nic-Can et al. 2013) may also affect the in vitro response.

Conclusions

The tissue culture procedure was effective for ISE establishment in the four *Coffea* under the same in vitro conditions, independently of the differences regarding (a) ploidy level, (b) nuclear DNA content, and (c) physiological conditions of the explant donors (greenhouse – *C. arabica* and *C. eugenioides*, or in vitro – *C. canephora* and HT 'CIFC 4106'). As summarized in Figures 3 and 7, the main karyotypic difference identified among the four *Coffea* was the ploidy level (diploids *C. canephora* and *C. eugenioides*; true allotriploid HT 'CIFC 4106'; and true allotetraploid *C. arabica*), which were related to the obtained ISE responses. Therefore, for future ISE studies in *Coffea*, it is imperative to establish the ploidy level background. Besides, other karyotype features should be investigated, particularly between *Coffea* with same ploidy level.

Acknowledgments

We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília – DF, Brazil, grant: 443801/2014-2), Fundação de Amparo à Pesquisa do Espírito Santo (FAPES, Vitória – ES, Brazil, grant:

65942604/2014) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília – DF, Brazil) for financial support.

Author contribution statement: The authors Sanglard NA, Sattler MC, Oliveira SCD and Clarindo WR conceived, designed and conducted the tissue culture experiments, flow cytometry and cytogenetic analyses. The authors Sattler MC and Oliveira SCD contributed with cytogenetic analyses. The author Carvalho CR contributed with flow cytometry analysis. The author Ferreira A performed the statistical analysis. For manuscript editing and revision, all authors equally contributed to the work and approved the final manuscript version for submission.

Conflict of interest: The authors declare that they have no conflict of interest.

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CAPÍTULO II

Title: 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 DOI 10.1007/s11240-017-1278-4
Received: 11 May 2017 / Accepted: 7 July 2017

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Abstract

Since 1966, chromosome doubling has been performed mainly in vitro, associating anti-tubulin treatment and different plant tissues showing proliferative cells. Despite the achieved improvements, some bottlenecks have been pointed out, such as the low rate of polyploids and high rate of mixoploid plantlets. To overcome these hurdles, some approaches have indicated that indirect somatic embryogenesis (ISE) constitutes an alternative trigger for chromosome doubling, especially for homoploid and anorthoploid germplasms. In this way, a guideline has been developed for hexaploidization of the *Coffea* line “Híbrido de Timor” (HT) 'CIFC 4106' (anorthoploid, 3x = 33 chromosomes, 1C = 2.10 pg, *Coffea canephora* x *Coffea arabica*) from friable embryogenic calli (FEC) treated with colchicine. From this, a relatively high percentage (49.3%) of HT hexaploids (6x = 66 chromosomes, 2C = 4.20 pg) was obtained, without recovery of mixoploids. Besides confirmation of endomitosis induction through the obtained hexaploids, SSR markers revealed that the FEC/colchicine strategy also resulted in loss of allelic diversity in 39 regenerated HT plantlets, demonstrating its genotoxic effect. Considering these results, the present procedure resolved the main bottlenecks for chromosome doubling, which have been reported since the discovery and isolation of the anti-tubulin colchicine in 1930. Hexaploid HT plantlets have enriched *Coffea* germplasm banks as a new genetic resource since the resolution of their karyotype and DNA sequence. Just as the true allotetraploid *C. arabica* and the allotriploid HT 'CIFC 4106', the hexaploid HT is relevant to investigate the genomic and phenotypic changes arising from polyploidization events.

Keywords: *Coffea*, in vitro polyploidization, indirect somatic embryogenesis, flow cytometry, cytogenetics, SSR markers.

Abbreviations:

2,4-D 2,4-dichlorophenoxyacetic acid

CIM callogenesis induction medium

FCM flow cytometry

FEC friable embryogenic callus

FEC-CT friable embryogenic callus – colchicine-treated

FEC-NCT friable embryogenic callus – not colchicine-treated

GA_3 gibberellic acid

GM germination medium

HT "Híbrido de Timor"

HT-ED "Híbrido de Timor" – explant donors

ISE indirect somatic embryogenesis

RM regeneration medium

SE somatic embryos

SSR simple sequence repeat

Introduction

In homoploids (hybrids with two genomes – AB) (Hegarty et al. 2008) and anorthoploids (auto- or allopolyploids with odd number of chromosome sets, e.g. 3x, 5x, 7x…,) (Levan and Müntzing 1963; Greilhuber and Doležel 2009), irregularities may occur during chromosome pairing and segregation in meiosis (Ramsey and Schemske 1998). Such abnormalities result in aneuploid reproductive cells, and hence infertility or semi-fertility (Xiong et al. 2011; Lloyd and Bomblies 2016). Accordingly, in vitro polyploidization has been accomplished with the perspective of restoring the fertility of homoploids (Song et al. 1997) and anorthoploids, mainly in allotriploids (Faleiro et al. 2016).

Considering this and other impacts on plant breeding, the application of in vitro chromosome doubling (also denominated in vitro polyploidization induction) gains importance (Dhooghe et al. 2011; Sattler et al. 2016b). Briefly, this application implies: (a) treatment of explants showing proliferative (totipotent) cells with anti-tubulinic compounds, (b) plantlet recovery through an in vitro morphogenic pathway, (c) screening of the regenerated polyploids, and (d) propagation of the selected polyploids. Various explants have been used for in vitro chromosome doubling, but calli (or aggregate cellular suspensions) stand out for presenting several totipotent cells, which have provided polyploid plantlets by indirect organogenesis (Song et al. 1997; Gao et al. 2002; Suzuki et al. 2005; Meyer et al. 2009) or indirect somatic embryogenesis (ISE) (Wu and Mooney 2002; Petersen et al. 2003; Zhang et al. 2007; Dutt et al. 2010; Faleiro et al. 2016).

