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UMA VISÃO PROTEÔMICA E EPIGENÉTICA DA EMBRIOGÊNESE SOMÁTICA DE *Theobroma cacao* L.

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Aos meus pais, Carlos e María, e a minha irmã, Geovanna.

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RESUMO

Theobroma cacao L. é uma espécie amplamente cultivada na zona tropical húmida do planeta. As sementes tem um elevado valor econômico e são a matéria prima para a produção de chocolate. No entanto, devido às características botânicas que esta espécie apresenta, associadas a um alto grau de auto-incompatibilidade, os plantios comerciais apresentam alta heterozigosidade genética, afetando diretamente a produtividade do cultivo. Por esta razão, sistemas de propagação clonal como estacas enraizadas e enxertia tem sido aplicados para a multiplicação de variedades de elite. Estas técnicas, contudo, apresentam alto custo de mão de obra e não garantem a qualidade sanitária das plantas. Neste contexto, técnicas de micropropagação *in vitro* associadas à embriogênese somática podem contribuir para produção em larga escala de novas variedades e de genótipos superiores, bem como para a conservação de germoplasma, além de servir como modelo para o estudo de processos fisiológicos, genéticos e bioquímicos envolvidos no desenvolvimento embrionário. A embriogênese somática a partir de partes florais tem sido o método mais eficiente para a regeneração *in vitro* de plântulas. Este sistema integra a embriogênese indireta com subsequente embriogênese secundária direta, a partir da qual o sistema se torna cíclico. Os protocolos de embriogênese somática apresentam lacunas no conhecimento que precisam ser preenchidas. Neste contexto, o presente trabalho teve como objetivos aprofundar nos mecanismos moleculares que determinam a qualidade dos embriões somáticos e estudar a relação da metilação do DNA e a capacidade de conversão de embriões somáticos com a embriogênese somática secundaria a longo prazo. Assim, utilizou-se a tecnologia de identificação multidimensional de proteínas ($HDMS^E$), para identificar o perfil proteômico de embriões do tipo branco e do tipo translúcido, os quais diferem amplamente pela capacidade de conversão. O grupo das proteínas diferencialmente expressas revelou que a capacidade de conversão dos embriões somáticos está relacionada com o metabolismo dos carboidratos e com a capacidade das culturas em manter um equilíbrio no sistema redox. A análise global dos níveis da metilação do DNA em embriões somáticos jovens e embriões somáticos envelhecidos sugere que a hipometilação do DNA observada em embriões envelhecidos é uma resposta adaptativa (acumulativa) das culturas às condições *in vitro*, enquanto que a perda do potencial embriogênico associada com a

embriogênese somática a longo prazo, é uma característica epigenética que pode ser reversível. Finalmente, o uso da droga demetilante 5-azcC mostrou uma correlação positiva com a recuperação do potencial embriogênico em embriões somáticos envelhecidos.

Palavras-chave: Micropropagação; embrião somático; epigenética; proteômica; rota morfogenética

ABSTRACT

Theobroma cacao L. is a plant species widely cultivated in the humid tropics of the planet. The cacao beans have a high economic value and they are the raw material for the chocolate industry. However, due to the botanical traits associated with a high degree of self-incompatibility, the commercial plantations present high genetic heterozygosity, directly affecting the crop yield. For this reason, clonal propagation systems such as rooted cuttings and grafting have been applied for the multiplication of elite varieties. However, these methods present a high labor cost and do not guarantee the plants sanitary quality. In this view, *in vitro* micropropagation methods associated with somatic embryogenesis can contribute to the large-scale propagation of improved varieties and genotypes, as well as the conservation of germoplasm, and this can uses as a model for the study of the physiological, genetic and biochemical processes involved in plant embryogenic development. Somatic embryogenesis using floral explants has been the most efficient method for regeneration in vitro seedlings. This system integrates indirect embryogenesis with subsequent direct secondary embryogenesis, from which the system becomes cyclic. Somatic embryogenesis protocols present hole in the knowledge, which need to be elucidated. In this context, the present work had as objective to deepen in the molecular mechanisms that determine the somatic embryo quality to study the relationship between the DNA methylation and the embryogenic potential of somatic embryos in the long-term secondary somatic embryogenesis. Thus, the multidimensional protein identification technology ($HDMS^E$) was used to identify the proteomic profiles of white and translucid somatic embryos, which are widely different in the conversion capacity. The somatic embryos conversion ability was related to carbohydrate metabolism and the ability of cultures to maintain equilibrium in the redox system. The global DNA methylation levels in young somatic embryos and aged somatic embryos suggested that hypomethylation of DNA in aged somatic embryos is an adaptive (cumulative) response to *in vitro* culture conditions, whereas the loss of the embryogenic potential associated with long-term somatic embryogenesis, is an epigenetic feature that can be reversible. Finally, the 5 -azaC showed a positive correlation with the recovery of the embryogenic potential in aged somatic embryos.

Keywords: micropropagation; somatic embryo; proteomics; epigenetic; *in vitro* morphogenesis

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1. INTRODUÇÃO, JUSTIFICATIVA E ANTECEDENTES

1.1 *Theobroma cacao*

Theobroma cacao L. (cacaueiro) é uma planta neotropical da Familia Malvacea. O gênero *Theobroma* compreende 22 espécies (Angiosperm Phylogeny Group, 2009), todas originárias dos bosques úmidos tropicais da América Equatorial (Thomas et al. 2012).

O cacaueiro é a espécie com alto valor econômico uma vez que a que suas sementes são a matéria prima principal para a elaboração do chocolate (Bartley 2005), o qual é rico em polifenóis, compostos que mostram efeitos benéficos em doenças cardiovasculares, atividade anti-inflamatória, função antiplaquetária e efeitos antioxidantes, definindo assim, o chocolate, seu principal produto alimento funcional (Martín et al. 2010).

A cultura do cacaueiro dinamiza a economia no mundial e a cadeia produtiva envolve diversos atores, tais como: produtores, compradores de amêndoas, indústrias e moageiras, empresas de importação e exportação, indústria chocolateira e de cosméticos, além de várias empresas fabricantes e distribuidoras de insumos e equipamentos envolvidos em toda a cadeia de produção (ICCO, 2009). Esta atividade agrícola representam uma importante fonte de trabalho e renda para milhões de pessoas e estima-se que o 70% da produção de cacaueiro provêm de pequenos agricultores, que possuem unidades produtivas menores do que cinco hectares (EUFIC, 2010). Além do alto valor econômico nas regiões onde é cultivado, e do forte componente social envolvido, a cultura do cacaueiro exerce também um papel importante na preservação ambiental. Seu cultivo associado às arvores nativas tem auxiliado na preservação de biomas ameaçados assim também, sistemas agroflorestais com o cacaueiro são considerados como uma alternativa sustentável para a recuperação de áreas degradadas (Niehaus 2012).

Como efeito da colonização, a cultura do cacaueiro se estendeu aos cinco continentes e as principais regiões produtoras situam-se na faixa de 15ºN e 20ºS de latitude do planeta (ICCO, 2011).

A sua origem é ainda controversa. Segundo Bartley (2005), o seu centro de origem é a região do Orinoco e a bacia Amazônica. De acordo com N´goran et al. (1994), o centro de origem está localizado no Alto

Amazonas, no limite Equador-Colômbia, no ecossistema formado pela influência da Cordilheira dos Andes. Nestas regiões as árvores de cacaueiro são encontradas em condições espontâneas no estrato inferior das florestas, em clareiras e beirando os grandes rios, onde predominam condições de temperatura e umidades elevadas (Bartley 2005).

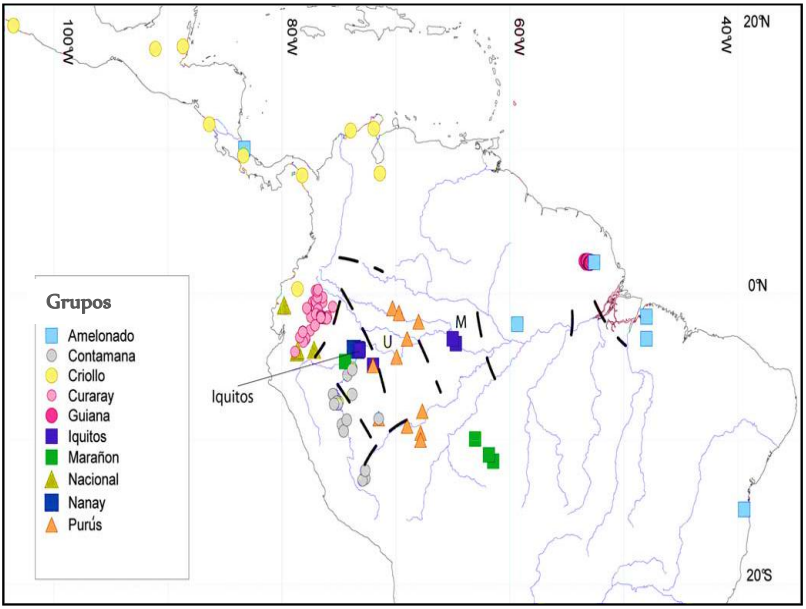

Fig. 1 Distribuição geográfica da diversidade genética de cacaueiro (*Theobroma cacao* L.). Fonte: Motamayor et al. 2008.

Provas da sua domesticação, baseadas nos escritos da civilização Maia mostraram que o cacaueiro era cultivado e que a partir de suas sementes torradas elaborava-se uma bebida amarga chamada "xocoatl", a qual, acreditava-se, combatia o cansaço e era atribuído um poderoso efeito afrodisíaco (Guiltinan et al. 2008). Aparentemente o uso social das amêndoas de cacao foi iniciado na cultura Mayo-Chinchipe-Marañón localizada no Equador há 5.500 anos (MINISTERIO DE CULTURA Y PATRIMONIO, 2015). Nessa perspectiva, acredita-se que o cacaueiro se espalhou em duas direções, dando origem a grupos genéticos distintos (N 'goran et al. 1994; Bartley 2005). O grupo dos Criollos se disseminou na direção à América Central e sul do México. Por sua vez, um o grupo dos

Forasteiros se disseminou até a Amazônia e o nordeste da América de Sul. Estudos com o auxílio de análises moleculares demonstraram que a diversidade do cacaueiro está representada por 10 grupos geneticamente diferentes e que a maior diversidade está localizada no oeste da Amazônia, onde localizam-se sete dos dez grupos, incluindo o grupo local chamado de Nacional (Motamayor et al. 2008).

1.2 Embriogênese somática

O cacaueiro é uma planta hermafrodita com flores pentâmeras, monoclinas, predominantemente alógama, apresentando auto-incompatibilidade gametofítica e esporofítica (De Nettancourt 2001). Esta característica determina que a propagação sexuada por sementes resulte na segregação da progênie, o que não garante a uniformidade dos cultivos. Assim, a propagação da espécie está baseada nos métodos assexuais clássicos, tais como a estaquia e a enxertia. A estaquia é realizada a partir de material ortotrópico, induzindo a produção de ramos, os quais em condições adequadas podem ter um crescimento ortotrópico vertical, no que a formação da copa é de arquitetura similar à de uma árvore produto de semente, mas com diferente formação da raiz (Maximova et al. 2005). No entanto, estas técnicas têm desvantagens associadas à demanda intensiva de mão de obra, altos custos, transmissão de doenças, taxas de propagação frequentemente baixas e padrões de crescimento indesejável das plantas. Assim, técnicas de micropropagação baseadas na embriogênese somática se configuram como uma ferramenta poderosa para a propagação em larga escala de genótipos elite no cacaueiro, com a possibilidade de capturar e fixar ganhos genéticos de plantas selecionadas (Maximova et al. 2002; Traore et al. 2003; Maximova et al. 2008).

A embriogênese somática (ES) é o processo de desenvolvimento através do qual as células somáticas indiferenciadas, em condições de indução adequadas, sofrem uma série de alterações morfológicas e bioquímicas, as quais resultam na formação de um embrião somático. A regeneração de plantas inteiras, a partir de células indiferenciadas, cultivadas *in vitro* é uma clara demonstração da plasticidade das células vegetais (Fehér 2003). O início da ES depende de uma rede complexa de interações entre reguladores de crescimento, origem do explante, composição do meio de cultura, fontes de nitrogênio e carbono e as

condições de cultura, as quais desencadeiam uma forte condição de estresse (Fehér 2015). Em resposta, as células adquirem competência para iniciar a mudança no programa genético das células somáticas, o qual começa pela fase de dediferenciação seguida pela rediferenciação, para finalmente chegar na implementação de uma nova via de desenvolvimento (Wagner 2003).

A ES no cacaueiro foi inicialmente descrita a partir de embriões zigóticos imaturos (Pence et al. 1980). Contudo, plântulas regeneradas a partir de da ES induzida utilizando embriões zigóticos possuem limitado valor, por serem derivadas de um genótipo desconhecido. Trabalhos posteriores foram conduzidos partir de tecidos nucelares (Figueira and Janick 1993), no entanto, a eficiência na obtenção de embriões somáticos bem como sua conversão em plântulas foi limitada

O principal protocolo da ES no cacaueiro está baseado no uso de tecidos somáticos de órgãos florais (pétalas e estaminoides) (Li et al. 1998; Maximova et al. 2002; Traore et al. 2003). Assim, duas rotas morfogenéticas para a ES no cacaueiro foram definidas: a primeira corresponde à ES indireta induzida a partir de tecidos florais (pétalas e estaminoides) na qual embriões somáticos se diferenciam a partir de calos; a segunda corresponde à ES direta induzida a partir de cotilédones de embriões somáticos, na qual embriões somáticos secundários são obtidos diretamente a partir de células epidérmicas sem a fase intermediária de calo (Maximova et al. 2002).

A embriogênese somática no cacaueiro foi testada em sistemas de imersão temporária em frascos duplos resultando em uma melhora nas taxas de multiplicação de embriões somáticos sem causar efeitos negativos nas características morfológicas (Niemenak et al. 2008). Por outro lado, a suplementação dos meios de cultura com diferentes concentrações de MgSO4 e K2SO4 mostrou um efeito positivo na redução de formação de calos e melhorou a diferenciação dos embriões somáticos em genótipos recalcitrantes (Minyaka et al. 2008).

