Joseph Francis Ree

IMPERMANENCE OF SOMATIC EMBRYOGENESIS: THE EFFECTS OF AND SOLUTIONS TO GRADUAL AGING OF PEACH PALM (BACTRIS GASIPAES KUNTH) CULTURES IN VITRO

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Impermanence of somatic embryogenesis: the effects of and solutions to gradual aging of peach palm (*Bactris gasipaes Kunth*) cultures *in vitro*

por

Joseph Francis Ree

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This work is dedicated to the many teachers who helped me get to where I am.

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I have not failed. I've just found
10,000 ways that won't work

-Thomas Edison

Our virtues and our failings are
inseparable, like force and matter.
When they separate, man is no more

-Nikola Tesla

RESUMO

Culturas embriogênicas podem ser cultivadas por anos, mas acumulam características deletérias ao longo do tempo: crescimento reduzido; aumento da necrose; estruturas aberrantes; maturação impedida; e, eventualmente, perda completa do potencial embriogênico. Esse fenômeno ocorreu em uma linha de tecido de pupunha (*Bactris gasipaes* Kunth) após oito anos de cultivo. Esta tese investigou esta ocorrência através de três diferentes abordagens: (i) o uso de 5-azacitidina para reverter a hipermetilação relacionada à idade e análise da abundância de proteína em culturas de dois anos e oito anos para determinar quais processos de plantas foram os mais afetados pelo envelhecimento; (ii) otimizar a conversão de plântulas de embriões somáticos com vasos selados ou ventilados, a fim de fornecer uma fonte de plântulas para a criação de novas linhas de cultura e avaliar o efeito do ambiente de cultura sobre bioquímica celular; e (iii) prevenir o envelhecimento através do desenvolvimento de novas estratégias para otimizar a vitrificação de embriões somáticos. A 5-azacitidina estimulou o crescimento de embriões somáticos em culturas de oito anos de idade. No entanto, o crescimento de culturas embriogênicas de dois anos de idade foi interrompido. A análise proteômica mostrou que todos os tecidos foram submetidos à hipóxia, sugerindo que todos os tecidos dependiam da fermentação como meio de geração de energia. A fermentação leva à acidificação citosólica, que pode causar danos a importantes processos celulares, como o metabolismo. A análise por HPLC-ELSD mostrou que as culturas de dois anos tinham quantidades detectáveis de ribose, arabinose e sacarose, mas nenhum carboidrato foi detectado em culturas de oito anos de idade. Além disso, muitos genes conservados relacionados ao controle epigenético foram acumulados em culturas de dois anos em comparação com culturas de oito anos de idade, sugerindo que as culturas mais jovens mantiveram uma maior quantidade de controle epigenético. No segundo capítulo, os vasos ventilados levaram a um menor crescimento de massa fresca comparado aos vasos selados de cultura, mas apresentaram maior massa seca e maior emergência de clorofila, sugerindo uma maior taxa de conversão embrionária. Além disso, maior abundância do total de poliaminas, e especialmente de putrescina e menor quantidade de frutose disponíveis e glicose sugerem que as culturas em vasos ventilados foram submetidos a grandes taxas de mudança, mas suas fontes endógenas de nutrientes pode ser insuficiente para abastecer a conversão completa de um embrião para um plantlet. No terceiro capítulo, várias estratégias de otimização mostraram grande potencial para

melhorar o protocolo de vitrificação da pupunha. Culturas vitrificadas descongeladas na temperatura mais quente (75 ° C) tiveram as maiores taxas de rebrotação. Uma solução de vitrificação planta três diluída a 80% da sua concentração, incluindo 0,1 M de KCl, MgCl2, MgSO4 ou K2H (PO4) melhorou recrescimento. Além disso, a desidratação parcial melhorou o recrescimento das culturas possivelmente porque foi menos traumático para o tecido em comparação com a exposição com alta concentração de solutos. Os resultados desses três capítulos oferecem uma explicação do motivo pelo qual as culturas embriogênicas envelhecem e várias estratégias distintas para evitá-las.

RESUMO EXPANDIDO

Introdução

A capacidade de cultivar plantas *in vitro* é uma tecnologia poderosa: plantas raras, economicamente importantes ou ameaçadas podem ser propagadas para conservação ou reflorestamento; genótipos valiosos podem ser produzidos em larga escala para a agricultura; e modelos biológicos para estudar vários aspectos da genética, bioquímica e morfologia de plantas podem ser estabelecidos e mantidos, teoricamente, indefinidamente. Culturas embriogênicas podem ser cultivadas por anos, entretanto, com tempo seu potencial de crescimento diminui; suas estruturas tornam-se malformadas; e começam a perder a capacidade de desenvolver estruturas especializadas. Essa seqüência de eventos ocorreu ao longo de oito anos em uma linha de tecido embriogênica de pupunha (Bactris gasipaes). Várias teorias sugerem que esse fenômeno ocorre devido a fatores como a habituação a reguladores de crescimento exógenos ou a hipermetilação de seções codificadoras de genes essenciais. Nenhum estudo analisou as mudanças subjacentes no acúmulo de proteína entre uma linha de tecido de dois anos de idade e uma linha de oito anos de idade, de modo que isso oferece uma nova visão sobre o que pode causar essa mudança gradual ao longo do tempo. Além disso, a substância química 5-azacitidina, por ser um inibidor da metilação, potencialmente poderia testar a teoria do envelhecimento in vitro como uma função da hipermetilação, mas o efeito caótico na regulação epigenética de culturas mais jovens pode ter um efeito deletério. Outra maneira de evitar o envelhecimento in vitro é fazer um ciclo de culturas: indução da embriogênese somática; multiplicação de culturas por vários anos; crescimento de plântulas dos embriões somáticos; e o uso de tecido das plântulas para induzir a embriogênese somática na próxima linha de tecido. Embora a embriogênese somática e a multiplicação de culturas