In vitro chromosome doubling in calli is conducted by exposing all proliferative cells to polyploidization medium supplemented with an anti-tubulin, such as colchicine

(Song et al. 1997; Gao et al. 2002; Wu and Mooney 2002; Petersen et al. 2003; Zhang et al. 2007; Dutt et al. 2010), oryzalin (Wu and Mooney 2002; Petersen et al. 2003; Meyer et al. 2009; Faleiro et al. 2016) or trifluralin (Faleiro et al. 2016). Subsequently, the calli are transferred to a new culture medium devoid of the anti-tubulin (Gao et al. 2002; Zhang et al. 2007).

From the treatment of calli with anti-tubulin, new approaches for in vitro chromosome doubling have been proposed applying ISE (Wu and Mooney 2002; Petersen et al. 2003; Zhang et al. 2007; Dutt et al. 2010; Faleiro et al. 2016). Because the somatic embryo commonly originates from a single cell of the callus (Steward et al. 1958; Féher et al. 2003), only stable polyploid plantlets have been regenerated with this method (Gmitter and Ling 1991; Gmitter et al. 1991; Gao et al. 2002; Dutt et al. 2010; Faleiro et al. 2016). Moreover, mixoploid plantlets, which find little use in breeding programs (Barrett 1974; Jaskani et al. 1996), are not formed this way.

Despite the advantages of ISE for in vitro chromosome doubling, occurrence of somaclonal variation (genetic and epigenetic changes) is common in these assays (Larkin and Scowcroft 1981; Song et al. 1997; Liu et al. 2009; Bairu et al. 2011). Furthermore, the anti-tubulinic agents used for in vitro chromosome doubling are toxic compounds that disrupt microtubule formation (Dutt et al. 2010), alter the DNA sequence (genotoxic effect) (Rauf et al. 2006; Khosravi et al. 2009; Liu et al. 2009; Temel and Gözükirmizi 2015), and promote retrotransposon activation (Temel and Gözükirmizi 2015).

In this study, insights regarding in vitro chromosome doubling are provided in order to: (a) optimize an ISE procedure for the natural allotriploid “Híbrido de Timor” (HT) ‘CIFC 4106’ (1C = 2.10 pg, 2n = 33 chromosomes, *Coffea arabica* L. *x Coffea canephora* Pierre ex A. Froehner) (Clarindo et al. 2013); (b) propose a guideline for

chromosome doubling using friable embryogenic calli (FEC) of HT; (c) screen triploid and hexaploid plantlets through determination of DNA ploidy level; (d) confirm the ploidy level by chromosome counting; and (e) evaluate the DNA sequence changes of the recovered HT plantlets by simple sequence repeat (SSR) codominant markers. As this study was conducted with an allotriploid, the term "chromosome doubling" was preferred to "in vitro polyploidization induction".

Materials and methods

Plant material

Three HT 'CIFC 4106' plantlets were obtained via direct somatic embryogenesis in previous experiments, and have been maintained in vitro (Universidade Federal do Espírito Santo, Espírito Santo, Brazil), according to conditions described by Sattler et al. (2016a). The ploidy level (3x = 33 chromosomes) and the nuclear genome size (1C = 2.10 pg) of these HT explant donors (HT-ED) were confirmed to be identical to the four HT 'CIFC 4106' plants (Clarindo et al. 2013) maintained in greenhouse.

FEC induction

Four leaf fragments (1 cm^2) of the three HT-ED were excised and placed into 60 x 15 mm Petri dishes containing callogenesis induction medium (CIM), composed of half-strength MS salts (Sigma®), 10 mL L^{-1} Gamborg's 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} casein, 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μM 6-benzylaminopurine and 2.8 g L^{-1} Phytagel (modified from van Boxtel and Berthouly 1996). The pH of all culture media

was adjusted to 5.6 prior to autoclaving. FEC induction was conducted in the dark at 25°C.

ISE chromosome doubling

Under aseptic conditions, individual FEC were transferred to new Petri dishes containing CIM. Next, 3 mL of filter-sterilized colchicine solution were added to each Petri dish. Colchicine concentrations of 0.0 (ISE-control), 0.5, 1.5 and 2.5 mM were applied for 96 h, with incubation in orbital shaker at 50 rpm. Eight repetitions were performed for each treatment (0.0, 0.5, 1.5 and 2.5 mM colchicine), with one FEC per Petri dish. After exposure to colchicine, each FEC was washed separately five times with autoclaved dH_2O and inoculated in regeneration medium (RM). Figure 1 shows a guideline summarizing the chromosome doubling procedure performed in HT FEC.

Somatic embryogenesis (SE) and plantlet regeneration

Colchicine-treated (FEC-CT) and not treated FEC (control, FEC-NCT) were transferred to RM composed of MS salts (Sigma®), 10 mL L^{-1} Gamborg's 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} casein, 4.44 μM 6-benzylaminopurine, 2.8 g L^{-1} Phytagel (modified by van Boxtel and Berthouly 1996) and 2 g L^{-1} activated charcoal (Fig. 1). All FEC were kept in RM until the SE reached the mature cotyledon stage. Only the mature cotyledonary somatic embryos were transferred to tubes containing germination medium, which consisted of MS salts (Sigma®), 10 mL L^{-1} Gamborg's B5 vitamins, 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel (modified from van Boxtel and Berthouly 1996) and 2.89 μM gibberellic acid (GA_3) (Fig. 1, 2a, b).