Plântulas de cacaueiro resultantes da ES demonstraram fenótipos normais em condições de campo e com características de crescimento semelhantes às plantas propagadas por métodos tradicionais (Maximova et al. 2008). Este resultado, suporta a observação feita por Rodríguez-López et al. (2010), na qual linhagens de células mutantes formadas como resultado da indução da ES somática no cacaueiro perdem progressivamente totipotência, de modo que as linhagens de células totipotentes e livres de mutações são as que predominam, originando embriões somáticos e plântulas sem divergência genética e epigenética da planta matriz.

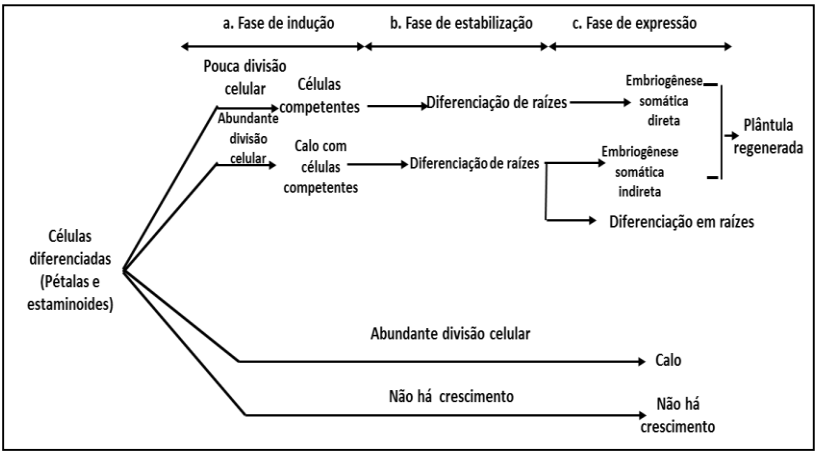

Fig. 2 Rotas morfogenéticas *in vitro* estabelecidas para o cacaueiro (*Theobroma cacao* L.). a- Crescimento de calo primário; b- Crescimento de calo secundário; c- Desenvolvimento de embriões (Niemenak et al. 2008).

O êxito da ES na regeneração de plantas pode ser avaliado não somente pela capacidade na formação de embriões, mas também pela frequência e a capacidade dos embriões de se converter em plantas. Assim, observou-se na ES do cacaueiro dois tipos diferentes de embriões somáticos, com características morfológicas similares, mas com coloração distinta: brancos e translúcidos. Os primeiros mostraram um maior potencial de conversão em plântulas entre 75 a 80% e os segundos mostraram um limitado potencial de conversão entre 15 a 25% (Pila 2013). Assim, estes resultados sustentam a necessidade de investigar as diferenças entre esses embriões somáticos, a fim de elucidar os mecanismos que afetam essas características, as quais podem representar marcadores relacionados com a qualidade dos embriões somáticos desta espécie.

1.3 Abordagens proteômicas e embriogênese somática

O desenvolvimento do mapa genético deesta espécie, representa um importante avanço do conhecimento na área genômica. No contexto

da ES, as "ômicas" tem aberto a possibilidade de estudos desde o ponto de vista da genômica funcional, que visa a identificação da sequência e função dos genes e proteínas através de estudos do transcriptoma e proteôma (Agout et al. 2011; Neelakandan et al. 2012).

Proteôma refere-se ao conjunto de proteínas expressas pelo genoma de um organismo num dado momento e sobre uma condição específica (Altelaar et al. 2013). As proteínas são moléculas que influenciam diretamente a bioquímica celular, portanto o estudo das proteínas leva a refletir com mais precisão o estado celular (Neelakandan et al. 2012).

Na ES, a proteômica vem se configurando como uma ferramenta robusta para identificar as alterações fisiológicas, bioquímicas e moleculares associadas a diferentes estádios de desenvolvimento.

A proteômica comparativa é a abordagem mais utilizada no estudo da ES, e pode auxiliar no entendimento da dinâmica temporal e espacial de processos morfogênicos e na determinação de padrões proteicos como marcadores de eventos relevantes dentro do processo embriogênico (Varhaniková et al. 2014). Um número significativo de estudos de proteômica comparativa está relacionado com a competência das culturas embriogênicas, onde são observadas diferenças significativas nos perfis proteicos de tecidos embriogênicos e não-embriogênicos (Shi et al. 2010; Dos Santos et al. 2016). Da mesma forma, as abordagens comparativas têm sido utilizadas para estudar a ES com foco na indução e desenvolvimento de embriões somáticos e as diferenças entre embriões somáticos e suas contrapartes zigóticas (Jin et al. 2014; Vale et al. 2015).

Estudos em cacaueiro, compararam os proteômas dos embriões somáticos com as suas contrapartes zigóticas em um estágio de desenvolvimento precoce. A diferença mais pronunciada entre os dois tipos de embriões refere-se ao metabolismo dos hidratos de carbono. O proteôma dos embriões zigóticos exibiu uma elevada atividade glicolítica, enquanto que o dos embriões somáticos caracterizou-se por intensa atividade da via aeróbia da respiração, apoiada pelo excepcional aumento das proteínas do ciclo TCA, assim como das proteínas relacionadas com a fosforilação oxidativa (Noah et al. 2013). Da mesma forma, Niemenak et al., (2015) comparou embriões somáticos com embriões zigóticos em diferentes estádios de desenvolvimento, observando que proteínas relacionas ao estresse foram mais expressas nos embriões somáticos do que nos embriões zigóticos, fato que se atribuí a resposta dos embriões somáticos as condições de estresse impostas pela cultura *in vitro*. Assim, apesar das semelhanças entre estes dois tipos de embriogênese, existem algumas diferenças importantes: os embriões zigóticos podem ser

nutridos via floema. Na ES, os embriões são dependentes do suprimento exógeno de carboidratos. Além disso, uma diferença marcada entre embriões somáticos e zigóticos é a disponibilidade de compostos de armazenamento, isto é, hidratos de carbono, lipídeos e proteínas (Rode et al. 2011). No entanto, estudos de proteômica focados em embriões somáticos com capacidade de conversão contrastante não foram realizados.

A ferramenta de identificação multidimensional de proteínas ($HDSM^E$) têm se tornado relevante no contexto da proteômica *shotgun*, possibilitando uma separação ortogonal de alta resolução por cromatografia líquida de alta performance acoplada a espectrometria de massa em tandem (Chen et al. 2006). Esta tecnologia tem facilitado a identificação de proteínas em larga escala e ao mesmo tempo aquelas pouco abundantes (Issaq and Veenstra 2008). Assim, uma abordagem baseada na proteômica comparativa, com a utilização de ferramentas proteômicas de alta resolução, pode auxiliar na elucidação dos aspectos moleculares relacionados com os dois tipos diferentes de embriões somáticos.

1.4 Mecanismos epigenético associados a embriogênese somática

É amplamente aceito que na desdiferenciação e diferenciação de células somáticas que permitem a regeneração de uma planta inteira, mecanismos epigenéticos operam modulando a expressão gênica (Miguel and Marum 2011). As variações epigenéticas em células vegetais cultivadas *in vitro* são o reflexo da adaptação das células ás diferentes condições ambientais e de estresse, as quais podem afetar a mudança do desenvolvimento nas células somáticas. A regulação epigenética em culturas *in vitro* é mediada pela metilação do DNA, remodelação da cromatina e uma pequena regulação mediada pelo RNA (Wang and Wang 2012).

A metilação da DNA em regiões codificadoras ou promotoras pode bloquear a expressão dos genes-alvo, enquanto a demetilação de DNA pode ser um dos passos necessários para a ativação da transcrição no genoma (Finnegan 2010). A metilação do DNA é o mecanismo epigenético melhor descrito, a metilação do DNA ocorre pela adição de um grupo metilo na posição 5' do anel pirimidina de citosina no DNA (5mdC) (De-la-Peña et al. 2015). Embora a função fisiológica da

metilação do DNA não tem sido completamente elucidada, a metilação de DNA tem sido associada a numerosos processos biológicos, incluindo o imprinting genômico, a regulação da transcrição da expressão genica, controle da mobilidade dos elementos transponíveis e silenciamento de genes, com o objetivo de preservar a integridade do genoma (Li et al. 2011; Finnegan 2010).

A variação na metilação nas células vegetais *in vitro* corresponde a uma resposta adaptativa às condições *in vitro* e ao programa de desenvolvimento (Nic-Can et al. 2015) Neste contexto, a dinâmica do metilação do DNA tem o potencial de desencadear variações fisiológica ou de desenvolvimento entre os regenerantes da ES. Assim, a propagação *in vitro* de células de vegetais em períodos de longo prazo tem sido frequentemente associada a desestabilizar o programa genético e epigenético, o que pode levar a variações cromossômicas e de sequência de DNA, variação de metilação, ativação do transposon e geração de variantes somaclonais (Smulders and de Klerk 2011).

2. OBJETIVOS

Objetivo Geral

Estudar os eventos bioquímicos, fisiológicos, genéticos e epigenéticos que influenciam embriogênese somática de *Theobroma cacao* L.

Objetivos específicos

Análise comparativa dos embriões somáticos de *Theobroma cacao* L. de aparência branca e translucida com o auxílio da tecnologia de identificação de proteínas multidimensional ($HDMS^E$).

Avaliar a relação da metilação do DNA com a embriogênese somática secundaria de *Theobroma cacao* L., assim como, o efeito da 5-azaC sobre a recuperação do potencial embriogênico em embriões somáticos secundários envelhecidos.

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CAPÍTULO 1 –

Advances in *Theobroma cacao* L. somatic embryogenesis: a proteomic view on the somatic embryo conversion potential.

Este manuscrito encontra-se submetido no periódico: *acta physiologiae plantarum*

Abstract

Somatic embryogenesis (SE) has been routinely used as a mean of mass micropropagation, and as a model system for investigating the structural, physiological, and molecular events occurring during somatic embryo development. Successful *in vitro* somatic embryogenesis is related to the quality and yield of somatic embryos obtained. In cacao (*Theobroma cacao* L.) SE, poor embryo conversion potential into viable plantlets hinders the use of this technique. In addition, during this morphogenetic route in this species, two morphological types of somatic embryos can be identified: white and translucent. The first type shows higher conversion potential into plantlets as compared to the translucent type. In order to identify the expressed proteins in white and translucent somatic embryos, mass spectrometry HDMS^E proteomic strategy was used. About 1330 proteins were identified in both samples. At least 60 proteins showed differences in abundance levels in cacao white somatic embryos as compared to translucent. Differences in protein abundance levels between embryos types are discussed in terms of GO functional categories. Using white somatic embryos as a model, we suggest that carbohydrate metabolism process and the redox regulation are involved in the control/regulation of somatic embryo quality. These new findings may improve cacao SE protocol**,** as well as the understanding of the role of pivotal metabolic pathways associated to this *in vitro* morphogenetic route.

Keywords: micropropagation; somatic embryogenesis; cacao; embryo quality; label-free proteomics.

Introduction

Cacao (*Theobroma cacao* L.) is a tropical plant, which play a relevant role in both the stability of tropical ecosystems and in the economy of millions of small-holder farmers (Argout et al. 2011). Cacao beans are rich source of polyphenols and present the main raw material for the multi-billion-dollar chocolate industry (Maximova et al. 2014)

Plant tissue culture techniques based on SE present several advantages associated to a mass clonal propagation, such as induction from a several explant source, high propagation efficiency, artificial seeds, among others (Zimmerman 1993). Cacao SE is well characterized (Alemanno et al. 1997; Li et al. 1998; Maximova et al. 2002; Maximova et al. 2008) and protocols for somatic embryos production have been established (Maximova et al. 2002). Somatic embryo-derived plants have been tested under field conditions, revealing similar growth patterns to those from plants propagated by seeds (Maximova et al. 2008). However, low efficiency of somatic embryo conversion potential into plantlets limit the commercial application of this technique (Maximova et al. 2002). Considering that somatic embryogenesis is an analogous process to zygotic embryogenesis, several studies have been carried out comparing zygotic and somatic embryos, using zygotic embryos as a reference model (Noah et al. 2013; Niemenak et al. 2015)

Somatic embryos developmental patterns are similar to zygotic embryos in terms of morphology, biochemistry, desiccation tolerance, and germination. Conceptually, developmental stages of somatic and zygotic embryo are divided into two stages: the first morphogenetic stage is characterized by cell division and the onset of cell differentiation; the second metabolic stage or maturation phase is characterized by biochemical activities which involves the accumulation of major storage products and the preparation for seed desiccation, dormancy, and germination (Alemanno et al. 1997). In the current phase, embryos achieve both “morphological” and “physiological” maturity, which, guarantee satisfactory post-embryonic performance.

Despite the similarities between these two types of embryogenesis, some key differences exist, and, zygotic embryos may be nourished via phloem and simultaneously development of a normal endosperm tissue. In SE, embryos are dependent on exogenous carbohydrate supply and morphogenesis occurs without the surrounding

embryo sac and the simultaneous development of a normal endosperm tissue. In addition, one marked difference between somatic and zygotic embryos is the availability of storage compounds, i.e. carbohydrates, lipids and proteins (Rode et al. 2012)

During cacao SE, it is possible to recognize two different types of normal somatic embryos: the white somatic embryo, showing enhanced conversion potential, and translucent embryos, which shows limited conversion potential (Li et al. 1998; Pila 2013). Thus, it is important to investigate the differences between those somatic embryos in order to elucidate the mechanisms affecting such features.

Proteomic studies comprise powerful tools for understanding the physiological status of plant cells and/or organs in specific development phases (Neelakandan and Wang 2012). Proteomic approaches have been used to study SE with focus in the induction and development in somatic embryos and the differences between somatic embryos and their zygotic counterparts in several plant species (Tzafrir et al. 2002; Winkelmann et al. 2006; Vale et al. 2014; Jin et al. 2014). For cacao, proteomic studies of somatic and zygotic embryos have been previously performed (Noah et al. 2013; Niemenak et al. 2015) bringing important insights about cacao SE.