tenham sido extensivamente estudadas, os últimos estágios da conversão de embriões em plântulas e aclimatação não são. O ambiente in vitro possui alta umidade, sem troca gasosa. O fornecimento de troca gasosa pode começar a processar a aclimatação da plântula ao ambiente ex vitro sem tanto estresse, e assim manter efetivamente um genótipo em vários estágios de crescimento. A criopreservação é uma abordagem mais direta porque uma linha de cultura pode ser mantida por anos sem acumular efeitos deletérios devido ao envelhecimento e também requer menos materiais para manter. Um método de vitrificação já foi desenvolvido para pupunha, mas muitos fatores podem melhorar ainda mais a baixa taxa de sobrevivência. Essas abordagens incluem o uso das propriedades físicas da água para melhorar as taxas de congelamento e descongelamento e diminuir o efeito toxico dos crioprotetores no tecido.

Objetivos

Nesta tese de doutorado, eu explorei o conceito de impermanência na cultura de tecidos, mais especificamente com a embriogênese somática de pupunha como um modelo. Esta tese é dividida em tres capítulos: (i) estudo sobre o uso de 5-azacitidina para reverter a perda de potencial embriogênico relacionada à idade, bem como a comparação de proteínas diferencialmente acumuladas entre uma linha de cultura de oito anos de idade e uma linha de cultura de dois anos de idade; (i) estudo comparativo da resposta de crescimento e bioquímica diferencial de culturas de pupunha em frascos ventilados e selados; e (iii) estudo de várias estratégias para melhorar a criopreservação de culturas embriogênicas de pupunha. O objetivo do primeiro capítulo foi avaliar as diferenças no acúmulo de proteínas e carboidratos entre uma linha de tecido de dois anos de idade e uma linha de tecido de oito anos para determinar quais os efeitos do tempo na bioquímica celular. Além disso, culturas de dois anos de idade e culturas de oito anos de idade foram tratadas com 5-azacitidina para avaliar seus efeitos na reversão da hipermetilação relacionada à idade e morfologia tecidual. O objetivo do segundo capítulo foi avaliar o efeito de recipientes selados ou ventilados na estimulação da conversão de embriões somáticos. Além disso, essas diferenças foram quantificadas através de uma análise de metilação global, poliaminas e carboidratos para avaliar os efeitos do ambiente sobre a atividade celular. O objetivo do terceiro capítulo foi avaliar novas abordagens para a criopreservação de embriões somáticos de pupunha e determinar quais abordagens serviriam de base para um protocolo otimizado.

Metodologia

Capítulo 1: culturas embriogênicas de dois anos de idade, culturas não embriogênicas com dois anos de idade e culturas de oito anos de idade foram cultivadas em meio de cultura com 0, 4, 16 ou 64 uM de 5-azacitidina por duas semanas. Elas foram então removidas para meio sem 5-azacitidina e avaliadas quanto ao tipo de crescimento tecidual e necrose. Amostras de cada tipo de cultura foram avaliadas para metilação global usando HPLC, e quantidades específicas de carboidratos foram avaliadas com HPLC-ELSD. Culturas embriogênicas de dois anos de idade, culturas de oito anos de idade e culturas embriogênicas desenvolvidas a partir de culturas de oito anos tratadas com 5-azacitidina foram avaliadas com proteômica *shotgun*.

Capítulo 2: Culturas embriogênicas foram maturadas em meio de cultura com 5 μM de ácido abscísico e, após, colocadas em frasco selado ou ventilado. Após 42 dias, foram avaliados o crescimento de massa fresca, o peso seco e o desenvolvimento de clorofila. Estas amostras foram então avaliadas quanto ao seu conteúdo de poliaminas, metilação global e carboidratos específicos com HPLC.

Capítulo 3: Culturas embriogênicas foram submetidas a uma série de experimentos de otimização baseados em protocolos de vitrificação estabelecidos. Diferentes temperaturas de descongelamento (30, 45, 60, 75 ° C) e duração (1, 2 ou 3 min) e diferentes métodos de criopreservação foram testados. A solução de vitrificação também foi testada com avaliação por substituição de água com água pesada; reduzir a concentração global dos solutos dentro da solução; e adicionando sais. A desidratação antes da vitrificação também foi testada.