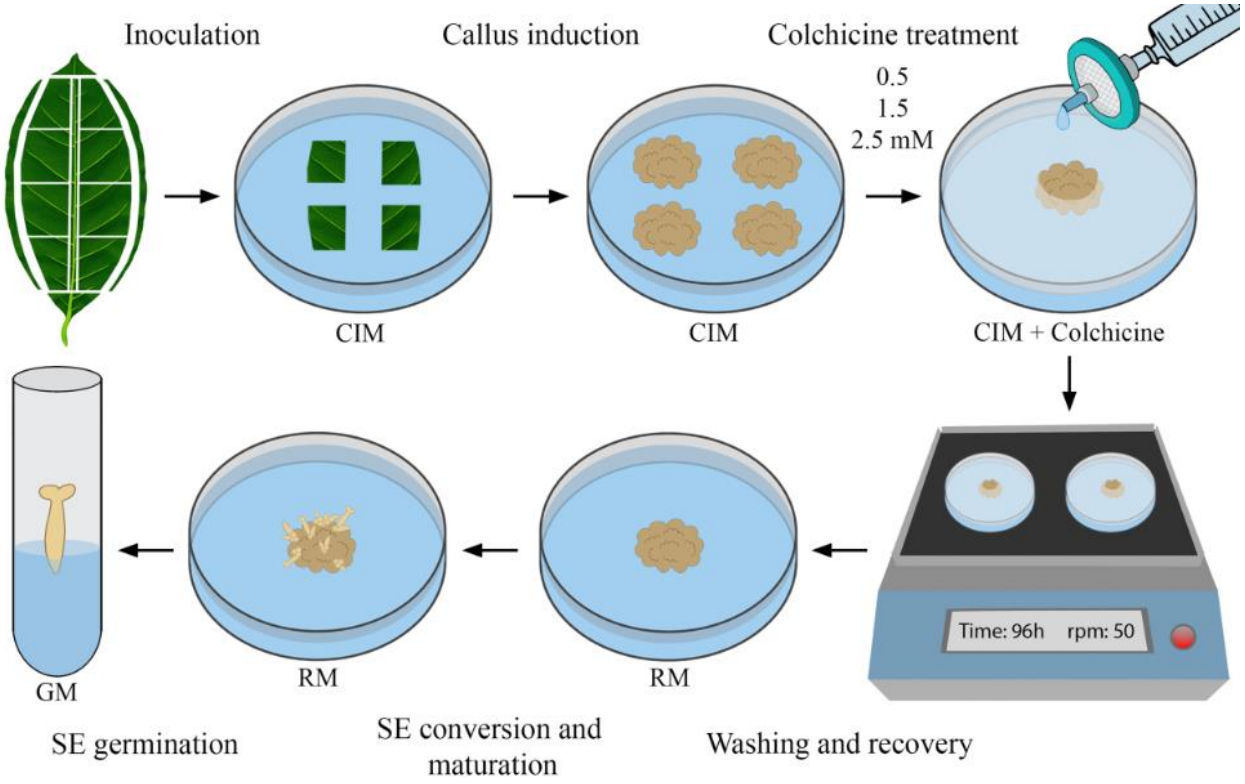
Inoculation
Callus induction
Colchicine treatment
0.5
1.5
2.5 mM
CIM
CIM
CIM + Colchicine
Time: 96h rpm: 50
RM
RM
GM
SE germination
SE conversion and maturation
Washing and recovery

Fig. 1 Guideline for chromosome doubling via FEC/anti-tubulin procedure. Explants of somatic tissue, such as leaf fragments (1 cm^2) of the allotriploid HT-ED, are excised, fragmented and inoculated in callus induction medium like the proposed CIM. After obtaining the calli, each FEC should be transferred to the same callus induction medium, and sterile filtered anti-tubulin solution at distinct concentrations (for instance the 3 mL of 0.5, 1.5 or 2.5 mM colchicine used here) should be added. As adopted in several studies, the anti-tubulin treatment is performed in pulse, exposing the FEC to the anti-tubulin for distinct times in orbital shaker at 40/50 rpm. After anti-tubulin treatment, the FEC are washed three times in sterile distilled water and inoculated in somatic embryo recovery medium like RM. The somatic embryos at mature cotyledonary stage are transferred to germination medium, and the plantlets are regenerated. The leaves of recovered plantlets are used for determination of DNA ploidy level, and the roots for chromosome counting (Fig. 2).

Hexaploid screening following ploidy level assessment

Using flow cytometry (FCM), DNA ploidy level and nuclear 2C value of the regenerated HT plantlets were determined from nuclei suspensions extracted and stained according to Otto (1990) and Praça-Fontes et al. (2011). Nuclei suspensions obtained from leaves of the three HT-ED as well as HT plantlets regenerated from FEC-NCT were used as control for DNA ploidy level determination. The suspensions were analyzed with a Partec PAS® cytometer (Partec® GmbH, Munster, Germany).

For determination of chromosome number (2n), metaphases were obtained according to Clarindo et al. (2013) from root meristems of HT plantlets previously screened by FCM. The slides were prepared by cell dissociation and air-drying, followed by hot plate at 50°C for 3 min, staining with 5% Giemsa and two washings with dH_2O. All slides were analyzed under a Nikon Eclipse Ci-S microscope (Nikon). Metaphase images were captured using 100x objective and a CCD camera (Nikon Evolution™) coupled to a Nikon 80i microscope (Nikon).