In the present study, we used a proteomic approach in order to compare the two above- mentioned different types of somatic embryos in cacao at the equivalent developmental stage (cotyledonary stage). $HDMS^E$ platform was used to quantify and identify proteins in those somatic embryos types.

Material and methods

Somatic embryos

Secondary SE was obtained from cotyledons of somatic embryos previously established *in vitro* as described by Maximova et al. (2002b), using cacao genotype EET 103. All culture media were composed by DKW (Phytotechnology Lab, Overland Park, KS, USA) basal salts, as described by Driver and Kuniyuki (1984) The embryo development (ED) culture medium was supplemented with MgSO4, as described by Minyaka et al. (2008). Cotyledons from mature somatic embryos were excised and subcultured in SCG (secondary callus growth) culture medium for 14 days. This culture medium was supplemented with DKW vitamins, 20 g L^{-1} glucose, 9 μM 2,4- dichlorophenoxyacetic acid (2,4-D Sigma-Aldrich), 1.2 μM kinetin (Kin; Sigma-Aldrich) and 0.2% (w/v)

Phytagel® (Sigma-Aldrich, St. Louis, MO, USA). Cultures from SCG culture medium were transferred to ED (embryo development) culture medium and subcultured every 21 days, where secondary somatic embryos regenerated. ED culture medium was supplemented with DKW vitamins, 30 g L^{-1} sucrose, 1 g L^{-1} glucose, 3.0 mM MgSO4 and 0.2% (w/v) Phytagel, and plant growth regulators-free. After 45 days in culture, normal cotyledonary somatic embryos were classified into two types: white and translucent (Fig. 1). Samples of both embryos types were collected and stored at -20°C for proteomic analysis.

Fig. 1 Somatic embryogenesis of cacao genotype EET 103. (A) Somatic embryos. (B) White somatic embryo at cotyledonary stage. (C) Translucent somatic embryo at cotyledonary stage (bar = 20.0 mm).

Proteomic analyses

Total protein extraction

Proteins extractions for each embryo type were carried out in biological triplicate (500 mg) following the method of (Carpentier et al. 2005) with modifications. Samples collected were shock frozen in liquid nitrogen, pulverized in a bead mill and subsequently transferred to clear 10-mL micro tubes containing 5 mL of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 2% (v/v) β-mercaptoethanol, 30% w/v sucrose, and 1 mM PMSF) and 5 mL of buffer-saturated phenol (pH 7.8). The extracts were homogenized by vortexing for 15 min and incubated on ice for 30 min followed by centrifugation at 12,000 xg for 30 min at 4°C.

The phenolic phase was recovered and homogenized with 5 mL of extraction buffer by vortexing for 15 min followed by centrifugation at 12,000 xg for 30 min at 4 °C. The phenol phase was collected and proteins

were precipitated with 5 mL of 100 mM ammonium acetate in methanol at −20 °C overnight. The resulting protein pellet was washed three times with cold 100 mM ammonium acetate and twice with cold acetone. Finally, the proteins were solubilized in 0.3 mL of solubilization buffer (7 M Urea; 2 M Tiourea; 2% IPG buffer; 3% CHAPS; 1.5% DTT) by mild vortexing and stored at −20°C until proteomic analyses. The protein concentration was estimated using the 2-D Quant Kit (GE Healthcare) using bovine serum albumin (BSA, GE Healthcare) as a standard. The three protein extracts resulting from each somatic embryos type were pooled, totaling 100 µg of protein (Luge et al. 2014; Heringer et al. 2015).

Protein digestion

Pool sample (100 µg) was prepared as described by Reis et al. (2016) and desalted on 5000 MWCO Vivaspin 500 membranes (GE Healthcare, Little Chalfont, UK). The membranes were saturated with 50 mM ammonium bicarbonate (Sigma-Aldrich) at pH 8.5 and centrifuged at 15,000 g for 20 min. at 8°C. This procedure was repeated three times. Finally, 50 µL of sample was left on the membrane, collected and used for tripsin digestion.

Trypsin protein digestion was carried out as described by Calderan-Rodrigues et al. (2014) For each 50 µL of sample, 25 µL of 0.2% (v/v) RapiGest® (Waters, Milford, CT, USA) was added, vortexed for 5 s and heated in an Eppendorf Thermomixer® Comfort device at 80°C for 15 min. Then, 2.5 µL of 100 mM dithiothreitol (DTT) was added and placed in the thermomixer at 60°C for 30 min. The tubes were placed on ice (30 s), and 2.5 µL of 300 mM iodoacetamide (IAA) was added, followed by vortexing for 5 s and incubation in the dark for 30 min at room temperature. The digestion was carried out by adding 20 µL of trypsin solution (50 ng μL^{-1}; V5111, Promega, Madison, WI, USA) prepared in 50 mM NH4HCO3 pH 8.5. The samples were placed in a thermomixer at 37°C overnight. For RapiGest precipitation, 10 µL of 5% (v/v) trifluoroacetic acid (TFA, Sigma-Aldrich) was added and vortexed for 5 s incubated at 37 °C for 90 min (without shaking) and centrifuged at 4000 xg for 30 min at 8 °C. Finally, 100 µL of supernatant was collected and transferred to the Total Recovery Vial (Waters, USA) for proteomics analysis.

Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for ESI-LC-MS/MS analysis. Peptide mixtures were separated by liquid chromatography using 1 µL of digested samples in scouting runs. Normalization among samples was based on total ion counts consisting of three replicates per pooled sample. The peptide mixture was first loaded into a nanoAcquity UPLC 5 µm C18 trap column (180 µm x 20 mm) and then into a nanoAcquity HSS T3 1.8 µm analytical reversed-phase column (100 µm x 100 mm) at 600 nL min^{-1}, with a column temperature of 60 °C.

For peptide elution, the binary gradient consisted of water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA) as mobile phase A, and acetonitrile (Sigma-Aldrich) and 0.1% formic acid as mobile phase B. Gradient elution started at 7% B up to 40% B in 90.09 min and from 40% B to 85% B until 94.09 min, maintained at 85% until 98.09 min, then decreased to 7% B until 100.09 min and maintained at 7% B to the end at 108.09 min.

Mass spectrometry was performed in positive and resolution mode, 35,000 FWMH, and the transfer collision energy ramped from 19 v to 45 v in high-energy mode; cone and capillary voltages of 30 v and 2,800 v, respectively; and a source temperature of 70°C. In TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50 to 2,000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) was used as an external calibrant. Data-independent acquisition (DIA) scanning with added specificity and selectivity of a non-linear 'T-wave' ion mobility device was performed (HDMSE) (Heringer et al. 2015).

Proteomics data analysis

Progenesis QI for Proteomics Software v.2.0 (Nonlinear Dynamics, Newcastle, UK) was used to process the MSE data. The analysis was performed following parameters: one missed cleavage, minimum fragment ion per peptide equal to 1, minimum fragment ion per protein equal to three, minimum peptide per protein equal to 1, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY) groups, and a default false discovery

rate (FDR) value at a 4% maximum, a score greater than five, and maximum mass errors of 10 ppm. A protein databank from *Theobroma cacao* was used, obtained from UniProt database (http://www.uniprot.org/taxonomy/3641). Label-free relative quantitative analyses were performed by the ratio of protein ion counts among contrasting samples. After the Progenesis analysis and to ensure the quality of results, only proteins present in 3 of 3 runs and with coefficients of variation less than 0.3 were selected. Proteins common to all treatments were filtered based on a fold change of log2 determined by the overall coefficient of variance for all quantified proteins across all replicates. Proteins differentially abundant were classified as up-regulated when log 1.2 was 2 or greater and as down regulated when log1.2 was -2 or less. Functional annotation based on protein gene ontology was performed using the Blast2Go software v3.0 PRO (Conesa et al. 2005) and UniProtKB (www.uniprot.org) databases. The subcellular localization of the proteins was predicted through TargetP and UniprotKB databases.

Results

Protein identification and differential expression analysis

Comparative proteomics analysis among white and translucent somatic embryos by HDMS^E revealed qualitative and quantitative differences among samples. Protein data from white somatic embryos were contrasted against protein data from translucent somatic embryos. From the total identified proteins, 1,339 were present in both samples and across all replicates. Proteins present in both samples were grouped according differences in abundance levels of at least 2-fold. Therefore, 25 proteins from white somatic embryos were up-regulated in relation to translucent somatic embryos, whereas 35 proteins from white somatic embryos were down-regulated when compared to translucent somatic embryos. Functional classification based on protein Gene Ontology (GO) was performed. Protein annotation was carried out according to biological processes (Table 1). Some proteins were associated with various biological process.

Table 1. List of proteins associated at main gen ontology (GO) functional categories and proteins with differences in abundances levels in cacao white somatic embryos and translucent somatic embryos.

SeqName	Peptide count	Unique peptides	Confidence score	Description	Biological process	Ratio	Tag expression
Carbohydrate metabolic process							
A0A061ENV0	10	2	85,17	Beta-glucosidase 44 OS=Theobroma cacao GN=TCM_019272 PE=3 SV=1	Carbohydrate metabolic process	4,78	Up
A0A061G3B6	2	2	12,06	3,4-dihydroxy-2-butanone kinase, putative OS=Theobroma cacao GN=TCM_013222 PE=4 SV=1	Metabolic process, phosphorylation	2,54	up
A0A061DYK6	4	2	33,83	Fructose-bisphosphate aldolase OS=Theobroma cacao GN=TCM_006785 PE=3 SV=1	Glycolytic process	2,45	up
A0A061DST1	17	9	152,41	Sucrose synthase OS=Theobroma cacao GN=TCM_004698 PE=3 SV=1	Seed maturation, starch metabolic process, sucrose metabolic process	2,31	up

Oxidation-reduction process							
A0A061EMC5	1	1	5,87	NAD(P)-linked oxidoreductase superfamily protein, putative OS=Theobroma cacao GN=TCM_020708 PE=4 SV=1	Energy metabolism	3,5	up
A0A061ECK6	3	1	28,13	Oxidoreductase, zinc-binding dehydrogenase family protein isoform 2 (Fragment) OS=Theobroma cacao GN=TCM_016876 PE=4 SV=1	Oxidation-reduction process	2,49	up
A0A061FF21	2	1	16,75	Cytochrome P450, family 706, subfamily A, polypeptide 6, putative OS=Theobroma cacao GN=TCM_034411 PE=3 SV=1	Oxidation-reduction process	12,65	down
S1SIP6	2	1	11,18	Cytochrome p450 79a2, putative OS=Theobroma cacao GN=TCM_046361 PE=4 SV=1	Oxidation-reduction process	8,66	down
A0A061DPK2	11	9	135,01	Geraniol dehydrogenase 1 OS=Theobroma cacao GN=TCM_000950 PE=3 SV=1	Oxidation-reduction process	3,63	down
A0A061F232	16	3	170,48	NAD(P)-linked oxidoreductase superfamily protein OS=Theobroma cacao GN=TCM_026371 PE=4 SV=1	Energy metabolism	3,26	down

A0A061G198	3	2	18,44	NADH-ubiquinone oxidoreductase-related OS=Theobroma cacao GN=TCM_015272 PE=4 SV=1	Oxidation-reduction process	2,92	down
Response to stimulus							
A0A061EIR4	2	1	20,33	RAB GTPase A5E isoform 1 OS=Theobroma cacao GN=TCM_020064 PE=3 SV=1	Protein transport	3,15	up
A0A061DHX	2	1	11,53	RAB GTPase H1E isoform 2 (Fragment) OS=Theobroma cacao GN=TCM_001112 PE=3 SV=1	Protein transport, small GTPase mediated signal transduction	4,16	down
A0A061EWI0	7	1	55,7	Pathogenesis-related protein 10.5 OS=Theobroma cacao GN=TCM_021284 PE=3 SV=1	Stress response, defense response, response to biotic stimulus	3,21	down
A0A061FUN2	4	4	35,62	Eukaryotic aspartyl protease family protein OS=Theobroma cacao GN=TCM_012281 PE=4 SV=1	Response to water deprivation, systemic acquired resistance, response to abscisic acid	2,62	down

Protein not assigned with the selected functional groups							
A0A061FDC9	2	2	11,63	Electron transfer flavoprotein alpha isoform 1 OS=Theobroma cacao GN=TCM_030993 PE=4 SV=1	Energy metabolism	9,31	UP
Protein not associated							
A0A061E8P3	3	3	21,33	Nucleoporin GLE1, putative isoform 1 OS=Theobroma cacao GN=TCM_010631 PE=4 SV=1	Seed development, embryo sac egg cell differentiation, mitotic recombination	8,73	UP
A0A061DYA8	4	2	28,31	PLC-like phosphodiesterases superfamily protein OS=Theobroma cacao GN=TCM_006664 PE=4 SV=1	Lipid metabolic process	5,71	UP
A0A061GGL7	2	2	12,35	Nudix hydrolase isoform 1 OS=Theobroma cacao GN=TCM_027573 PE=4 SV=1	Metabolic process	5,62	UP
A0A061E0D3	3	2	22,88	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase 1 isoform 1 OS=Theobroma cacao GN=TCM_006702 PE=4 SV=1	Metabolic proecess, nucleoside metabolic process	4,37	UP
A0A061FVE9	2	1	16,11	CCHC-type integrase, putative OS=Theobroma cacao GN=TCM_013205 PE=4 SV=1	Metabolic process	3,95	UP