Resultados e Discussão

Capítulo 1: as culturas de pupunha tratadas com 5-azacitidina, um potente inibidor da metilação da citosina, mostraram vários resultados importantes: os tecidos embriogênicos de dois anos de idade tendem a perder a regulação morfológica com concentrações altas de 5-azacitidina, resultando na formação de massas amorfas de tecido não embriogênico e calos amarelos; aumento de tecido necrosado; desenvolvimento em 8,80% de embriões somáticos pela primeira vez após dois anos, tratadas com 16 μM de 5-azacitidina, nas culturas com oito anos de idade. Culturas embriogênicas de dois anos de idade, culturas de oito anos de idade e embriões derivados da mesma cultura tratadas com 5-azacitidina, foram comparadas usando *shotgun proteomics*. Todas as três culturas mostraram

sinais crescimento sob condições hipóxicas na presença de piruvato descarboxilase e álcool desidrogenase, mas observou-se que o tecido embriogênico era melhor equipado para essas condições através de proteínas acumuladas envolvidas na manutenção do estado redox celular e regulação epigenética. Culturas de oito anos de idade mostraram uma *down-accumulation* de proteínas envolvidas em muitos aspectos do metabolismo. Uma análise de carboidratos com HPLC-ELSD mostrou que as culturas de oito anos de idade não continham quantidades detectáveis de oito carboidratos comuns, como sacarose, glicose ou frutose. No entanto, culturas embriogênicas e não embriogênicas de dois anos de idade mostraram quantidades detectáveis de ribose, arabinose e sacarose. Além disso, muitas proteínas relacionadas à parede celular foram acumuladas em tecido de oito anos de idade, possivelmente sugerindo uma conexão entre a textura fibrosa do tecido e seu estado celular. Esses resultados mostram que, ao longo do tempo, condições hipóxicas contínuas criaram um ambiente insalubre onde a célula foi forçada a gerar energia com pouco oxigênio disponível - levando a um citosol acidificado. Esta produção de energia diminuída pode levar a uma perda na capacidade da célula de manter seu estado redox, que pode exacerbar ou ser exacerbado por uma quebra no controle epigenético, levando à ruptura do comportamento celular e à perda do potencial embriogênico.

Capítulo 2: embriões somáticos colocados em vasos ventilados tiveram menor ganho de massa fresca em comparação com aqueles em frascos fechados, mas essas mesmas culturas apresentaram peso seco total mais alto, maior número de embriões fotossintéticos em desenvolvimento e continham concentrações mais altas de poliaminas totais. Além disso, embora estivesse na fronteira de significância estatística, embriões colocados em ventilação poderiam ter utilizado algumas de suas reservas de energia para desenvolver cloroplastos, como sugerido por uma diminuição nas concentrações médias de glicose ($p = 0,0624$) e frutose ($p = 0,0587$). A metilação global entre os dois tecidos não foi significativa, mas ambas as culturas em vasos selados ou ventilados apresentaram taxas de metilação global mais altas em comparação com culturas em multiplicação.

Capítulo 3: a velocidade com que um tecido é descongelado é tão importante quanto a temperatura em que o tecido é congelado: o tecido descongelado a 70°C apresentou o maior crescimento após 12 semanas, e o tecido descongelado a 30°C teve o menor recrescimento,

independentemente da duração do descongelemento. O tamanho do *cluster* embriogênico também teve um impacto significativo na regeneração: apesar dos *clusters* de menor tamanho absorverem a solução de vitrificação mais rapidamente, tanto as culturas vitrificadas como as não vitrificadas sofreram severas quedas na regeneração à medida que a duração da incubação aumentou. *Clusters* maiores, no entanto, tiveram taxas de regeneração mais altas em todas as durações. Foram também tentativas para melhorar a solução de vitrificação 3 (PVS3; 50% w/v glicerol, 5-% w/v sacarose)—uma solução, embora tóxica, necessária para a criopreservação de vitrificação de pupunha usando duas abordagens gerais: o uso de sais dissolvidos e uma diluição de PVS3 para 80% da sua concentração inicial; e o uso de água pesada com cloreto de magnésio. Em comparação com o PVS3, 80% PVS3 resultou em taxas de regeneração inicialmente menores, embora o potencial de regeneração em ambos fossem menores do qualquer uma das misturas de 80% de PVS3 com 0,1 M KCL, $MgCl_2$, $MgSO_4$ ou $K_2H(PO_4)$. Isto pode ser devido à utilização de várias partículas carregadas na manutenção da conformação da macromolécula, manutenção do equilíbrio de carga elétrica entre a célula e a solução para evitar a perda de íons com a difusão de água para fora das células durante a incubação de PVS3; ou um outro fator. O resultado do experimento com água pesada foi menos pronunciado: o $MgCl_2$ não teve efeito significativo, e a água pesada, apesar de ter um efeito significativo positivo, não resultou em taxas de regeneração mais altas, mas não por muito. Os pré-tratamentos de desidratação por uma ou duas horas melhoraram as taxas de regeneração das culturas incubadas por 60 ou 120 minutos em PVS3, todavia, desidratações mais longas e períodos de incubação mais longos mostraram uma grande perda na regeneração. Com base nesses resultados, o protocolo de criopreservação melhorado tem os seguintes resultados: taxas de descongelamento mais rápidas; pouca manipulação do tecido; concentração reduzida de PVS3 e com 0,1 M de qualquer um dos quatro sais dissolvidos testados; e desidratação parcial.