Evaluation of genetic stability by SSR markers

Molecular analysis was performed using leaves of three HT-ED (control), five HT plantlets regenerated from ISE without colchicine treatment (ISE control), and 42 HT plantlets recovered from FEC treated with colchicine. The genomic DNA was extracted following Doyle and Doyle (1990), with addition of 7.5 M $C_2H_3O_2NH_4$ and excluding the overnight period for DNA precipitation. DNA concentration and purity were estimated by NanoDrop (Thermo Scientific® 2000c), and amplification was performed using ten SSR primers previously selected from 19 primers developed and validated for *Coffea arabica* L. (Missio et al. 2009). These ten SSR primers (SSRCa002, SSRCa021, SSRCa045, SSRCa091, SSRCa006, SSRCa084, SSRCa085,

SSRCa087, SSRCa088 and SSRCa095, Missio et al. 2009) provided amplification products in all HT plantlets. PCR reactions were conducted in a final volume of 20 µL containing 2.0 µL of 10X buffer, 150 µM of dNTPs, 0.2 µM of each primer, 50 ng of DNA, 1.6 mM of $MgCl_2$ and 1 U of Taq DNA polymerase, and the remaining volume was completed with dH_2O. The reactions were carried out in a Bio-Rad® 96-Well Thermal Cycler C1000™ by touchdown PCR procedure. The PCR program consisted of initial denaturation at 94°C for 5 min, followed by ten denaturation cycles at 94°C for 30 s, annealing at 66°C–57°C for 30 s (decreasing 1°C at each cycle) and extension at 72°C for 30 s. The last 30 cycles were performed at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 8 min. The samples were applied to electrophoresis on 6% polyacrylamide gel in 1X TBE buffer for 4 h at 100 V. The gels were stained in ethidium bromide solution (0.25 mg mL^{-1}) for 20 min. The images of the fragments obtained in the gels were photo-documented (Bio-Rad®, C1000™ Thermal Cycler) using the Image Lab program. The amplification patterns were codified considering the number of bands.

Statistical analysis

Considering each SSR primer as a trait, multivariate clustering was accomplished in order to group individuals by similarity in number and class of loci. The Euclidean distance and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) were applied. Analyses were performed using the software R (R Core Team 2016).

Based on the SSR amplifications, the colchicine treatments (0.0, 0.5, 1.5 and 2.5 mM) were compared using Tukey's test ($p < 0.05$).

Results

In vitro plant recovery from FEC

FEC were obtained after 60 days in CIM, being clearly identified by their pale yellow color and friable aspect (Fig. 2b). In RM, SE were regenerated from all FEC (FEC-NCT and FEC-CT). The SE presented distinct development stages (globular, heart-shaped, torpedo and cotyledonary), evidencing an asynchronized response of HT 'CIFC 4106' during ISE (Fig. 2b). Since inoculation of the FEC in RM, the ISE showed continuous production of SE with potential of recovering plantlets over several months.

Hexaploid screening following ploidy level assessment

The DNA ploidy level of the plantlets regenerated from FEC (FEC-NCT and FEC-CT) was determined upon comparison with the G_0/G_1 peak of the three HT-ED (1C = 2.10 pg, 2n = 33 chromosomes; Fig. 2d, g). The five plantlets regenerated from FEC-NCT displayed the same DNA ploidy level as the HT-ED (Fig. 2d, g). Of the 65 FEC-CT plantlets evaluated by FCM, the number of individuals showing DNA ploidy level equivalent to the triploid and hexaploid condition were, respectively: 4 (50%) and 4 (50%) for 0.5 mM, 10 (77%) and 3 (23%) for 1.5 mM, and 19 (43%) and 25 (57%) for 2.5 mM of colchicine. Considering the present procedure adapted for FEC, independently of the colchicine concentration, the rate of hexaploidy was 49.3%. All hexaploid plantlets showed 2C = 4.20 ± 0.025 pg (Fig. 2).

The triploidy and hexaploidy were confirmed by chromosome counting. Triploid plantlets exhibited 2n = 33 and hexaploids 2n = 66 chromosomes (Fig. 2d–f).

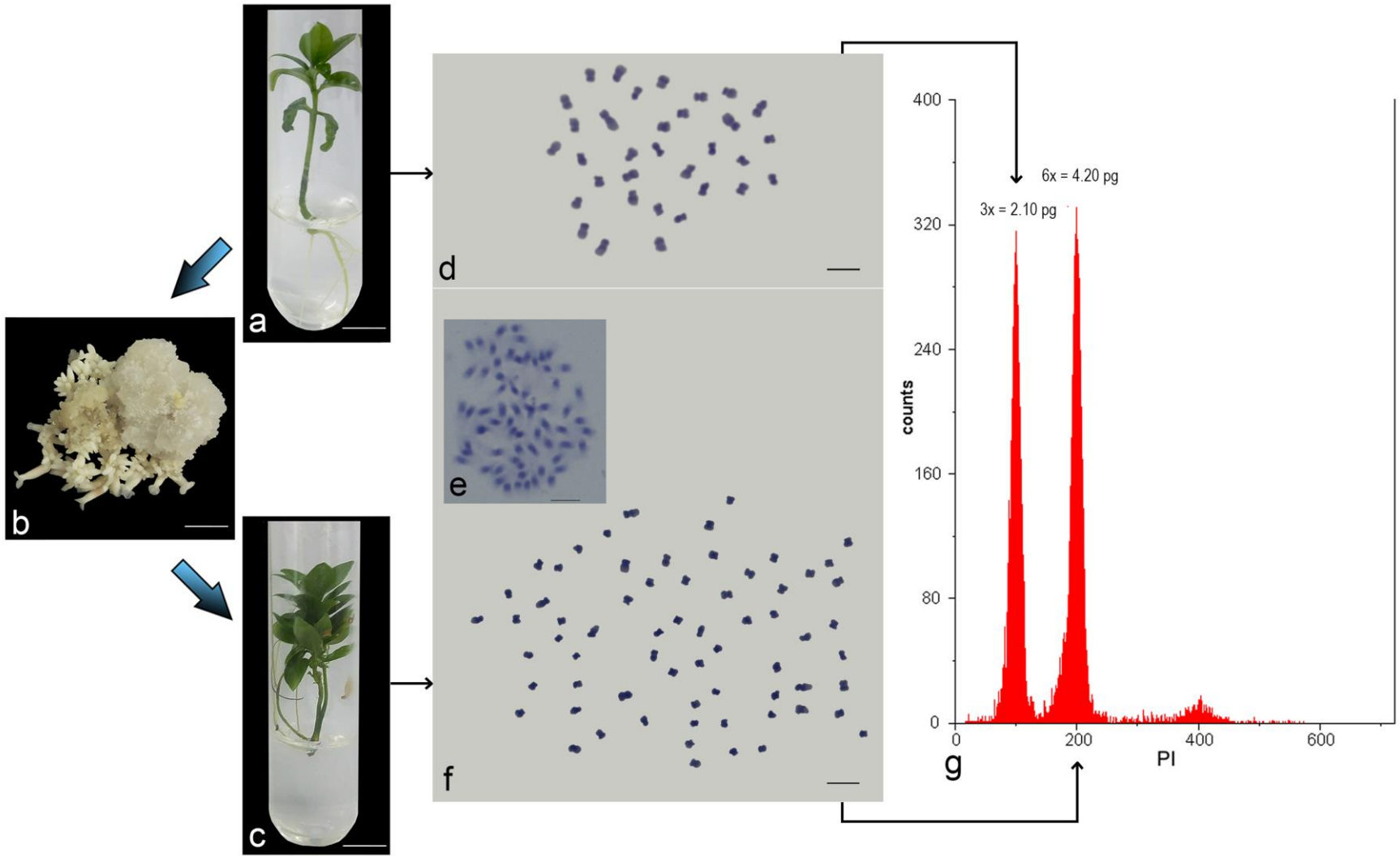
a
b
c
d
e
f
g
6x = 4.20 pg
3x = 2.10 pg
counts
PI
0
80
160
240
320
400
0
200
400
600