A0A061G4G8	2	2	16,61	Tobamovirus multiplication 2A isoform 1 OS=Theobroma cacao GN=TCM_015779 PE=4 SV=1	Viral replication complex formation and maintenance	3,71	UP
A0A061GZE2	3	3	17,04	Pyrimidine 2 isoform 2 OS=Theobroma cacao GN=TCM_040900 PE=4 SV=1	Metabolic process	3,64	UP
A0A061EUN5	1	1	5,98	Pentatricopeptide (PPR) repeat-containing protein, putative isoform 1 OS=Theobroma cacao GN=TCM_022424 PE=4 SV=1		3,62	UP
A0A061GDF7	1	1	5,71	DNA binding protein, putative OS=Theobroma cacao GN=TCM_029637 PE=4 SV=1	Genetic information processing	2,31	DOWN
A0A061FMP5	3	3	20,69	Uncharacterized protein isoform 1 OS=Theobroma cacao GN=TCM_042648 PE=4 SV=1		2,33	DOWN
A0A061G8J4	4	1	31,11	Uncharacterized protein isoform 1 OS=Theobroma cacao GN=TCM_026904 PE=4 SV=1		2,33	DOWN
A0A061G5Y3	3	3	17,14	Hydroxymethylglutaryl-CoA synthase / HMG-CoA synthase / 3-hydroxy-3-methylglutaryl coenzyme A synthase OS=Theobroma cacao GN=TCM_016163 PE=4 SV=1	Isoprenoid biosynthetic process	2,39	DOWN
A0A061H049	6	2	59,60	Ribosomal L29 family protein OS=Theobroma cacao GN=TCM_041920 PE=3 SV=1	Translation	2,40	DOWN

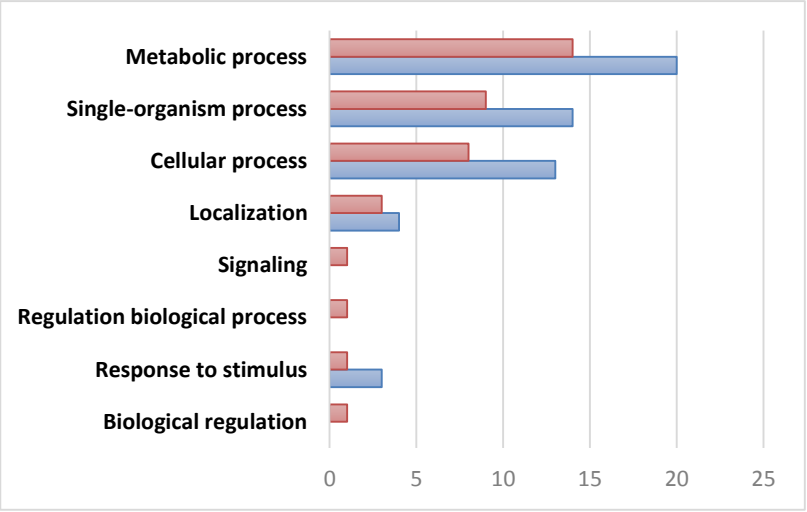
A0A061FUV3	2	2	11,75	Pathogenesis-related thaumatin superfamily protein OS=Theobroma cacao GN=TCM_012750 PE=4 SV=1	Stress response	2,48	DOWN
A0A061DVN6	1	1	6,04	RNA-binding family protein OS=Theobroma cacao GN=TCM_005932 PE=4 SV=1	Photosynthetic electron transport in photosystem	2,48	DOWN
A0A061GBZ8	13	12	101,33	Receptor-like protein kinase-related family protein OS=Theobroma cacao GN=TCM_029030 PE=4 SV=1	Phosphorylation	2,49	DOWN
A0A061E6K3	1	1	5,41	Papain family cysteine protease OS=Theobroma cacao GN=TCM_006819 PE=3 SV=1	Proteolysis	2,49	DOWN
A0A061E105	5	4	38,48	UDP-glycosyltransferase 73B4, putative OS=Theobroma cacao GN=TCM_007099 PE=4 SV=1	Carbohydrate metabolic process	2,56	DOWN
A0A061DYN7	19	2	106,54	Coatomer subunit alpha OS=Theobroma cacao GN=TCM_006812 PE=4 SV=1	Intracellular protein transport	2,56	DOWN
A0A061FHY0	3	1	22,67	Long chain acyl-CoA synthetase 9 isoform 1 OS=Theobroma cacao GN=TCM_035112 PE=4 SV=1		2,60	DOWN
A0A061GPW5	1	1	6,04	Voltage dependent anion channel 4 OS=Theobroma cacao GN=TCM_039252 PE=4 SV=1	Regulation of anion transmembrane transport	2,63	DOWN

A0A061GR28	14	1	126,93	Tubulin alpha-5 OS=Theobroma cacao GN=TCM_040014 PE=3 SV=1	Microtubule-based process, protein polymerization	2,68	DOWN
A0A061EXA3	4	2	35,23	Transport protein particle (TRAPP) component isoform 1 OS=Theobroma cacao GN=TCM_024391 PE=4 SV=1	Vesicle-mediated transport	2,71	DOWN
A0A061ELW2	7	5	54,89	Pseudouridine synthase/archaeosine transglycosylase-like family protein OS=Theobroma cacao GN=TCM_020727 PE=4 SV=1	Sulfate assimilation	2,72	DOWN
A0A061EQ03	4	1	32,52	Nucleic acid-binding proteins superfamily, putative isoform 2 OS=Theobroma cacao GN=TCM_021358 PE=4 SV=1	Genetic information processing	2,84	DOWN
A0A061FTX7	3	2	19,54	Actin binding-like protein OS=Theobroma cacao GN=TCM_012244 PE=4 SV=1		3,05	DOWN
A0A061ECX9	12	4	104,35	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent OS=Theobroma cacao GN=TCM_016784 PE=4 SV=1	Glucose catabolic process	3,28	DOWN
A0A061FK70	1	1	5,95	Mitogen-activated protein kinase kinase kinase 21, putative OS=Theobroma cacao GN=TCM_036235 PE=3 SV=1	Phosphorylation	3,47	DOWN
A0A061FP97	13	2	75,79	Phenylalanine ammonialyase OS=Theobroma cacao GN=TCM_043179 PE=3 SV=1		3,95	DOWN

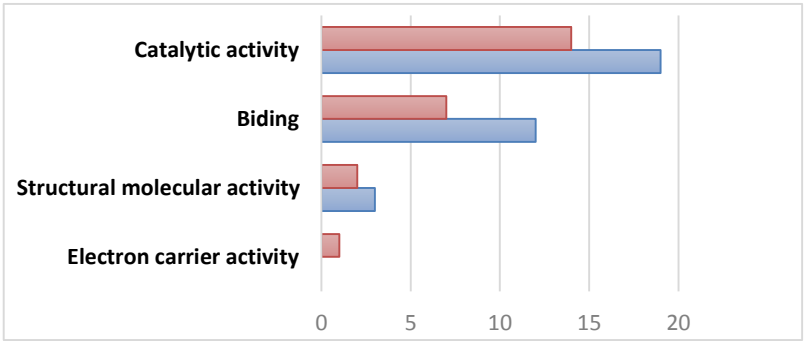
A0A061GQP5	8	1	62,63	Plasma membrane ATPase 4 isoform 2 OS=Theobroma cacao GN=TCM_039126 PE=3 SV=1	ATP biosynthetic proces	4,63	DOWN
A0A061FP20	1	1	6,24	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, putative OS=Theobroma cacao GN=TCM_043741 PE=4 SV=1	Lipid metabolic process	5,72	DOWN
A0A061EL22	8	1	68,58	Eukaryotic aspartyl protease family protein isoform 2 OS=Theobroma cacao GN=TCM_020529 PE=4 SV=1	Proteolysis	5,87	DOWN
A0A061DP55	2	1	18,35	Uncharacterized protein OS=Theobroma cacao GN=TCM_002872 PE=4 SV=1		6,85	DOWN
A0A061EQB8	8	1	47,60	Eukaryotic translation initiation factor 2 gamma subunit, GAMMA isoform 1 OS=Theobroma cacao GN=TCM_021231 PE=4 SV=1	Genetic information processing	17,94	DOWN

Protein groups with differences in abundance levels were associated with GO categories on Level 2. GO functional categories indicated that proteins associated with “metabolic process”, “single-organism process”, “cellular process", "localization”, “signaling”, “regulation biological process”, “response to stimulus” and “biological regulation” were annotated according to “biological processes” category. “Catalytic activity”, “binding”, “structural molecular activity” and “electron carrier activity” were the most representative categories annotated according to “molecular function” category. In addition, “cell part”, “membrane”, “cell”, “membrane part”, “organelle part”, “macromolecular complex” and “supramolecular fiber” were the main annotated categories according to “cellular component” category (Fig. 2).

A. Biological process



B. Molecular Funtion



C. Cellular component

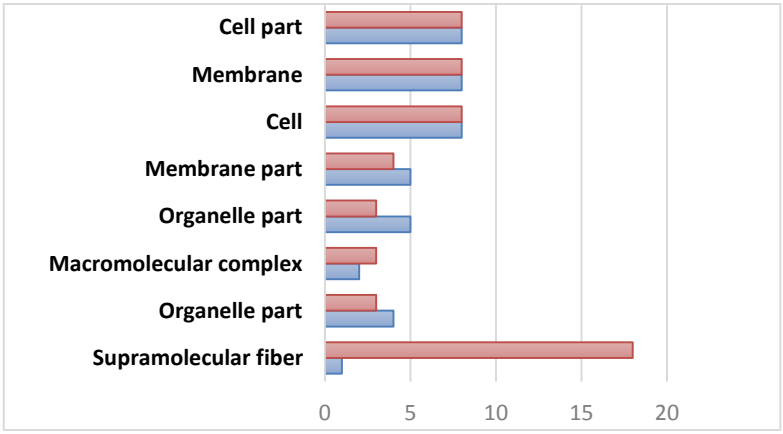


Fig. **2** Functional classification of proteins with difference in abundance level in cacao somatic embryos by Blast2GO software based on universal gene ontology (GO) annotation terms. Up-regulated and down-regulated proteins in white somatic embryos compared to translucent somatic embryos. The proteins were related to at least one annotation term within

the GO category: (A) Biological process. (B) Molecular Function. (C) Cellular component. The histograms represent the number of proteins associated to level 2 GO categories. (Colors: rose up-regulated; blue down-regulated)

Finally, proteins with differences in abundance levels were classified into 3 main GO functional categories: “carbohydrate metabolic process”, “oxidation-reduction process” and “response to stimulus”. The distribution of proteins in the different functional categories was associated with biological process (Table 1).

The dominant functional GO category in up-regulated proteins was associated with “carbohydrate metabolic process”. However, the down-regulated proteins group contained more proteins associated with “oxidation-reduction process” and “response to stimulus” GO categories (Table 1).

Discussion

Cacao SE represents an important method for clonal propagation and provides an *in vitro* experimental system for studying embryo development (Zimmerman 1993). However, many cacao genotypes are recalcitrant to SE (Minyaka et al. 2008), being the embryo conversion the most inefficient step of this process (Traore et al. 2003).

In the present work, cacao embryo development culture medium (ED) was supplemented with sulphate ($MgSO_4$), which was effective to overcome cacao genotypes recalcitrance to SE improving the overall proportion of somatic embryos obtained (Minyaka et al. 2008; Emile et al. 2010). ED was also supplemented with sucrose and glucose (Maximova et al. 2002), carbohydrates involved in multiple roles during embryo development processes (Iraqi and Tremblay 2001; Konrádová et al. 2002; Koch 2004; Lema-Rumińska et al. 2013).

In this context, a comparative label free proteomic analysis was performed to investigate the molecular and functional proteomic aspects in mature somatic embryos. The results will be discussed exploring the biological process associated with white and translucent somatic embryos. The proteins with differences in abundance levels are discussed from 3 main functional GO categories: "carbohydrate metabolic process", "oxidation-reduction process" and "response to stimulus".

Carbohydrate metabolic process

In the present work, proteins associated to carbohydrate metabolism (Table 1) were significantly up-regulated in white somatic embryos when compared to translucent somatic embryos. Within this functional category, enzymes involved in sucrose metabolism (sucrose synthase, 3.21-fold) and in energy metabolism (beta-glucosidase, 4.78-fold), fructose-bisphosphate aldolase, 3.45-fold and 3,4-dihydroxy-2-butanone kinase, 2.54-fold were identified.

Sucrose can be incorporated into the cell metabolism only after the hydrolysis (Tognetti et al. 2013); the first step is the cleavage of the glycosidic bond by either sucrose synthase (SuSy) or invertase (Inv) resulting in the hexoses production. These hexoses are essential energy sources for the deposition of different storage products and tissue growth (Rolletschek et al. 2004).

Somatic embryo development is a complex multi-step process which demand high energy (Lipavská et al. 2000; Iraqi and Tremblay 2001; Konrádová et al. 2002; Dinakar et al. 2012). During early stages, establishment and growth of embryo structures prevails (Hakman 1993) while later stages, such as maturation, are characterized by deposition of storage compounds (Konrádová et al. 2002; Lipavská and Konrádová 2004; Iraqi et al. 2005; Verdier and Thompson 2008). Thus, a transition from metabolic to storage status has been proved to be accompanied by changes in activities of sucrose metabolism enzymes (Iraqi 2001). Metabolic status is mainly characterized by high Inv activities (Winter and Huber 2000; Konrádová et al. 2002), while storage status is characterized by SuSy activity (Déjardin et al. 1997a; Déjardin et al. 1997b; Businge et al. 2013; Yaseen et al. 2013). In addition to a function in starch biosynthesis, SuSy is also correlated with cell wall synthesis (Delmer and Amor 1995; Koch 2004; Tognetti et al. 2013) and play a role in the respiratory pathway (Ishizaki et al. 2006).

In the present study, SuSy proteins were up-regulated in white somatic embryos (2.31 fold) in comparison to translucent somatic embryos. Comparative proteomics analysis of cacao somatic and zygotic embryogenesis showed that SuSy enzyme was expressed in lower abundance in somatic embryos (Noah et al. 2013). This fact was ascribed to a possible disturbance in carbohydrate metabolism, resulting in irregularities in storage compound and cell wall metabolism. However, these results are possibly a consequence of culture conditions.