Considerações Finais

Estes resultados mostram que o envelhecimento durante a cultura de tecido é potencialmente evitável. A cultura contínua em um ambiente no qual os tecidos são submetidos a culturas de forças de hipóxia depende da fermentação, causando ruptura dos processos celulares internos. Embora

a 5-azacitidina tenha estimulado o retorno do crescimento somático em culturas com oito anos de idade, ela interrompeu as culturas mais jovens, tornando seu uso limitado. Permitir uma maior circulação de ar no recipiente de cultura provavelmente reduzirá o envelhecimento, fornecendo mais energia aos tecidos para se desenvolver. Conversão melhorada de culturas em frascos ventilados oferece um potencial para melhorar a geração de plântulas, que pode então servir como uma fonte de tecido para criar uma nova linha de culturas quando as culturas estabelecidas começam a mostrar idade. No entanto, mais trabalho é necessário porque os resultados sugerem que carboidratos endógenos são outro fator importante. Embriões somáticos no processo de conversão tiveram menos reservas, o que pode reduzir sua capacidade de se desenvolver em plântulas. Este trabalho deve servir como base para um estudo maior para melhorar este fator com o resultado esperançoso de aumentar o crescimento das plântulas. Pois forneceria um ciclo de renovação que permita que as culturas sejam mantidas in vitro, mas às custas de exigir várias etapas. Em contraste, a vitrificação oferece um meio de preservar o tecido sem muita manutenção, especialmente com as abordagens mais bem sucedidas usadas separadamente e, potencialmente, juntas. Estas abordagens oferecem várias variações nas quais uma linha de tecido vegetal embriogênico pode ser mantida in vitro permanentemente.

Palavras-chave: embriogênese somática; especificação celular; envelhecimento *in vitro*; perfil bioquico; criopreservação

ABSTRACT

The ability to grow plants *in vitro* is a powerful technology: rare, economically-important, or endangered plants may be propagated for conservation or reforestation; valuable genotypes may be mass-produced for agriculture; and models for studying various aspects of plant genetics, biochemistry, and morphology can be maintained indefinitely—or nearly. Cultures may be grown for years, but most begin to show their age; they begin to grow slower; their structures become malformed; and they begin to lose the ability to grow specialized structures. In this report, I explore the concept of impermanence in tissue culture using the model species peach palm (*Bactris gasipaes*), with a focus on studying and exploring means around impermanence. This doctorate dissertation is divided into four sections: (i) a bibliographic review; (ii) a study on the use of 5-azacytidine to reverse age-related loss of embryogenic potential and a comparison of differentially-accumulated proteins between an eight-year-old and a two-year-old culture line; (iii) a study comparing the growth response and differential biochemistry of mature peach palm cultures in ventilated and sealed vessels; and (iv) a study of several little-studied or novel factors to improve cryopreservation of embryogenic peach palm cultures. Peach palm cultures treated with 5-azacytidine, a potent inhibitor of cytosine methylation, showed several key results: two-year-old embryogenic tissue tended to lose morphological regulation with increasing concentrations, leading to the growth of non-embryogenic tissue and fast-growing yellow callus; overall increased tissue necrosis in two-year-old embryogenic tissue; and the growth of somatic embryos in 8.80% of eight-year-old-cultures treated with 16 μM 5-azacytidine—the first growth of somatic embryos in this culture line in two years. Two-year-old embryogenic cultures, old cultures, and the embryos derived from 5-azacytidine-treated old cultures were compared using shotgun proteomics. All three cultures showed signs that they were growing under hypoxic conditions by the presence of pyruvate decarboxylase and alcohol dehydrogenase, but either embryogenic tissue was better equipped for these conditions through up-accumulated proteins involved in maintaining the cellular redox state and epigenetic regulation. Old cultures showed a further down-accumulation of proteins involved in many aspects of metabolism, and an HPLC carbohydrate showed no detectable amounts of any of any carbohydrate in eight-year-old cultures, though young embryogenic and non-embryogenic cultures alike showed detectable amounts of ribose, arabinose, and sucrose. Further, many cell wall-related proteins were up-accumulated in eight-year-old tissue,

