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EMBRIOGÊNESE SOMÁTICA E CRIOPRESERVAÇÃO DE EMBRIÕES SOMÁTICOS EM PUPUNHA (*Bactris gasipaes* Kunth)

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À Julia Py B. Teixeira,
Por seu amor e carinho,
Ofereço.

Á minha Família Marília, Caide e Camila,
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1. Resumo Geral

A pupunha (*Bactris gasipaes* Kunth) é uma palmeira nativa da Amazônia, pertencente à família Arecaceae, sendo considerada uma árvore multi-propósito e desempenha um papel importante como um componente de agrossistemas. A produção de frutos e palmito para o mercado nacional são um dos seus usos mais importantes. Ela se tornou uma alternativa para a produção de palmito cultivado, apresentando diversas vantagens comparativamente a outras espécies de palmeiras (ex.: *Euterpe edulis* e *E. oleracea)* usadas para produção deste produto, tais como um curto ciclo de vida, presença de ramificações, níveis mais elevados de açúcares. Além disso, esta palmeira apresenta baixas concentrações das enzimas peroxidases e polifenoloxidase, permitindo o comércio *in natura* sem que haja oxidação do produto. O desenvolvimento de protocolos de regeneração *in vitro* se configura como uma eficiente ferramenta de apoio à conservação e programas de melhoramento genético da espécie. Entre as várias técnicas disponíveis, a embriogênese somática oferece vantagens como a produção automatizada em grande escala, com culturas cíclicas, e estabilidade genética das plântulas regeneradas. O Sistema de Imersão Temporária (SIT) é relevante para a ampliação ou melhoria de protocolos de regeneração *in vitro* e também abre possibilidades para automatizar algumas fases da cultura. Além disso, a embriogênese somática pode ser acoplada a programas de conservação por meio, por exemplo, da criopreservação de embriões somáticos. Sabe-se que o *pool* de genes da pupunha cultivada e selvagem é rico em diversidade, porém, esta sujeito à erosão genética, necessitando de urgente desenvolvimento de estratégias eficientes de conservação de germoplasma à longo prazo, sendo a criopreservação a estratégia mais adequada para este fim. A criopreservação é definida como a conservação de material biológico em temperaturas ultrabaixas (-196°C ou -150°C) e duas técnicas, vitrificação e vitrificação-droplet, estão sendo amplamente utilizadas para diversas espécies, sendo que as duas objetivam a obtenção de um estado vítreo de líquidos celulares, obtida por concentrações suficientemente altas de solutos (crioprotetores) e resfriamento rápido. Entretanto, a capacidade de recrescimento e estabilidade genética em plantas criopreservadas estão associadas a mudanças no DNA global celular, pricipalmente em nível epigenético, alterando o padrão de

metilação global do DNA celular. Além disso, um aspecto crítico para se obter um protocolo de criopreservação de uma determinada espécie é diminuir a injuria na ultraestrutura da célula, que normalmente é causada pela técnica, e análises ultraestruturais e epigenéticas durante as etapas do protocolo de criopreservação se tornam importantes para aprimoramento destas técnicas.

Considerando os aspectos supramencionados, o objetivo geral deste trabalho foi o de otimizar o protocolo de embriogênese somática para pupunha utilizando SIT nas diferentes fases deste, bem como desenvolver um protocolo para a criopreservação de embriões somáticos desta espécie, elucidando aspectos ultraestruturais e epigenéticos dos embriões somáticos submetidos a estas técnicas.

A primeira parte do trabalho teve como objetivo específico identificar o melhor sistema de cultivo para cada etapa do protocolo de embriogênese somática de pupunha, aliado à análise bioquímica e epigenética, bem como avaliar os efeitos do ABA no processo de maturação dos embriões somáticos. Embriões somáticos multiplicados em aparatos RITA® apresentaram os melhores parâmetros analisados, ou seja, uma multiplicação elevada com o incremento significativo de proteínas totais (4,07 mg g^{-1}), amido (1,34 mg g^{-1}) e atividade enzimática da álcool desidrogenase (ADH)(3,65 OD min^{-1} mg^{-1} de proteína), ligado a um baixa taxa de metilação de DNA global (27,52%), indicando um possível avanço no desenvolvimento de embriões somáticos. O ABA juntamente com o meio de cultura semi-sólido, foi importante para a maturação de embriões somáticos, ligado a uma queda na metilação global do DNA (27,28%), o que provavelmente permitiu que a expressão de proteínas essenciais para a maturação dos embriões somáticos. No passo inicial da conversão, o cultivo em placas de Petri com meio de cultura isento de fitoreguladores proporcionou o desenvolvimento de um grande número de embriões verdes (105,25) que, em seguida, quando transferidos para os aparatos RITA® resultaram em um grande número de plântulas (315,7) em um tempo curto se comparado com os outros sistemas. Na aclimatização, a sobrevivência (83,3%) e enraizamento (94,4%) foram melhores para plântulas inicialmente com 7 cm, indicando a importância do tamanho da planta nesta etapa final.

A segunda parte do trabalho avaliou a dinâmica da metilação global do DNA associada com as taxas de recrescimento de embriões

somáticos durante as etapas do protocolo de criopreservação pela técnica de vitrificação-*droplet*. *Clusters* de embriões somáticos (SEC), submetidos a solução de vitrificação PVS3 (*Plant Vitrification Solution* 3) por diferentes períodos (0, 60, 120, 180 e 240 min) apresentaram diferentes taxas de recrescimento. A maior taxa (52,4%) foi obtida em resposta à técnica de vitrificação-*droplet*, combinado com um tempo de incubação de 120 minutos. A dinâmica de metilação do DNA global foi afetada tanto pela solução crioprotetora quanto pela submissão ao protocolo de criopreservação. Por exemplo, a incubação de SEC em PVS3, independente do tempo, não só reduz as taxas de recrescimento, como imediatamente aumenta os níveis de metilação global do DNA em relação aos SEC multiplicados no sistema de imersão temporária (controle). Porém, SEC que foram submetidos a criopreservação e posteriormente recuperados tiveram uma maior variação nas taxas de metilação global do DNA, e a exposição de SEC em PVS3 durante 120 min foi a única que conseguiu restabelecer o padrão inicial de metilação global após 24 semanas de recrescimento. Assim, durante a criopreservação de embriões somáticos, mudanças epigenéticas causadas pela alteração do estado de metilação global do DNA podem apresentar consequências fisiológicas implicando na conservação do germoplasma dessa importante espécie de palmeira.

Para estabelecer um protocolo de criopreservação adequado, a terceira parte do trabalho objetivou avaliar as características ultraestruturais de SEC de *B. gasipaes* submetidos a criopreservação pela técnica de vitrificação. Assim, SEC foram incubados em diferentes tempos (0, 60, 120, 180 e 240 min) na solução PVS3. Em geral, as células submetidas a esta solução apresentaram características de células viáveis associadas a um núcleo proeminente, numerosas mitocôndrias e as paredes celulares bem preservadas. Células não incubadas em PVS3 não sobreviveram após o processo criogênico, apresentando células ultraestruturalmente colapsadas. O melhor tempo de incubação para a técnica de vitrificação foi de 240 minutos, resultando numa taxa de recrescimento de 37%. Nestes casos, várias características foram indicativas de um metabolismo celular ativo, incluindo núcleos intactos e paredes de células preservadas, um grande número de mitocôndrias e corpos lipídicos, bem como a presença de muitos grânulos de amido e cromatina condensada. Assim, a análise ultraestrutural revelou que as estruturas celulares totais foram conservadas depois do tratamento

criogênico aliado a solução PVS3, validando o uso da técnica de vitrificação para a criopreservação de genótipos elite de pupunha, bem como os genótipos selvagens, que possuem uma ampla base genética muito rica e que devem ser conservados.

Em conclusão, o protocolo de embriogênese somática desenvolvido neste trabalho mostra uma alta eficiência para a multiplicação, maturação dos embriões somáticos e produção de plântulas de pupunha e enfatiza a importância de modificar sistemas de cultivo nas diferentes etapas do protocolo resultando em alta qualidade de embriões somáticos, encurtando o tempo e aumentando o número de plântulas no final do processo. Com relação à criopreservação dos embriões somáticos, os acréscimos nas taxas de metilação global do DNA podem explicar as altas taxas de recrescimento desses embriões somáticos e estudos adicionais podem elucidar se estas variações epigenéticas podem ser herdadas pela geração seguinte e se as mudanças de metilação global do DNA causados durante criopreservação podem influenciar nas plântulas regeneradas a partir destes embriões somáticos criopreservados. Quanto às análises estruturais associadas à criopreservação, as mesmas revelaram estruturas celulares conservadas depois do tratamento criogênico e assim validando a técnica de vitrificação para a criopreservação de genótipos de pupunha.

2. Objetivo Geral

Otimizar o protocolo de embriogênese somática para pupunha (*Bactris gasipaes* Kunth), no que tange principalmente aos pontos de controle relacionados às fases de multiplicação, maturação e conversão de embriões somáticos, utilizando sistemas de imersão temporária, bem como desenvolver um protocolo para a criopreservação de embriões somáticos desta espécie, estudando também aspectos morfo-histoquímicos e epigenéticos dos embriões somáticos submetidos a estas técnicas.

Objetivos Específicos:

a) Otimizar o protocolo multiplicação, maturação e conversão de embriões somáticos em sistemas de imersão temporária;

b) Caracterizar as principais alterações bioquímicas dos embriões somáticos posteriormente aos tratamentos de maturação e conversão;

c) Estudar fatores que afetam a criopreservação de embriões somáticos por meio das técnicas de vitrificação e vitrificação-droplet;

d) Estabelecer um protocolo de criopreservação de embriões somáticos;

e) Caracterizar as principais alterações na metilação global do DNA dos embriões somáticos submetidos à técnica de criopreservação;

f) Caracterizar as principais alterações ultraestruturais dos embriões somáticos submetidos à técnica de criopreservação.

CAPÍTULO I:
Estado da arte e situação do problema

1. Pupunha (*Bactris gasipaes*)

1.1 Descrição Botânica da espécie

A família Arecaceae é um dos grupos mais importantes das plantas tropicais (Balick 1988), sendo constituída por cerca de 2800 espécies subdivididas em cinco subfamílias, Calamoideae, Nypoideae, Coryphoideae, Ceroxyloideae e Arecoideae. A subfamília Arecoideae, a qual o gênero *Bactris* pertence, é bem distribuída em todas as regiões tropicais e subtropicais. Porém ela está em maior concentração na América Central e do Sul e no Caribe (Henderson 2000). A Pupunha (*Bactris gasipaes* Kunth) também tem uma ampla distribuição geográfica, porém limitada as Américas, desde a região central da Bolívia ao nordeste de Honduras, da foz do rio Amazonas e as Guianas até a costa do Pacífico do Equador e Colômbia (Figura 1; Mora-Urpí et al. 1997).

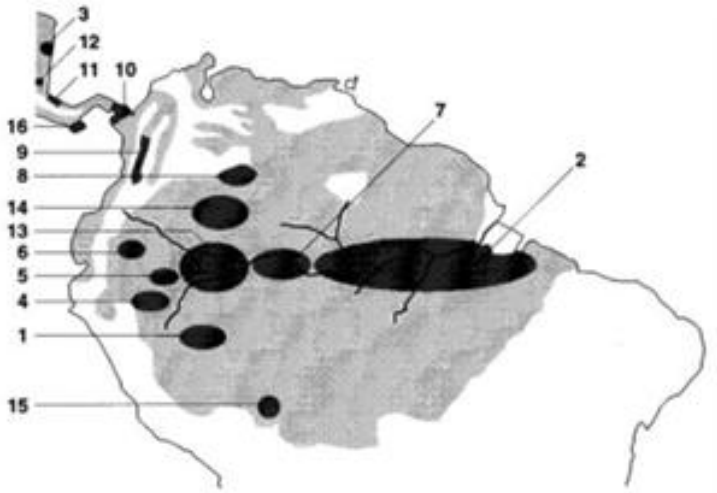

Figura 1. Distribuição geográfica de pupunha (*Bactris gasipaes* Kunth) e suas diferenças raças: Microcarpa (1) Juruá, (2) Pará, (3) Rama, (4) Azuero; Mesocarpa (16) Pampa Hermosa,(5) Tigre, (6) Pastaza, (7) Solomões, (8) Inirida, (9) Cauca, (10) Tuira, (11) Utilis, (12) Guatuso; Macrocarpa (13) Putumayo, (14) Valpés. Fonte Mora-Urpí et al. (1997).

A planta adulta pode chegar a 20 m de altura (Figura 2A), com diâmetros da haste entre 15 e 30 cm e entrenós entre 2 e 30 cm. É uma

planta cespitosa, o que significa que se ramifica no nível do solo formando um aglomerado de hastes (Figura 2B). Os entrenós possuem numerosos espinhos rígidos, que variam, na coloração, de preto ao marrom. No entanto, existem mutações apresentando hastes sem espinhos. Os ápices costumam conter de 15 a 25 folhas pinadas, com folíolos inseridos em diferentes ângulos. Essa espécie é monóica e suas inflorescências aparecem nas axilas das folhas senescentes. Após a polinização, ocorre a formação de cachos, com 50-1000 frutos, pesando entre 1-25 kg e cada um destes frutos pesando entre 0,5-120 g (Mora-Urpi et al. 1997; Clement 2008) (Figura 2C). O fruto maduro é composto de um exocarpo fibroso que pode ser vermelho, laranja ou amarelo (Figura 2C), e um mesocarpo rico em amido e óleo (Arkcoll e Aguiar 1984; Mora-Urpí et al. 1997; Yuyama et al. 2003). Durante o desenvolvimento dos frutos ocorrem mudanças como na cor do exocarpo, bem como alterações nas sementes, incluindo lignificação do endocarpo associados à sua mudança de cor e endurecimento do endosperma gelatinoso. O embrião zogótico é inicialmente globular e durante a diferenciação do meristema apical ele se alonga atingindo 1,5-2 mm no seu estado maduro com um formato cônico de forma oblíqua em relação aos cotilédones e o procâmbio (Steinmacher et al. 2007a)

É uma espécie que apresenta elevada taxa de crescimento mesmo em solos pobres (Mora-Urpi et al. 1997), possivelmente devido a características morfológicas, como a arquitetura das folhas pinadas e do sistema radicular, para a captura de luz solar, água da chuva e nutrientes do solo. Raízes adventícias da pupunha produzem um espesso tapete superficial que pode se estender 4-5 metros ao redor da planta e com 20 cm no horizonte do solo, porém podendo se estender a uma profundidade de 2 m de profundidade (Mora-Urpi et al 1997; Emmerich 2002). Não há a presença de pêlos radiculares e a exoderme consiste de grandes células globosas ligeiramente achatadas, e essas células estão posicionadas em uma espiral em torno do cilindro de raiz o que aumenta significativamente a área específica da raiz e faz com que haja espaços intercelulares facilitando a associação com microorganismos (Göllnitz et al. 2000; Emmerich 2002).

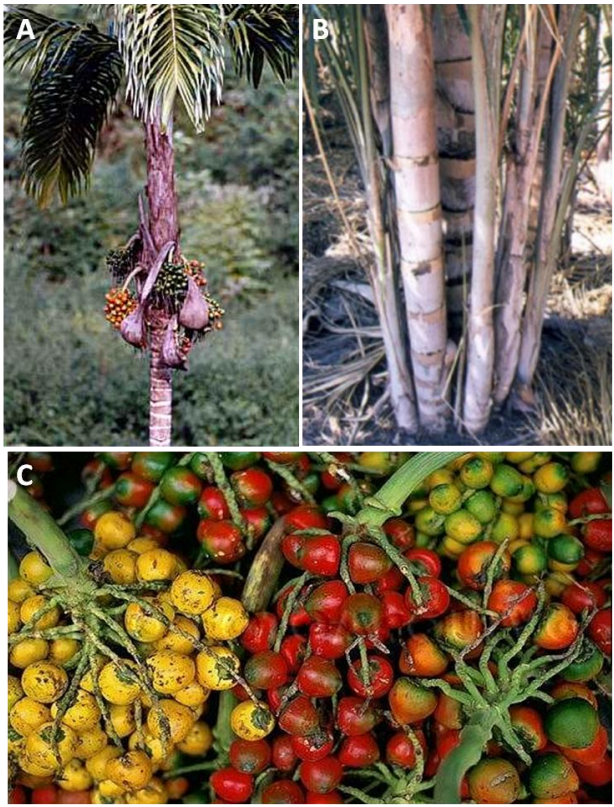

Figura 2. Aspectos da pupunha **A)** Planta adulta de pupunha com frutos **B)** Touceira de pupunha **C)** Frutos de pupunha de diferentes colorações.

Na ponta da raiz é observado um tipo de célula chamada de *border-cells* que são células vivas que se desprendem da coifa à medida que se aproximam da sua periferia (Hamamoto et al. 2006) Um possível papel destas células é a liberação de substâncias para a rizosfera, incluindo proteínas arabinogalactanas que podem atrair microorganismos para este local (Vicre et al. 2005), e essa associação da pupunheira com microorganismos do solo pode explicar o notável crescimento desta planta, inclusive em solos pobres (Emmerich 2002; Göllnitz et al. 2000; Silva Junior e Cardoso 2006).

1.2 Importância Econômica da espécie

Vários usos potenciais e tradicionais são atribuídos a esta espécie (Clement e Mora-Urpí 1987). O uso da sua madeira está reaparecendo como um mercado atraente (Mora-Urpí et al. 1997) e essa madeira pode ser utilizada na indústria moveleira, produção de instrumentos musicais e artesanato. A aplicação de fibras de pupunha para reforçar compósitos de poliéster é também um uso alternativo (Santos et al. 2008). No entanto, os seus principais usos estão associados ao consumo dos seus frutos, moderadamente popular em toda a região de distribuição tradicional, e do palmito, um produto *gourmet* extraído do ápice dos seus brotos.

Seus frutos são item de maior comércio nacional na Colômbia, Costa Rica e Panamá, os quais são também exportados na forma processada para outros países da América Central, para os Estados Unidos e Canadá (Mora-Urpí et al. 1997). Tradicionalmente os frutos são consumidos após o cozimento, mas pratos refinados também são preparados com a pupunha. No entanto, por várias razões que ainda não se sabe, os frutos da pupunha ainda não atraiu a atenção do setor privado (Clement et al. 2004) e estes frutos são destinados principalmente para os mercados locais. Muitos agricultores reconhecem o valor potencial de seus frutos (Mora-Urpi et al. 1997), que são explorados por pequenos agricultores em hortas e roças, com alguns pequenos pomares perto das áreas de maior consumo (Clement 2008). Sugere-se que 50% da produção de frutos é comercializado nos mercados locais, enquanto o outro 50% é utilizado para a subsistência, seja diretamente ou como ração animal. As estimativas sugerem que a produção total de frutos é de cerca de 120.000 toneladas por ano (Clement et al 2004; Clement 2008). Geralmente os frutos são comercializados em cachos, cada um pesando 2-5 kg que vale cerca de US$ 0,50-1,00 para os agricultores, e no mercado de cachos são vendidos a US$ 1,00-3,00, resultando em um valor de mercado de US$ 30 milhões por ano (Clement 2008).

O palmito é composto por folhas juvenis e tecido subapical e é considerado um produto *gourmet* com grande potencial para o mercado internacional. O mercado do palmito tem um *status* já estabelecido econômicamente e importante na América Latina. Para este mercado, a pupunha apresenta vantagens sobre outras espécies de palmeiras usadas para produção de palmito, tal como um curto ciclo de vida, presença de

ramificações e um produto apreciável (Mora-Urpí et al 1997; Clement 2008). Níveis mais elevados de açúcar também foram encontrados no seu palmito em comparação com os de *Euterpe edulis* e *E. oleracea* (Clement et al. 1993). Além disso, o palmito normalmente é vendido em vidro ou em lata como pickles. Entretento eles apresentam baixas concentrações das enzimas peroxidases e polifenoloxidase, permitindo o comércio in natura (Clement et al. 1993), atributo que poderia abrir um novo nicho de mercado para este produto.

O Brasil é o maior produtor e consumidor de palmito, e as estimativas revelaram um aumento da produção durante os últimos anos, a partir de 27.031 ton em 1990 para 51.376 toneladas em 2003 (Rodrigues e Durigan 2007). Porém o Equador e Costa Rica são os principais países exportadores de palmito, com a maioria de produção baseada em pupunha. Já o Brasil exporta menos de 10% do total de sua produção (Clement 2008). No entanto, o mercado para este produto também atraiu a atenção de produtores fora da América Latina e, atualmente, a pupunha está sendo cultivada no Havaí / EUA, Ilhas da Reunião / França, Indonésia e Malásia.

1.3 Recursos genéticos e programas de conservação de pupunha

A pupunha é a única palmeira domesticada na América tropical, sua madeira e seus frutos já eram consumidos por comunidades ameríndias da região neotropical de planície úmida a mais de 10.000 anos atrás, tendo sua origem provavelmente no sudoeste da Amazônia (Clement 2008; Clement 2009).