Corroborating our results, SuSy had a prominent role in the transition from metabolic to storage status in Norway spruce (Konrádová et al. 2002). Therefore, these authors concluded that low Inv activity accompanied by high SuSy activity could be considered as an indicator of embryo quality (Konrádová et al. 2002).

Likewise, studies in Spruce embryo development showed that an imbalance on Inv and SuSy activities by replacing sucrose by glucose and fructose in the maturation medium, resulted in an alteration of storage accumulation and, consequently, in a reduction of somatic embryo production and germination capacity (Iraqi 2001). These findings, suggest that a high SuSy activity result in to a suitable starch accumulation in somatic embryo. Consequently, the deposited starch in the mature somatic embryo will expected to provide energy to the embryo metabolism during conversion and thereby enhance plantlets vigor.

In the present work, specific enzymes for glycolysis (beta-glucosidase (4.78-fold), fructose-bisphosphate aldolase (3.45-fold) and 3,4-dihydroxy-2-butanone kinase (2.54-fold)) were up-regulated in white somatic embryos. In a similar way, Noah et al. (2013) and Niemenak et al. (2015) found that proteins involved in metabolic processes, including glycolysis were expressed in zygotic and somatic embryos of cacao, suggesting that glycolysis enzymes activity could be associated with the energy demand required for embryo development in the storage phase.

Beta-glucosidase, one of the enzymes responsible for glycolysis, was up-regulated in white somatic embryos. This enzyme plays important roles in plant physiology, including the conversion of plant growth regulators such as cytokinin, gibberellin and auxin, into an active form (Brozobohaty el al 1993) as well as the activation of chemical defense compounds (Jones et al., 2000; Halkier and Ger- shenzon, 2006; Suzuki et al., 2006). Fructose-bisphosphate aldolase was also found up-regulated in white somatic embryos. This enzyme plays different roles in the processes of plant cell proliferation, growth, development, photosynthesis and stress resistance (Konishi et al. 2004; Lu et al. 2012; Zeng et al. 2013).

Beta-glucosidase and fructose-bisphosphate aldolase are enzymes widely expressed in development processes in seeds and somatic embryos (Stitt 1990; Winkelmann et al. 2006; Morant et al. 2008; Ketudat Cairns and Esen 2010; Lu et al. 2012; Noah et al. 2013; Zeng et al. 2013). It is clear that enzymes of this pathway have a key role in both types of

embryos development. Therefore, it is possible that the glycolytic enzymes are associated with generation and acquisition of energy supplies for embryo development, and, at the same time, expressed as stress responses.

Starch accumulation, energy supplies and stress tolerance are associated with high activity of proteins related to carbohydrate metabolic process. Based on the results of the present study we can conclude that white somatic embryos managed to accumulate large amounts of storage compounds when compared to translucent somatic embryos, positively affecting its conversion potential to plantlets.

Oxidation-reduction process

Recent evidences revealed that cellular signaling pathways are regulated by the intracellular redox state (Kamata and Hirata 1999). The plant cell apparently has redox sensors, which detect deviations from redox homeostasis prior to the development of major imbalances (Dietz and Scheibe 2004). These redox changes affect growth and development through signaling networks due to the reprogramming of the transcriptome, proteome and metabolome (Go and Jones 2013; Dietz 2014). The model for redox homeostasis depends on the reactive oxygen species (ROS) activity which, acts as a metabolic interface for signals derived from metabolism and the environment (Dietz 2003; Foyer and Noctor 2005). In SE, it has been shown that redox state represents a key metabolic switch which triggers the induction phase, by means of the regulation of embryogenic competence (Kamata and Hirata 1999; Stasolla 2010; Vieira et al. 2012; Go and Jones 2013; Mohamed and Stasolla 2015; Dos Santos et al. 2016) and embryo development (Stasolla et al. 2004; Pullman et al. 2015).

In the present study, the white somatic embryos proteins associated with redox process category (Table 1) were both up and down-regulated, although these groups were represented by different proteins. On one hand, the up-regulated proteins were represented by (NAD(P)-linked oxidoreductase superfamily protein (2.49-fold) and oxidoreductase, zinc-binding dehydrogenase (3.5-fold). On the other hand, the down-regulated proteins were represented by NADH-ubiquinone oxidoreductase-related (2.92 fold), NAD(P)-linked oxidoreductase superfamily protein (3.26-fold), Geraniol dehydrogenase 1 (3.63-fold), Cytochrome p450 79a2, putative (8.66 fold) and Cytochrome P450, family 706, subfamily A, polypeptide 6, putative (12.5 fold).

These results revealed the simultaneous presence of both oxidized and reduced forms of electron carriers in somatic embryos. Enzymes such NAD(P)-linked oxidoreductase, NADH-ubiquinone oxidoreductase-related and oxidoreductase, zinc-binding dehydrogenase are continuously produced not only as products of various metabolic processes but also in response to specific signals from culture conditions (Gäbler et al. 1994; Downs and Heckathorn 1998; Kamata and Hirata 1999; Ishizaki et al. 2006; Kocsy et al. 2013). This enzymes-set has also been found present in different somatic embryos developmental stages in other species (Lindemann and Luckner 1997; Morel et al. 2014), and the equilibrium promote the optimal growth and development (Kocsy et al. 2013). Accordingly, the imbalance of these enzymes can affect both redox state of antioxidants and ROS formation, which, through a redox signaling pathway, leads to the metabolism reprogramming of many compounds, including sulphur, nitrogen and carbohydrates containing organic compounds (Kocsy et al. 2013).

Another proteins widely up-regulated in translucent somatic embryos was Cytochrome P450 (12.65 fold and 8.66 fold). Cytochromes P450 represent a family metabolic enzymes, found in all kingdoms showing expressive diversity in their chemical reactions (Bolwell et al. 1994; Schuler et al. 2006; Mizutani and Ohta 2010; Mizutani 2012). Cytochromes P450 (P450s) are involved in essential housekeeping functions and metabolism of most phytohormones, including auxins, gibberellins, cytokinins, brassinosteroids, abscisic acid, and jasmonic acid, as well as many secondary metabolites (Werck-Reichhart et al. 2002). P450s also play a significant role in plant defense responses (Schuler 1996; Matthes et al. 2011). Redox regulation is a central control element in P450 metabolic pathways, since cytochrome P450 oxygenase reactions require cytochrome P450 reductase to transfer two electrons from NADPH to their substrate (Xu et al. 2015).

Thus, it can be suggested that the up-regulation of P450 in translucent somatic embryos, when compared to white somatic embryos, might generate a redox imbalance, which often trigger an oxidative burst (Dietz 2003; Foyer and Noctor 2005; De Gara et al. 2010). Plant hormonal imbalance is another possibility that cannot be excluded, since several evidence indicate that P450 is a key regulator in the biosynthesis or catabolism of plant hormones (Mizutani and Ohta 2010) which function directly in somatic embryo maturation (Zimmerman 1993a; von Arnold

et al. 2002; Maximova et al. 2002b; Fehér et al. 2003).

In addition, several studies on seed development indicated that P450 is associated with phytohormones pathways to promote cell proliferation and expansion, leading to growth and grain filling (Chen et al. 2014). In *Arabidopsis*, it was suggested that the overexpression of P450 proteins was associated with seed growth (Fang et al. 2012). Although, P450 proteins functions have been underreported in SE process, the results of the present work suggest that P450s are closely involved in metabolic pathways, which regulate cacao somatic embryo growth and maturation.

Finally, we can conclude that an intensive redox activity is apparently necessary for optimal somatic embryo maturation, since enzymatic apparatus are carefully balanced. Thus, any imbalance in redox homeostasis have crucial consequences into cell function and it may induce severe damage in the embryo development and therefore in the embryo quality.

Response to stimulus

Transition of single cells to somatic embryos involves a series of events associated with the molecular recognition of internal signals and external stimuli, mainly comprised "response to chemical stimulus", "response to stress", "response plant growth regulators" and "response to abiotic stimulus" (Zeng et al. 2007; Jin et al. 2014). The perception of /and response to these stimulus sets off various signal cascades that eventually might result in healthy somatic embryos (Zimmerman 1993; Fehér 2003; Fehér 2005).

In our results, we observed the expression of two subunits of RAB GTPase protein: RAB GTPase A5E isoform 1, which was up-regulated (3.15 fold), and RAB GTPase H1E isoform 2 (Fragment), which was down-regulated (4.12 fold). RAB GTPases proteins are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and McBride 2001). Membrane trafficking is required for a variety of cellular functions, such as storage-protein accumulation, cell differentiation and growth, secretion of protein and polysaccharide components of the cell wall and cell plate, and for the morphogenesis, which depends on spatial and quantitative control of cell expansion (Rutherford and Moore 2002; Lycett 2008).

In addition, it has been evidenced that Rab GTPases expression is modulated in response to variations in levels of phytohormones such as

ethylene (Moshkov et al. 2003) and abscisic acid during plant development and germinating seedlings (Nishimura 2004; Cui et al. 2013). In maritime pine embryogenesis, RAB GTPases were expressed in early stages of embryo development; the transcript was detected in early developmental stages of zygotic embryogenesis and the amount of transcript was progressively reduced in later stages (Tzafrir et al. 2002; Gonçalves et al. 2007).

In the present study, RAB GTPases were down-regulated in white somatic embryos when compared to translucent embryos. RAB GTPases seem to play a role during embryo development; however, the higher levels of these proteins are related with early developmental embryo stage. Thus, we can suggest that the translucent somatic embryo were unable to follow a complete developmental program, which is a requisite for embryo conversion ability.

Also in the present study, eukaryotic aspartyl protease (APs) family protein (2.62-fold) showed a similar expression pattern with pathogenesis-related (PR) proteins (3.21-fold). Both were up-regulated in translucent somatic embryos compared to white somatic embryos.

Aspartyl protease has been studied in different plant species. Nevertheless, their biological functions are not as well identified. In general, it is related with plant senescence, programmed cell death, stress responses and plant reproduction (Simões and Faro 2004) Besides, plant aspartyl protease participation in storage protein degradation during the mobilization of reserve proteins in seed germination was proposed in wheat (Belozersky et al. 1989), rice (Asakura et al. 1997) and cacao (Voigt et al. 1997). On the other hand, the high activity of these enzymes is common in non-germinated seeds, but nevertheless in suitable concentrations their activity has been proved to be essential during the seed development and germination (Palma et al. 2002).

During *Arabidopsis* embryogenesis, an aspartic protease was identified, which play a significant role as an anti-cell-death component by processing and activating a polypeptide that functions as a survival factor (Ge et al. 2005). Comparative proteomic studies of cacao somatic and zygotic embryos revealed that, aspartic proteases were more abundant in the torpedo stage of zygotic embryos. Therefore it was suggested that this protein could be a marker of the onset of embryo maturation (Noah et al. 2013; Niemenak et al. 2015) since that aspartic protease play important role for storage protein processing during seed development

(D'Hond et al. 1993; Voigt et al. 1997).

In the present study, we analyzed mature somatic embryos. However, the accumulation of aspartic protease in translucent somatic embryo might be an indication that although both somatic embryos types were collected in the same morphological developmental stage, they may not be in equivalent physiological stages. Translucent somatic embryo has accumulated aspartic protease proteins in similar patterns with early zygotic embryos.

Pathogenesis-related (PR) proteins have been identified as produced by the host plant, but induced by several pathogens (van Loon 1985). However, recent evidences show that these proteins display additional functions, including response to environmental stress and oxidative signals (Mur et al. 2004; Sabater-Jara et al. 2014), hormones signaling (Sessa et al. 1995), role in developmental processes and enzymatic activities in secondary metabolism (Liu and Ekramoddoullah 2006). Direct SE in *Cichorium* was accompanied by an increase in the level on PR proteins expression in the culture medium, suggesting that these proteins could be correlated with SE process (Helleboid 2000).

In fact, plant somatic cells respond to biotic and abiotic stress factors by activating an array of defense mechanisms, which, switch their developmental program to a specific physiological state that allows the reprogramming of gene expression and therefore the acquisition of embryonic competence (van Loon 1985; Sabater-Jara et al. 2014). Previous studies by Noah et al. (2013) and Niemenak et al. (2015) found that cacao somatic embryos were more stressed than zygotic embryos and PR proteins were expressed in higher abundance. In addition, a large number of differentially regulated genes that encode for transcription factors were related to stress responses in cacao somatic embryos when compared to zygotic embryos, suggesting that restrict cotyledon development in somatic embryos is related with gene stress responses expression (Maximova et al. 2014). Based on these results, it is hypothesized that PR proteins expression are involved in cacao somatic embryo maturation pathways, where a subtle imbalance could lead to abnormal embryo development.

In summary, it is possible to suggest a correlation between the stress response protein and somatic embryos development since that an increase of level of these proteins may generate signal molecules for embryo maturation and/or influence metabolism pathways. In this sense, the white somatic embryos were less stressed and consequently showed improved conversion ability when compared to translucent embryos.

Protein not assigned with the selected functional groups

In this work, electron transfer flavoprotein alpha isoform 1 (ETF) (9.31-fold) was a highly up-regulated protein in white somatic embryos that was not assigned in the main selected functional groups. Electron transfer flavoprotein belongs to "energy metabolism" category and is associated with oxidative phosphorylation.

During oxidative phosphorylation, ETF protein serves as a specific electron acceptor for several dehydrogenases, including five acyl-CoA dehydrogenases, glutaryl-CoA and sarcosine dehydrogenase. ETF protein transfers electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase. These redox reactions release energy, which is used to form ATP (Christie et al. 2015). In addition, ETF protein was found in plants under stress conditions and when carbohydrates as respiratory substrates are limiting. The ETF protein provides an alternate electron supply to the mitochondrial electron transport chain, either by supplying alternative substrates: protein and lipids or by promoting the metabolism of toxic products thereof, or both (Ishizaki et al. 2006; Araújo et al. 2011; Buer et al. 2013).