possibly hinting at a connection between the tissue's fibrous texture and its cellular state. These findings show that, over time, continuous hypoxic conditions create an unhealthy environment where a cell must conduct cytosol-acidifying cellular reactions to produce energy, and this decreased energy production may lead to a loss in ability for the cell to maintain its redox state, which may exacerbate or be exacerbated by a breakdown in epigenetic control, leading to cell behavior disruption and a loss of embryogenic potential. The second chapter details an analysis of the effects of a ventilated or closed environment in stimulating somatic embryo conversion to plantlets. Somatic embryos placed in ventilated vessels had overall less fresh mass gain, but those same cultures had overall higher dry weight, increased number of developing photosynthetic embryos, and contained overall higher concentrations of polyamines. In addition, though it was at the border of statistical significance, embryos placed in ventilation may have expended some of their energy reserves, as suggested by a decreased mean reduction in glucose ($p=0.0624$) and fructose ($p=0.0587$). Global methylation between either tissue was not significant, but both cultures in sealed or ventilated vessels had higher global methylation rates compared to cultures undergoing multiplication. The third chapter contains a series of experiments aimed at improving previously-established cryopreservation protocols through two general perspectives: the physics of water phase transitions and the biological aspect of cellular integrity. Speed is of utmost importance in both freezing and thawing: the faster a sample was thawed, the more likely that it'd regrow, and, consequently, the faster it was frozen, the more likely it would regrow as suggested by the decreased regrowth when droplet vitrification aluminum strips were replaced with polystyrene or less-conductive stainless steel. Embryogenic cluster size likewise had a significant impact on regrowth: though smaller sized clusters seemed to absorb vitrification solution faster, both vitrified and non-vitrified cultures alike faced severe drops in regrowth as incubation duration increased. Large cultures, however, had high regrowth rates at all durations. I also made two general attempts at improving plant vitrification solution 3 (PVS3; 50% w/v glycerol, 50% w/v sucrose)—a required, though toxic solution required for peach palm culture vitrification cryopreservation—using two general approaches: the use of dissolved salts in a reduced-concentration form of PVS3, and the use of heavy water and magnesium chloride. Compared to PVS3, 80% PVS3 showed initially lower regrowth rates, but this climbed as incubation duration increased, though regrowth potential in either were less than any of the 80%PVS3 mixtures with 0.1 M KCl, $MgCl_2$, $MgSO_4$, or $K_2H(PO_4)$.

This may be due to the use of various charged particles in maintaining macromolecule conformation, maintenance of electrical charge balance between the cell and the solution to prevent electrostatic-driven loss of dissolved ions; or maintenance of the charged phospholipid bilayer during rapid changed in a high-osmotic environment, but, simply put, there's no way to know until a more in-depth experiment is conducted. The result of the heavy water experiment was less pronounced: $MgCl_2$had no significant effect, and heavy water, though having a significant positive effect when $MgCl_2$ was ignored as a factor, was not large enough to justify its costly use as part of a protocol. Dehydration pre-treatments for one or two hr improved regrowth rates for cultures incubated for 60 or 120 min. in vitrification solution, but longer dehydrations and longer incubation periods showed a large loss in regrowth. Based on these results, an improved cryopreservation protocol includes the following: faster thaw rates; vitrification strips made from thermally-conductive materials; little tissue manipulation; reduced 80%PVS3 concentration with 0.1 M of any of the four tested dissolved salts; and partial dehydration.

Key words: somatic embryogenesis; cell fate; *in vitro* aging; biochemical profile; cryopreservation

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2,4-D—2,4-dichlorophenocyacetic acid
5-azac—5-azacytidine
ABA—Abscisic acid
AC—Activated charcoal
BAP—6-benzylaminipurine
CV—closed vessel
DW—Dry weight
FW—Fresh Weight
GA3—Gibberellic acid
EC—Embryogenic culture
IAA—Indole-3-acetic acid
IBA—Indole-3-butyric acid
LN—liquid nitrogen
MW—Morel and Wetmore vitamins
MS—Murashige and Skoog media
NAA—1-naphthaleneacetic acid NAA
NEC—Non-embryogenic culture
NO—nitric oxide
OC—Eight-Year-Old Cultures
OC-E—embryogenic cultures derived from OC
PGR—Plant Growth Regulator
PUT—putrescine
PVS3—plant vitrification solution3
ROS—Radical Oxidative Species
SE—Somatic Embryogenesis
SPD—spermidine
SPM—spermine
VV—ventilated vessel