Ela é considerada uma árvore multi-propósito (Clement e Mora-Urpi 1987) e desempenha um papel importante como um componente de agrossistemas (Clement 1989). Ela foi listada como prioridade número um para o desenvolvimento de espécies de árvores agroflorestais, com base na preferência dos agricultores realizadas pelo Centro Internacional de Pesquisa Agroflorestal (ICRAF) (Mora-Urpí et al. 1997). A produção de frutos hoje em dia para a subsistência e mercado e a produção de palmito para o mercado são os usos mais importantes da *Bactris gasipaes* (Clement 2008). Portanto, programas de conservação são necessários como reservatório de genes para melhorar ainda mais e atender às demandas dos agricultores e consumidores para esta espécie. Idealmente, programas de melhoramento devem começar a partir de variedades locais com

características semelhantes ao ideótipo desejado no final. Por exemplo, as *landraces* Pampa Hermosa, Putumayo e Guatuso têm características ideais para a produção de palmito. Por outro lado, Putumayo e Vaupés são recomendados para a produção de farinha, pois seus frutos são maiores e possuem mais conteúdo de amido. O tipo Pará tem características desejáveis para a produção de óleo (Clement 1997). No entanto, até agora não há cultivar moderna de pupunha (Clement 2008), apesar de algumas instituiçoes possuírem programas de melhoramento genético para esta espécie. A pupunha cultivada é um grupo complexo e diverso com alta variabilidade genética (Mora-Urpí et al. 1997), porém também estão sujeitas a erosão genética, essa vulnerabilidade fica por conta principalmente pelo desmatamento para agricultura e pastagem e também aspéctos biológicos incluindo doenças e pragas, falta de conhecimento sobre sua biologia reprodutiva, aspectos político-institucionais e política do uso do solo (Clement 1996), criando uma necessidade muito grande de um programa eficiente para conservação do seu germoplasma (Clement 2009; Mora-Urpí et al. 1997).

A conservação *on farm*, é também um método efetivo para conservação de *Bactris gasipaes*, e os resultados apontaram que as comunidades de agricultores na Amazônia peruana estão mantendo uma base genética relativamente ampla em suas populações de pupunheira (Adin et al. 2004). Elas estão baseadas em intensa troca de sementes, coleta de sementes de palmeiras selecionadas nas fazendas e nas fazendas vizinhas, bem como a partir de frutas selecionadas nos mercados locais para plantio em suas fazendas. No entanto, evidencias também apontam que os agricultores usam uma média de apenas quatro variedades de pupunha para fornecer semente para a criação de seus jardins (Cole et al. 2007), que pode causar significativa endogamia. Portanto, tem sido sugerido que algum tipo de rede para a conservação *on farm* seja estabelecida, de forma a guiar um grau adequado de troca de material genético necessário para o estabelecimento de programas de conservação da pupunheira (Cole et al. 2007).

Até o momento os programas de conservação para pupunha são direcionados para o estabelecimento de bancos ativos de germoplasma de campo, que são adequados para uso e pesquisa da espécie, mas não são uma opção viável de abordagem para a conservação a longo prazo (Mora-Urpí et al. 1997). A pupunha tem sementes recalcitrantes (Bovi et al. 2004) e portanto, estudos sobre a regeneração *in vitro* para fins de

conservação são necessárias. A criopreservação de embriões zigóticos desta espécie também já foi descrita (Steinmacher et al. 2007a), mas um protocolo confiável tanto para embriões zigóticos como para embriões somáticos ainda precisa ser estabelecido. O desenvolvimento de protocolos de regeneração *in vitro* pode se constituir em uma ferramenta eficaz de apoio à conservação e programas de melhoramento genético da espécie.

1.4 Micropropagação da pupunha

Dada a importância do palmito e do fruto provindos da pupunha,várias Instituições latino-americanos estabeleceram programas de melhoramento para um ou ambos os usos, onde a regeneração *in vitro* de plântulas é considerada importante. Técnicas de cultura de tecidos se constituem em estratégias mais eficientes para a regeneração clonal de plântulas desta espécie, bem como para sua conservação genética (Mora-Urpí et al. 1997). A principal aplicação das técnicas de regeneração *in vitro* em programas de melhoramento é a fixação do ganho genético, pela captura aditiva e não aditiva dos componentes de variabilidade genética (Guerra et al. 1999). Entre várias técnicas, a embriogênese somática oferece vantagens como a produção automatizada em grande escala e estabilidade genética das plântulas regeneradas (Steinmacher et al. 2007c; Steinmacher et al. 2011). Além disso, a embriogênese somática tem a possibilidade de ser acoplada a programas de conservação através, por exemplo, da criopreservação de embriões somáticos (Steinmacher et al. 2007a).

A regeneração *in vitro* de plântulas de pupunha foi descrito pela primeira vez por organogênese indireta (Arias e Huete 1983), mais tarde, por embriogênese somática (Stein e Stephens 1991; Valverde et al 1987), e por organogênese direta (Almeida e Kerbauy 1996). Arias (1985) descreveu respostas morfogenéticas em diferentes tecidos, sem reportar a regeneração de plântulas. Depois disso, Valverde et al. (1987) descreveu um protocolo para a regeneração *in vitro* de pupunha, embora apenas dez calos embriogênicos foram obtidos, cada um calo produzindo 2-8 embriões somáticos. No protocolo descrito por Stein e Stephens (1991), apenas quatro culturas de calos foram estabelecidas, produzindo mais de 10 mudas por cultura. Usando inflorescências de pupunheira imatura como explantes, Almeida e Kerbauy (1996) descreveram um protocolo para a regeneração por meio da

organogênese em baixa frequência com um pequeno número de mudas sendo regeneradas.

Um protocolo completo regenerativo *in vitro* utilizando embriões zigóticos maduros como explantes foi descrito em pupunha como o primeiro passo para o desenvolvimento de um protocolo confiável para a multiplicação *in vitro* desta espécie (Steinmacher et al. 2007b). Neste estudo, análises histológicas mostraram que a regeneração das plântulas ocorreu através da embriogênese somática e plântulas regeneradas foram aclimatizadas com sucesso (Steinmacher et al. 2007b). Steinmacher et al. (2007c) descreveram a indução, desenvolvimento e conversão de embriões somáticos de pupunha usando inflorescências como explante. O uso da técnica de camada fina de células também foi descrito e alta frequencia de embriogênes somática foi reportada como resultado (Steinmacher et al. 2007d). Este protocolo utilizou a base de folhas jovens e ápices caulinares como explantes (Steinmacher et al. 2007d). A formação de embriões somáticos ocorreu em clusters, o que sugeriu a ocorrência de embriogênese somática secundária.

Mais recentemente Steinmacher et al. (2011) comprovou a formação de aglomerados de embriões somáticos de pupunha a partir da embriogênese somática secundária. Neste trabalho também foi observado que as culturas quanto inoculadas em Sistemas de Imersão Temporária (TIS) aumentavam a sua capacidade regenerativa. Após a maturação dos embriões somáticos a taxa de conversão ficou em 30%, sendo que nenhuma das as plântulas obtidas em placas de Petri atingiu mais de 6,4cm, enquanto que 51% das plântulas obtidas em TIS apresentavam tamanho maior que 6,4 cm.

2. Embriogênese Somática

A Embriogênese Somática (ES) é uma tota morfogenética na qual células isoladas ou um pequeno grupo de células somáticas dão origem a embriões somáticos (Tautorus et al. 1991), num processo análogo à embriogênese zigótica. O processo se inicia com a desdiferenciação dos tecidos do explante até a formação de estruturas embrionárias bipolares. Isso demonstra a permanência da totipotência nas células de plantas superiores, onde uma célula adulta consegue dar origem a uma planta inteira (Guerra 1989).

O primeiro relato de ES ocorreu em 1958, quando Steward e colaboradores (1958) obtiveram embriões somáticos a partir de culturas celulares de cenoura (*Daucus carota*) em suspensão. A partir daí protocolos regenerativos baseados nesta rota morfogenética foram desenvolvidospara um expressivo número de plantas (Schmidt 1997).

Há dois tipos de ES, a direta na qual se originam diretamente do explante, sem a ocorrência de uma fase interveniente de calos; e a indireta, na qual ocorre a formação de uma fase intermediária de calos antes do desenvolvimento dos embriões somáticos (Sharp et al. 1980).

A origem do embrião somático pode se dar a partir de dois padrões distintos, unicelular ou multicelular. Quando os embriões têm origem unicelular ocorrem divisões celulares coordenadas e o embrião muitas vezes fica conectado com o tecido de origem por uma estrutura semelhante ao suspensor. Em contraste, na origem multicelular não é observada essa proliferação coordenada de células e o embrião fica fusionado ao tecido de origem (Williams e Maheswaran 1986).

A ES envolve a aquisição de competência embriogênica, a indução e a determinação. Competência embriogênica da célula é a sua capacidade para responder a sinais específicos (reguladores de crescimento, condições de cultura). Indução de embriogênese somática ocorre quando o sinal dado produz uma única resposta de desenvolvimento. E determinação é um processo no qual o destino de desenvolvimento da célula (ou grupo de células) torna-se fixo e limitado a um caminho particular de desenvolvimento (Yeung 1995).

A morfogênese implica primeiramente na predeterminação das células que sofrem mudanças morfológicas, como a desdiferenciação, para posterior diferenciação e crescimento (Guerra 1989). Desdiferenciação celular é um processo pelo qual o caminho do desenvolvimento celular pode ser alterado, permitindo que as células definam um novo desenvolvimento padrão após a divisão celular (Feher et al. 2003). Entretanto, para que ocorram esses processos, é necessário que haja competência celular, ou seja, a capacidade de um certo tecido, ou parte deste, expressar um potencial inerente. A resposta da ES se manifesta com a bipolaridade de um único eixo, sem comunicação com o tecido de origem, diferenciando da organogênese (Guerra et. al. 1999). Células embriogênicas apresentam características que estão normalmente presentes, como tamanho pequeno com o citoplasma

denso e pequenos vacúolos, núcleo centralizado com nucléolos proeminentes, e alta razão núcleo:citoplasma (Verdeil et al. 2001).

A aquisição de competência embriogênica e o próprio desenvolvimento embrionário também dependem da combinação da expressão temporal e espacial de genes específicos. Vários genes têm sido relacionados como promotores da embriogênese somática. O gene Somatic Embryogenesis Receptor Kinase (SERK) é o mais estudado e melhor caracterizado e foi isolado pela primeira vez em células embriogênicas de cenoura (Schmidt et al. 1997) e com isso tem sido relacionado um forte marcador para embriogênese somática por promover a formação de células competentes (Schmidt et al. 1997). O gene Leafy Cotyledon genes (LEC) também desempenha um papel chave na regulação na aquisição de competência embriogênica (Harada 2001). Ele foi primeiramente isolado a partir de *Arabidopsis thaliana* sendo especificamente expresso em sementes (Stone 2001). Do mesmo modo o gene Wurchel (WUS) também foi isolado a primeira vez em *A. Thaliana* que promove a transição do estado vegetetivo para o estado embriogênico (Zuo et al. 2002). Há também determinados genes que atuam na repressão da embriogênese somática como os genes Primordia Timing (PT), Clavata (CLV) 1 and 3, and Pickle (PKL) (Kwaaitaal and de Vries 2007), também sendo sendo importântes para essa regulação.

As proteínas Arabinogalactanas (AGPs) são uma classe de proteoglicanas distribuidos em todo o reino vegetal (Nothnagel 1997; Seifert and Roberts 2007). Tem sido demonstrado que as AGPs são moléculas que apresentam um papel na sinalização célula-célula, atuando na divisão, expanção e diferenciação celular, e também participando da cascata de sinalização dos reguladores vegetais (Mashiguchi et al. 2008), podendo também atuar durante a indução e o desenvolvimento de embriões somáticos (Poon et al. 2012). Steinmacher et al. (2012) mostrou por anticospos monoclonais que as AGPs tem um papel específico na embriogêne se somática de pupunha, e sua expressão se dá no início da formação de setores que iram dar origem à embriões somáticos. Então ele proprõe que a adição de AGPs no meio de cultura pode ser uma interresante estratégia para aumentar a indução da embriogênese somática em culturas não responsivas ou em genótipos de baixa taxa embriogênica. Um trabalho recente mostrou que AGPs produzidas por culturas embriogênicas de algodão (*Gossypium hirsutum*) quando isoladas, mesmo que desglicolizadas, e inoculadas em

meio de cultura contendo culturas não-embriogênicas da espécie, a embriogênese somática é promovida e há a formação de embriões somáticos a partir destas (Poon et al. 2012), comprovando a importância destas proteínas na indução da embriogênese somática.

Os principais fatores que afetam a ES são a origem do explante e a composição do meio de cultura, principalmente os reguladores de crescimento. As condições apropriadas de cultivo são importantes para o sucesso da técnica, principalmente no que se refere à aquisição de competência embriogênica (Guerra et al. 1999). Porém mudanças no padrão de desenvolvimento e aquisição da competência na embriogênese somática podem ocorrer previamente à exposição dos explantes a um novo ambiente ou a condições de cultivo (Yeung 1995). Dado que os tecidos são explantados e cultivadas geralmente sob condições sub-ótimas e desequilibras (ou seja, concentrações não-fisiológicas de reguladores crescimento da planta), tem sido proposto que a embriogênese somática seria o último resultado possível de adaptação ao novo ambiente (Dudits et al. 1995; Feher et al. 2003). Conseqüentemente, postulou-se que uma das principais forças que faz com que uma célula somática volte a um estado embrionário seria o nível de estresse a que esta célula é submetida (Dudits et al. 1995; Feher et al. 2003). Assim, se o nível de estresse excede, a célula entrará em colapso e irá morrer, enquanto o oposto seria aumentar o metabolismo, provocando mecanismos de adaptação que levaria a embriogênese somática (Davletova et al. 2001; Feher et al. 2003).

Mais recentemente Smulders et al. (2011) salientaram que as culturas *in vitro* são submetidas a muitos estresses, como por exemplo, altas doses de auxinas que normalmente são utilizadas para indução da embriogênese somática, resultando em alterações nos padrões de metilação do DNA, o que possibilitar a indução de células embriogênicas. As auxinas além de serem um dos principais reguladores de crescimento das plantas atuando na divisão, diferenciação e no ciclo celular (Fehér et al. 2003), também alteram o padrão de metilação do DNA das células (Smulders et al. 2011). Estudos em epigenética, que identificam a mudança na expressão da informação do DNA causado por alterações no padrão de metilação de bases nitrogenadas ou mudanças nas histonas ou em ambos, são importantes para monitorar a mudança no padrão de transcrição dos genes, que muitas vezes podem

ser temporárias, mas algumas vezes são de longa duração, podendo até serem passadas durante a propagação sexual (Smulders et al. 2011).

De forma geral, o protocolo de ES é dividido em duas fases de acordo com a figura 3, baseada em Durzan (1989): o primeiro ciclo compreende a indução e multiplicação das culturas suplementadas com uma auxina forte (ex.: 2,4-D, Picloram, Dicamba). A segunda fase compreende a etapa de desenvolvimento e maturação dos embriões somáticos. No final deste processo os embriões somáticos são obtidos, os quais então podem então ser convertidos em plantas ou então gerar sementes sintéticas por meio da técnica de encapsulamento em alginato.

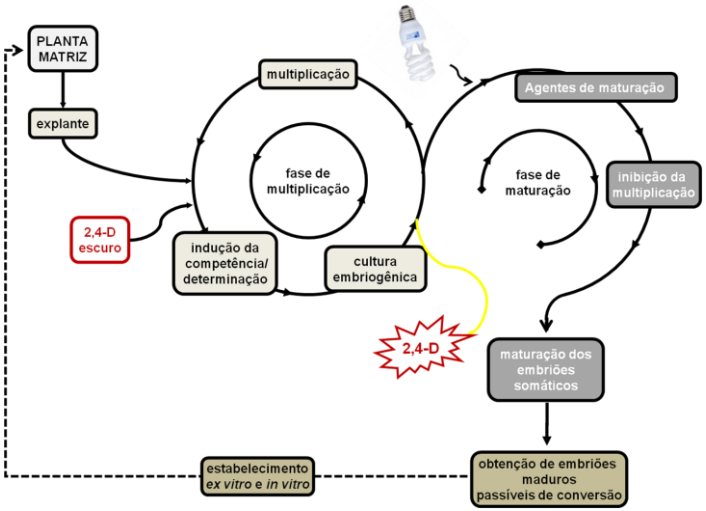

Figura 3. Esquema mostrando a modulação da Embriogênese somática, apresentado suas duas fases, primeiramente a fase de multiplicação e a logo após a segunda fase de maturação. Modificado de Durzan (1989).

As principais vantagens da ES em relação às outras técnicas de propagação *in vitro* são: a obtenção de uma grande quantidade de propágulos (embriões somáticos); permite um alto grau de automatização (biorreatores), reduzindo o custo; produção de embriões somáticos de forma sincronizada, com alto grau de uniformização e pureza genética, pois captura componentes aditivos e não aditivos da variância genética; apresenta-se como uma ferramenta de possível integração a programas de melhoramento genético, regenerando plantas

geneticamente modificadas, poliplóides ou híbridos somáticos (Steiner et al. 2008). Além de tudo esta rota morfogenética se configura como um modelo biológico para estudos fisiológicos e bioquímicos durante o metabolismo celular e morfogênese vegetal.

2.1 Maturação

Durante o estágio de maturação, os embriões somáticos sofrem alterações morfológicas e bioquímicas. Os cotilédones se expandem concomitantemente com a acumulação de substâncias de armazenamento, tais como proteínas, lipídeos e aminoácidos, os quais serão úteis na conversão dos embriões somáticos em plântulas (Thomas 1993). No entanto, a quantidade de um produto de armazenamento pode variar entre embriões somáticos e zigóticos (Merkle et al. 1995).

Em várias espécies, é necessário suplementar as culturas embriogênicas com ABA para estimular a maturação. Em outros casos o ácido abscísico (ABA) é utilizado para reduzir o processo de embriogênese secundária ou para inibir a germinação precoce. Em geral, o tratamento não deve ser longo e exceder a um um mês, uma vez que a exposição prolongada ao ABA pode ter um efeito negativo sobre o crescimento de plantas (Bozhkov e Von Arnold 1998).

2.1.1 ABA

O ABA está envolvido no controle de muitos processos fisiológicos, tais como a abertura de estômatos, a síntese de proteínas de estoque de sementes, a inibição da germinação de embriões imaturos, o estresse hídrico e a tolerância ao déficit de água. Na cultura de tecidos o este composto está envolvido principalmente na maturação de embriões obtidos na embriogênese somática, sendo um composto termoestável e fotossensível. ABA é essencial para o crescimento normal de embriões somáticos e em presença eles se assemelham no desenvolvimento e estrutura dos embriões zigóticos. A alteração de níveis endógenos e/ou exógenos de ABA aumenta a freqüência de embriões que atingem a maturidade e pode auxiliar na otimização de propagação em massa a partir de embriões somáticos de uma determinada espécie (Ammirato 1988).

A função do ABA no desenvolvimento dos embriões somáticos foi comprovada por Ammirato (1974), onde 10^{-7} M de ABA esteve associado com a eliminação de formas anormais de embriões somáticos

de *Carum carvi* L. A eficácia de ABA na diminuição da proporção de embriões anormais e na sincronização da maturação do embrião já foi confirmada em outras espécies. Em cenoura, Jimenez e Bangerth (2001) mostraram níveis muito mais elevados de ABA em calos embriogênicos em relação aos não-embriogênicos. Kim et al., (1998) mostrou que a maturação com ABA dos embriões de *Larix leptolepis* foi benéfica na redução da germinação precoce. É também evidente a partir do trabalho de Faure et al. (1998) e Gawronska et al. (2000), trabalhando com *Vitis vinifera* e *Cucumis sativus* L. respectivamente, que embriões somáticos e zigóticos diferem significativamente em seu conteúdo de ABA, baixa e alta respectivamente, indicando que o ABA é essencial na maturação dos embriões, evitando assim sua má formação.

A adição de ABA exógeno tem sido usada para maturação de embriões somáticos de outras palmeiras tais como *Phoenix canariensis* (Huong et al. 1999), *Cocos nucifera* L.(Fernando e Gamage 2000), *Phoenix dactylifera* (Zouine et al. 2005; Othmani et al. 2009) mas até o momento são há trabalhos relacionando esse composto com a maturação de embriões somáticos em pupunha. Sua utilização poderá significar um passo importante para o aprimoramento da fase de maturação dos embriões somáticos desta espécie, já que os protocolos de maturação e conversão tem que ser aprimorados em relação aos trabalhos existentes até o presente momento (Steinmacher et al. 2011).

2.2 Conversão dos embriões somáticos

Apenas os embriões maduros, que acumularam substância de armazenamento suficiente e adquirem tolerância à dessecação no final de maturação, se desenvolvem em plantas normais (Ammirato 1974). Além disso, uma alteração marcada em meio basal é muitas vezes necessária. Para algumas espécies, a inclusão de compostos extra, como glutamina e hidrolisado de caseína, também é necessária (Steinmacher et al. 2007b; Steinmacher et al. 2011).