In cacao somatic embryos, the intensive energy metabolism may be the result of enzymatic activity, which is induced in order to break down of the carbohydrate supply in culture medium. According to Noah et al. (2013), this fact might be related to the absence of dormancy in cacao somatic embryos. On the other hand, we cannot exclude the possibility that under stress conditions or when the carbohydrate supply is inadequate, the metabolism of plant cells is modified and alternative respiratory substrates are metabolized to carry out the embryo development.

Conclusion

In this study, we report for the first time a comparative proteomic data between two different cacao somatic embryos types with relevant differences in somatic embryo conversion potential. Cacao SE is multi-step complex process where embryo induction, development and maturation depend on a series of factors encompassing salt composition, plant growth regulators, culture conditions and internal response to this stimuli. The proteomic analysis showed a large number of differentially

regulated proteins between withe and translucent somatic embryos.

Considering white somatic embryos as model, we found important differences between the accumulation patterns of the proteins related to carbohydrate metabolic process, which are involved in synthesis storage compounds. The accumulation of storage product is an important event during embryo maturation and could be considered a marker for somatic embryo quality. At the same time, stress responses and oxidation-reduction proteins were down-regulated in white somatic embryos. Congruent with these results, it was observed that the same proteins were down-regulated in later development stages of zygotic embryos, but in later somatic embryo development these proteins continued up-regulated. Based on these data, we can suggest that the cacao somatic embryos are able to develop efficient redox homeostasis system for controlling oxidative stress in order to reach the full development, which determines the conversion potential.

In addition, we hypothesized that translucent somatic embryos do not reach physiological maturity. This could be triggered by sub-optimal media composition or inadequate culture conditions. Knowledge about the differences in white somatic embryos compared to translucent somatic embryos might serve as a basis for manipulation of the culture conditions in order to increase the frequency of white somatic embryos and improve the cacao SE protocol, as well as to understand the role of pivotal metabolic pathways associated to this *in vitro* morphogenetic route.

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CAPÍTULO 2 –

Epigenetic of long-term somatic embryogenesis in *Theobroma cacao* L.: DNA methylation and recovery of embryogenic potential.

Este manuscrito encontra-se submetido no periódico: *Plant cell, tissue and organ culture*

Abstract

In *Theobroma cacao* L., declined embryogenic potential was observed in regenerated somatic embryos from long-term secondary somatic embryogenesis (SE). In order to explore the relationship between DNA methylation and the long-term secondary SE, the embryogenic potential and global DNA methylation levels in young, aged and regenerated somatic embryos subjected to 5-Azacytidine (azaC) treatment were comparatively assessed. Global DNA methylation levels increased in aged somatic embryos with long-term *in vitro* culture, but 5-azaC-supplemented treatments resulted in unaltered levels. In addition, DNA methylation pattern during SE was not affected by 5-azaC. DNA methylation increased during SE expression. Finally, recovery of embryogenic potential in aged somatic embryos in response to 5-azaC-supplemented treatments was observed. The outcome of this study suggested that the long-term SE in cacao induced the decline on embryogenic potential, which can be reversible trough 5-azaC supplementation. Besides, increased DNA methylation levels might be a response to the stress conditions that plant cells were exposed to during SE.

Keywords: Somatic embryo development; 5-Azacytidine; DNA methylation inhibitor; Subcultures; *In vitro* morphogenesis.

Introduction

Somatic embryogenesis (SE) in *Theobroma cacao* L. (cacao) is considered a powerful tool for mass clonal propagation (Maximova et al. 2005; Maximova et al. 2008). In secondary SE, the high embryogenic potential observed in the cotyledon epidermal cells allows cyclic somatic embryos regeneration. Somatic embryos are obtained directly from epidermal cells without the intermediate callus phase, which is commonly associated with higher levels of epi/genetic variation (Maximova et al. 2002; Traore et al. 2003). However, the *in vitro* propagation of plant cell over long-term periods has often been associated to the destabilization of the genetic and epigenetic program, which can lead to chromosomal and/or DNA sequence variations, methylation variation, transposon activation, and generation of somaclonal variants (Smulders and de Klerk 2011; Neelakandan and Wang 2012). Thus, to deepen on the causes of variation is important for the establishment of reliable protocols based on this morphogenetic route.

The *in vitro* regeneration of whole plants from differentiated cells is a clear demonstration of plant cells plasticity (Fehér 2003). SE onset depends on a complex network of interactions among plant growth regulators, origin of the explant, culture medium composition, nitrogen and carbon sources, and the *in vitro* culture conditions, which trigger a strong stress condition (Fehér 2015). In response to signals, cells acquire competence to initiate the change in the genetic program of somatic cells, accomplished by dedifferentiation process followed by the implementation of a new developmental pathway (Wagner 2003). These events are accompanied by changes at the chromatin level and gene expression reprogramming, highlighting the central role of epigenetic regulation in these processes (Miguel and Marum 2011). In this sense, epigenetic variation in *in vitro* plant cells is an adaptive response to cell culture conditions and to the developmental program (De-la-Peña et al. 2015).

DNA methylation is the best described epigenetic mechanisms and consists in the addition of a methyl group at the 5' position of the pyrimidine ring of cytosine in the DNA (5mdC). Cytosine methylation has been implicated in silencing of transposable elements and endogenous genes to permit genome integrity (Finnegan 2010; Zhang et al. 2010). DNA methylation is strongly involved in SE, during dedifferentiation and

redifferentiation of plant cells, expression of responsive genes can be altered by locus-specific modulation of DNA methylation (Fehér 2015). In this view, DNA methylation dynamics has the potential to trigger physiological or developmental variation among regenerants from *in vitro* tissue culture.

Studies in *Arabidopsis thaliana* (Tokuji et al. 2011) and *Pinus pinaster* Ait. (Klimaszewska et al. 2009) suggested that declined embryogenic potential in long-term *in vitro* cultures could be partly attributed to increased DNA methylation level. At the same time, these authors showed that exposition of aged cultures to hypomethylation agent, such as 5-azacytidine (5-azaC), can partially recover this ability. The 5-azaC is structurally similar to cytosine molecule, and in contact with cells, it may lead to a rapid decrease in DNA methyltransferase activity, causing hypomethylated DNA synthesis (Goffin and Eisenhauer 2002).

Previous studies with cacao SE showed that, although aged cacao callus yielded fewer somatic embryos induction, the late regenerants exhibited significantly less genetic and epigenetic mutations (Rodríguez López et al. 2010). In addition, according to Fang et al. (2009), cacao secondary SE resulted in true-to-type somatic embryos. Morphological and genetic fidelity has been extensively studied (Minyaka et al. 2008; Fang et al. 2009; Rodríguez López et al. 2010; Adu-Gyamfi et al. 2016), however no information about the long-term culture and DNA methylation is available for cacao.

In this context, the aim of the present work was to evaluate the relationship between long-term cacao SE and global DNA methylation levels, as well as, the effect of 5-azaC on the recovery of embryogenic potential in aged secondary somatic embryos.

Material and methods

Plant material

Primary SE were induced from surface-sterilized staminodes of immature flower buds of cacao genotype EET 103, as previously described by Maximova et al. (2005).

Secondary somatic embryogenesis

Secondary SE was performed from cotyledons of young somatic embryos (12 months-old) previously *in vitro* established, as described by Maximova et al. (2002) and Minyaka et al. (2008). Briefly, cotyledons were excised and subcultured in SCG (secondary callus growth) induction culture medium for 14 days. This culture medium was composed by DKW basalt salts (Driver and Kuniyuki 1984) supplemented with DKW vitamins, 20 g L^{-1} glucose, 9 μM 2,4- dichlorophenoxyacetic acid (2,4-D, Sigma-Aldrich®, St. Louis, MO, USA), 1.2 μM kinetin (Kin, Sigma-Aldrich®) and 0.2% (w/v) Phytagel™ (Sigma-Aldrich®). Cultures from SCG culture medium were transferred to ED (embryo development) culture medium.

ED culture medium was composed by DKW basal salts, DKW vitamins, 30 g L^{-1} sucrose, 1 g L^{-1} glucose, 3.0 mM MgSO4 and 0.2% (w/v) Phytagel™ and plant growth regulators-free. The cultures were subcultured every 14 days, where secondary somatic embryos were obtained. After 60 days of culture, cotyledonary-staged somatic embryos were counted and the embryogenic potential (number of somatic embryos per explant) was estimated. Samples (about 300 mg) were frozen in liquid nitrogen and stored at −80 °C for subsequent global DNA methylation analysis. For each collection, three replicates were analyzed.

Scanning electron microscopy

Representative samples from the control (5-azaC-free) and each 5-azaC-supplemented treatments were collected at 14, 28 and 42 days in culture. The samples were processed according to (Horridge and Tamm 1969). Briefly, the samples were fixed in phosphate buffer 0.1 M (pH 7.2) containing 2.5 % paraformaldehyde overnight at 4 °C. Subsequently, the samples were dehydrated in an increasing ethanol series, dehydrated in CO2 critical point dryer EM-CPD-030 (Leica®, Heidelberg, Germany), and gold sputter-coated prior to in the scanning electron microscope JSM6390 LVSEM (JEOLLtd., Tokyo, Japan) at 10 kV.

Global DNA methylation

Global DNA methylation levels and effects of 5-azaC during secondary

somatic embryogenesis from aged somatic embryos.

Secondary SE induction was performed from aged somatic embryos 36 month-old (12 sub-cultures) as described above (= control treatment) and in the 5-azaC-supplemented treatments (10 µM, 20 µM and 30 µM). Aliquots of filter sterilized 5-azaC solution were added to the SCG induction medium. Cultures from SCG culture medium were transferred to ED culture medium 5-azaC-free and subcultured every 14 days. After 60 days in culture, cotyledonary-staged somatic embryos were counted and the embryogenic potential (number of somatic embryos per explant) was estimated. Data were analyzed using Statistica® (Statsoft Inc., Tulsa, USA) for Windows® version 7.0 and submitted to ANOVA. The treatments were compared by the Tukey test ($p<0.05$).

Samples from the donor explant were collected prior 5-azaC-supplementstion. Samples from the control and each 5-azaC-supplemented treatments were collected after 14, 28 and 42 days in culture. After 60 days in culture, cotyledonary-staged somatic embryos were collected separately. Samples (about 300 mg) were frozen in liquid nitrogen and stored at −80 °C for subsequent global DNA methylation analysis. For each collection, three replicates were analyzed.

Somatic embryogenesis induction from regenerated somatic embryos of 5-azaC-supplemented treatments

An extra secondary SE was performed from regenerated somatic embryos of 5-azaC-supplemented treatments included the control. SE (5-azaC free) was induced as previously described above. After 60 days in culture, cotyledonary-stage somatic embryos were counted and the embryogenic potential (number of somatic embryos per explant) was estimated. Data were analyzed using Statistica® (Statsoft Inc., Tulsa, USA) for Windows® version 7.0 and submitted to ANOVA. The treatments were compared by the Tukey test ($p< 0.05$).

Genomic DNA extraction and HPLC determination of global DNA methylation levels

DNA was extracted from 300 mg tissue samples according to Doyle and Doyle (1987), with minor modifications. Briefly, frozen samples were ground into a fine powder with liquid nitrogen. Extraction buffer was composed of 2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 2 % polyvinylpyrrolidone and 3 % 2-mercaptoethanol.

The re-extraction buffer was composed of 50 µl of 10 % CTAB and 1.4 M NaCl. The extracted DNA was precipitated at −80 °C with 2/3 volume of isopropanol by a period of 4 h. The DNA pellet was washed twice in 70 % ethanol and once in 95 % ethanol and then dried at ambient temperature followed by dissolution in 100 µl of water. Nucleid acids were quantified in a spectrophotometer Nanodrop® 1000 (NanoDrop Technologies, Inc.).

Digestion procedures were performed according to Johnston et al. (2005) and Fraga et al. (2012). Briefly, 100 µl of nucleic acids (1 µg µl $^{-1}$) were incubated for 5 min at 65 °C and then for 10 min at 4 °C. Subsequently, 10 µl of RNase A (10 µg µl $^{-1}$, Sigma-Aldrich®) and 10 µl of RNase T1 (20 µg µl $^{-1}$, Sigma-Aldrich®) were added and incubated for 17 h at 37 °C. Then, 20 µl of sodium acetate (3 M, pH 5.4) was added. DNA and RNA resistant to RNase were precipitated at -20 °C with 136 µl of cold isopropanol for 30 min. Pellets were dried at ambient temperature and re-suspended in 100 µl of ultrapure water. The DNA concentration was adjusted to 0.25 µg µl $^{-1}$.

Finally, DNA samples was digested with 10 µl of nuclease P1 (1.0 U ml^{-1} in 30 mM NaOAc, pH 5.4, Sigma-Aldrich®) for 17 h at 37 °C. Then, 10 µl of 0.5 M Tris–HCl (pH 8.3) and 10 µl of alkaline phosphatase (10 U ml^{-1} $(NH4)_2SO_4$ 2.5 M, Sigma-Aldrich®) were added and incubated for 2 h at 37 °C. The supernatant was collected and stored at -20 °C until HPLC analysis.

HPLC analysis was performed according to Johnston et al. (2005). A HyperClone™ 5 µm ODS (C18) 120 Å, LC Column 250 x 4.6 mm (Phenomenex®, Torrance, USA), guard column (4.0 × 3.0 mm) (Phenomenex®), and UV detector (280 nm) were used. The gradient program consisted of 3 min with 100% buffer A (0.5 % v/v methanol in 10 mM KH_2PO_4 adjusted to pH 3.7 with phosphoric acid, 0.22 µm filtered), followed by a linear gradient from 3 to 20 min to 100% of buffer B (10 % v/v methanol in 10 mM KH_2PO_4 adjusted to pH 3.7 with phosphoric acid, 0.22 µm filtered), followed by 20–25 min with 100 % of buffer B. A flow rate of 1 ml min^{-1} and 20 µl of sample injection volume were applied.