CONTENTS

1 BIBLIOGRAPHIC REVIEW

1.1 THE DEVELOPMENT OF SOMATIC EMBRYOGENESIS AS A TOOL

Under the right conditions, plant cells are highly plastic; many—but not all—types are able to change their epigenetic, genetic, biochemical, and morphological states from that of a specialized tissue to a pluripotent state; reenter the cell cycle; and form specialized structures. One of the most useful applications of this principle is somatic embryogenesis (SE)—the creation of an embryo from a somatic cell (Fehér, 2015). This is a complex procedure that, more often than not, requires specific culturing conditions to induce competent cells into making these extensive changes to their chemistry and morphology (Pasternak et al., 2002; Jiménez, 2005). Even after a cell has reverted to an embryogenic state, it must then divide; establish bipolar root/shoot meristem tissues; develop nascent embryo organs; convert into a plant; and survive acclimatization from *in vitro* conditions to the less-humid conditions of a greenhouse. Cultures may be kept *in vitro* for many successive subcultures, but time itself adds another layer of complexity: cultures become less embryogenic over time (Dunstan et al., 1996; Smýkal et al., 1998; Breton et al., 2006; Landey et al., 2015). Aging peach palm (*Bactris gasipaes*) cultures began to show the typical symptoms of age: embryogenic tissues failed to mature despite previously-established optimized protocols (Heringer et al., 2014); cultures began to grow more slowly; aberrant structures began to form with increasing regularity; cultures oxidized frequently, turning necrotic starting from the point of any wounds; and, eventually, stopped forming somatic embryos entirely. By the eighth year, only fibrous, slow-growing tissue with a tendency to turn necrotic—so much so that multiplication cycles often yielded a net decrease of cultures—remained of the previously-vigorous culture line. Time, therefore, is a problem, both for the cultures themselves and for the researchers, propagators, and conservationists who require cultures for their work. Here, I focus on this problem through three distinct pathways: first, the role of 5-azacytidine (5azac), a potent inhibitor of cytosine methylation (Kiziltepe et al., 2007), in the rejuvenation these eight-year-old cultures (OC) by reversing age-related hypermethylation and compare them to younger, two-year-old tissues of the same genotype; second, the role of closed or ventilated tissue culture vessels on conversion rate and biochemical characteristics of mature somatic embryos for establishing plants in the field to serve as tissue sources for new culture lines; and,

third, an improved vitrification cryopreservation protocol with the goal of exploring relatively-understudied factors that may prove useful not just for peach palm, but also useful for other species. These three approaches are quite different in both scope as well as the underlying scientific knowledge. Thus, the following bibliographic review was written to acquaint the reader with the epigenetic, genetic, biochemical, morphological, and, especially in the case of cryopreservation, physical aspects of each of these three strategies.

Six decades have passed since the first observations of SE in *Oenanthe aquatica* (Miettinen and Waris, 1958), and nearly five since the first description of SE in the first Arecaceae, oil palm (*Elaeis guineensis*) (Racechault et al., 1970). Since then, tissues from other plant species have likewise been induced to undergo SE, resulting in the formation of semi-reliable means of mass-propagating single genotypes far beyond what classical propagation is able to generate. These species range in diversity, encompassing the vast majority of plant types, including monocots such as palms (Ree and Guerra, 2015), dicots such as *Acca sellowiana* (Cruz et al., 1990; Cangahuala-Inocente et al., 2009a), gymnosperms such as *Araucaria angustifolia* (Astarita and Guerra, 1998; Guerra et al., 2000), and pteridophytes such as *Cyathea delgadii* (Mikuła et al., 2015). Despite the physiological and genetic differences between such groups, several general trends have emerged: SE requires some degree of abiotic stress; exogenous auxin is often required in the culture medium; genotype is often the main determiner of SE induction success; and SE is morphologically, but often not biochemically, similar to zygotic embryogenesis (ZE) (reviewed in Feher et al., 2002; Jiménez, 2005; Mahdavi-Darvari et al., 2014; Fehér, 2015a). Successful SE induction, however, is often unpredictable despite an ever-growing knowledge base. Some genotypes are simply recalcitrant; the tissue from one cultivar may ready produce multitudes of somatic embryos, but tissue from another may simply turn necrotic or produce non-embryogenic callus tissue. Recalcitrancy, just as much as high-embryogenic potential, demonstrates a complex set of genetic and biochemical conditions that help explain why one tissue behaves one way and another behaves differently.

1.2 BASIC TISSUE CULTURE REQUIREMENTS FOR BACTRIS GASIPAES SE INDUCTION

SE induction requirements are complex and often depend on a long list of factors, such as explant type, explant cell biochemistry, genotype, and explant cell epigenetic state. By providing a tissue culture environment that optimizes these factors, SE can be induced in a wide number of