A conversão de embriões somáticos de palmeiras parece ser positivamente influenciada pela presença de citocinicas no meio de cultura (Aberlenc-Bertossi et al. 1999; Karun et al. 2004). Por isso, muitos autores recomendam a sua adição ao meio de conversão, como é o caso de Guerra e Handro (1998), que propõe um meio de cultura suplementado com citocinina para a conversão de embriões somáticos de *E. edulis*.

As citocininas são derivadas da adenina (aminopurina) e têm um papel fundamental na diferenciação e regeneração de plantas na maioria das espécies (Santiago 2001). Induzem a divisão celular, proliferação e morfogênese da parte aérea. As citocininas mais usadas em cultura de tecidos são a cinetina (CIN), benziladenina (BA), zeatina (Zea), isopentenil adenina (2ip) e thidiazuron (TDZ).

3. Biorreatores de imersão temporária

Biorreator é um sistema automatizado de cultura de células cuja sua principal função é fornecer um ambiente controlado, a fim de alcançar as condições essenciais para o crescimento celular. Células vegetais foram primeiramente cultivadas em biorreatores na década de 1960, adaptados a partir de biorreatores que eram usados para cultivo de células animais (Payne et al. 1991).

A sua utilização pode estar ligada a diversas utilidades, como ao cultivo de células vegetais para a obtenção de biomassa ou metabólitos secundários, automatização das culturas, controle dos parâmetros internos da cultura. Suas vantagens em relação ao cultivo de células vegetais em erlenmeyers se resumem basicamente em duas, maior controle das culturas em relação ao ambiente e escalabilidade, em que no biorreator se apresenta bem maior.

Esses biorreatores também são classificados em dois tipos, sistema de imersão continua onde o material em cultivo permanece imerso continuamente no meio de cultura. Essa imersão contínua causa problemas de hiperhidratação dos tecidos, órgãos e plântulas. Dependendo da espécie e do tipo de meio utilizado, a hiperhidratação dos tecidos pode causar distúrbios fisiológicos sérios, que irão afetar o crescimento e desenvolvimento do material em cultivo. O segundo tipo é o sistema de imersão temporária (TIS) (Alvard et al. 1993). Nesse tipo de biorreator, o meio de cultura permanece em contato com o explante por um período predeterminado. Em seguida, o meio é drenado e o explante deixa de ficar em contato direto com o meio de cultura. TIS combina as vantagens da cultura em meio de cultura líquidos e semisólidos. Estes podem ser associados a melhor suprimento de nutrientes, tendo, portanto uma ausência de gradientes de nutrientes que é comum no meio semi-sólido. Outra vantagem é a ausência de anoxia nos explantes e as taxas mais baixas de hiperhidricidade, normalmente

observado em meio de cultura líquido (Etienne e Berthouly 2002; Murch et al. 2004). Existem diferentes formas e modelos de sistema de imersao temporaria ja desenvolvidos. Entre estes se destaca o sistema RITA® (Figura 4A) e os Frascos Duplos (Figura 4B).

O sistema RITA® (Figura 4A) é um biorreator com sistema de imersão temporária do tipo airflit, e é muito utilizado para culturas de células vegetais. O seu princípio de funcionamento (Figura 4C) é baseado no bombeamento de ar na câmera inferior, onde esta o meio de cultura, forçando assim esse meio a inundar a câmara superior, onde estão localizadas as culturas celulares. Essas duas câmaras estão separadas por uma peneira fina, que sustenta as células vegetais. Assim as culturas são nutridas e areadas no decorrer deste processo. Esgotado o tempo, o ar sai da câmara inferior e faz com que o meio retorne para esta, deixando as culturas celulares sem contato com o meio de cultura por mais um tempo (Teisson et al.1995). As inundações e tempo destas podem ser ajustados de acordo com cada tipo de cultura.

Este tipo de biorreator está sendo utilizado em várias espécies vegetais, como por exemplo na multiplicação de brotos de maçã (Li-Hua Zhu et al. 2005), na produção de mudas de eucalipito para fins comerciais (Mcalister et al. 2005), na regeneração de brotos de morango (Hanhineva 2005), na multiplicação massal de cana de açúcar (Mordocco et al. 2009) e para multiplicação de embriões somáticos em café (Etienne-Barry et al. 1999).

Já o sistema de Frascos Duplos (Figura 4B) também utiliza o sistema de imersão temporária, porém nele estes dois frascos são conectados por um cano de silicone. Em um dos frascos são inoculadas as culturas, sendo o meio de cultura adicionado no outro frasco. Seu princípio de funcionamento (Figura 4D) é baseado no bombeamento de ar no frasco do meio de cultura, fazendo com que este suba pelo cano de silicone e vá para o outro frasco, onde estão as culturas. Assim as culturas são nutridas e areadas no decorrer deste processo. Esgotado o tempo, o bombeamento de ar agora é feito no sentido contrário, fazendo com que o meio de cultura volte para seu frasco. Assim como no RITA® o bombeamento de meio e tempo destas podem ser ajustados de acordo com cada tipo de cultura.

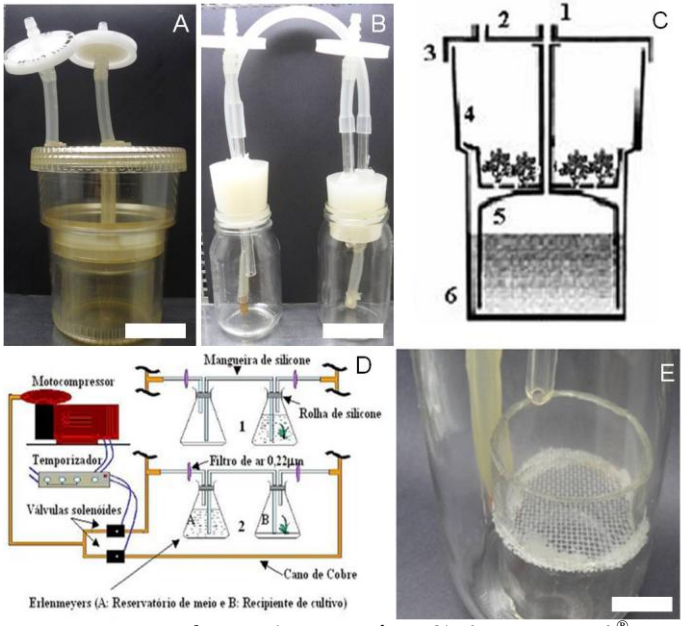

Figura 4. Biorreatores de imersão temporária. **A)** Aparatos RITA® em imersão temporária. Barra = 5 cm **B)** Frascos Duplos em sistema de imersão temporária; **C)** Princípio de funcionamento de sistema de imersão temporária por aparatos RITA® (legenda: 1 entrada de ar, 2 saída de ar; 3 tampa de vedação, 4 câmara de crescimento, 5 base interna, 6 base do aparato). Fonte: Scheidt et al. 2009. Barra = 5 cm **D)** Princípio de funcionamento de sistema de imersão temporária por Frascos Duplos; **E)** Suporte-peneira adaptado no sistema de Francos Duplos Modificado (FDM). Barra = 1,5 cm

Há também maneiras de adaptar esses sistemas de acordo com a necessidade da cultura. Na figura 4E observa-se que foi colocado um suporte atravessado por uma peneira no frasco onde se depositam as as culturas, e com isso estas ao invés de permanecer no fundo do vidro elas permanecem sobre a peneira. Assim as culturas se mantêm longe da lâmina de meio de cultura restante no frasco e somente entram em contato com o meio de cultura nos tempos programados, semelhante ao que ocorre no RITA®. Essa adaptação foi chamada de Frascos Duplos Modificado (FDM).

4. Criopreservação

O pool de genes da pupunha cultivada e selvagem é rico em diversidade, porém, sofrem alta erosão genética, necessitando de urgente desenvolvimento de estratégias eficientes de conservação de germoplasma à longo prazo (Mora-Urpi et al. 1997; Clement et al. 2009). Os programas de conservação da pupunha atualmente são voltados ao estabelecimento de bancos de germoplasma *on farm*, porém, esta não é uma alternativa viável para a conservação à longo prazo (Mora-Urpí et al. 1997). Além do mais, a pupunha produz sementes recalcitrantes a desidratação (Bovi et al. 2004), de forma que a conservação por sementes não é uma alternativa valida.

Para a conservação *ex situ* de plantas cujas sementes são recalcitrantes, como a pupunha, a criopreservação é tida como uma aproximação segura para a conservação em longo prazo e o desenvolvimento de protocolos para esta técnica vem sendo realizado para diferentes espécies vegetais. Segundo Steinmacher (2007a), a técnica de criopreservação pode ser considerada uma alternativa para a conservação da pupunha, complementando as demais técnicas existentes.

A criopreservação é definida como a conservação de material biológico em temperaturas ultra baixas, usualmente em Nitrogênio Líquido (NL) a -196°C, ou em sua fase de vapor a -150°C (Santos 2000). Uma coleção de germoplasma em nitrogênio líquido é considerada uma coleção base e atua com caráter complementar, pois garante a estabilidade genética e a segurança do material conservado (Santos 2000). Dentre as vantagens deste sistema de conservação está o custo, posteriormente ao aperfeiçoamento da técnica tornou desnecessária a utilização de resfriamento programado, e após a criopreservação do material vegetal, a utilização de energia elétrica e subcultivos são dispensáveis (Engelmann 1997; Valois 2001; Engelmann 2004).

Para o sucesso da técnica, as células devem ser condicionadas para sobreviverem após a exposição ao NL, através da manipulação da quantidade de água (Engelmann 1997). Neste contexto, várias técnicas e protocolos têm sido sugeridos, contudo, estes são divididos em três linhas gerais, correspondendo ao congelamento lento, à vitrificação ou à desidratação. O principal desafio a ser contornado num processo de

criopreservação é alcançar o congelamento evitando a formação de cristais de gelo intracelular, pela nucleação dos cristais de gelo (Santos 2000). Esta nucleação causa a ruptura das membranas celulares, resultando em colapso celular (Engelmann 1997).

4.1 Criopreservação por Vitrificação

O estado vítreo de líquidos celulares é obtido pelo uso de concentrações suficientemente altas de solutos (crioprotetores) e resfriamento rápido. A alteração das condições osmóticas é muito mais forte do que no congelamento lento. Portanto, várias etapas de desidratação podem ser necessárias. Dependendo do seu tamanho molecular, os solutos penetram no espaço intracelular (por exemplo, DMSO, glicerol), ou permanecem na parede celular (por exemplo, sacarose e outros carboidratos). Assim, eles afetam os processos de vitrificação em diferentes compartimentos do tecido. O equilíbrio ideal é atingido quando uma mistura de substâncias crioprotectoras é utilizada. Algumas misturas padrão são publicadas e são empregados extensivamente, muitos dos quais são chamados PVS (plant vitrification solution). A mistura mais usada é PVS2, consistindo de sacarose, glicerol, etilenoglicol e DMSO (Sakai et al. 1990). Como certos componentes do PVS podem ser tóxicos, como o DMSO no caso de pupunha, utiliza-se uma mistura crioprotetora PVS3, que é composta somente por sacarose e glierol. É essencial que o reaquecimento das amostras seja rápida, assim evitando a re-cristalização de solutos celulares.

4.2 Criopreservação por Vitrificação-*Droplet*

A Vitrificação-*Droplet* é uma técnica relacivamente recente, derivada da técnica *droplet-freezing* de congelamento lento e desenvolvida por Kartha et al. (1982) para criopreservação de brotos mandioca. Nesta técnica a amostra a ser criopreservada é tratada anteriormente com PVS3, posteriormente é acomodada individualmente em gotículas (5-10 µl) de crioprotetor pipetadas sobre uma tira de folha de alumínio e imersa em NL. O interese principal desta técnica é a obtenção de taxas de congelamento/descongelamento muito elevadas, devido ao pequeno volume de crioprotetor utilizado e também pelas propriedades de alta tranferência de calor do alumínio (Sakai e Elgenmann 2007).

5. Epigenética

Estudos em epigenética, que identificam a mudança na expressão da informação do DNA causado por alterações no padrão de metilação de bases nitrogenadas ou mudanças nas histonas ou em ambos, são importantes para monitorar a mudança no padrão de transcrição dos genes, que muitas vezes podem ser temporárias, mas algumas vezes são de longa duração, podendo até serem transmitidas durante a propagação sexual (Smulders et al. 2011).

A metilação de bases nitrogenadas é um importante mecanismo de regulação de genes em inúmeros processos biológicos. Assim pesquisadores da área buscam controlar as respostas morfogenéticas em cultura de tecidos vegetais para manter a estabilidade genética (Kaeppler et al. 2000). Há também o interesse no estudo destes padrões de metilação na conservação *ex situ* de um germoplasma vegetal pelas técnicas de criopreservação (Harding 2004).

Os principais alvos da metilação são os resíduos de citosina nas sequências de GpC e GpXpC formando 5-metilcitosina (Gruenbaum et al. 1981). A presença destes grupamentos metil afeta a ligação dos fatores de transcrição impedindo assim a expressão do gene. Esses padrões são dinâmicos e podem mudar durante o crescimento e desenvolvimento da planta pela ação das metiltransferases base específicas (Finnegan e Kovac 2000).

A quantificação dos padrões de metilação do DNA total é uma técnica utilizada para detecção de variações epigenéticas em maior escala e o HPLC (High Performance/Pressure Liquide Chromatography) é considerado a técnica mais precisa e sensível para determinar essas variações, que envolve a digestão do DNA para nucleotídeos, nucleosídeos ou bases, separação pela coluna do HPLC e quantificação por Ultravioleta (UV). O equipamento também pode detectar bases nitrogenadas modificadas, como a 5-metilcitosina, permitindo fazer uma relação das bases e identificar então o padrão de metilação total da amostra (Johnston et al. 2005).

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CAPÍTULO II:

Embriogênese Somática

High Efficiency Somatic Embryogenesis Protocol of Peach Palm (*Bactris gasipaes* Kunth)

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Abstract

Bactris gasipaes Kunth (Arecaceae) is cultivated for its fruit and heart of palm, and somatic embryogenesis can offer automated production and large-scale genetic stability of regenerated clonal plantlets of this species. The objective of this study is to identify the best culture system for each step of peach palm somatic embryogenesis protocol, ally with biochemistry and molecular analysis, and observe the action of ABA in the process of somatic embryo maturation. Somatic embryos multiplied in RITA system had a high multiplication with significant increase of protein (4.07 mg g^{-1}), starch (1.34 mg g^{-1}) and enzyme ADH active (3.65 OD min^{-1} mg^{-1} protein), connected to a low DNA global methylation rate (27.52%), indicating a possible advanced step in the development of somatic embryos. The ABA (abscisic acid) together with the semi-solid medium culture was very important for maturation of somatic embryos, connected to a fall in global methylation (27.28%), probably permitting the essential protein express for somatic embryo maturation. In the conversion into Petri dishes, free of plant growth regulators, a large number of green embryos was formed (105.25) which then transferred to the RITA showed a large number of plantlets (315.7) converted in a shorter time. In the acclimatization,

survival (83.3%) and rooting (94.4%) were better for plantlets initially to 7 cm, indicating the importance of plant size in this final step.

Keywords somatic embryogenesis, temporary immersion system, peach palm, abscisic acid, DNA methylation

Introduction

Peach palm (*Bactris gasipaes* Kunth), belongs to Arecaceae family, one of the most important tropical plant group (Balick 1988), is a caespitose and multipurpose palm tree. This palm became an alternative for the commercial production of cultivated heart of palm (Clement 2008), because it has many of advantages, such as perennial production from off-shoots and rapid growth rate even in poor soils (Mora-Urpi et al. 1997). This gourmet product has advantages as high levels of sugar and low concentrations of peroxidase and polyphenoloxidase enzymes compared with those of *Euterpe edulis* and *E. oleracea*, possibility to be fresh or minimally processed commercialized.

It is known that the gene pool of cultivated plants and their wild relatives is rich in diversity, but also subject to genetic erosion, creating an urgent need to collect and conserve the germplasm (Mora-Urpi et al. 1997; Steinmacher et al. 2011). Tissue culture comprise several techniques to be used for the regeneration of elite plants of peach palm as well as for genetics conservation (Mora-Urpí et al. 1997), and somatic embryogenesis is one of the most promising biotechnological tools.

Somatic embryogenesis has been studied for a wide diversity of species since its discovery in 1958 (Steward et al. 1958). Tautorus et al. (1991) defined this biotechnology technique as an analogous process to zygotic embryogenesis which gives rise to somatic embryos (Tautorus et al. 1991). This technique offers advantages such as automated production and large-scale genetic stability of regenerated plantlets (Steinmacher et al. 2011). Moreover, somatic embryogenesis has the possibility to be coupled to conservation programs by, for example, the cryopreservation of somatic embryos. The first protocol described for peach palm somatic embryogenesis (Valverde et al. 1987) showed a low efficiency. Steinmacher et al. (2007) described a complete protocol based on this morphogenetic route, and subsequently the same author described a protocol connecting somatic embryogenesis to temporary immersion system (TIS), increasing the regenerative capacity of cultures

and still proving by histomorphological analyzes the occurrence of secondary embryogenesis, important for a large-scale automated production (Steinmacher et al. 2011). In order to improve each step of the protocol, biochemical and molecular analysis combined with morphological analysis may identify points of protocol to be improved.

Stress is an important factor related to the acquisition of embryogenic competence (Pasternak et al. 2002) and different culture systems create different forms of stress which is sensed by the cells as a sort of stress triggering short-term metabolic adaptations, which can alter the proteins pattern expression, the reserve compounds accumulation, as well the DNA expression. Recently, Smulders et al. (2011) described that *in vitro* cultures are subjected to many forms of stresses to the plant tissue which expressions various responses, one being the modification of DNA methylation pattern, which allows for the acquisition of cell embryogenic competence. Thus, the study of these metabolic adaptations in different multiplication culture systems can improve the protocol of somatic embryogenesis for this species.

Another important step on the protocol of somatic embryogenesis in peach palm is the somatic embryo maturation phase. The transference to maturation conditions prior to conversion can still be considered as a bottleneck to a successful protocol in peach palm (Steinmacher et al. 2011). Changing endogenous and/or exogenous ABA (abscisic acid) levels increases the frequency of matured embryos and can assist in optimizing mass propagation from somatic embryos (Ammirato 1988), generating a greater number of plantlets in the somatic embryos conversion step thereof. The exogenous supplementation of ABA to the maturation culture medium has already been used in other palms, such as *Phoenix canariensis* (Huong et al. 1999), *Cocos nucifera* L. (Fernando and Gamage 2000), *Phoenix dactylifera* (Zouine et al. 2005; Othmani et al. 2009), but to date there are no studies relating this phytohormone with the maturation of somatic embryos in peach palm.

The objective of this study is to identify the best culture system for each step (multiplication, maturation and conversion) of peach palm somatic embryogenesis protocol. Biochemical and epigenetic analysis were also performed as well as the role of ABA in the process of embryo somatic maturation, enabling a new design of a high efficiency protocol for mass multiplication of this species.

Material and Methods

Somatic embryos induction and multiplication

Somatic embryogenesis induction was performed by Steinmacher et al. (2007). Briefly, zygotic embryos were inoculated in Petri dishes containing 25 ml of MS (Murashige and Skoog 1962) medium culture supplemented with Morel and Wetmore (1951) vitamins, 3% (w/v) sucrose, 500 mg l^{-1} glutamine (Sigma), 2.5 g l^{-1} Sigma-Phytagel™, 1 µM $AgNO_3$ and 10 µM Picloram [4-amino-3,5,6-trichloropicolinic acid (Sigma)]. The multiplication of the somatic embryos clusters (SEC), obtained in induction, occurred in five different culture systems (Fig. 1) inoculated with 1 g of fresh mass (FM) in dark condition at 25±2 °C. The systems were Petri dishes, Erlenmeyer Flasks (EF) (250 ml) on orbital shaker at 90 rpm (Fig. 1A), RITA® System (Teisson and Alvard 1995)(Fig. 1B), Twin Flasks System (TF) (67,2 mm x 129,3 mm / 350 ml)(Fig. 1C), model described by Escalona et al. (1999) and improved by Scherer et al. (2012), and Modified Twin Flasks (MTF) system with a sieve-like support in the cultures flasks (Fig. 1D), simulating the RITA® system. The culture medium used in all systems was the same used in the somatic embryos induction without $AgNO_3$, and addition of 2.5 g l^{-1} Sigma-Phytagel™ only in Petri dishes system, which the culture medium is semi-solid. After 6 weeks, the cultures were weighed for compare the initial fresh mass (IFM) with the final fresh mass (FFM).

Somatic embryos maturation

For SEC maturation, 0.2 g of material coming from the multiplication were maintained in the same culture system origin, but with a maturation culture medium consisting of MS culture medium supplemented with Morel and Wetmore vitamins, 3% (w / v) sucrose, 500 mg l^{-1} glutamine (Sigma) and 5 µM ABA [abscisic acid (Sigma)], for 2 weeks in dark condition at 25±2 °C. In order to evaluate the effect of ABA in maturation medium culture, cultures were also inoculated into ABA-free culture medium for 2 weeks in the same conditions, as a control.