The dNTPs (Fermentas®, Hanover, MD, USA) used as standards (dA, dT, dC and dG) and 5mdC were digested for 2 h with alkaline phosphatase (10 U ml^{-1}) and Tris-HCl (0.5 M, pH 8.3) at 37 °C to obtain the nucleosides. The standard nucleosides (5-50 mM) were prepared in

deionized H_2O and stored at -20°C. 5mdC quantification (%) was performed according to 5mdC concentration divided by 5mdC concentration plus dC concentration multiplied by 100. The obtained peak area was analyzed by LC Solution software (Shimadzu®, Kyoto, Japan).

Changes in global DNA methylation levels (% mdC) in 5-azaC-supplemented treatments were compared with the control and between the treatments at 14, 28 and 42 days in culture. The global DNA methylation levels from regenerated somatic embryos of 5-azaC-suplemented were also compared with the control and between treatments. Additionally, regenerated somatic embryos of 5-azaC-suplemented treatments were compared with aged somatic embryos used as explantes (donor sample) included the control. In addition, young, aged and regenerated somatic embryos from 5-azaC-supplemented treatments were compared. Global DNA methylation levels (% mdC) were compared by the Tukey test ($p< 0.05$).

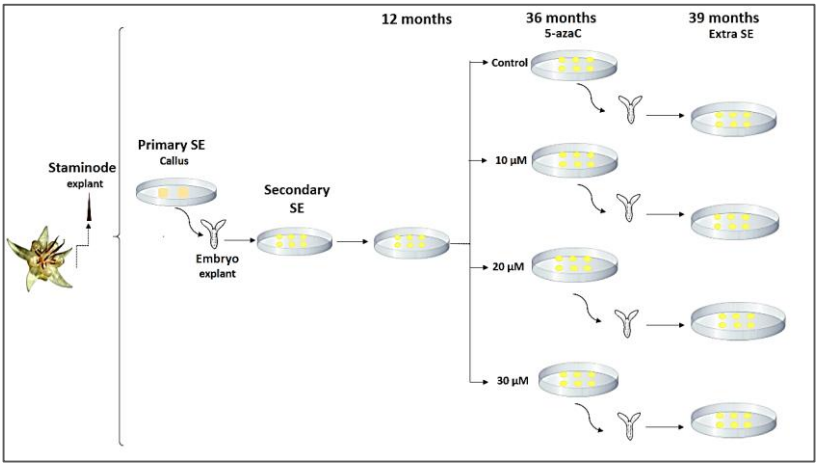

Fig. 1 Schematic representation of the experimental design based on the establishment and long-term secondary somatic embryogenesis maintenance at different culture ages (12, 36, and 39 months).

Results

Secondary somatic embryogenesis

Secondary SE performed from cotyledons of young somatic embryos (12 months-old) produced 18 somatic embryos, after 60 days in culture. The global DNA methylation levels was 17.49 ± 0.3% (Table 1).

Table 1 Global DNA methylation levels (%) in cacao cotyledonary-staged somatic embryos.

Culture conditions	Somatic embryo	DNA global methylation levels in somatic embryos (%)
	Young	17,49 ± 0,3
	Aged (donor explant)	27,06 ± 1,3
5-azaC (μM) treatment	Control	27,53 ± 1,9 a
	10 μM	27,38 ± 6,1 a
	20 μM	26,90 ± 4,2 a
	30 μM	27,57 ± 5,3 a

Different letters within each column indicate statistical difference between treatments submitted and between embryos type to ANOVA and Tukey mean separation test ($p < 0.05$).

Global DNA methylation levels and effects of 5-azaC during secondary somatic embryogenesis from aged somatic embryos

SE induction in the presence of 5-azaC in the SCG culture media affected the global DNA methylation levels (% mdC) and the embryogenic capacity of cultures. At 14 days of induction, the global DNA methylation levels in the treatments did not show a significant difference compared to donor sample (27.06 ± 1.29%). Compared to the control (27.06 ± 1.29 %), the global DNA methylation levels slightly

decreased in treatments supplemented with 10 µM (25.3 ± 0.6 %) and 20 µM (26.4 ± 0.4 %) 5-azaC, whereas the treatment supplemented with 30 µM of 5-azaC the levels remained unchanged (Fig. 2). In addition, oxidation of explants was observed in all treatments (Fig.3).

At 28 days culture, the global DNA methylation levels significantly increased compared with to 14 days culture. Compared to the control, the global DNA methylation levels increased in the treatment supplemented with 20 µM 5-azaC from 46.0 ± 1.4 % to 50.9.0 ± 0.7 % and decreased in the treatments supplemented with 10 µM and 30 µM 5-AzaC to 37.1 ± 5.7.4 % and 29.1.0 ± 0.9 %, respectively. At 42 days in culture, the global DNA methylation levels decreased compared to 28 days in culture, but slightly increased compared to 14 days in culture. Compared to the control, the global DNA methylation levels slightly increased in the treatments supplemented with 10 µM and 30 µM 5-azaC from 27.9 ± 1.8 % to 29.3 ± 1.4 % and 29.20 ± 3.2 %, respectively and decreased in the treatment supplemented with 20 µM 5-azaC to 26.9 ± 4.8 %. At this time, the somatic embryos expression stopped and the explants showed an abundant phenolic oxidation (Fig 3).

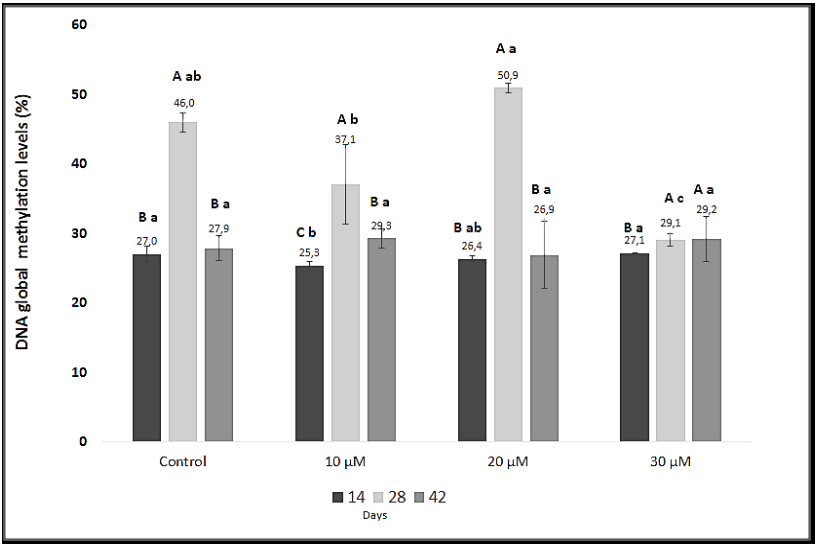

Fig. 2 Global DNA methylation levels during cacao somatic embryogenesis. Percentage of global DNA methylation levels during SE in normal conditions (control) and treatments supplemented with 10 µM,

20 μM and 30 μM 5-azaC. At 14, 28 and 42 days in culture. Mean values followed by standard deviation (vertical bars). Means followed by uppercase letters are significantly different among days-culture (14, 28 and 420 days) according to the Tukey test ($p < 0.05$). Means followed by lowercase letters are significantly different among treatments according to the Tukey test ($p < 0.05$).

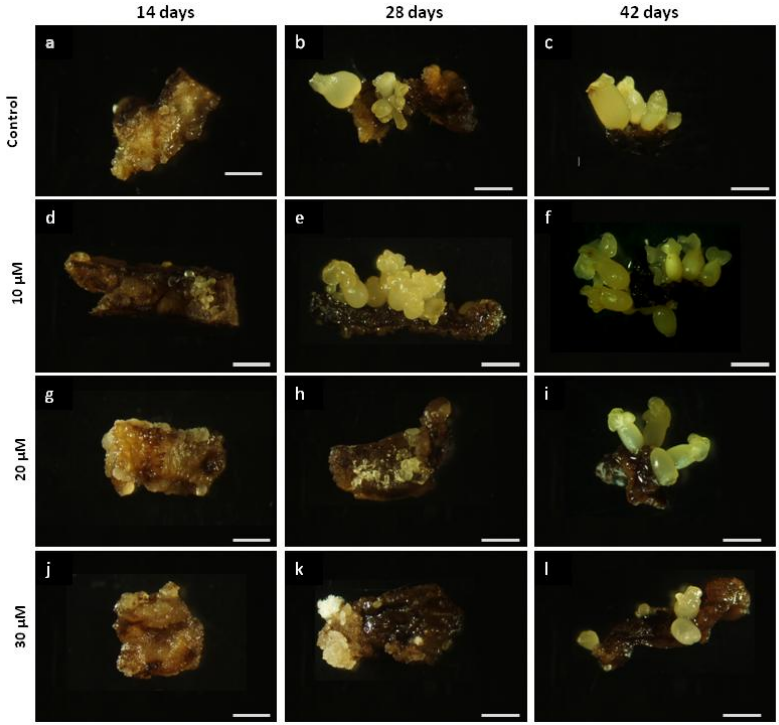

Fig. 3 Morphological features of cacao somatic embryogenesis. Explants from aged somatic embryos under normal conditions (control), treatments supplemented with 10 μM, 20 μM and 30 μM 5-azaC during 42 days-culture. Bars = 2 mm.

Aged somatic embryos (donor explant) indicated 27.06 ± 1.29 % global DNA methylation, this level was significant higher than young

somatic embryos (17.49 ± 0.33 %). Global DNA methylation levels of regenerated somatic embryos from 5-azaC-supplemented treatments slightly decreased from 27.53 ± 1.9 % in the control to 27.38 ± 6.1 %, 26.90 ± 4.2,9 % and 27.57 ± 5.3 % in the treatments supplemented with 10 µM, 20 µM and 30 µM 5-azaC respectively, without significant difference. Similarly, the global DNA methylation levels on regenerated somatic embryos from 5-azaC-supplemented treatments also were not significantly different, when compared with donor explant (Table 1).

After 60 days in culture, the regeneration of somatic embryos was significantly affected by the presence of 5-azaC in the SCG culture media. On average, the control treatment produced 10 somatic embryos per explant. Embryogenic potential was increased in the treatments supplemented with 10 µM and 20 µM 5-azaC to 12 and 14 somatic embryos per explant, respectively and decreased in the treatment supplemented with 30 µM 5-azaC to 8 somatic embryos per explant (Fig. 4).

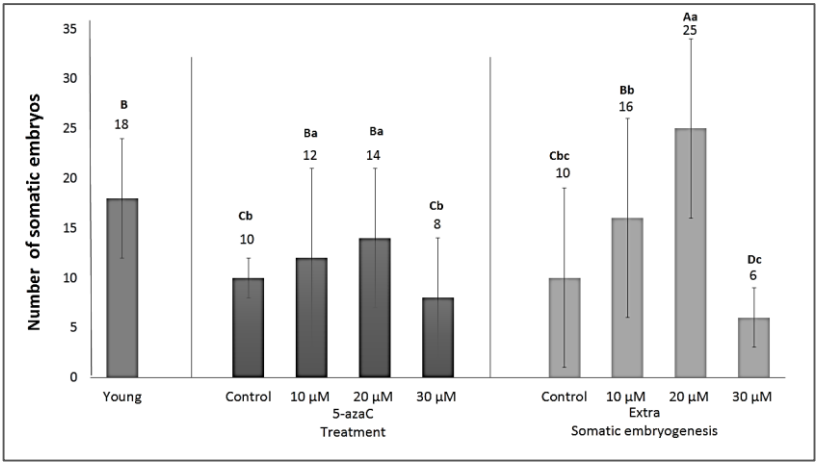

Fig. 4 Effect of 5-azac in cacao embryogenic potential. Number of somatic embryos from in young, aged and regenerated somatic embryos subjected to 5-Azacytidine (azaC) treatment. The number of every cotyledonary-staged somatic embryos were counted after 60 days-culture. Mean values followed by standard deviation (vertical bars). Means followed by different uppercase letters are significantly different among somatic embryos young, aged, somatic embryos from 5-azaC-treatments

and somatic embryos from 5-azaC-free treatments according to the Tukey test ($p < 0.05$).Means followed by lowercase letters are significantly different among treatments according to the Tukey test ($p < 0.05$).

Scanning electron microscopy analysis

In order to anatomically evaluation SE development in 5-azaC-suplemented treatments, this process was registered every 14 days by scanning electron microscopy (SEM) (Fig. 5). In the control treatment, at 14 days in culture, the first morphological change observed in the explants was a small amount of growth of dedifferentiated tissue around the explant (Fig. 5a). In the explants in 5-azaC treatments, the most evident morphological change was the wounds on the explant surface (Fig. 5b to 5d). In addition, in 5-azaC-supplemented treatment with 10 μM 5-azaC was observed structures that could correspond to early embryonic stages such as globular somatic embryos (Fig 5b).

At 28 days culture, it was possible to observe somatic embryos on the globular and heart embryonic stage, which were connected to explant surface with the suspensor (Fig. 5f). In 5-azaC-supplemented treatments with 10 μM and 20 μM, the somatic embryos expression on the globular and heart embryogenic stage was more evident, compared with the control and 5-azaC treatment with 30 μM (Fig. 5f-h). At 42 days in culture, somatic embryos have detached from the suspensor structure (Fig. 5i-l).