Fig. 2 Hexaploidization of the allotriploid HT 'CIFC4106' via FEC/colchicine strategy. **a)** True allotriploid HT 'CIFC 4106' plantlet used as explant donor for FEC induction. Bar = 1 cm. **b)** Representative FEC in regeneration medium (RM) after treatment with colchicine. Note the *pale yellow color* and friable aspect of the FEC, as well as the large number of SE at distinct developmental stages: globular, heart-shaped, torpedo and cotyledonary. Bar = 0.5 cm. **c)** Hexaploid HT plantlet recovered from a mature cotyledonary somatic embryo in germination medium (GM). Bar = 1 cm. **d)** Common karyotype (2n = 3x = 33 chromosomes) of the three HT-ED, the five plantlets recovered from FEC-NCT, and 50.7% of the plantlets regenerated by FEC-CT. Bar = 5 µm. **e)** Initial prophase and **f)** metaphase with 2n = 6x = 66 chromosomes found in hexaploid HT plantlets recovered from FEC-CT. Through this result, the endomitotic effect of colchicine could be confirmed. Bar = 5 µm. **g)** FCM histogram exhibiting peaks corresponding to the G_0/G_1 nuclei of the allotriploid HT-ED and FEC-NCT plantlets (channel 100, 1C = 2.10 pg), and of the hexaploid HT plantlets recovered from FEC-CT (channel 200, 2C = 4.20 pg).

Evaluation of genetic stability by SSR markers

The DNA sequences of 56 regenerated plantlets (three from HT-ED, five from FEC-NCT and 48 from FEC-CT) were amplified with ten SSR primers. The number of alleles varied from one (SSRCa045 and SSRCa095) to three (SSRCa084 and SSRCa087) for the HT-ED and HT plantlets regenerated from FEC-NCT. For those obtained from FEC-CT, the allele number ranged from one (SSRCa045) to 4 (SSRCa021).

The primer SSRCa045 was monomorphic for all HT plantlets (HT-ED, FEC-NCT and FEC-CT). Differently, the most polymorphic primers were SSRCa021, with occurrence of two allelic forms in all HT plantlets (HT-ED, FEC-NCT and FEC-CT), and SSRCa084 and SSRCa087, with three allelic forms for most HT plantlets.

Considering the used SSR primers and the three HT-ED, emergence and above all disappearance of alleles were verified in some HT plantlets recovered from FEC-NCT and FEC-CT. Only one HT plantlet regenerated from FEC-NCT revealed disappearance of allele for the SSRCa088 primer. No DNA sequence change was detected for 14 of the FEC-CT plantlets, and at least one alteration was observed for the other plantlets (Fig. 3).

Based on SSR amplicons and UPGMA clustering, five groups were formed. In relation to HT-ED, the mean values of Euclidean distances evidenced that group I was the most genetically divergent, followed by groups II, III and V, with group IV being the most identical to HT-ED. Triploid and hexaploid HT plantlets were randomly distributed among the five groups, pointing to the occurrence of DNA sequence changes regardless of ploidy level (Fig. 3).

The allele variation found in the plantlets regenerated from FEC-CT was compared by Tukey's test. The colchicine concentration of 2.5 mM promoted more

DNA sequence changes in relation to the treatments with 1.5 and 0.5 mM, which were statistically identical.