Somatic embryos conversion and plantlets acclimatization

For SEC conversion, 0.2g of matured cultures were maintained in the same culture system, but now subjected to conversion culture media

in light conditions (40-60 mmol m^{-2} s^{-1}) provided by fluorescent lamps at 25±2 °C. Cultures were maintained for 4 weeks in the conversion culture media consisting of MS supplemented culture medium with Morel and Wetmore vitamins, 3% (w/v) sucrose, 500 mg l^{-1} glutamine (Sigma) and 20 µM 2-iP (2-isopentyladenine) and 0.5 µM NAA (α-naphthaleneacetic acid), adapted from Steinmacher et al. (2007), subculturing to the same culture medium free of growth regulators for 4 weeks. In order to evaluate the effect of 2-iP and NAA in conversion medium culture, cultures were also inoculated into culture medium free from 2-iP and NAA for 8 weeks in the same conditions, as a control treatment. After 8 weeks the number of green embryos was counted.

The best conversion system for obtaining green embryos was chosen, and then it was established a new experiment to evaluate the best system for obtaining plantlets from these greens embryos. Four culture systems were used, flasks with a culture medium semi-solid such as Petri dishes, flasks with a liquid culture medium in permanent immersion, TF and RITA. The culture medium used was the conversion culture medium without growth regulators supplemental. After 12 weeks it was quantified the number of plantlets, which were classified by size.

Plantlets were inoculated into flasks containing culture medium for elongation and rooting, consisting of MS culture medium supplemented with Morel and Wetmore vitamins, 3% (w / v) sucrose, 500 mg l^{-1} glutamine (Sigma), 2.5 g l^{-1} Sigma-Phytagel™, 1.5 g l^{-1} Activated Charcoal (AC), for 8 weeks, until it reaches a size greater (between 5 – 7 cm) and rooted, may be acclimatized.

The acclimatization procedure was adapted from that of Steinmacher et al. (2007), keeping the plantlets in a high humidity environment for the first 3 weeks. The entire acclimatization step was carried out in a growing room at 25 ºC under 16-h light (100–130 µmol m^{-2} s^{-1}) periods. To maintain the humidity, 30 plantlets of each size (5 and 7 cm) were transferred to expanded polystyrene trays, with 5×5-cm cells, containing commercial substrate (PlantMax® Paulinia, SP, Brazil) and carbonized rice straw (1:1), and watered with distilled water. The trays were placed inside a plastic box covered with glass to allow the entry of light and reduce water exchange. After 3 weeks the plantlets were watered every other day with distilled water. After 3 months survival and rooting of the plantlets were evaluated.

Protein and starch extraction and quantification

SEC from five different multiplication systems were collected after 6 weeks for protein and starch quantification. Proteins (200 mg) were extracted with a phosphate buffer (pH 7.5), containing 0.05 M of sodium phosphate dibasic (pH 7.0), 1.5% (v/v) 2-mercaptoethanol, 0.5% (w/v) sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride (PMSF) the supernatants containing proteins were stored at –20 ºC. Pellets were collected for starch extraction using MCW (methanol/chloroform/water 12/5/3 v/v) buffer resuspending in perchloric acid (PCA) 30%. Supernatants containing the starch were stored at –20 ºC. Protein concentrations were determined using Bradford's method (1976), with bovine serum albumin as a standard and starch concentrations were determined according to McCready et al. (1950) using glucose as standard.

Alcohol dehydrogenase enzyme extraction and quantification

Protein extracts were prepared in biological triplicates (300 mg FM) for each multiplication system. The extraction of proteins was done according to the method described by Alfenas et al. (2006), using 1,5 ml of solution 1 described by the author. The supernatants (100 µl) containing the proteins were added to 2,9 ml of reaction buffer (0.1M Tris-HCl buffer pH 8.0 + 20 mg NAD^+ (β-nicotinamideadeninenucleotide) + 3 ml absolute ethanol + distilled water to 50 ml) and incubated at 30 ºC for 10 mim. Absorbance reading at 340 nm was done every 10 sec during 180 sec, varying according to the NAD^+ reduction. The enzymatic activity was quantified relative total protein quantification from the same sample.

Global Methylation Analyzes

DNA extraction and digestion

SECs coming from five different systems multiplication and SECs maturated in 5µM ABA were collected after 6 weeks and 2 weeks, respectively, to be quantified DNA global methylation rate. DNA extraction was performed according to Doyle and Doyle (1987). A total of 150 mg of fresh matter were ground in LN. Then, 700 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 2% polyvinylpyrrolidone and 3% 2-mercaptoethanol) were added, transferred to a microtube (2 ml), and incubated in a water bath (60-

65°C) for 1h. Subsequently, 600 µl of chloroform-isoamyl alcohol (CIA) (24:1) were added and subjected to stirring for 5 min by inversion and centrifuged at 15000×g for 8 min. The aqueous phase was transferred to a new tube to which was added 50 µl of CTAB 10% and NaCl 1.4 M. The extraction procedure with 600 µl of CIA was repeated again to ensure the DNA quality.

After the second extraction, DNA was precipitated with 2/3 volume of isopropanol by a period not less than 2h at -20°C. The sample was centrifuged at 7500×g for 10 min, for pellet formation. The supernatant was discarded and the pellet washed twice in 70% ethanol and once in 95% ethanol and the pellet was allowed to dry for approximately 30 min. Afterwards, the pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the total nucleic acids amount was quantified in Nanodrop® 1000 (NanoDrop Technologies, USA).

Digestion procedures were based on the protocol described by Johnston et al. (2005) and Fraga et al. (2012). Digestions with RNase were performed with 100 µl of nucleic acids (1 µg μl^{-1}) in TE buffer. Samples were heated to 65°C for 5 min to denaturation and incubated at 4°C for 10 min. Subsequently, 10 µl of RNase A (Sigma-Aldrich®, St Louis, USA) (1 µg μl^{-1}) and 10 µl of RNase T1 (Sigma-Aldrich®) (20 µg μl^{-1}) were added, vortexed and incubated at 37°C for 17h.

After digestion, 20 µl of sodium acetate (3M, pH 5.4) were added to a final concentration of 0.3 M and the microtubes were vortexed. DNA and RNA resistant to RNase were precipitated with 136 µl of cold isopropanol for 30 min at -20°C. Samples were centrifuged at 5000×g for 10 min to form a pellet, washed with ethanol 70% (v/v) and re-centrifuged at 5000×g for 5 min. The pellets were dried at room temperature and resuspended in 100 µl of deionized and autoclaved H_2O. Nucleic acids concentration was again determined as described above and concentrations of samples were adjusted to 0.25 µg μl^{-1} with H_2O.

Digestions with nuclease P1 and alkaline phosphatase (Sigma-Aldrich®) were performed using 25 µg of nucleic acid in 100 µl of deionized H_2O. Nucleic acids were denatured by heating at 100°C for 2 min and incubated at 4°C for 5 min. 5 µl of $ZnSO_4$ 10 mM and 10 µl of nuclease P1 (1.0 U ml^{-1} in 30 mM NaOAc, pH 5.4) were added followed by sample vortexing and incubating for digestion reaction at 37°C for 17h. After this period, 10 µl of Tris-HCl 0.5 M (pH 8.3) and 10 µl of

alkaline phosphatase (10 U ml^{-1} $(NH_4)_2SO_4$ 2.5 M) were added and incubated for 2h at 37°C. Samples were centrifuged at 10.000×g for 5 min and the supernatant was stored at -20°C until HPLC analysis.

HPLC analysis

HPLC analysis was based on the protocol described by Johnston et al. (2005). It was used a Hyperclone column 5 µm C18 (250 × 4.6 mm) (Phenomenex®, Torrance, USA), guard column (4.0 × 3.0 mm) (Phenomenex®) and UV detector (280 nm). The gradient program consisted of 3 min with 100% of 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 was applied with triplicates of each sample.

The dNTPs (Fermentas®, Hanover, USA) used as standards (dA, dT, dC and dG) and 5mdC were digested for 2h 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 peak area obtained was analyzed by LC Solution software (Shimadzu®, Kyoto, Japan).

Statistical analysis

Data were evaluated by analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) post hoc test, both with 95% reliability. All experiments were performed in triplicate. Data were analyzed using Statistica® (Statsoft Inc., Tulsa, OK, USA) for Windows® version 7.0.

Results and Discussion

Multiplication of the SECs

The multiplication of cultures characterized mostly by somatic embryos clusters (SEC), presented different responses to different

culture systems. The conventional Petri dishes showed the proliferation of somatic embryos after 6 weeks of culture, with 6.158g of FFM (Final Fresh Mass)(Table 1). There was also the formation of non-embryogenic structures which compose much of the acquired FM (Fig. 2A). The RITA® system showed a clear multiplication of somatic embryos with 7.350g of FFM (Table 1), and unlike the Petri dish cultures, somatic embryos showed a whitish appearance (Fig. 2B). Steinmacher et al. (2011) also observed that peach palm embryogenic cultures growing in a system similar to RITA® the showed increased embryogenic capacity as compared cultures growing in Petri dishes. The same authors reported a decreased occurrence of spongy structures that do not exhibited embryogenic capacity, characteristics of cultures resulting from Petri dishes. The cultures from MTF system were morphologically similar to RITA® cultures, but the multiplication was lower resulting in a FFM of 2.010 g (Fig. 2C)(Table 1).

Otherwise, in the TF system, cultures showed a 4.297g of FFM (Table 1) with evident oxidation as compared with the other systems, indicating the presence of large amounts of phenolic compounds (Fig. 2D). The role of phenolic compounds appears to be most likely related to the cellular dedifferentiation process (Alemanno et al. 2003) and the early stages of somatic embryo differentiation (Reis et al. 2008). But a high phenolic content and oxidation of these compounds could be a limiting factor preventing proper tissue multiplication and maintenance (Alemanno et al. 2003). In this study, the oxidation caused by TF system damaged the proliferation of SECs, preventing its subsequent conversion into plantlets.

Cultures from EF showed the highest FFM (13.525 g)(Table 1) when compared to other systems. Morphologically it was not observed an expressive proliferation of somatic embryos but the hypertrophy of existing somatic embryos, composing most of the FFM (Figure 2E) and indicating a excessive accumulation of reserve materials. Thus, considering the embryogenic capacity, obtainment of high FFM without generating many of non-embryogenic structures, oxidation and excessive accumulation of reserve materials it was possible to conclude that the RITA® system was the best system culture for the multiplication of peach palm SEC.

Protein and global methylation analyzes

SEC coming from Petri dishes showed a DNA global methylation rate of 28.7% (Fig. 3) and 3.4 mg g^{-1} of total proteins (Fig. 4). SEC coming from RITA® showed the highest content of total protein after 6 weeks in culture (4.07 mg g^{-1}) as compared to the other systems (Fig. 4). This system showed the accumulation of proteins which may be related to the synthesis of LEA (late embryogenesis abundant) proteins. These proteins are associated with the acquisition of tolerance to the desiccation of seeds, and some of them are induced by cold or by osmotic stress (Welin et al. 1994). Their exact function is still unknown, but it has been shown that they are expressed in late embryo developmental phase (Hundertmark and Hincha 2008). The stress caused by RITA® may be responsible by the more advanced developmental stages reached by the embryos of *Bactris gasipaes*, increasing the embryogenic capacity (Steinmacher et al. 2011) and allowing to obtain a much larger number of plants at the end of the process. Besides, these SEC showed a low global DNA methylation rate of (27.52%), as compared to the SECs coming from Petri dishes (28.7%)(Fig. 3). DNA methylation has been associated with altered gene expression in many plant species, increasing the variation of quantitative traits, since many genes may be affected simultaneously (Johannes et al. 2009). The decrease of DNA global methylation rate is fully connected to the high content of protein of these SEC, and these proteins can be important for the development of somatic embryo, and thus explaining the increased embryogenic capacity. This fact was evidenced when comparing the data of total protein and DNA global methylation rate of the RITA®, EF and TF systems (Fig 3, 4). Unlike the RITA® the EF and TF showed the highest rates of DNA global methylation (29.19% and 28.76%, respectively) (Fig. 3) and consequently the lowest rate of total protein content (1.82 mg g^{-1} and 2.66 mg g^{-1}, respectively) (Fig. 4), again showing the inverse relationship rate global DNA methylation in expression of proteins.

The SEC originated from RITA® and supplemented with ABA in Petri dishes for 2 weeks showed an slight decrease in the global methylation rate (27.28%) as compared to SEC not submitted to ABA (27,52%)(Fig. 3). ABA regulates plant development through genetic and epigenetic mechanisms by direct induction of gene expression or histone ubiquitination, acetylation and methylation-dependent chromatin

remodeling (Chinnusamy et al. 2008). These epigenetic processes might regulate ABA levels through changes in the expression of genes involved in ABA metabolism (Chinnusamy et al. 2008). The decrease in global methylation rates can promote or increase expression of proteins essentials for the maturation of somatic embryos, increasing the number of plants converted to the end of the process. It is not known whether ABA-induced epigenetic changes in response to stresses are heritable through mitosis as well as meiosis and their adaptive advantages (Chinnusamy et al. 2008). If these changes is heritable, the embryos that are matured may by means of secondary embryogenesis, to give rise to a new embryos with the same global methylation rate, facilitating the maturation of the new somatic embryos, which could explains the greatest number of plants obtained comparing to de somatic embryos not matured in the presence of ABA.

Starch and alcohol dehydrogenase enzyme analyses

The SEC coming from RITA® system showed increased starch content (1.34 mg g^{-1}, Fig. 5), as compared to SEC coming from Petri dishes (1.02 mg g^{-1}), which is considered an important feature to provide ATP for the somatic embryos development. Cell divisions necessary for somatic embryo development, demands a high amount of carbon in order to supply the ATP necessary for cellular metabolism (Martin et al. 2000). Starch accumulation is considered a marker for embryogenic capacity in several systems, including oil palm and coconut (Schwendiman et al. 1988; Verdeil et al. 2001). This confirms the postulation of Steinmacher et al. (2011) that peach palm somatic embryos coming from TIS adopt the strategy of carbohydrate-conserving response to hexose sugars and starch reserve for use in aerobic metabolism upon recovery from the stress, as proposed by Fukao and Bailey-Serres (2004). However, in peach palm starch accumulation could not be systematically correlated to somatic embryo development, as some cells accumulated starch without developing into somatic embryos (Steinmacher et al. 2011). In the present work SEC coming from EF had the highest rate of starch (1.94 mg g^{-1}) (Fig. 5) of all culture systems, surpassing including the rate of total protein content (1.82 mg g^{-1})(Fig. 4). This excessive accumulation of starch decreased the embryogenic capacity, as seen in the data of multiplication, where the FM was more attributed to the accumulation of reserve materials

than multiplication of somatic embryos. This high accumulation of reserve could also hinder the conversion of the somatic embryo in a normal plantlet, preventing this type of system for multiplication of the peach palm SEC.

As stated previously, stress is an important factor related to the acquisition of embryogenic competence (Pasternak et al. 2002) and the temporary flooding in TIS (RITA®,TF and MTF) could create different forms of stress which is sensed by the cells as a sort of stress triggering short-term metabolic adaptations. One of those metabolic adaptations was observed in the altered expression of the alcohol dehydrogenase (ADH) enzyme in different culture systems. Induction of ADH by flooding or anaerobiosis has been observed in many plant species (Newman and Van Toai 1991), and this enzyme appears to be related to zygotic embryos development of angiosperms an anaerobic environment (Boyle and Yeung 1983). SEC coming from Petri dishes showed low activity of this enzyme (0.49 OD (Optical Density) min^{-1} mg^{-1} protein) (Fig. 6). On the other hand SEC coming from RITA®, TF and EF had an increase in this enzyme activity, with 3.65, 5.17 and 4.92 OD min^{-1} mg^{-1} protein (Fig. 6), respectively. This confirms the supposition that TIS could create different forms of stress, such as temporary hypoxia, which is sensed by the cells as a sort of stress triggering short-term metabolic adaptations, as postulated by Steinmacher et al. (2011). However, anaerobiosis can be caused by intense multiplication provided by TIS too, which does not occur in cultures coming from Petri dishes and EF. This enzyme has been related with embryogenic cultures and embryo development in plants. In *Vitis rupestris* there was an increase in ADH banding intensity between primary and embryogenic calli, which was much larger than the increase in protein concentration between the two sets of samples (Martinelli et al. 1993). According to these authors, at least for this enzyme it can thus be concluded that ADH specific activity rises as soon as the embryogenic path is undertaken. In other work anaerobic conditions also prevailed during somatic embryo development and a lowering of oxygen in suspension cultures of carrot was found to promote embryogenesis (Kessel et al. 1977). In the present work the cultures coming from TIS resulting somatic embryos exhibited a different form (Fig. 2B) that was not observed in the Petri dishes (Fig. 2A), which may be associated with more advanced stages of promoting a efficient conversion as seen in the above data, proving TIS importance

for the multiplication and development of the somatic embryos to be converted into plantlets.

Maturation and conversion of SECs

In the maturation phase the cultures submitted to system using liquid culture medium supplemented with ABA (RITA®, TF, MTF and EF) presented high oxidation and in the end of the process (data not shown), proved to be unviable for further somatic embryos conversion. Only cultures matured in Petri dishes showed cultures with suitable characteristics for a subsequent conversion of somatic embryos. ABA and the water content of the embryo are also known to regulate the cell-cycle progression and radicle protrusion (Gendreau et al. 2008). When barley (*Hordeum vulgare*, cv Pewter) dormant grains had a partial hydration they had a ABA content decreases (Hoang et al. 2012), as an example for the inverse relationship with the ABA keeps the water content in the embryo. Apparently when the cultures are supplemented with ABA in liquid medium, the cells accumulates many phenolic compounds as a defense to this adverse situation, which ultimately is very toxic to the cells causing them to death.

As cultures maturated in liquid cultures supplemeted with ABA did not originated viable cultures for somatic embryos conversion, conversion occurred only in Petri dishes. Accordingly, the cultures subjected to culture medium supplemented with 5 µM ABA and subsequently converted on MS culture medium free of plant growth regulators for 8 weeks showed the best results with 105 green embryos (Fig. 7A) and 5.05 g FFM per Petri dishes (Table 2). Since cultures originated from maturation with ABA and converted into the MS culture medium free of NAA and 2-Ip showed a more intense green color and green embryos clearly evident (Fig. 7A), it is possible to infer that these cultures synthesized greater amount of chlorophyll. Cultures converted on MS medium supplemented with NAA and 2-Ip showed the predominance of white or green less intense color, with a conversion of somatic embryos longer and increasing the chances of having an abnormal conversion. Both treatments conversion supplemented with NAA and 2iP, matured in the presence or not of ABA resulted in low number of green embryos (48 and 48.25, respectively) as compared with cultures converted in MS medium free of NAA and 2iP, matured in the presence or absence of ABA (105.25 and 79.20, respectively)

(Table 2). Thus, we conclude that the maturation culture medium supplemented with ABA followed by the conversion culture medium free of NAA and 2-iP were the most effective to yield green embryos. The transference to maturation conditions prior to conversion can still be considered as a bottleneck to a successful protocol in peach palm (Steinmacher et al. 2011), being confirmed by the data presented in this paper.

The culture systems used to obtain plantlets from green embryos coming from Petri dishes influenced the number of plantlets obtained after 8 weeks. The best system used was the RITA®, wherein it resulted in 315 plantlets at the end of 8 weeks (Fig. 7B) and the majority (265 plantlets) those with a length between 1 and 2 cm, but with 34 between 2 and 3 cm (Table 3). The cultures which were maintained in semi-solid medium in Petri dishes showed a mean number of 29 plantlets (Fig. 7C), with the majority (22 plantlets) between 1 and 2 cm (Table 3), indicating the importance of the exchange culture system at this step of the protocol. The second best system was the TF which resulted in 78 plantlets (Fig. 7D) at the end of the process; 67 of them ranging from 1 to 2 cm (Table 3). These values were below from those reported in response to the RITA® system, but highlighting the importance of a temporary immersion system (TIS) in this step of the protocol. Scherer et al. (2012) showed that pineapple (*Ananas omosus* var. *comosus*) nodule cluster cultures (NC) fresh weight increased ratio was more efficient in Twin Flasks System, but the microshoots development, coming from NC, was better in RITA® System, demonstrating the importance of changing the culture systems in the different steps of the protocol. In the present work, the permanent immersion into flasks resulted in a total of 53 plantlets (Fig. 7E), with 51 plantlets ranging from 1 to 2 cm (Table 3). These values were the double of the values obtained in response to semi-solid medium culture, demonstrating the importance of the liquid culture medium in this step of the protocol. The plantlets obtained (1 to 2 cm) were transfer for 8 weeks to flasks with culture medium supplemented with activated charcoal to promote growth and rooting (Fig. 8A) passing to 3 to 4 cm after 4 weeks (Fig. 8B) and after 8 weeks the plantlets had 5 to 7 cm (Fig. 8C). Plantlets with 5 (Fig. 8D) and 7 cm were separated to be acclimatized.