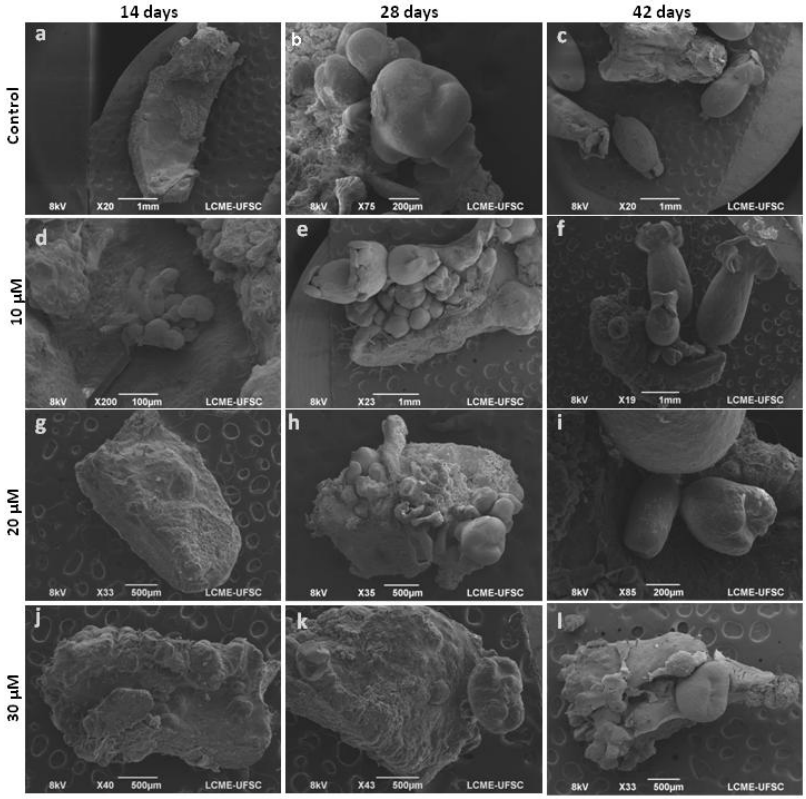

Fig. 5 Scanning electron microscopy (SEM) of cacao somatic embryogenesis. Explants from aged somatic embryos under normal conditions (control), treatments supplemented with 10 µM, 20 µM and 30 µM 5-azaC during 42 days-culture.

Somatic embryogenesis from regenerated somatic embryos of 5-azaC-treated tissue

Regenerated somatic embryos of 5-azaC-supplemeted treatments, including those of the control, were used as explant to secondary SE induction. Thus, after 60 days in culture, the control produced 10 somatic embryos per explant, while explants derived from treatments supplemented with 10 µM and 20 µM 5-azaC resulted in a higher number of somatic embryos (16 and 26 somatic embryos per

explant, respectively). Explants from treatment supplemented with 30 μM 5-azaC produced 6 somatic embryos per explant, decreased compared to the control (Fig 3B).

Discussion

During cyclic secondary cacao SE, we observed a clear and progressive decrease of embryogenic potential as a function of time and number of subcultures. A low production of somatic embryos was registered in 36 month-old cultures (12 subcultures) compared to 12 months-old cultures (4 subcultures). Similar results has been earlier reported for a significant number of species (Breton et al. 2006; Klimaszewska et al. 2009; Bobadilla Landey et al. 2015; Bradaï et al. 2016). Olive (*Olea europaea* L.) embryogenic cultures modified proliferation pattern leading to a decrease embryogenic potential after repetitive subcultures (Bradaï et al. 2016). In the same way, in maritime pine (*Pinus pinaster*), a complex embryogenic culture organization was observed, which affected the morphology and subsequent maturation of early somatic embryos (Breton et al. 2006).

Although variation in embryogenic potential is associated to genotypic background, which affected all the steps of the SE (Konan et al. 2009; Park et al. 2011; Bradaï et al. 2016), the loss of regeneration ability in long-term subcultures is associated with a decline in tissue quality due to culture aging (Breton et al. 2006). These age-associated alterations on tissue culture are based on the accumulation of cell damage achieved through repetitive subcultures, which inactivates randomly genes important for the functioning of the somatic cells (Dubrovina and Kiselev 2016). Accordingly, Fraga et al. (2002) reported that in *Pinus radiata* morphological changes and loss of morphogenic ability during cell plant aging are associated to changes in gene expression caused by DNA methylation.

In the present study, we observed that, in addition to progressive decrease of embryogenic potential, the aged somatic embryos (36 month-culture) showed increased global DNA methylation levels compared to young somatic embryos (12 month-culture). It is important to mention that in cyclic cacao SE, regenerated somatic embryos were used as explants to induce each new SE cycle. In this view, we speculate that the high global DNA methylation levels in aged somatic embryos of cacao

might be an accumulative effect as response to stress imposed by *in vitro* culture conditions. This fact might be a mechanism of plant cells to protect the genome integrity.

DNA methylation plays an important role in preservation of genome integrity by controlling the appearance of aberrant recombinant events (Finnegan 2010) and silencing mobile elements such as transposons (Ikeda and Nishimura 2015). Several authors pointed out that the ability of plant cells to modulate genome plasticity through DNA methylation reflects the adaptation and tolerance to stress (Boyko and Kovalchuk 2008; Sahu et al. 2013). *In vitro* SE is a complex process, which involve reprogramming of cells in response to various stress factors and DNA methylation operate by modulating gene expression (Zavattieri et al. 2010; Mahdavi-Darvari et al. 2015; Fehér 2015).

In support to our results, Adu-Gyamfi et al. (2016) found that cacao somatic embryos showed higher epigenetic variability after cryopreservation, which may be an adaptive response to high stress induced by the cryopreservation process. Although this epigenetic variability was decreased after an extra SE, it was significantly higher compared to donor samples (somatic embryos). These authors suggested that epigenetic variability in the cacao somatic embryos is not stochastic event, but probably resulted as adaptive response to stress conditions that plant cells are exposed during *in vitro* culture.

A growing number of studies reveals that genomic DNA methylation is involved in the modulation of gene expression during SE (LoSchiavo et al. 1989; Yamamoto et al. 2005; Che et al. 2006; Zeng et al. 2007; Miguel and Marum 2011; Elhiti et al. 2013). For instance, Li et al. (2011) showed that during *de novo* shoot regeneration in *Arabidopsis thaliana*, DNA methylation and histone modifications are able to regulate regeneration through modulating WUSCHEL transcription factor and auxins signaling. Rival et al. (2013) reported that DNA hyper-methylation is a time-dependent process during long-term *in vitro* proliferation of oil palm embryogenic suspension cultures. In addition, these authors showed that genetic background also affect the changes in the DNA methylation.

Smýkal et al. (2007) assessed the genetic and epigenetic stability of clonally propagated multiple shoot pea culture, which had been maintained *in vitro* for 24 years. Although, the fertility and senescence problems of regenerants were observed, DNA methylation levels did not show significant differences respect to *in vitro*-derived and seed-derived plants, as well as low level of polymorphisms were detected through the AFLP technique. Klimaszewska et al. (2009) showed that young and aged embryogenic cultures of *Pinus pinaster* did not show statistically

significant differences in the global DNA methylation levels, although qualitative alterations in specific DNA target sequences were detected by MSAP analysis.

Contrarily, in *Coffea arabica* L. aged cell culture, low levels of methylation variation were found. The authors concluded that genetic polymorphisms and epigenetic changes are particularly limited during cell culture ageing and they pointed out that the aneuploidy plays a more relevant role in the genetic variation (Bobadilla Landey et al. 2015; Etienne and Bertrand 2016). At the same way, previous studies in cacao SE showed that aged callus exhibited reduced embryogenic potential, yielded somatic embryos containing less genetic and epigenetic aberrations (Rodríguez López et al. 2010).

The results of the present work showed that 5-azaC-supplemented treatments in the induction culture medium (SCG), at moderate concentrations, did not influence the global DNA methylation levels as compared to control. Interestingly, at neither 14 nor 42 days-culture the global DNA methylation levels showed values significantly differed compared to donor sample. Thus, we can suggest that the lack of change of global DNA methylation at 14 and 42 days in culture is associated with the direct SE, in which a few portion of epidermal cells in appropriate induction conditions initiate an embryonic development program, resulting in somatic embryo differentiation, without the intermediate callus phase.

At 28 days culture in culture, the global DNA methylation levels increased, and this fact may be ascribed to somatic embryos differentiation (Fig. 5). Several authors showed that increased global DNA methylation during somatic embryos differentiation are directly associated to DNA replication and cell division, accompanied by activation of embryogenic gene expression program (Chakrabarty et al. 2003; Che et al. 2006).

The DNA hypomethylating drug 5-azaC is a cytidine analogue, in which the carbon at position 5´of the pyrimidine ring is replaced by nitrogen (Goffin and Eisenhauer 2002). 5-AzaC can be also incorporated into RNA. The incorporation of 5-azaC into RNA produces a ribosome malfunction and inhibits protein synthesis (Santini et al. 2001). 5-azaC exhibits cytotoxicity, which would be through the damage to the structural stability of DNA (Kiziltepe et al. 2007).

Studies on *Arabidopsis* showed that increase DNA methylation

at *in vitro* long-term cultures decreased the shoot-forming ability, and the addition of 5-azaC to induction culture medium can partially recover this ability through modulating *CUC1, CLV1, CLV3, ESR1*, and *WUS* mRNA levels (Tokuji et al. 2011). In the same way, studies with *Pinus pinaster* indicated that 5-azaC hypomethylating activity did not change the global DNA methylation levels of aged embryogenic cultures. However, somatic embryo regeneration ability was increased compared to 5-azaC-free treatments (Klimaszewska et al. 2009).

Finally, we observed that SE induction on SCG culture medium supplemented with 5-azaC-caused a clear increase in the embryogenic potential, compared to 5-azaC free treatment. However, higher concentrations of 5-azaC showed to be inhibitory to the somatic embryo formation. Several authors reported cytotoxicity effects associated to high levels of 5-azaC, during SE high concentrations of this hypomethylating drug may affect the somatic cell transition to embryogenic state and restricting the somatic embryos formation (Yamamoto et al. 2005; Klimaszewska et al. 2009; Teyssier et al. 2014). Surprisingly, 5-azaC hypomethylating activity did not influence the global DNA methylation levels in regenerated cacao somatic embryos, which were not different, compared to control and to donor sample (Table 1). However, the lack of quantitative differences in global DNA methylation does not excludes a locus-specific methylation process (Alvarez-Venegas et al. 2014).

Interestingly, an extra SE (5-azaC free) using somatic embryos regenerated from 5-azaC-sumplemented treatments maintained the embryogenic potential showed during SE with 5-azaC-supplemented treatments. In this view, we can suggest that loss of embryogenic potential associated to long term *in vitro* culture may be reversible, supporting the hypothesis of it being epigenetic. According to Feng and Jacobsen (2011), epigenetic reprogramming events are needed to erase the effects of epigenetic marks caused by external stimuli.

Conclusion

Our results evidenced a clear loss of embryogenic potential and an increased global DNA methylation in aged somatic embryos as a result to long-term secondary SE. Thus, we can suppose that DNA hyper-methylation in aged somatic embryos may be an accumulative effect in response to *in vitro* stress conditions, which may be preserving the genome integrity. At the same time, we showed that SE induction in 5-azaC-supplemented treatments from aged somatic embryos, yield a higher number of somatic embryos compared to the control. Interestingly,

an extra SE induction (5-azaC free) showed that aged somatic embryos can recovery the embryogenic potential. This fact prove that embryogenic potential is epigenetic reversibly feature. Thus, in order to minimize the long-term effects, the mechanism trough which DNA methylation is involved in long-term *in vitro* culture requires further studies.

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CONSIDERACOES FINAIS E PERSPECTIVAS FUTURAS

O presente estudo contribuiu para ampliar os conhecimentos sobre os mecanismos que regulam a qualidade e a capacidade de conversão nos embriões somáticos de cacaueiro, usando como foco a embriogênese somática secundária por meio de abordagens de proteômica e da epigenética. No capítulo 1, os resultados das análises de proteômica *shotgun* indicaram diferenças nos perfis de expressão das proteínas nos embriões somáticos de coloração branca quando comparados com os embriões somáticos translúcidos. Considerando os embriões somáticos brancos como modelo, por apresentar uma capacidade superior de conversão, foram encontradas diferenças importantes entre os padrões de acumulação das proteínas relacionadas ao processo metabólico dos carboidratos, os quais estão diretamente envolvidos na síntese de compostos de armazenamento. A acumulação de compostos de armazenamento durante a maturação do embrião garante o sucesso da conversão e, portanto, esta característica pode ser considerada como marcador confiável para a qualidade do embrião somático. Ao mesmo tempo, as proteínas relacionas a respostas de estresses e de processos oxidação-redução foram sub-expressas nos embriões somáticos brancos. Congruente com estes resultados, diferentes autores observaram que as mesmas proteínas foram sub-expressas em estádios tardios de desenvolvimento de embriões zigóticos. Com base nesses dados, sugere-se a qualidade dos embriões somáticos de cacaueiro depende da capacidade de desenvolver um sistema de homeostase redox eficiente para controlar o estresse oxidativo, próprio da cultura *in vitro* a fim de atingir o pleno desenvolvimento, o qual determina o potencial de conversão. Esta informação poderia ser utilizada para ajustar e otimizar a composição dos meios de cultura com o objetivo de aumentar a frequência de embriões somáticos brancos.

O estudo da embriogênese somática secundaria apresentado no capitulo 2, evidenciou que embriões somáticos envelhecidos e resultantes de repetidas subculturas perdem parcialmente o potencial embriogênico, ao mesmo tempo em que aumentam os níveis na metilação global do DNA. Assim pode-se supor que a hipermetilação do DNA em embriões somáticos envelhecidos corresponderia a um efeito cumulativo em resposta a condições de estresse repetitivo impostas pela cultura *in vitro*, o que poderia estar preservando a integridade do genoma. Ao mesmo

tempo, mostrou-se que a embriogênese somática induzidas a partir de embriões somáticos envelhecidos, quando expostas a um meio de cultura suplementado com 5-azaC, produziu maior número de embriões somáticos quando comparada a culturas que não foram tratadas com esta substância, revelando assim recuperação do potencial embriogênico. Este fato comprova que a diminuição e mesmo perda de potencial embriogênico pode ser reversível. Estes resultados motivam o desenvolvimento de mais estudos sobre os mecanismo pelo qual a metilação do DNA está envolvida na cultura *in vitro* a longo prazo.

Tomando em conjunto, os resultados obtidos e apresentados neste trabalho de tese contribuíram para elucidar os mecanismos que determinam a qualidade dos embriões somáticos de cacaueiro assim como também delimitar as implicações da ES secundaria no longo prazo. Estes resultados contribuem para o aprimoramento do protocolo da ES de *Theobroma cacao* L. e podem auxiliar os estudos de outras de espécies com características similares.