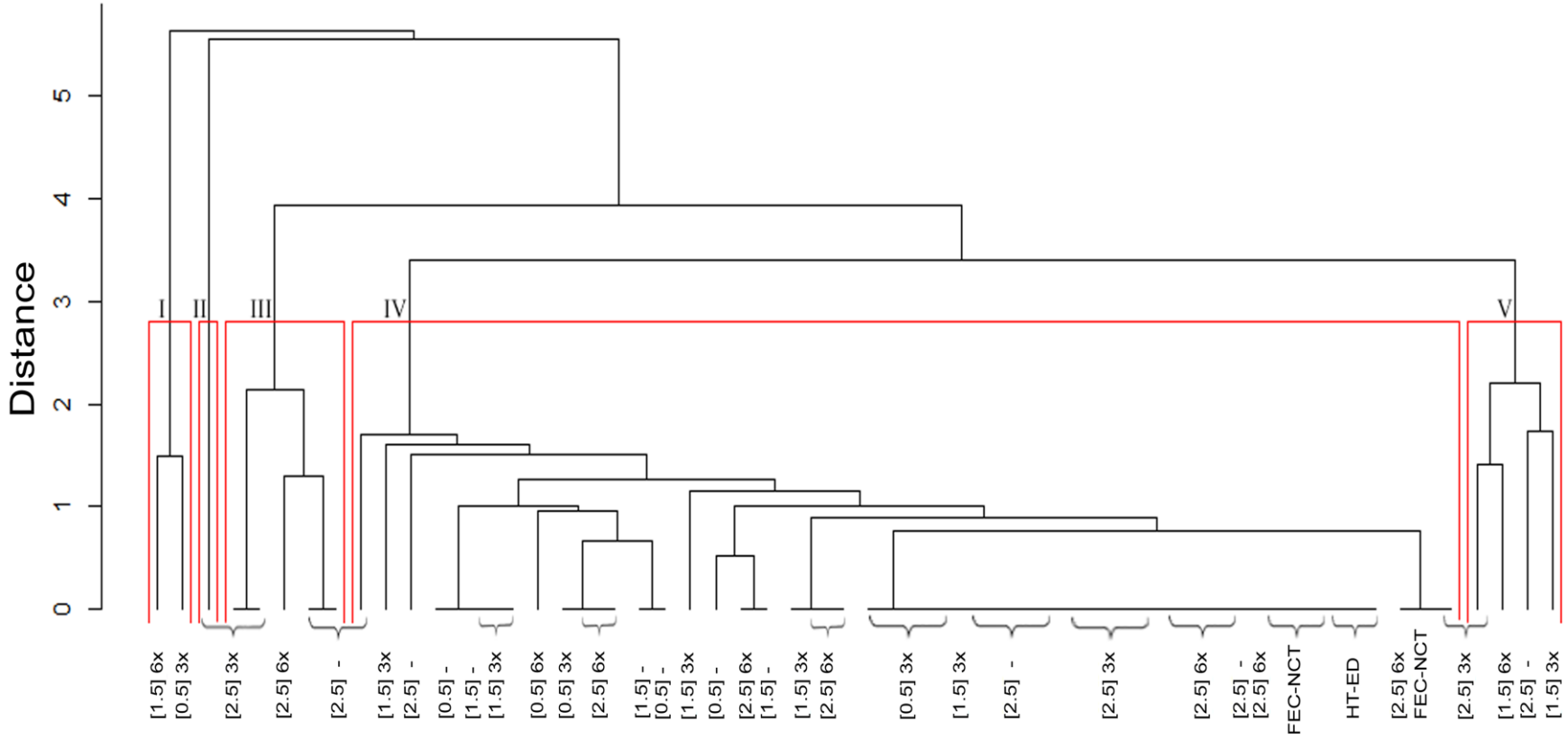
Distance
0
1
2
3
4
5
I
II
III
IV
V
[1.5] 6x
[0.5] 3x
[2.5] 3x
[2.5] 6x
[2.5] -
[1.5] 3x
[2.5] -
[0.5] -
[1.5] -
[1.5] 3x
[0.5] 6x
[0.5] 3x
[2.5] 6x
[1.5] -
[0.5] -
[1.5] 3x
[0.5] -
[2.5] 6x
[1.5] -
[1.5] 3x
[2.5] 6x
[0.5] 3x
[1.5] 3x
[2.5] -
[2.5] 3x
[2.5] 6x
[2.5] -
[2.5] 6x
FEC-NCT
HT-ED
[2.5] 6x
FEC-NCT
[2.5] 3x
[1.5] 6x
[2.5] -
[1.5] 3x

Fig. 3 UPGMA clustering of 56 HT plantlets based on allele variation evidenced by the ten SSR markers. The allele variability verified in the regenerated HT (HT-NCT and HT-CT) plantlets in relation to HT-ED resulted in five groups. Group I is composed by the two plantlets most divergent in relation to HT-ED. In contrast, 14 HT plantlets, including four HT-NCT and ten HT-CT, exhibited the same allele forms as HT-ED. Allotriploid and hexaploid HT plantlets were randomly distributed among the five groups, indicating that DNA sequence changes occur independently of the chromosome doubling. Individuals with the *dash symbol* (-) were not submitted to ploidy level assessment by FCM due to the absence of suitable leaves. *Brackets include different plantlets* recovered from the same colchicine concentration and the same ploidy level.

Discussion

In the present work, ISE was established for HT based on an improved and reproducible procedure, involving three steps: FEC induction, SE recovery and plantlet regeneration (Fig. 2). Initially, FEC were obtained in the relatively short time of two months. The time for FEC establishment varies in *Coffea*, ranging from two (for *C. canephora* and *C. arabica*; Samson et al. 2006) to six months (for *C. canephora*; Ducos et al. 2003). The cellular mass of the HT 'CIFC 4106' FEC increased over this period, indicating the totipotency of the cells (Fig. 2b). This in vitro response was influenced by chronological, physiological and ontogenetic characteristics of the leaf explants, which were obtained from HT-ED (Fig. 2a) propagated in vitro. In addition, the CIM was formulated based on the most frequent chemical and physical conditions for *Coffea* species (van Boxtel and Berthouly 1996). Among the chemical compounds, 2,4-D stands out as predominant for FEC induction in *Coffea*. This agent stimulates the increase in endogenous levels of natural auxins, culminating in cell proliferation (Fig. 2a) and resulting in pro-embryogenic mass (callus) formation (Fehér et al. 2003).