species. In our lab, our main focus has been on the Amazonian palm *Bactris gasipaes*, also called *pupunha* or peach palm—so called because its fruits have a red-pink hue. Currently, it is widely cultivated due to its ability to grow multiple stems, and thus survives the harvest of heart-of-palm—the interior leaves of the plant, which are consumed as a vegetable (Mora-Urpí and Clement, 1988). Peach palms grow several shoots, making it more desirable for sustainable heart-of-palm production compared to palms with a single shoot; harvest kills the latter, but the former recovers. *In vitro* techniques offer a means of growing a larger population of clean and healthy plants to supply farmers compared to conventional propagation. Valverde et al. (1987) were the first to induce SE in peach palm zygotic embryos; convert the resulting somatic embryos; and transfer them to greenhouse conditions. Various aspects of SE in peach palm have already been studied in our lab: explant choice and response; media composition; the effects of plant growth regulators; cryopreservation; plantlet production; and histological examination of explants and somatic embryos at various stages of development (Steinmacher et al., 2007a, 2007b; Heringer et al., 2013a, 2013b; Nascimento-Gavioli et al., 2017). Many of the results from these studies, as well as the other ~20 palm species with published SE protocols, have shown several general requirements for culture medium able to support palm SE (Ree and Guerra, 2015): presence of high concentrations of artificial auxins, such as picloram or 2,4-D; activated charcoal as an antioxidant; 3% sucrose as the energy source; and Murashige and Skoog (Murashige and Skoog, 1962) basal salts with vitamins. Each of these additives serve specific requirements, which will be summarized briefly at present and discussed more thoroughly in subsequent sections. Exogenous auxins indirectly influence endogenous concentrations of the auxin indole-3-acetic acid (IAA) by disturbing the endogenous auxin metabolism and increasing IAA concentrations (Dudits, 1995), which, in turn, stimulate specific genes required for embryo dedifferentiation. Activated charcoal serves as an antioxidant, which is required because excised explants are subjected to numerous types of stress during inoculation *in vitro*. Physical removal of the explant and subsequent decontamination causes significant stress on the tissue, leading to formation of radical oxidative species (ROS), which then lead to tissue necrosis. Sucrose provides an energy source and osmotic stress, which alters plasmodesmatal connections between cells and disrupts cell-to-cell communication, thus isolating the cell and allowing it to dedifferentiate with reduced influence from neighboring cells (Karami and Saidi, 2010). Basal salts and vitamins provide the elements that plants require for

normal growth. Even with these basic elements, a given tissue may or may not undergo SE. Further success depends on a series of interwoven factors: endogenous PGRs; epigenetic regulation and gene expression; and explant source.

1.2.1 Successful SE induction is closely related with the endogenous biochemistry of competent cells

Auxins, cytokinins, abscisic acid (ABA), gibberellic acid (GA3), and ethylene, often referred to as the 'classical' PGRs, actively affect gene transcription and cell fate (Gaspar et al., 1996). Endogenous auxin in the form of indole-3-acetic acid (IAA) guides cell fate by binding to proteins, such as auxin response factors, which promote auxin-regulated genes that affect many processes, including cell elongation, cell division, cell fate, and, after a cell has reentered the cell cycle, establishing embryo bilateral symmetry through polar auxin transport (Liu et al., 1993; Jiménez, 2005). Because of this, changes in total IAA concentration in a given tissue, or differences in IAA between different tissues, is frequently a marker of wide-ranging and contrasting changes in cellular behavior. Auxin concentration gradients, transport, and role in changing the expression of several key genes, such as *AUX* and *PIN*, are considered responsible for establishing cell polarity—the division between the regions of a developing embryo that devoted to root and shoot development (reviewed in Boutte et al., 2007). In a similar study, high IAA in early *Ocotea catharenensis* zygotic embryogenesis (ZE) corresponded with formation of bilateral symmetry (Santa-Catarina et al., 2006). Disruption to auxin signaling may cause detrimental physiological development, as shown by the treatment of *Picea abies* cultures with N-1-naphthylphthalamic acid, a polar auxin inhibitor, which caused malformed embryos with irregular cotyledons, poor shoot apical meristem development, expanded procambium, and a broader root apical meristem (Hakman et al., 2009). *Geranium* cultures treated with TDZ and maintained in continuous darkness, showed significantly decreased IAA and ABA concentrations and decreased embryogenic potential compared to cultures maintained in the light (Hutchinson et al., 2012). Based on this principle, auxin is most often the first factor to be tested when establishing a new SE protocol. Exogenously-applied auxins likely affect cell behavior by stimulating greater synthesis of endogenous auxins (Dudits, 1995). Endogenous IAA concentrations were seven-times greater in carrot (*Daucus carota*) cultures in the presence of 2,4-D than cultures without 2,4-D (Michalczuk et al., 1992a), and a later study supported this evidence by finding that exogenous 2,4-D, IAA, or NAA significantly increased endogenous IAA

in carrot cultures (Ribnicky et al., 1996). In palms, exogenous auxins have been found to be essential for SE induction for coconut (*Cocos nucifera*) (Karunaratne and Periyapperuma, 1989), areca (*Areca catechu*) (Wang et al., 2003), oil (da Silva Guedes et al., 2011), and peach palms (Steinmacher et al., 2007c). However, these observations are not universal to all palms. Two interspecific *Elaeis oleifera x E. guineensis* hybrids cultured in the presence of 2,4-D showed inverse relations: one genotype had the highest embryogenic tissue formation on medium with the lowest 2,4-D concentration and the other on the highest concentration (Alves et al., 2012). Therefore, levels of endogenous auxin alone, though often linked with embryogenic potential, is not the only factor governing it. Date palm leaf explants treated with 1 μM NAA developed organogenic roots, whereas higher concentrations promoted callus growth (Gueye et al., 2009a). A range of 2,4-D concentrations on seven wheat (*Triticum aestivum*) genotypes found that low concentrations promoted growth of watery, transparent, and friable non-embryogenic cultures; however, increasing concentrations promoted increasing proportions of yellow-white friable embryogenic cultures (Filippov et al., 2006). Unlike many other species, sunflower (*Helianthuis annus*) was capable of producing somatic embryos without exogenous auxins. IAA accumulated during SE induction in a way that mimicked the pulse of IAA usually provoked by exogenous auxin application, leading the authors to suggest that endogenous auxin pulse is among the first signals of SE induction (Thomas et al., 2002). In support of this, IAA was found to accumulate in both embryogenic and non-embryogenic carrot lines; however, IAA declined throughout embryogenesis in the embryogenic line but the non-embryogenic line maintained high IAA levels (Michalczuk et al., 1992b). This led the authors to suggest that high IAA levels may be necessary for SE induction but is not sufficient for causing the initial events in plant embryogenesis. Similarly, *Pinus taeda* zygotic embryos contained high concentrations of IAA at the globular stage, which decreased as the embryo developed (Silveira et al., 2004).