The acclimatization step was influenced by the initial size of plantlets (Fig. 8E). When acclimatized with a size of 7 cm, plantlets showed a survival rate of 83.3% and rooting of 94.4% (Fig. 8F), while the plantlets with 5 cm had a survival rate of only 53.3% and rooting of 55.6% (Table 4). Steinmacher et al. (2011) showed that plantlets coming from semi-solid culture medium had a greater survival rate (97%) as compared to plantlets coming from TIS (67%). However in the present work the plantlets coming from TIS (RITA) had a high survival rate (83.3%) after 3 months of acclimatization when acclimatized with 7cm with 94.4% of rooting rate. This fact may be related to the long period that the seed germinated plantlets depend on nutrients emanating from the gradual digestion of the endosperm, by the haustorial structure (Silva et al. 2006). Regarding to the supply of nutrients coming from the culture medium with the endosperm, the plantlets is dependent on the supply of nutrients to reach a relatively large size (7 cm) being hampered their development when the supply of nutrients is removed early (plantlets with 5cm). Furthermore, after three months, plantlets initially with 7cm reached an average height of 10.56 cm, with a root mean size of 2.87 cm and 2.18 roots per plant (Table 4). On the other hand the plantlets initially with 5 cm had an average height of only 6.05 cm, with a mean size of 1.18 cm and 1.16 roots per plant (Table 4), getting down on all the parameters analyzed comparing to plantlets initially with the plantlets with 7 cm in the initial of the process.

In conclusion, the best system to be used on each step of the protocol is described in schematic diagram (Fig. 9). In short, SEC are subjected to the multiplication phase by means of RITA and then to petri Dishes containing culture medium supplemented with ABA (5 µM) during 2 weeks for somatic embryos maturation, and finally to Petri dishes with culture medium free of plant growth regulators during 8 weeks. Green somatic embryos obtained in this step are transferred to the RITA® apparatus for more 12 weeks. Plantlets 7 cm long area acclimatized. This protocol showing high efficiency emphasizes the importance of changing culture systems in different steps of the protocol thus resulting in high quality of somatic embryos, shortening the time to obtain these somatic embryos and increasing the number of plantlets as final result.

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Figures

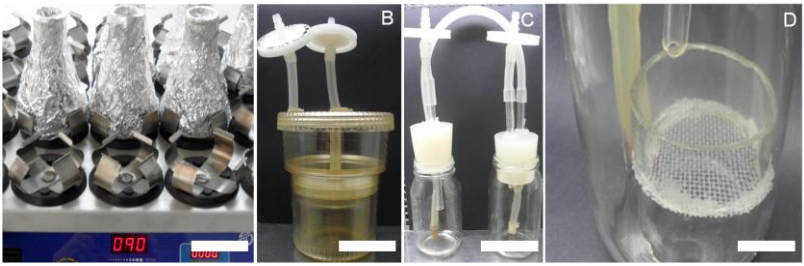

Figure 1 Different culture systems used in the peach palm (*Bactris gasipaes*) somatic embryogenesis protocol. A - Erlenmeyer Flasks (EF) (250 ml) on orbital shaker at 90 rpm; B - RITA® System; C - Twin Flasks System (TF), model described by Escalona et al. (1999); D - Modified TF System (MTF) with a sieve-like support in the cultures flasks, simulating the RITA® System

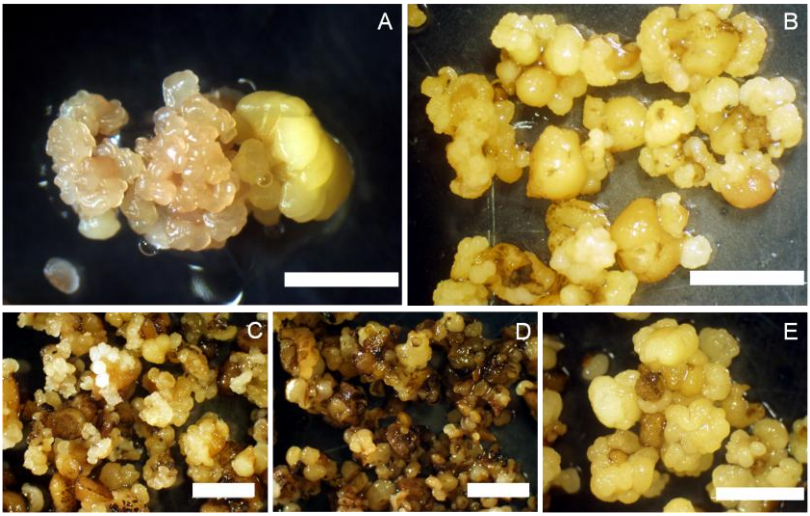

Figure 2 Peach palm (*Bactris gasipaes*) somatic embryo clusters **(**SEC) morphology in different culture systems for 6 weeks. A – SEC multiplication in Petri dishes; B – SEC multiplication in RITA® System; C – SEC multiplication in Modified Twin Flasks System (MTF); D – SEC multiplication in Twin Flasks System (TF), note cultures oxidation; E – SEC multiplication in Erlenmeyer Flasks (EF) (250 ml) on orbital shaker at 90 rpm, note the hypertrophy of the somatic embryos

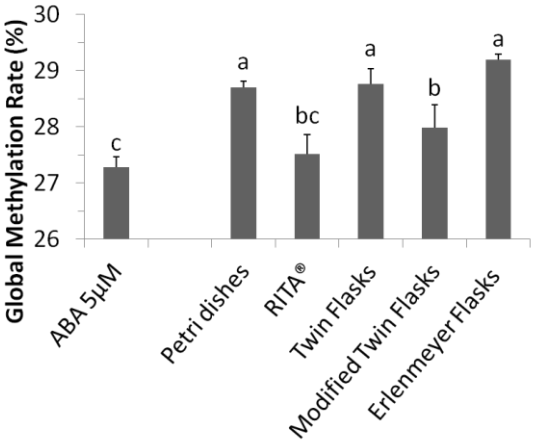

Figure 3 DNA global methylation rate of somatic embryos clusters (SEC) of peach palm (*Bactris gasipaes*) multiplied in different culture systems for 6 weeks and SEC maturated in petri dishes supplemented with ABA 5µM for 2 weeks. Means followed by different letters within each culture system are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 0.72%, n = 3)

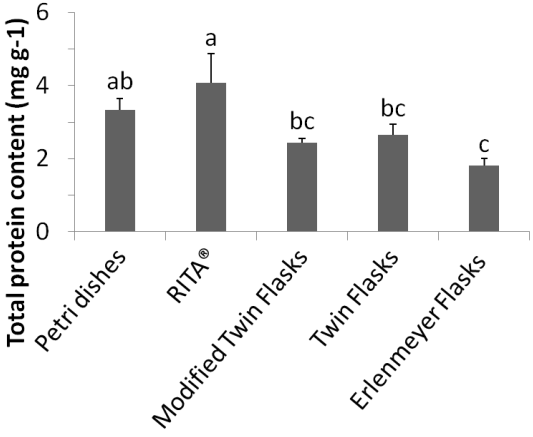

Figure 4 Total protein content of SEC of peach palm (*Bactris gasipaes*) multiplied in different culture systems for 6 weeks. Means followed by different letters within each culture system are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 0.89%, n = 3)

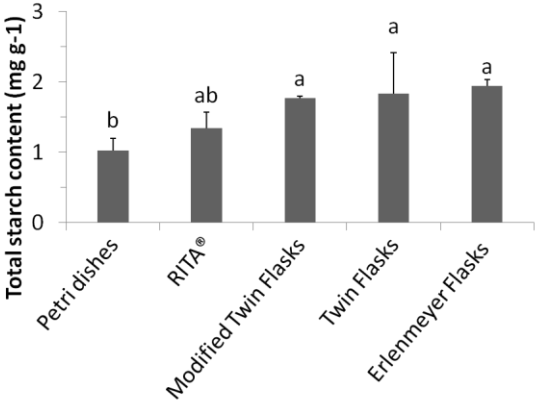

Figure 5 Starch content of SEC of peach palm (*Bactris gasipaes*) multiplied in different culture systems for 6 weeks. Means followed by different letters within each culture system are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 0.47%, n = 3).

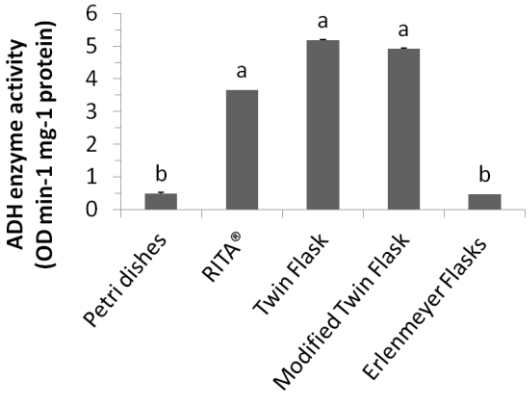

Figure 6 Alcohol dehydrogenase **(**ADH) enzyme activity of SEC of peach palm (*Bactris gasipaes*) multiplied in different culture systems for 6 weeks. Means followed by different letters within each culture system are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 0.02%, n = 3).

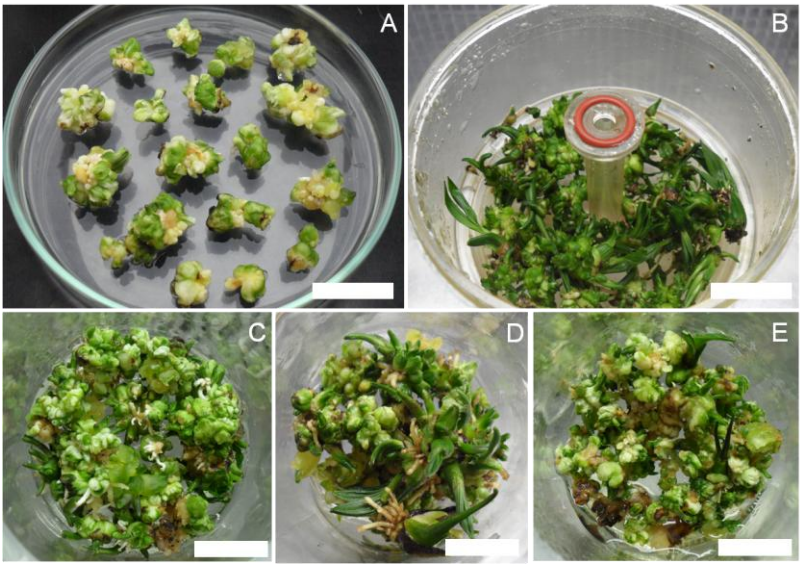

Figure 7 Peach palm (*Bactris gasipaes*) SEC conversion in culture medium free of plant growth regulators after being submitted to maturation step in culture medium supplemented with supplemented with ABA (5 µM) for 2 weeks. A - Green embryos obtained in Petri dishes with semi-solid culture medium at week 8; B - Plantlets obtained in RITA® system from the green embryos at week 12; C – Semi-solid culture medium flask at week 12; D – Twin Flasks System at week 12 (TF); E - Liquid culture medium flask at week 12.

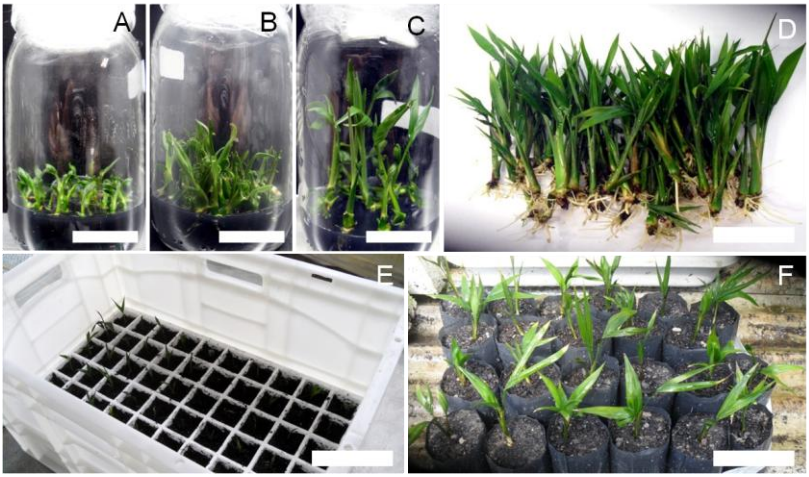

Figure 8 Peach palm (*Bactris gasipaes*) plantlets derived from somatic embryos submitted to acclimatization. Flasks with culture medium supplemented with activated charcoal (AC) to plantlets growth and rooting: A – Plantlets 1 to 2 cm long on the first day; B – Plantlets 3 to 4 cm long after 4 weeks; C – Plantlets 5 to 7 cm long after 8 weeks; D - The plantlets with 7 cm separated to be acclimated, presenting a good uniformity; E - Polystyrene trays containing commercial substrate (PlantMax® Paulinia, SP, Brazil) and carbonized rice straw (1:1) inside a plastic box covered with glass to allow the entry of light and reduce water exchange; F – Plantlets acclimatized after 3 months

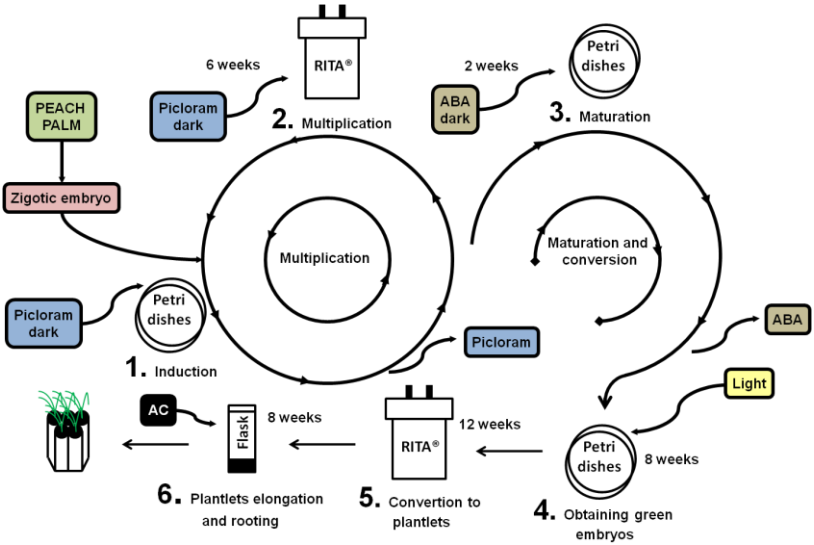

Figure 9 Schematic diagram of peach palm (*Bactris gasipaes*) high efficiency somatic embryogenesis protocol. (Durzan 1989 modified).

Tables

Table 1. Somatic embryos multiplication of peach palm in different culture systems during 6 weeks

Culture System	IFM (g)	FFM (g)	Principal caracteristics
Petri dishes	1.0	6.16c	spongy structures formation
RITA®	1.0	7.35b	intense multiplication
MTF	1.0	4.30d	similar to RITA®'s cultures
TF	1.0	2.01e	Intense oxidation
EF	1.0	13.16a	somatic embryos hipertrophy

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level according to the SNK test ($p < 0.05$) (CV = 1.73%, n = 3). FFM: Final Fesh Mass; IFM: Initial Fresh Mass

Table 2. Obtaining green embryos of peach palm in different culture medium (Petri dishes) during 8 weeks

Culture medium	Green embryos (No.)	IFM (g)	FFM (g)
MS/MS	79.20b	0.2	4.58ab
MS/2ip + NAA	48.25c	0.2	4.85a
ABA/MS	105.25a	0.2	5.05a
ABA/2ip + NAA	48.0c	0.2	3.68b
CV(%)	3.56	-	0.14

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level according to the SNK test ($p < 0.05$) ($n = 3$). FFM: Final Fesh Mass; IFM: Initial Fresh Mass

Table 3 Obtaining peach palm plantlets from green embryos in different culture systems for 12 weeks

Culture System	$1 < x < 2$	$2 < x < 3$	$3 < x < 4$	$x \geq 4$	Total
RITA	265.3a	34a	10.3a	6a	315.7a
Twin Flask	67.3b	7.7b	3b	0.7a	78.7b
Liquid	51.3b	1.7b	0c	0a	53c
Semi-solid	22.7c	6.7b	0c	0a	29.3c

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level according to the SNK test ($p < 0.05$) ($n = 3$)

Tabela 4 Peach palm plantlet growth and survival rate after 3 months of acclimatization

Initial plantlets height (cm)	Suvival (%)	Rooting (%)	Height (cm)	Root size (cm)	Roots per plantets
5	53.3b	55.6b	6.05b	1.18b	1.16b
7	83.3a	94.4a	10.56a	2.87a	2.18a
CV(%)	-	-	1.13	0.42	0.32

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level according to the SNK test ($p < 0.05$) ($n = 30$). Commercial substrate (PlantMax® Paulinia, SP, Brazil) and carbonized rice straw (1:1)

CAPÍTULO III:
Dinâmica epigenética durante a criopreservação

Dynamics of global DNA methylation levels of peach palm (*Bactris gasipaes* Kunth) somatic embryos are affected by cryoprotectants and droplet-vitrification cryopreservation

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Abstract

Regrowth capacity and genetic stability in plants recovered from cryopreserved germplasm are associated with changes in DNA, especially at the epigenetic level by altered DNA methylation status. The present work evaluated the global DNA methylation dynamics associated with the regrowth rates of peach palm (*Bactris gasipaes*) somatic embryos during cryopreservation by the droplet-vitrification technique. Somatic embryo clusters (SEC) submitted to PVS3 vitrification solution for different periods (0, 60, 120, 180 and 240 min) presented different regrowth rates. The highest rate of regrowth (52.4%) was obtained in response to droplet-vitrification combined with an incubation time of 120 minutes. Global DNA methylation dynamics were affected by both cryoprotectants and the droplet-vitrification cryopreservation protocol. For example, incubation of SECs in PVS3 vitrification solution in a time-independent manner not only reduced the regrowth rates, but immediately increased DNA methylation levels when compared to SECs multiplying in a temporary immersion system (control). In addition, although SECs that underwent cryopreservation and recovery had greatest variation in genomic and DNA methylation rates, exposure of SECs to PVS3 solution during 120 min succeeded in re-establishing the initial pattern of global methylation after 24 weeks of regrowth. Thus, during the cryopreservation of somatic embryos, epigenetic changes caused by the altered state of DNA methylation have

physiological consequences with further implications in the germplasm conservation of this important palm species.

Keywords: cryopreservation, somatic embryogenesis, *Bactris gasipaes*, global DNA methylation, epigenetics

Key Message Exposure time to vitrification solution before cryopreservation affects the global DNA methylation levels in peach palm somatic embryos.

Introduction

The peach palm belongs to the Arecaceae family, one of the most important tropical plant groups (Balick 1988), and it has become an alternative for the commercial production of cultivated heart of palm (Clement 2008). In fact, Brazil is the largest producer and consumer of heart of palm, the soft edible young leaves of several palm species. Aside from the cultivation of its fruit for subsistence and the local market, heart of palm production is much appreciated in the gourmet food market and, as such, is produced for export (Mora-Urpí et al. 1997). Thus, in the absence of a modern peach palm cultivar, the establishment of conservation programs for this species is required (Clement 2008). In the past, such programs have mainly relied on the establishment of gene banks (Mora-Urpí et al. 1997), and in the case of peach palm, they are currently focused on the establishment of on-farm gene banking (Mora-Urpí et al. 1997). It should be noted that peach palm seeds are recalcitrant to dehydration (Bovi et al. 2004), making seed storage an unavailable option. Most recently, cryopreservation-based techniques have been considered more productive approaches for peach palm long-term conservation.

Cryopreservation is defined as the conservation of biological material at ultra-low temperatures, usually at -196ºC, the temperature of liquid nitrogen (LN) (Engelmann 2004). Cryopreservation techniques have been developed for different plant species. In particular, Steinmacher et al. (2007a) established a cryopreservation protocol of peach palm zygotic embryos based on the encapsulation-dehydration technique, obtaining 29% of regrowth survival. It can thus be considered an alternative for peach palm conservation, complementing other approaches (Steinmacher et al. 2007a). However, the conservation of peach palm zygotic embryos has some limitations based on unknown

genotypic background resulting from uncontrolled crosses. Therefore, as a complement to other techniques, cryopreservation by droplet-vitrification allows a better option for the long-term conservation of this species. Moreover, this technique is considered promising for large-scale cryopreservation (Sakai and Engelmann 2007; Gonzalez-Arnao et al. 2008), and its success largely stems from the freezing and thawing rate provided by foil strip properties of rapid temperature exchange (Yi et al. 2012), decreasing the risk of large ice crystal formation that would cause cell collapse. However, the use of such approaches should take into careful consideration the genetic stability of regenerants recovered from cryopreserved plant material, as this has been associated with changes in DNA methylation (Mikula et al. 2011).