The proliferative condition (totipotency, cell cycle) of the FEC cell mass was fundamental for chromosome doubling and, after colchicine treatment, for somatic embryo and plantlet regeneration from HT 'CIFC 4106' (Fig. 2). Associating these requirements with the endomitotic induction by colchicine, hexaploid HT plantlets were recovered after successive routine subcultures (every two months) of FEC-CT. Thereby, the chromosome doubling procedure associating FEC/colchicine (Fig. 1) represents an improved application for recovering plantlets with a new karyotype over a relatively long time.

Differently from the procedures starting with shoot apices (seedlings), routinely applied in chromosome doubling (Dhooghe et al. 2011), the FEC and, consequently, the ISE were established from few ED individuals (only three plantlets of the allotriploid HT 'CIFC 4106') (Clarindo et al. 2013). Therefore, this application can be adapted for other germplasms that are often semi-fertile or sterile (Lloyd and Bomblies 2016), as was the case of the homoploid *Allium fistulosum* L. *x Allium cepa* L. hybrids (Song et al. 1997) and the anorthoploid *Pennisetum purpureum* Schum. *x Pennisetum glaucum* L. (3x = 21) (Faleiro et al. 2016). Homoploids and anorthoploids are generally semi-fertile or sterile due to meiosis irregularities (Sattler et al. 2016a), causing limitation of biological material and making the use of shoot apices unfeasible. For the present work, employing shoot apices would require at least 65 individuals or buds. In contrast, only three ED were used.

Another hurdle related to homoploids and anorthoploids is that, considering a semi-fertility condition, the offspring often presents an aneuploid karyotype, as reported for the HT 'CIFC 4106' progeny (Clarindo et al. 2013). Therefore, explants of the offspring were replaced by other explants of the homoploids or anorthoploids, like the leaf fragments used for ISE in this study. This way, the formed hexaploid HT individuals are karyotypically pure (Fig. 2f), solving the problem related to aneuploidy arising in seminal propagation.

The combination FEC/colchicine treatment (0.5–2.5 mM for 96 h) provided a high rate of chromosome doubling for HT 'CIFC 4106' (49.3% of hexaploids), corroborating other research that used the same strategy: 35.1% of tetraploids for *Gladiolus tristis* L., 42.9% of tetraploids for *Gladiolus priorii* (N.E.Br.) Goldblatt & M.P.de Vos (1.25 mM colchicine for 72 h; Suzuki et al. 2005) and 34.7% of tetraploids for *Citrus reticulata* 'Blanco' (2.5 mM for 96 h; Dutt et al. 2010). Considering all used

explant types (summarized by Dhooghe et al. 2011), chromosome doubling rates vary from 15 to 55%.

In addition to a high percentage of chromosome doubling, only pure HT hexaploids were obtained, with no mixoploid plantlets being regenerated from HT FEC-CT. SE, and consequently plantlets, are usually regenerated from a single cell of the FEC (Barrett 1974; Jaskani et al. 1996; Féher et al. 2003). Exploiting this aspect, ISE has also been promising to provide pure plantlets (Suzuki et al. 2005; Dutt et al. 2010). Hence, an in vitro germplasm bank of HT comprising individuals with pure ploidy level (3x and 6x) has been established.

As reported by Doležel et al. (1997) and Dhooghe et al. (2011), FCM has been a practical and rapid tool for DNA ploidy level determination and nuclear 2C value measurement, allowing a large-scale screening of the hexaploid HT plantlets regenerated via FEC-CT (Fig. 2). The ploidy level of the putative allotriploid and hexaploid HT plantlets was confirmed by chromosome counting, with 2n = 3x = 33 or 2n = 6x = 66 chromosomes, respectively (Fig. 2d–f). Therefore, chromosome counting was an unambiguous method to confirm the ploidy level (Doležel et al. 2007).

The hexaploidy of 32 HT plantlets was clearly confirmed. However, the genomic (auto- or allopolyploid) origin of these synthetic polyploids is important for their classification. HT ‘CIFC 4106’ originated from a natural crossing between *C. canephora* and *C. arabica*. The true allotetraploid *C. arabica* perhaps arose from a crossing 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). The center of origin and native geographic distribution of *C. eugenioides* (East Africa) and *C. canephora* (West and Central Africa) (Anthony et al. 2010) also support

these species as progenitors (Hamon et al. 2015). This way, the allotriploid HT 'CIFC 4106' can be reported as CC^aE^a – diploid *C. canephora* (CC) x true allotetraploid *C. arabica* ($C^aC^aE^aE^a$). Therefore, the synthetic chromosome doubling of HT 'CIFC 4106' resulted in a new karyotype with six chromosome sets: $CCC^aC^aE^aE^a$. In this context, for classification of genomic origin (auto- or allopolyploid) of the hexaploid HT, future studies are needed to reveal the chromosome pairing and segregation during meiosis, mainly between the CC^a genomes that occur in this karyotype.

The genotoxic effect of the chromosome doubling procedure (Fig. 1, FEC/colchicine) was demonstrated via SSR markers. Among the molecular markers, SSR were chosen for their codominance, specificity, large distribution in the genome, high polymorphism and reproducibility (Missio et al. 2009; Bairu et al. 2011). Nine of the ten SSR primers were polymorphic for the regenerated HT plantlets (HT-ED, FEC-NCT and FEC-CT), exhibiting 2–4 allelic forms. Besides, the primers SSRCa084 and SSRCa087 showed three allelic forms. This observed polymorphism corroborates the polyploid karyotype nature of the HT plantlets (2n = 3x or 2n = 6x).

The SSR markers evidenced that 70% of the plantlets recovered from FEC-CT showed allelic variation in relation to HT-ED. However, disappearance of SSR alleles was the predominant change in HT plantlets regenerated from FEC-CT (Fig. 3). Therefore, the genetic diversity related to allelic forms decreased in relation to HT-ED and HT-NCT. Antimitotic agents like colchicine promote the appearance and disappearance of alleles. Distinct mechanisms culminate in microsatellite variants: (a) instability related to slippage of the DNA polymerase, affecting repair during DNA replication (Wells and Jakupciak 1999; Fenech 2000); (b) unequal crossing over (Métais et al. 2002); and/or (c) punctual changes in the DNA sequence or chromosomal

rearrangements (Atienzar and Jha 2006). These mechanisms interfere with the primer stability, resulting in loss or gain of alleles in tandem repeat regions (Fenech 2000).

As for the hexaploidy rate (50% for 0.5 mM, 23% for 1.5 mM and 57% for 2.5 mM colchicine), the rate of DNA sequence changes varied according to the colchicine concentration, being statistically greater for 2.5 mM colchicine. However, the rates of hexaploidy and DNA sequence changes have been currently evaluated in our routine due to asynchronized response of the ISE in HT. Hence, further comparisons between the colchicine treatments should be performed.

Conclusions

The new hexaploid HT plantlets, which were obtained by FEC-CT, contribute for the enrichment of *Coffea* germplasm banks, mainly because HT shows resistance to *Hemileia vastatrix*, the main pathogen of *Coffea*. This work shows, for the first time:

(a) the establishment of ISE for an anorthoploid *Coffea* species, HT 'CIFC 4106', allowing the mass propagation of this semi-fertile germplasm (Fig. 2);

(b) induction of chromosome doubling in a *Coffea* species from FEC, yielding a high rate of pure hexaploids;

(c) an evaluation of the genotoxic effects of the FEC/colchicine strategy (Fig. 1), revealing via SSR markers that the disappearance of alleles (Fig. 3) was the prevalent change in the DNA sequence.

Besides their relevance for *Coffea* breeding programs, the hexaploid HT plantlets and the allotriploid HT 'CIFC 4106' represent a potential genotype for further investigation on the genomic (genetic and epigenetic) and phenotypic (physiological and morphological) changes brought about by polyploidization.

Acknowledgments

We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília – DF, Brazil, grants: 443801/2014-2), the Fundação de Amparo à Pesquisa do Espírito Santo (FAPES, Vitória – ES, Brazil, grant: 65942604/2014) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília – DF, Brazil) for financial support. We also thank Eveline Teixeira Caixeta (Embrapa Café, Empresa Brasileira de Pesquisa Agropecuária, BIOAGRO, Laboratório BioCafé, Universidade Federal de Viçosa, MG, Brazil) for providing leaves in 2011 of the "Híbrido de Timor" 'CIFC 4106 plants.

Author contribution statement: The authors Sanglard NA, Sattler MC and Clarindo WR conducted the tissue culture experiments and in vitro chromosome doubling. Clarindo WR and Oliveira SC carried out the cytogenetic analyses. Amaral-Silva PM and Soares TCB executed the SSR molecular analyses. Clarindo WR and Carvalho CR conducted the flow cytometry analysis. Nunes ACP did the statistical analysis. All authors equally contributed for manuscript editing and revision and approved the final manuscript for submission.

Conflict of interest: The authors declare that they have no conflict of interest.

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Plant Cell Tiss Organ Cult (2017) 131:223–231
DOI 10.1007/s11240-017-1278-4

ORIGINAL ARTICLE

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)

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Received: 11 May 2017 / Accepted: 7 July 2017 / Published online: 15 July 2017

4. CONSIDERAÇÕES FINAIS

A estratégia adotada de ESI reproduziu para *C. eugenioides* e HT 'CIFC 4106' as mesmas condições in vitro usadas para as espécies mais importantes de café (*C. canephora* e *C. arabica*). Dessa forma, as primeiras plântulas de *C. eugenioides* e HT 'CIFC 4106' foram regeneradas por meio da ESI.

Considerando o limitado número de sementes produzido pelo acesso alotriploide, HT 'CIFC 4106', o estabelecimento da ESI dessa importante fonte de genes de resistência, representa um avanço na multiplicação e conservação de acessos elite de *Coffea*. Além disso, um banco de germoplasma in vitro para *C. eugenioides, C. canephora,* HT e *C. arabica* vem sendo estabelecido, proporcionando uma nova coleção genética de *Coffea.*

A série euploide característica dos *Coffea* do presente estudo, permitiu, pela primeira vez, relacionar características do cariótipo (número cromossômico, nível de ploidia, valor 2C nuclear) com a ESI, avançando na compreensão das consequências da poliploidia. Com base nos resultados, o nível de ploidia possui relação com a ESI.

A partir do conhecimento básico gerado com o estabelecimento da ESI em *Coffea,* a sua aplicabilidade foi explorada na duplicação cromossômica in vitro do HT 'CIFC 4106'. Essa metodologia resolveu os principais gargalos da duplicação cromossômica: baixa taxa de poliploides, alto número de mixoploides e alta taxa de mortalidade.

As plântulas hexaploides regeneradas a partir do HT 'CIFC 4106' e os outros *Coffea* com diferentes níveis de ploidia (o alotriploide verdadeiro HT 'CIFC4106', os diploides *C. canephora* e *C. eugenioides*, e o alotetraploide verdadeiro *C. arabica*) poderão ser utilizados para a compreensão do padrão de resposta morfogênica in vitro,

considerando os aspectos do genoma (número de cópias gênicas e metilação do DNA) e do transcriptoma (nível de expressão gênica).