Methylation modulates gene expression by blocking access of transcription factors to promoter sequences and by altering DNA secondary structure (Ng et al. 1999). DNA methylation has been associated with altered gene expression in many plant species, increasing the variation of quantitative traits, since many genes may be affected simultaneously (Johannes et al. 2009). These changes in the patterns of gene transcription can often be temporary, but they are sometimes protracted since they may be transmitted by sexual reproduction (Smulders et al. 2011).

Genetic stability in plants recovered from cryopreserved germplasm is also associated with changes in DNA, especially at the epigenetic level as a result of altered DNA methylation status (Hao et al. 2001). Specifically, DNA methylation involves post-synthesis deoxycytosine methylation at the 5' position of the pyrimidine ring of cytosine, forming methyldeoxycytosine. This modification can occur in any sequence context in plant DNA, but the patterns of methylation transmission can only occur in symmetrical CG sequences, CHG or CHH, where H can be any deoxynucleotide (Finnegan 2010). In plants, DNA methylation is more common in CG islands, characteristic of transposons, contributing to increased levels of cytosine methylation, especially from the high presence of these elements in plant genomes (Valledor et al. 2007; He et al. 2011).

The objective of this study was to establish a cryopreservation protocol for peach palm somatic embryos and embryogenic cultures by means of the droplet-vitrification technique, as well as examine

variations in concomitant global DNA methylation rates in relation to the genetic stability of regenerated cultures.

Material and Methods

Plant Material

Somatic embryogenesis induction was performed as described by Steinmacher (2007b). Briefly, zygotic embryos were inoculated in Petri dishes containing 25 ml of MS (Murashige and Skoog 1962) medium culture supplemented with Morel and Wetmore (1951) vitamins, 3% (w/v) sucrose, 500 mg l^{-1} glutamine (Duchefa, Haarlem, the Netherlands), 2.5 g l^{-1} Gelrite®, 1 µM $AgNO_3$ and 10 µM Picloram [4-amino-3,5,6-trichloropicolinic acid (Duchefa)]. Somatic embryo clusters were subjected to multiplication cycles (six weeks) in temporary immersion bioreactors (TIB), as described by Steinmacher et al. (2011). These TIBs were based on the twin-flask model described by Escalona et al. (1999) where each unit, consisting of two 250 ml flasks, was inoculated with 1 g of fresh mass (FM) of somatic embryos in one flask, while other received 30 ml of culture medium. Culture medium was liquid MS supplemented with Morel vitamins (Morel and Wetmore 1951), 3% (w/v) sucrose, 500 mg l^{-1} glutamine and 10 µM Picloram. Culture medium pH was adjusted to 5.8 prior to autoclaving during 15 min at 1.5 atm and 121°C. Cultures were maintained in a growth room at 24°C ± 2°C in the dark. Culture immersion cycle was 3 min in duration every 3h. After this period, somatic embryo clusters (SEC) obtained from secondary embryogenesis were individualized, containing three to five embryos at different developmental stages, and these were employed in subsequent experiments.

Cryopreservation

Peach palm SECs were first preconditioned in 30 ml of liquid MS culture medium supplemented with 0.3 M sucrose and, subsequently, in 0.6 M for one hour at each concentration. Subsequently, they were incubated in PVS3 vitrification solution (50% w/v sucrose, 50% w/v glycerol) (Nishizawaetal et al., 1993) for 0 (PVS0), 60 (PVS60), 120 (PVS120), 180 (PVS180) and 240 min (PVS240).

For the droplet-vitrification technique, droplets (5-10 µL) pretreated with PVS3 vitrification solution were transferred to

aluminum foil strips (0.5 cm x 3.0 cm), and each SEC (0.1 g FM) was individually transferred to these droplets and incubated as described previously. The strips were immersed in liquid nitrogen (LN) and then placed in sterile polypropylene cryovials (2 ml) previously frozen in LN. For each treatment, a corresponding sample was established, but only incubated in PVS3 and not subjected to LN. After 24 h, the cryovials were removed from LN and thawed in a water bath at 40°C for 2 min. Then, cultures were transferred to Petri dishes containing 25 ml of liquid MS culture medium supplemented with 0.3 M of sucrose for 20 min. Thereafter, the cultures were inoculated in Petri dishes containing 25 ml of regrowth gelled culture medium (2.5 g l^{-1} Gelrite®) (Steinmacher et al. 2011), followed by incubation in the dark in a biological oxygen demand (BOD) incubator at 25ºC. Samples were collected at different points during the experiment, as shown in Figure 1, for subsequent DNA extraction and HPLC analysis of global DNA methylation.

DNA extraction and digestion

DNA extraction was performed according to Doyle and Doyle (1987). A total of 150 mg of fresh matter were ground in LN. Then, 700 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 2% polyvinylpyrrolidone and 3% 2-mercaptoethanol) were added, transferred to a microtube (2 ml), and incubated in a water bath (60-65°C) for 1h. Subsequently, 600 µl of chloroform-isoamyl alcohol (CIA) (24:1) were added and subjected to stirring for 5 min by inversion, followed by centrifugation at 15000×g for 8 min. The aqueous phase was transferred to a new tube to which 50 µl of cetyltrimethyl ammonium bromide (CTAB) 10% and NaCl 1.4 M were added. The extraction procedure with 600 µl of CIA was repeated again to ensure the DNA quality.

After the second extraction, DNA was precipitated with 2/3 volume of isopropanol during 2h at -20°C. The samples were centrifuged at 7500×g for 10 min, the supernatant discarded and the resulting pellet washed twice in 70% ethanol and once in 95% ethanol, finally allowing the pellet to dry for 30 min. Afterwards, the pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the total nucleic acids were quantified in Nanodrop® 1000 (NanoDrop Technologies, USA).

Digestion procedures were based on the protocol described by Johnston et al. (2005) and Fraga et al. (2012). Digestions with RNase were performed with 100 µl of nucleic acids (1 µg μl^{-1}) in TE buffer. Samples were heated to 65°C for 5 min to denaturation and incubated at 4°C for 10 min. Subsequently, 10 µl of RNase A (Sigma-Aldrich®, St Louis, USA) (1 µg μl^{-1}) and 10 µl of RNase T1 (Sigma-Aldrich®) (20 µg μl^{-1}) were added, vortexed and incubated at 37°C for 17h.

After digestion, 20 µl of sodium acetate (3M, pH 5.4) were added to a final concentration of 0.3 M, and the microtubes were vortexed. DNA and RNA resistant to RNase were precipitated with 136 µl of cold isopropanol for 30 min at -20°C. Samples were centrifuged at 5000×g for 10 min to form a pellet, washed with ethanol 70% (v/v), and recentrifuged at 5000×g for 5 min. The pellets were dried at room temperature and resuspended in 100 µl of deionized and autoclaved H_2O. The concentration of nucleic acids was again determined as described above, and concentrations of samples were adjusted to 0.25 µg μl^{-1} with H_2O.

Digestions with nuclease P1 and alkaline phosphatase (Sigma-Aldrich®) were performed using 25 µg of nucleic acid in 100 µl of deionized H_2O. Nucleic acids were denatured by heating at 100°C for 2 min and incubated at 4°C for 5 min. 5 µl of $ZnSO_4$ 10 mM and 10 µl of nuclease P1 (1.0 U ml^{-1} in 30 mM NaOAc, pH 5.4) were added, followed by sample vortexing and incubating for digestion reaction at 37°C for 17h. After this period, 10 µl of Tris-HCl 0.5 M (pH 8.3) and 10 µl of alkaline phosphatase (10 U ml^{-1} $(NH_4)_2SO_4$ 2.5 M) were added and incubated for 2h at 37°C. Samples were centrifuged at 10,000×g for 5 min, and the supernatant was stored at -20°C until HPLC analysis.

HPLC analysis

HPLC analysis was based on the protocol described by 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 with triplicates of each sample.

The dNTPs (Fermentas®, Hanover, MD,USA) used as standards (dA, dT, dC and dG) and 5mdC were digested for 2h 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 peak area obtained was analyzed by LC Solution software (Shimadzu®, Kyoto, Japan).

Statistical analysis

Data were evaluated by analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) post hoc test, both with 95% reliability. All experiments were performed in triplicate. Data were analyzed using Statistica® (Statsoft Inc., Tulsa, OK, USA) for Windows® version 7.0.

Results and Discussion

Morphological aspects and cryopreservation regrowth rates

Somatic embryos cultured on gelled medium (Fig. 2A-B) did not survive to cryopreservation, presenting a whitish appearance after LN removal (data not shown). Thus, only the somatic embryos coming from TIS (Fig. 2C) were further submitted to statistical analyses. These results suggest that the culture conditions prior to cryopreservation affect regrowth survival post-LN. Apparently, the somatic embryos resulting from liquid culture medium acquired essential characteristics required to support the stress caused by LN. It has been postulated that the TIS culture system induces stresses on cultures and that one of these is temporary hypoxia, which requires rapid metabolic adaptation of the cultures (Steinmacher et al. 2011). During hypoxia, plant cells adopt a carbohydrate-conserving response to store starch and hexose sugars for subsequent use in aerobic metabolism upon recovery from the stress (Fukao and Bailey-Serres 2004). In peach palm, embryogenic cultures cultivated in TIS apparently adopt this strategy, and, according to histological analyses, enhanced starch accumulation was observed in

TIS-cultivated cultures, but not in gelled cultures (Steinmacheret al. 2011). This factor may have influenced cellular responses to cryopreservation, since a greater amount of reserve compounds corresponds to lowest free water content, thus avoiding large ice crystal formation, the main cause of cell death at cryogenic temperatures.

Treatment of somatic embryo clusters for different incubation times in PVS3 resulted in different regrowth rates of control cultures (-LN). In cultures without PVS3 treatment, the regrowth rate was 80.9% and 95.2% after 6 and 12 weeks of culture, respectively (Table 1). However, an inverse correlation between incubation period and regrowth rate of the cultures was observed when samples were subjected to cryoprotectant. Initially, 66.6% of the cultures treated for 60 minutes in PVS3 solution and then inoculated in the regrowth medium for 6 weeks showed the development of new somatic embryos, decreasing to 16.6% regrowth rate when treated for 240 minutes, with a similar rate after 12 weeks (Table 1). Increasing incubation time in PVS3 vitrification solution normally decreases cell viability (Kohmura et al. 1992). Studying oil palm (*Elaeis guineensis*) polyembryoid cryopreservation, Suranthran (2012) observed that incubation in PVS2 vitrification solution for over 10 min caused their death. However, even when these polyembryoids were not incubated in PVS2 vitrification solution, but were, instead, immersed in LN, they also died. Meanwhile, incubation for 5 min in PVS2 resulted in a 45% survival rate in regrowth after cryopreservation. These results suggest that the optimal incubation time in vitrification solutions should be evaluated for each biological system in order to minimize potential toxicity and irreversible cell damage (Kohmura et al. 1992).

The droplet-vitrification technique was efficient for peach palm somatic embryo cryopreservation, resulting in 33.3% regrowth rate after 6 weeks in culture (Fig. 2I) and 52.4% after 12 weeks (Fig. 2J) when associated with PVS120 treatment (Table 1). It is important to stress that the minimal regrowth rate acceptable for a cryopreserved germplasm bank is 30% (Gonzalez-Arnao *et al*. 2008). Considering this, in the present work, the results obtained for regrowth of the peach palm somatic embryos (52.4%) are above the minimum expected regrowth rate.

In the present work, PVS3 was efficient as a cryoprotectant for cryopreservation of peach palm somatic embryos. This solution is

dimethylsulfoxide (DMSO)-free. DMSO is, however, a component of PVS2 (Plant vitrification solution 2) solution (Sakai et al. 1990). Nonetheless, Al-Bahrany and Al-Khayri (2012), studying date palm (*Phoenix dactylifera*) callus cryopreservation, showed that DMSO was required for effective callus cryopreservation. Although DMSO is toxic to some plant species, no conclusive studies have reported peach palm sensitivity to this compound. Our preliminary data, however, suggest that this system is more sensitive to the presence of DMSO (data not shown).

However, after 12 weeks of culture, the regrowth rate in all treatments was greater than, or equal to, the rates observed after 6 weeks, independent of incubation time in the PVS3 vitrification solution. In our laboratory, peach palm SECs (Fig. 2A) were maintained in Petri dishes containing multiplication medium (Steinmacher et al. 2011) with subcultures every 6 weeks, the time required for culture growth (Fig. 2B). SECs incubated in PVS120, but not subjected to LN (Fig. 2D), also showed quite evident regrowth after 6 weeks (Fig. 2E), with progressive multiplication after 12 weeks (Fig. 2F) and 24 weeks (Fig. 2G). However, when the SECs were incubated in PVS120 and also submitted to LN (Fig. 2H), only slight regrowth was observed after 6 weeks (Fig. 2I), giving evidence of a regrowth delay when compared to cultures not cryopreserved. Normal regrowth was observed only after 12 weeks (Fig. 2J), maintaining this pattern after 24 weeks (Fig. 2K). At -196ºC, cell metabolism and growth are paralyzed (Benson 2008), entering into a theoretically unlimited steady state. After recovering the somatic embryos from LN, their cellular metabolism slowly reactivates, requiring some time to resume normal metabolism, which could explain the differences observed in the growth rate between 6 and 12 weeks.

The aim of cryopreservation is to preserve a germplasm that is ready, at any moment, to be recovered and reintroduced into the field (Sershen and Pammenter 2010). Considering this, the vigor and physiological conditions after cryopreservation are important because the regenerated plantlets are subjected to several environmental *ex vitro* conditions (Sershen and Pammenter 2010). Additionally, regenerated plantlets may differ from those not submitted to cryopreservation. Thus, Steinmacher et al. (2007) showed that partial dehydration and cryopreservation of peach palm zygotic embryos resulted in smaller plantlets deficient in haustorium development. In the cryopreservation

of *Amaryllis belladonna* zygotic embryo, it was shown that both partial drying alone, as well as drying followed by exposure to cryogenic temperatures, reduced dry matter accumulation and partitioning to roots during the regrowth phase as compared to the control (Sershen and Pammenter 2010). These authors also reported that cryopreservation would be potentially damaging to the plant's capacity to acquire resources as a consequence of abnormal development. In the present work, however, it was shown that the droplet-vitrification technique associated with PVS3 vitrification solution was effective in cryopreservation of the peach palm somatic embryos which had a high regrowth rate.

Apart from the issue of cryopreserved regrowth delay, clear plant materials are also subjected to changes in DNA, especially at the epigenetic level, as a result of altered DNA methylation status. Thus, a global methylation analysis was performed in order to identify possible variations and the implications for plant physiology.

Changes in global DNA methylation levels

SEC incubation in PVS3 solution for 0 and 60 min were not part of this analysis since no sufficient material resulted from regrowth. It was found that global DNA methylation rates varied according to the stage of cryopreservation. Incubation of SEC samples in PVS3 solution in a time-independent manner increased the methylation levels as compared to SEC multiplying in TIS (control), which presented an average methylation rate of 24.56%. Similarly, SEC incubation in PVS3 solution for 120 min, 180 min and 240 min resulted in mean global DNA methylation values of 25.83%, 25.28% and 25.73%, respectively (Fig. 3). Thus, it was shown that immediate submission of SEC to vitrification solution affected the overall DNA methylation rates. Similar results were found by Kaity et al. (2008) in cryopreserved papaya shoot tips and the effects on DNA methylation pattern, as assessed by the Amplified Methylation Polymorphism Polymerase Chain Reaction (AMP PCR) technique. In this case, the different methylation pattern observed in regenerated plants was attributed to a possible increase in DNA methylation levels in shoot tips originally subjected only to vitrification solution (Kaity et al. 2008), a finding which supports evidence that cryprotectant solutions used in

cryopreservation protocols may also alter the DNA methylation pattern (Wang and He 2008).

All treatments showed the same DNA methylation pattern of immediate response to inoculation in PVS3 solution. However, the three above-mentioned treatments showed different global DNA methylation dynamics when the SECs were recovered and then inoculated in the multiplication culture medium for 12 and 24 weeks in the absence of cryopreservation. After 12 weeks, SECs submitted to PVS3 for 120 min and 180 min showed an increase in global methylation rates, with values of 29.60% and 28.94%, respectively. In contrast, SECs submitted to PVS3 for 240 min showed a 25.5% methylation rate. After 24 weeks, SECs submitted to PVS3 for 120 min showed a slight decrease (28.59%), while SECs submitted to PVS3 for 180 min maintained the value (29.13%), and those submitted to treatment for 240 min showed an increase (29.10%) in DNA methylation rate (Fig. 3). Taken together, all treatments resulted in a DNA methylation rate of about 29%. However, this increase was faster in the SECs submitted to PVS3 for treatments of 120 min and 180 min, followed by PVS3 treatment for 240 min, which only reached a high methylation level after 24 weeks.

On the other hand, SECs incubated in PVS3 solution and then recovered from LN presented an increase in the global methylation rate, but only in response to PVS3 treatment for 120 min (27.56%) and 180 min (25.87%)(Fig. 4). SECs submitted to treatment for 240 min maintained their methylation rate after thawing (25.37%) (Fig. 4). After 12 weeks in regrowth, cryopreserved SECs submitted to PVS3 treatment for 180 min and 240 min showed an increase in global methylation rate (29.65% and 28.05%, respectively), followed by a slight decrease in response to treatment for 180 min (28.97%) (Fig. 4).

On the other hand, the lower exposure to vitrification solution (PVS3 for 120 min treatment) resulted in a different pattern of response. SECs in multiplication maintained a mean value of 27.87% global methylation rate at week 12, decreasing to 25.23% at week 24(Fig. 4). It is important to emphasize that only cryopreservation with PVS3 for 120 min was able to re-establish the pattern of DNA global methylation found in SECs maintained in multiplication cycle. Also, the global DNA methylation pattern of SECs submitted to the 240 min treatment, whether cryopreserved or not, presented a slow, but persistent, increase (Fig. 3 and 4).

It is well known that cryopreservation may induce DNA alterations, especially at epigenetic level, accomplished by altered DNA methylation dynamics (Urbanová et al. 2006). Johnston et al. (2009) reported increased global DNA methylation rate in cryopreserved *Ribes nigrum* shoots during cryopreservation protocol steps. This alteration was not attributed to any specific protocol step. However, after two subcultures (18-20 weeks), the authors also observed that the methylation rate declined at basal values close to those found in control cultures (Johnston et al. 2009). This result agrees with the response of cryopreserved peach palm somatic embryos treated with PVS3 solution for 120 min, as observed in the present work. These facts reinforce the possibility that the dynamics in the global methylation rate observed after submission of cultures to a cryopreservation protocol is a highly reversible epigenetic mechanism (Harding 2004).

Comparative responses to cryopreservation of SEC submitted to PVS3 treatments are shown in Figure 4. SECs submitted to PVS3 for 120 min showed a significantly higher global DNA methylation rate at day 0 after cryopreservation, followed by an equally significant decrease, with the lowest level at week 24, when compared with other treatments (Fig. 4). It is known that the cellular metabolism of SEC after cryopreservation is slowly reactivated, requiring some time to resume normalcy. In previous experiments (data not shown), we also observed that SECs subjected to vitrification solution for 120 min showed the highest regrowth rate (52.4%), while treatment for 180 min and 240 min resulted in 42.9% and 9.5% regrowth rates, respectively. In the present work, the initial high methylation rate found in response to treatment for 120 min may be closely related to the above-mentioned regrowth rates if it can be hypothesized that the initial high rate results first from the stabilizing effect of DNA protection, then later reverting to the original DNA methylation state and, as a consequence, allowing the percentage of culture regrowth to reach high values.

Methyltransferases can methylate DNA regions that preferentially change their state of euchromatin to heterochromatin, making this stretch of DNA packaged and silenced (Finnegan and Kovac, 2000). Such packaging can also protect the cell, thus hampering DNA cleavage by endonucleases (Noyer-Weidner and Trautner 1993), but also protecting cells against stressful conditions, such as PVS3 incubation, freezing and thawing, common features in a cryopreservation protocol.

In this sense, cultures exposed to cryoprotectant solution and LN may have their DNA methylated in order to gain protection from stressful conditions during cryopreservation. DNA methylation in repeated regions, as in the case of centromeres, is more related to DNA stability than chromosomal gene silencing (Jones, 2012). This increase in global methylation rate is also important for the maintenance of chromosomal structure of the cells subjected to cryopreservation, allowing their survival and subsequent regrowth after recovering from LN. Johnston et al. (2009) observed that cryotolerant genotypes of *Ribes nigrum* shoots showed increased DNA methylation levels; however, such changes in DNA methylation levels did not persist after the second subculture cycle. In the present work, significant changes in the pattern of SEC global DNA methylation were observed during different stages of cryopreservation, reaching up to 5.17% during incubation for 120 min in PVS3 solution and 12.21% after removal of LN (Fig. 4).

Based on these results, it can be concluded that the global DNA methylation rate of peach palm SECs depends on their response at each stage of the cryopreservation protocol. Specifically, SEC incubation in PVS3 solution, independent of time, increased global methylation levels as compared to SECs maintained in TIS. In contrast, the exposure of SECs to vitrification solution for 120 min resulted in a 52.4% regrowth rate and was the only treatment that almost re-established the pattern of DNA global methylation found in SECs during multiplication cycles. The initial high methylation rate found in response to PVS3 for 120 min may be closely related to the higher regrowth rates found in response to this treatment, as explained in detail above. This finding prompts us to postulate that such methylation could protect the DNA of SECs during the freezing and thawing steps, subsequently reverting to the initial stabilizing DNA methylation state. This process may, in turn, be responsible for the high regrowth rates observed in these cultures.

In summary, the present study demonstrated the dynamics of DNA methylation, as well as the survival and regrowth of somatic embryos, of the Amazonian native palm peach palm (*Bactris gasipaes*) in response to a protocol of cryopreservation. Somatic embryos that underwent cryopreservation and recovery had the highest levels of genomic and DNA methylation variations. Additionally, cryoprotectants based on the PVS3 vitrification solution not only negatively affected the regrowth rates of somatic embryos but also induced DNA methylation

changes. The enhanced rates of DNA methylation might explain the expressive survival regrowth rates of these somatic embryos. Further investigations may elucidate whether these epigenetic variations can be inherited by the next generation and whether the DNA methylation changes caused during cryoprotection might influence the regenerated plants from these somatic embryos.

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Figures

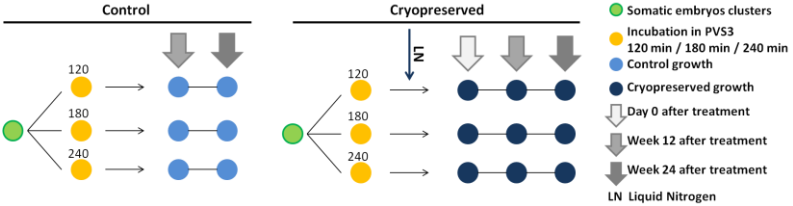

Figure 1 Schematic diagram of collection points during cryopreservation experiment of peach palm somatic embryo clusters for global DNA methylation analysis. Each colored ball represents a point of sample collection.

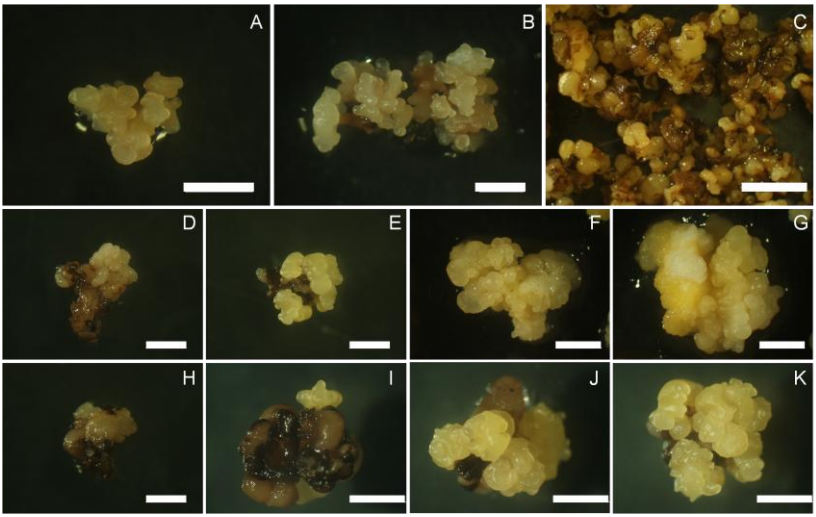

Figure 2 Peach palm somatic embryo clusters (SEC). (A) SEC on the first day after exchanging the multiplication culture medium (B) and after 6 weeks. SEC coming from TIS after 6 weeks (C). SEC submitted to PVS3 solution for 120 min (D), after culture in multiplication medium for 6 weeks (E), 12 weeks (F), and 24 weeks (G). Cultures submitted to PVS3 for 120 min and LN (H), after culture in multiplication medium during 6 weeks (I), 12 weeks (J) and 24 weeks (K). Bar: 2.0 mm

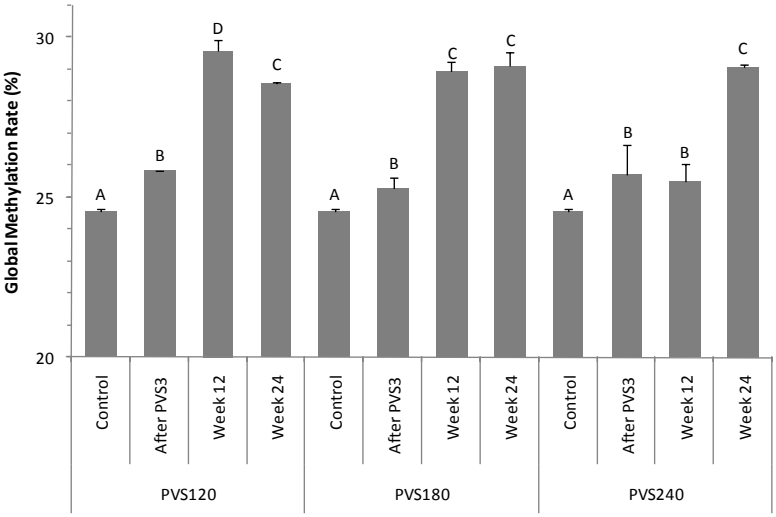

Figure 3 Global methylation rate of peach palm somatic embryo clusters non-cryopreserved and submitted to different incubation times in PVS3 solution. Samples were collected in multiplication medium (control) after PVS3 treatment (after PVS3), then recovered and inoculated in multiplication culture medium during Week 12 and Week 24. Mean values followed by standard deviation (vertical bars). Means followed by different letters within each incubation time in PVS3 solution are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 1.24%, n = 3). PVS120: incubation in PVS3 solution for 120 min; PVS180: incubation in PVS3 solution for 180 min; PVS240: incubation in PVS3 solution for 240 min.

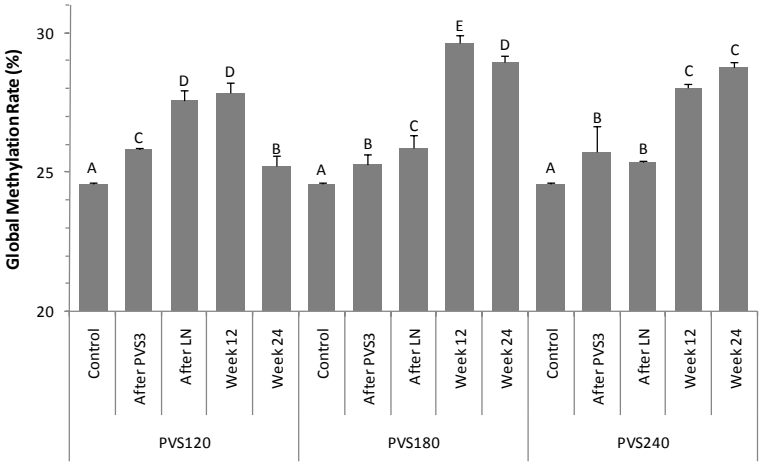

Figure 4 Global methylation rate of peach palm somatic embryo clusters (SEC) submitted to different incubation times in PVS3 vitrification solution and then liquid nitrogen (LN). Samples were collected in multiplication medium (control) after PVS3 treatment (after PVS3) and after recovery from LN and then inoculated in multiplication culture medium at Week 0 (after LN), Week 12 and Week 24. Mean values followed by standard deviation (vertical bars). Means followed by different letters within each incubation time in PVS3 solution are significantly different among data collection times according to the SNK test ($p < 0.05$) (CV = 1.17%, n = 3). PVS120: incubation in PVS3 solution for 120 min; PVS180: incubation in PVS3 solution for 180 min; PVS240: incubation in PVS3 solution for 240 min.

Tables

Table 1 Regrowth rate (%) of peach palm somatic embryos subjected to PVS3 incubation and Cryopreserved (+LN) using Droplet-Vitrification technique.

Time in	Control		Droplet-Vitrification	
	6(weeks)	12(weeks	6(weeks)	12(weeks)
0 min	80.9a	95.2a	0b	0b
60 min	61.9ab	66.6b	0b	4.8b
120 min	47.6b	57.1b	33.3a	52.4a
180 min	19.1c	19.1c	33.3a	42.9a
240 min	14.3c	14.3c	4.8b	9.5b

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level (n = 7).

CAPÍTULO IV:
Análise ultraestrutural durante a criopreservação

Survival and Ultrastructural Features of Peach Palm (*Bactris gasipaes*, Kunth) Somatic Embryos Submitted to Cryopreservation Through Vitrification

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Abstract
Bactris gasipaes (Arecaceae), also known as peach palm, was domesticated by Amazonian Indians and is cultivated for its fruit and heart of palm, a vegetable grown in the tree's inner core. Currently, the conservation of this species relies on *in situ* conditions and field gene banks. Complementary conservation strategies, such as those based on *in vitro* techniques, are indicated in such cases. To establish an appropriate cryopreservation protocol, this study aimed to evaluate the ultrastructural features of *B. gasipaes* embryogenic cultures submitted to vitrification and subsequent cryogenic temperatures. Accordingly, somatic embryo clusters (SEC) were submitted to Plant Vitrification Solution 3 (PVS3). In general, cells submitted to PVS3 had viable cell characteristics associated with numerous mitochondria, prominent nucleus and preserved cell walls. Cells not incubated in PVS3 did not survive after the cryogenic process in liquid nitrogen. The best incubation time for the vitrification technique was 240 minutes, resulting in a survival rate of 37%. In these cases, several features were indicative of quite active cell metabolism, including intact nuclei and preserved cell walls, a number of mitochondria and lipid bodies, and the presence of many starch granules and condensed chromatin. Moreover, ultrastructure analysis revealed that overall cellular structures had been

preserved after cryogenic treatment, thus validating the use of vitrification in conjunction with cryopreservation of peach palm elite genotypes, as well as wild genotypes, which carry a rich pool of genes that must be conserved.

Keywords long-term conservation, PVS3 solution, pejibaye, *in vitro* conservation, histomorphology, cryogenics

Abbreviations CBB - Coomassie Brilliant Blue; DMSO - Dimethylsulfoxide; FM - Fresh mass; LN - liquid nitrogen; MS - Murashige and Skoog; Picloram - 4-amino-3,5,6-trichloropicolinic acid; PAS - Periodic Acid-Schiff; PVS - plant vitrification solution; SEC - somatic embryo clusters; SNK - Student-Newman-Keuls; TEM - Transmission Electron Microscopy; TIS - temporary immersion system.

Introduction

Cryopreservation is considered a pertinent and safe approach for long-term conservation of plants with recalcitrant seeds, such as the peach palm. This technique is defined as the conservation of biological material at ultra-low temperatures, usually with liquid nitrogen (LN), at -196ºC (Engelmann 2004). The development of cryopreservation protocols has been performed for different plant species, including the peach palm (Steinmacher et al. 2007), and this technique can be considered an alternative for peach palm conservation, complementing other approaches. A cryopreservation protocol for peach palm zygotic embryos based on the encapsulation-dehydration technique has already been established (Steinmacher et al. 2007), resulting in 29% survival regrowth rate. However, conservation of peach palm zygotic embryos has some limitations based on unknown genetic background resulting from uncontrolled crosses. Currently, new cryopreservation protocols based on vitrification solutions are well established, and most of them include the vitrification technique (Gonzalez-Arnao et al. 2008). These solutions enable the intracellular solute to acquire a glassy state when exposed to low temperatures, resulting from high concentrations of cryoprotectant solutes and the rapid cooling of samples in LN (Engelmann 2000).

The key component for successful cryopreservation of plant cells is the degree of injury to cell microstructure. Understanding and minimizing the damage to cell microstructure during cryopreservation will improve plant cryopreservation techniques (Yil et al. 2012). Many

studies suggested that the physical and metabolic stresses, as well as consequent lesions, induced by cryopreservation protocols may predispose tissues to further damage and loss of viability with the progression of each protocol step, making the question of cryoinjury a complex and multifaceted one (Benson and Bremner 2004; Wen et al. 2012). Light microscopy combined with double-staining with Acid-Schiff (PAS) / Coomassie Brilliant Blue (CBB) (Schmidt et al. 2012) enables analysis of cell wall structure and compartmentalization of total cellular proteins, including nuclear proteins, allowing further evaluation of nuclear integrity during the cryopreservation protocol. Changes in the ultrastructure of cryopreserved material have also been reported in some recent studies (Sershen et al. 2012a; Sershen et al. 2012b; Wen et al. 2012), showing structural changes attributed to cryoprotectants and the cryogenic process in LN. Thus, investigations to evaluate ultrastructure changes caused by cryoprotectants and the cryogenic process are useful tools to improve cryopreservation protocols (Kaviani, 2011).

The peach palm is native to the Amazon basin (Arecaceae), and, as a multipurpose tree, it plays an important role as a component of agroforestry systems (Clement and Mora-Urpi 1987). Fruit production for local consumption and heart-of-palm production are its most important uses (Mora-Urpí et al. 1997). It is known that the gene pool of cultivated plants and their wild relatives is rich in diversity, but also subject to genetic erosion, creating an urgent need to collect and conserve the germplasm (Mora-Urpi et al. 1997; Steinmacher et al. 2011). Since their seeds are recalcitrant (Bovi et al. 2004), peach palm conservation programs have been directed to the establishment of active field gene banks (Mora-Urpí et al. 1997).

An effective strategy merging different technologies would include the use of somatic embryogenesis obtained from selected genotypes associated with cryopreservation. Steinmacher et al. (2011) demonstrated the formation of clusters of peach palm somatic embryos resulting in secondary cycling of somatic embryogenesis which could be obtained and kept in temporary immersion systems (TIS). If somatic embryos recovered from LN, as confirmed by ultrastructure analysis, might give rise to secondary somatic embryos, then cryopreservation could also be validated as a successful technique. Cryopreservation is also important to avoid the continuous culturing of somatic embryo

clusters, further suggesting that cryopreservation is an effective strategy for planning the production of *in vitro* plantlets.

In the present work, we investigated the effects of vitrification solution and cryopreservation by liquid nitrogen on the cell structure and ultrastructure of *Bactris gasipaes* somatic embryos to better understand cell survival capacity following the cryogenic process, as well as establish a suitable cryopreservation protocol for this species.

Material and Methods

Plant Material

Somatic embryos were obtained as previously described by Steinmacher et al. (2011). Briefly, zygotic embryos were inoculated in Petri dishes containing 25 ml of MS (Murashige and Skoog 1962) medium culture supplemented with Morel and Wetmore (1951) vitamins, 3% (w/v) sucrose, 500 mg L^{-1} glutamine (Duchefa, Haarlem, the Netherlands), 2.5 g L^{-1} Gelrite® (Duchefa), 1 µM AgNO3 and 10 µM Picloram [4-amino-3,5,6-trichloropicolinic acid] (Duchefa). Somatic embryo clusters were multiplied in TIS in small 250 ml culture flasks using approximately 1 g Fresh Mass (FM) of somatic embryos, inoculated in the same induction medium free of Gelrite®. Somatic embryo clusters obtained from gelled culture medium were also used in the present study. All the cultures were obtained after 6 weeks of culture growth. After this period, clusters of somatic embryos resulting from secondary embryogenesis, as defined by Steinmacher et al. (2011), were separated into smaller clusters composed of three to five somatic embryos at different developmental stages (globular to elongated embryos) and then used in the present study.

Vitrification technique

Peach palm somatic embryos (0.1g FM) were first preconditioned in liquid MS culture medium supplemented with 0.3M and, subsequently, 0.6M sucrose for one hour in each concentration. Subsequently, they were incubated for 0, 60, 120, 180 and 240 min in PVS3 (Plant Vitrification Solution 3) (50% w/v sucrose, 50% w/v glycerol; Nishizawa et al. 1993). For the vitrification technique, nine small clusters of somatic embryos were incubated in each cryovial (2 ml) containing 0.5 ml of cryoprotective solution, followed by immersion in LN. For each treatment, a corresponding control not subjected to LN

was established. After 24 hours, the cryovials were removed from the LN and thawed in water bath at 40°C during 2 minutes. The cultures were then transferred to Petri dishes containing 25 ml of liquid MS culture medium supplemented with 0.3M of sucrose for 20 minutes to rehydrate them. Thereafter, the cultures were inoculated in Petri dishes containing 25 ml of regrowth gelled culture medium (Steinmacher et al. 2011) and kept in culture room at 25ºC in the dark. After 6 and 12 weeks *in vitro*, responses and regrowth of the cultures were evaluated.

Histological procedures

Light Microscopy

Samples from treatments at different incubation times in PVS3 (0, 180 and 240 min) and samples from previously described treatments subjected to LN were used for histological analysis. Samples were fixed in paraformaldehyde (2.5%) in sodium phosphate buffer 0.2 M (pH 7.2) for 16-18h, followed by dehydration in increasing series of ethanol aqueous solutions. After dehydration, samples were infiltrated with Historesin (Leica® Historesin, Heidelberg, Germany). Sections of 5 µm were obtained using a Slee Technik® microtome, distended on slides with a drop of water and kept at a temperature of 42°C ± 2°C. After water evaporation, sections were submitted to PAS/CBB double-staining (Schmidt et al. 2012). PAS was used to identify the presence of neutral polysaccharides (starch grains and cellulose), and CBB was used to detect total protein presence. Relevant aspects have been identified and captured using an Olympus® DP 71 camera attached to an Olympus® BX-40 microscope.

Transmission Electron Microscopy (TEM)

TEM was performed according Schmidt et al. (2012). The same samples used in light microscopy were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight. The material was post-fixed with 1% osmium tetroxide for 4 h, dehydrated in a graded acetone series, and embedded in Spurr's resin. Thin sections were stained with aqueous uranyl acetate, followed by lead citrate, according to Reynolds (1963). Two samples per replication were then examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV). Similarities based on the comparison of individual treatments with replicates suggested that the ultrastructure analysis was reliable.

Data analysis

The data are presented as the means and were analysed by ANOVA ($P<0.05$), followed by the SNK (Student-Newman-Keuls) test ($P<0.05$) (Sokal and Rohlf 1995). Experiments were replicated three times. The means and standard errors were also used to analyze the data when ANOVA and the SNK test were not used. Data were analyzed using Statistica® (Statsoft Inc., Tulsa, OK, USA) for Windows® version 7.0.

Results and Discussion

PVS3 vitrification solution was used based on previous results showing that peach palm somatic embryos are more sensitive to DMSO, which is present in the PVS2 vitrification solution (data not shown). To prevent cellular damage by chemical toxicity or excessive tension during osmotic dehydration, vitrification techniques require careful control of highly concentrated solutions (Fábián et al. 2008). Thus, in any cryogenic procedure, finding the optimal incubation time in vitrification solution requires establishing the correct balance between toxicity and adequate cellular dehydration such that vitrification can occur upon rapid cooling in LN without undergoing lethal intracellular freezing (Fábián et al. 2008). However, it should be noted that studies on cryopreservation of callus and polyembryoids of date palm and oil palm, respectively, have successfully used PVS2 as the vitrification solution (Al-Bahrany and Al-Khayri 2012; Suranthran et al. 2012).

Treatment of somatic embryo clusters for different periods of incubation in PVS3 resulted in different regrowth rates compared to control cultures (no LN). In control cultures without PVS3, the regrowth rate was 85.2% and 96.3% after 6 and 12 weeks of culture, respectively (Table 1). An inverse correlation between incubation time and regrowth rate of the cultures was observed. Initially, 70.4% of the cultures submitted to PVS3 during 60 min and inoculated in the regrowth medium for 6 weeks showed the development of new somatic embryos, decreasing to a regrowth rate of 25.9% when treated during 240 minutes, with a similar rate after 12 weeks (Table 1). Increasing the incubation time in PVS3 normally decreases cell viability (Kohmura et al. 1992). However, after 12 weeks of culture for all treatments, the regrowth rate was greater than, or equal to, the rates observed after 6

weeks, independent of the incubation time in PVS3. In the cryopreservation of oil palm (*Elaeis guineensis*) polyembryoids, incubation in PVS2 in excess of 10 minutes caused their death (Suranthran 2012). However, when these polyembryoids were not incubated in PVS2 and further immersed in LN, they also died. Incubation during 5 minutes in PVS2, however, resulted in a survival rate of 45% in regrowth after cryopreservation. As stated by Niino et al. (1992), the time of incubation in vitrification solution is dependant not only on weight and size, but it is also species-specific.

The vitrification technique (Fig. 1a) also showed differences according to the time in PVS3 prior to the immersion of somatic embryos in LN. Specifically, when no incubation was used or the incubation time was reduced (60 minutes), no regrowth was observed (Table 1). However, after an incubation of 120 minutes, the cultures survived the LN, resulting in 11.1% of regrowth after 12 weeks. The best incubation time associated with the vitrification technique was 240 minutes, resulting in a regrowth rate of 25.9% after 6 weeks (Fig. 1b) and 37% after 12 weeks (Fig. 1c; Table 1). Cellular metabolism is practically null at -196 ° C (Benson 2008), entering in a theoretically unlimited steady state. After recovering the somatic embryos from LN, cell metabolism slowly reactivated, albeit requiring some time to resume normal metabolism, which may explain the observed differences in growth rate between 6 and 12 weeks.

It is important to stress that the minimal regrowth rate acceptable for a cryopreserved germplasm bank is 30% (Gonzalez-Arnao et al. 2008). In the present work, the results obtained for regrowth of the peach palm somatic embryos at 37% are, therefore, above the minimum expected regrowth rate.

Histological and ultrastructural analysis

As shown in Figure 2a, somatic embryos not submitted to PVS3 or LN were structurally preserved, presenting a correct delimitation of nuclear proteins, with no apparent cell wall disruption, and showing starch granules and dense cytoplasm. Ultrastructure analyses of these somatic embryos showed small cells with small vacuoles and lipid bodies (Fig. 3a), a structurally preserved cell wall with a middle lamella (Fig. 3b), numerous visible mitochondria, rough endoplasmic reticulum (Fig. 3c), Golgi bodies (Fig. 3d) and a large central nucleus with

prominent nucleolus (Fig. 3e). Taken together, such features suggest an active cell metabolism associated with viable embryogenic cells. These cells present the same characteristics as those described by Steinmacher et al. (2011), who demonstrated the multicellular origin of somatic embryos from epidermal and subepidermal cells of peach palm secondary somatic embryos.

When these SEC were not cryopreserved and only submitted to PVS3 during 180 and 240 min (Fig. 2b and 2c, respectively), they showed cell disruption, albeit at a lesser intensity than SEC submitted to LN. In these cells, the main change was a less dense cytoplasm and increases in the extent of vacuolation and vacuolar fusion, characteristics also observed in cryopreserved *Amaryllis belladonna* zygotic embryos (Sershen et al. 2012a). These authors showed that the proportion of the cytoplasm occupied by vacuoles was increased upon cryoprotection with either glycerol or sucrose. These events generally occur prior to autophagy and were previously described in recalcitrant embryonic axis cells of a number of species in response to desiccation stress (Wesley-Smith et al. 2001). In another work, madder (*Rubia akane* Nakai) roots exposed to various cryoprotectant solutions showed some plasmolysed cells (Yil et al. 2012).

In the present work, the ultrastructure analysis showed viable cell characteristics associated with numerous mitochondria and prominent nucleus (Fig. 4a), preserved cell walls (Fig. 4b) and rough endoplasmic reticulum (Fig. 4c) after PVS3 incubation. However, in some cells, unpreserved structures associated with cell wall disruption (Fig. 4d), changes in the nuclear envelope with expansions in the nuclear cisternal spaces (Fig. 4e) and, in the worst case, the presence of disrupted cells (Fig. 4f) were all observed. Cisternal spaces may be induced by the rupture of several nearby nucleus pore complexes, as previously observed in PVS2-dehydrated rice embryogenic cells (Wang et al. 1998). *A. belladonna* zygotic embryos treated during one hour with 5% glycerol did not show ultrastructural abnormalities in nuclei (Sershen et al. 2012a). In the present work, changes in the nuclear envelope were also observed (Fig. 4e) in response to PVS3 (240 min), which contains 50% glycerol. Helliot et al. (2003) cryopreserved banana meristems that showed some effects of PVS2 associated with a range of minor, mild or severe plasmolysis in cells of the corpus after incubation for 2h, with most of the cells showing dense nuclei. Therefore, it is important to

determine the optimal exposure time to PVS3 before cryopreservation, allowing the cryoprotectant solution to enter cells without causing damage (Kohmura et al. 1992).

Somatic embryos submitted to LN without incubation in PVS3 cryoprotectant solution showed collapsed cells (Fig. 2d), a phenomenon evidenced by nucleus disruption, spreading of protein bodies and disintegration of cell walls (Fig. 2d). Ultrastructurally, it was possible to observe the disruption (Fig. 5a) and disintegration of the cell wall (Fig. 5b). Cell wall disruption was also observed in cryopreserved cultures of *Rubia tinctorum* (Yil et al. 2012) and protocorm-like bodies of Vanda Kaseem's Delight orchid (Poobathy et al. 2012). In both cases, these effects were attributed to the rapid freezing method used in the cryopreservation technique based on vitrification. Most likely, cell wall rupture explains viability and function losses in some cryopreserved cells. As shown in the present work, the structure of the nucleus is also affected by disruption of the nuclear envelope (Fig. 5c), releasing its contents into the cytoplasm. In *Gentiana* spp., ultrastructure analysis revealed the leakage of nuclear proteins across the cell nuclear envelope after LN removal (Mikula et al. 2006).

Other cellular organelles, such as mitochondria, were also damaged by cryopreservation (Fig. 5d). Large vacuoles were observed (Fig. 5e); in contrast, only small vacuoles appeared in cells not treated with either PVS3 or LN (Fig. 3a). The middle lamella was also greatly affected by LN (Fig. 5e). The proportion of heterochromatin to euchromatin was increased, possibly as a cell defense mechanism, as observed in the unstructured nucleus (Fig. 5f). Cryopreserved *Livistona chinensis* zygotic embryos presented similar cellular features after freezing, including mitochondrial swelling, nuclear shrinkage and chromatin condensation, rupturing of the nuclear envelope, as well as increased heterochromatin (Wen et al. 2012).

Vitrification circumvents the problems associated with ice formation (Benson 2004). Figures 2e and 2f show that the SEC tissues submitted to PVS3 during 180 and 240 min were structurally more preserved after LN as compared to cells not incubated in PVS3 (Fig. 2d). In addition, the preserved nuclear and nucleoli organization of the cells showed a dense cytoplasm.

It is important to stress that the observed cellular collapse cannot be attributed to the direct effects of LN alone since PVS3 can also result

in cell structure degradation. In the present work, cells were greatly influenced by PVS3, showing degradation in their structure, as the incubation time increased (Fig. 4). However, the highest regrowth rate of cryopreserved SEC (37%) occurred in response to the longest incubation time (240 min). This was confirmed by the ultrastructure analysis of these cells. The SEC incubated in PVS3 during 180 min and then cryopreserved showed structurally preserved nuclei (Fig. 6a) and cell walls (Fig. 6b), as well as most uncondensed chromatin (Fig. 6c). However, some features associated with cell degradation were demonstrated, such as the appearance of a space between the protoplasm and the cell wall (Fig. 6a-b), possibly indicating plasmolysis. On the other hand, SEC incubated during 240 min and subjected to LN were structurally more preserved showing intact nuclei and cell walls (Fig. 6d), a number of mitochondria and lipid bodies (Fig. 6e), presence of many starch granules (Fig. 6f), and condensed chromatin. All these features indicate a quite active cell metabolism unlike that observed in cells incubated in PVS3 for a shorter time (180 min). Embryogenic peach palm cultures cultivated in TIS also showed greater starch accumulation as compared to gelled cultures (Steinmacher et al. 2011). This may have influenced the responses to cell cryopreservation since a greater content of storage compounds is associated with a low content of free water, thus avoiding the formation of ice crystals, the main cause of cell collapse at cryogenic temperatures.

Based on the results of the present work, we can conclude that the cells of somatic embryogenic clusters of *Bactris gasipaes* not incubated in PVS3 did not survive after cryopreservation in liquid nitrogen. On the other hand, cells submitted to PVS3 had viable cell characteristics associated with numerous mitochondria, prominent nucleus and preserved cell walls. The best incubation time for vitrification was 240 minutes, resulting in a survival rate of 37%. Moreover, these cells showed intact nuclei and preserved cell walls, a number of mitochondria and lipid bodies, presence of many starch granules and condensed chromatin, all features indicating active cell metabolism and a preserved cellular activity in cells successfully recovering from the cryogenic process. Ultrastructure analysis revealing overall cellular structures preserved after the cryogenic treatment validates the vitrification technique for the cryopreservation of peach palm elite genotypes, as

well as wild genotypes which carry a rich pool of genes that must be conserved.

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

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Figure

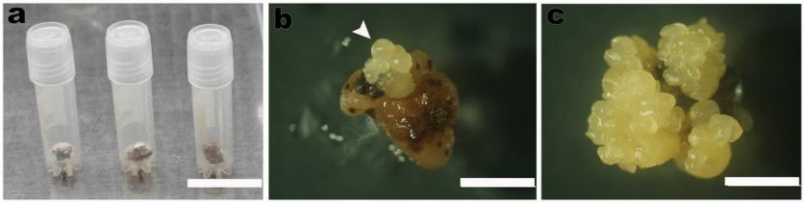

Figure 1 Somatic embryo clusters (SEC) of *Bactris gasipaes* recovered from liquid nitrogen. a) Cryovials containing SEC immersed in PVS3 (bar = 2 cm). b) Regrowth of SEC (arrow) submitted to PVS3 (240 min) and cryopreserved during 6 weeks (bar = 2 mm) and c) after 12 weeks (bar = 5 mm)

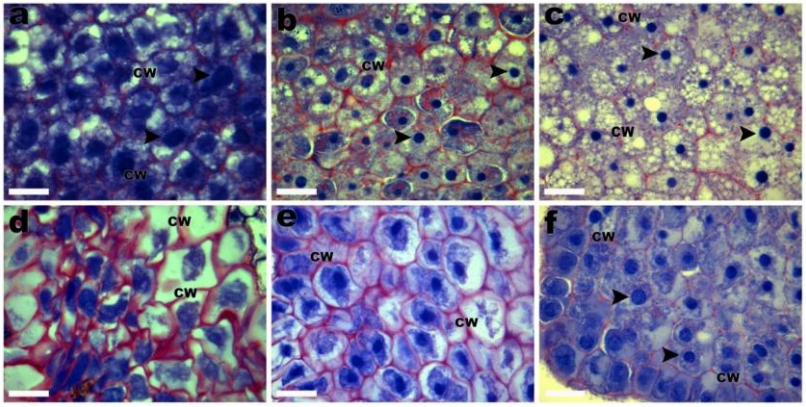

Figure 2 Light microscopy of transversal sections of *Bactris gasipaes* somatic embryo clusters (SEC). a) SEC cells not submitted to liquid nitrogen (LN) or PVS3, showing organized nuclei and preserved cell wall; b-c) Cells of SEC not submitted to LN, but incubated in PVS3, during 180 and 240 min, respectively, showing nuclear disintegration and cell wall disruption; d) Cells of SEC submitted to LN and not incubated in PVS3, showing nuclear disintegration and cell wall disruption; e-f) Cells of SEC submitted to LN and incubated in PVS3 during 180 min and 240 min, respectively, showing organized nuclei and preserved cell wall; Bars: 50 µM; CW: cell wall; arrow: nuclei

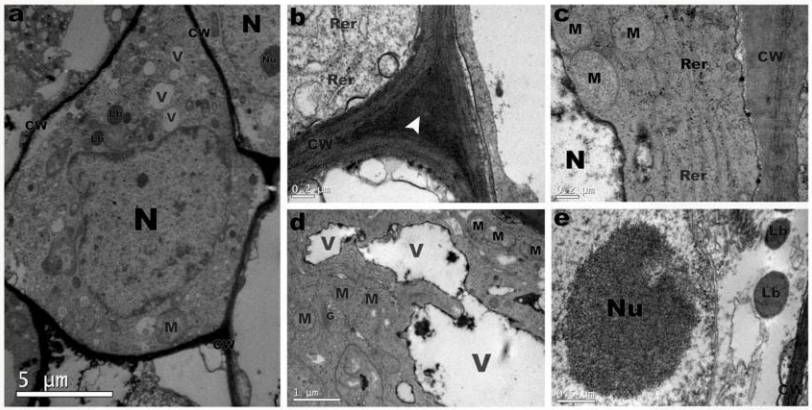

Figure 3 Transmission electron microscopy of *Bactris gasipaes* somatic embryo clusters (SEC) not submitted to PVS3 or liquid nitrogen (LN). SEC presenting small cells with preserved structures, such as a) numerous small vacuoles and lipid bodies; b) integrated cell wall with middle lamella; c) numerous and visible mitochondria, as well as rough endoplasmic reticulum; d) Golgi bodies; and e) large central nucleus with prominent nucleolus. CW: cell wall; G: Golgi body; Lb: lipid body; M: mitochondria; N: nucleus; Nu: nucleolus; Rer: rough endoplasmic reticulum; V: vacuole; arrow: middle lamella

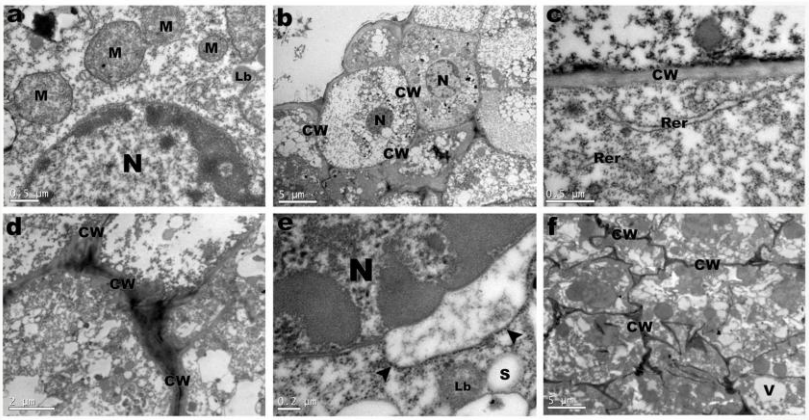

Figure 4 Transmission electron microscopy of *Bactris gasipaes* somatic embryo clusters (SEC) not cryopreserved, but submitted to PVS3. SEC cells showing well-preserved features before PVS3 incubation: a) numerous mitochondria and prominent nucleus; b) preserved cell wall; c) rough endoplasmic reticulum. SEC presenting some unpreserved structures in a few cells before PVS3 incubation: d)

cell wall disruption; e) changes in the nuclear envelope; f) presence of disrupted. CW: cell wall; Lb: lipid body; M: mitochondria; N: nucleus; S: starch; V: vacuole; arrow: changes in the nuclear envelope

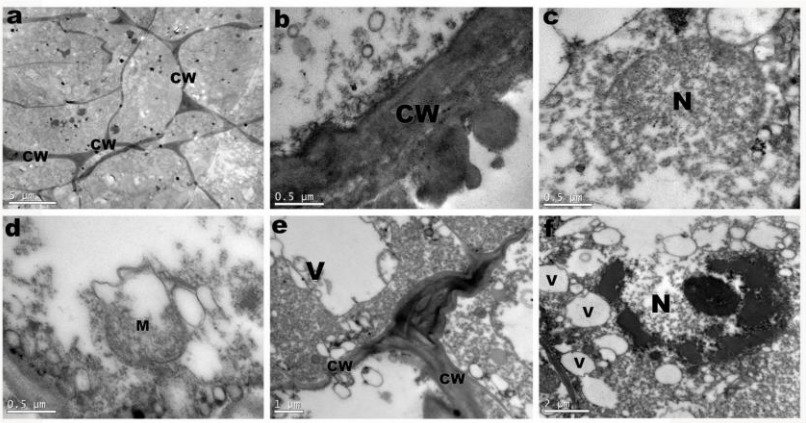

Figure 5 Transmission electron microscopy of *Bactris gasipaes* somatic embryo clusters (SEC) cryopreserved, but not submitted to PVS3. SEC cells presenting unpreserved structures, including a) complete cell disruption; b) destructuring of the cell wall; c) disruption of the nuclear envelope, releasing its contents into the cytoplasm; d) disruption of mitochondria; e) presence of large vacuoles; f) unstructured nucleus. CW: cell wall; M: mitochondria; N: nucleus; V: vacuole

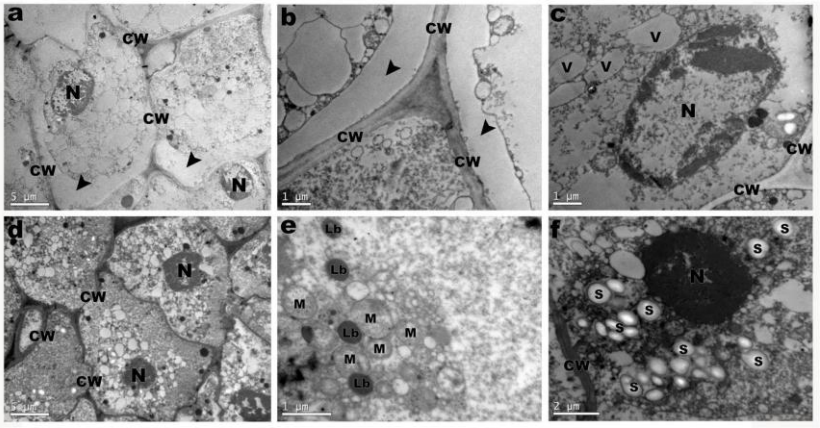

Figure 6 Transmission electron microscopy of *Bactris gasipaes* somatic embryo clusters (SEC) submitted to PVS3 solution during 180 and 240 min and cryopreserved. SECs submitted to PVS3 for 180 min and cryopreserved presenting a) structurally preserved nuclear envelope, space between the protoplasm and cell

wall; b) preserved cell walls; c) inconspicuous chromatin. SECs submitted to PVS3 during 240 min and cryopreserved showing d) preserved nuclei and cell walls; e) large number of mitochondria and lipid bodies; f) starch granules near the nucleus. CW: cell wall; Lb: lipid body; M: mitochondria; N: nucleus; V: vacuole; arrow: space between the protoplasm and the cell wall

Tables

Table 1 Regrowth rate (%) of *Bactris gasipaes* somatic embryo clusters (SEC) submitted to PVS3 and cryopreserved in liquid nitrogen (+LN) by means of vitrification

Time in PVS3	Control (-LN)		Vitrification (+LN)	
	6 weeks	12 weeks	6 weeks	12 weeks
0 min.	85.2a	96.3a	0c	0b
60 min.	70.4ab	74.1b	0c	0b
120 min.	59.3b	66.7b	3.7c	11.1b
180 min.	37c	37c	14.8b	29.6a
240 min.	25.9c	25.9c	25.9a	37.0a

*Values followed by the same letter in the same column are not significantly different at the 0.05 probability level (n = 9).

Considerações Finais e Perspectivas Futuras

O presente trabalho apresentou resultados relevantes para a multiplicação massal da pupunha a partir da embriogênese somática (ES) aliada ao sistema de imersão temporária (SIT), o que pode ajudar na conservação e melhoramento genético da espécie. A multiplicação de embriões somáticos apresentou melhores características morfológicas e bioquímicas quando multiplicados em SIT, comprovando a importância desses sistemas na otimização de um protocolo de ES. Na etapa de maturação, o ABA em meio de cultura semi-sólido resultou na uniformização dos embriões somáticos e facilitando as etapas posteriores, mostrando que a maturação é uma etapa fundamental, sendo o gargalo do protocolo de ES para esta espécie. A etapa inicial da conversão, sem a suplementação de fitoreguladores e em meio semi-sólido, possibilitou a obtenção de grande número de embriões verdes, que posteriormente foram subcultivados para SIT originando um grande número de plântulas e mostrando a importância da mudança de sistema de cultivo para acelerar determinada rota morfogênica. E na última etapa do processo, a aclimatização, os tamanhos iniciais das plântulas a serem aclimatizadas influenciaram fortemente no resultado final, sendo os melhores resultados obtidos com plântulas com altura média de 7 cm. Novos trabalhos devem ser conduzidos com outros genótipos da espécie, avaliando assim o papel do genótipo na embriogênese somática de pupunha.

Eficientes estratégias para a conservação de embriões somáticos à longo prazo também foram abordadas no trabalho, sendo um resultado inédito, podendo representar uma nova abordagem para conservação do germoplasma da pupunha. A solução crioprotetora PVS3 foi fundamental para a convervação dos embriões somáticos submetidos ao nitrogênio líquido, não havendo sobrevivência sem esta. A incubação dos embriões somáticos em solução crioprotetora PVS3 também modificou as células sob o prisma epigenetico e ultraestrutural, mostrando a importância de definir um tempo de incubação que permita conservar as estruturas celulares sem apresentar toxidez e possibilitando às mesmas retornarem ao seu metabolismo original. As análises de metilação global do DNA e de ultraestrutura celular possibilitaram avaliar o comportamento das células durante os protocolos de criopreservação e saber por que estas sobreviviam ou não quando eram

submetidas a essas técnicas. Novos trabalhos devem ser conduzidos para analizar diferenças morfo-histoquímicas das plântulas regeneradas de embriões somáticos criopreservados em comparação com os não criopreservados do mesmo genótipo.

E por fim, a técnica de vitrificação-*droplet* se mostrou a mais eficiente na criopreservação de embriões somáticos de pupunha, apresentando altas taxas de recrescimento, provando ser a técnica mais atual e eficiente do momento. Novos trabalhos devem ser conduzidos com outros genótipos desta espécie, avaliando assim o papel do genótipo na criopreservação de embriões somáticos de pupunha.