In addition to the auxin pulse, relative concentrations of other PGRs may serve as a marker of embryogenic potential. In both hazelnut (*Corylus avellana*) (Centeno et al., 1997) and rubber tree (*Hevea brasiliensis*) (Etienne et al., 1993), a higher IAA/ABA ratio was a significant indicator of embryogenic potential. In the latter report, the non-embryogenic tissue contained higher ABA concentrations, leading the authors to suggest non-embryogenic cultures may have been under stress. Additionally, non-embryogenic cultures initially contained higher IAA concentrations, but

embryogenic tissue eventually accumulated more IAA than non-embryogenic tissue. High initial IAA and ABA levels characterized the globular stage of *Pinus taeda* zygotic embryo development, but ABA levels declined during development only to accumulate again during maturation (Silveira et al., 2004). Though ABA has been most related to embryo maturation, precocious germination prevention, and abiotic stress, it affects the expression of numerous genes (Rai et al., 2011). This suggests a possible interaction either directly and indirectly between IAA and ABA. Higher endogenous IAA concentrations were found in embryogenic *Picea abies* cell lines that, when cultured continuously with ABA, produced higher-quality embryos than cultures without ABA (Vágner et al., 1998). Addition of ABA to date palm cultures increased somatic embryo growth (Zouine et al., 2005). Addition of fluridone (100mg/L) inhibited ABA synthesis in *Pennisetum purpureum* leaf explants, causing complete loss of embryogenic potential (Rajasekaran et al., 1987). This suggests that, in addition to the high IAA concentration pulse, that SE induction also requires ABA due to its role in stress response.

Ratios between other growth regulators are associated with embryogenic potential. Cytokinins, a group of PGRs involved in many diverse processes, but possibly most known for role in promoting cell division through modifying gene expression (Mazri, 2014), may have a part in inducing SE in palms. Treatments of BAP and NAA led to greater date palm somatic embryo numbers than with NAA alone (Kurup et al., 2014). Auxins and cytokinins actively interact, mutually regulating each other through an antagonistic relationship, making their relative abundances important factors in cellular fate and gene transcription (Moubayidin et al., 2009). Embryogenic cotton (*Gossypium hirsutum*) cultures displayed higher IAA and zeatin levels compared to a recalcitrant line (Xu et al., 2013). Cellular dedifferentiation of *Gossypium hirsutum* occurred at the same time that the ratio between IAA to isopentenyladenosine groups increased sharply (Zeng et al 2007). Non-embryogenic orchard grass (*Dactylis glomerata*) genotypes were found to contain 3 or 4-fold higher total cytokinins than the only studied embryogenic genotype, but there were no significant differences in IAA concentrations between the genotypes (Wenck et al., 1988). The cytokinin BAP was instrumental in establishing the shoot meristem in coconut somatic embryos (Verdeil et al., 1994) and *Ocotea catharinensis* (Santa-Catarina et al., 2006). Coconut water, a known source of cytokinins, has been shown to improve date palm SE (Al-Khayri, 2010; Khierallah and Hussein, 2013). Park et al.

(2010) suggested that another marker of embryogenic potential based on plant growth regulators was the ratio of cytokinins to ABA. Six-month-old highly-embryogenic *Kalopanax septemlobus* cultures were compared to 2.5-year-old cultures with diminished embryogenic potential; the younger cultures had a higher ratio of cytokinins to ABA, as well as increased total protein, mRNA, increased mitotic activity, and dense cell cytoplasmic content compared to 2.5-year-old cultures. The cross-talk between auxin, cytokinin, and ABA is a complex web of interactions with likely the greatest impact on guiding cell fate; however, they are not the only factors.

Ethylene, a gaseous growth regulator has likewise been associated with embryogenic potential. Non-embryogenic *Picea abies* cultures produced forty-times more ethylene than the embryogenic line (Vágner et al., 1998). Silver nitrate, a potent ethylene inhibitor, promoted tissue growth in *Coffea canephora* (Kumar et al., 2007) and date palm (Al-Khayri and Al-Bahrany, 2001). Other highly influential growth regulators, such as salicylic acid, brassinosteroids, and jasmonic acid, have been recently shown to affect homeostasis pathways that might be involved with embryogenesis (Elhiti et al., 2013), but their role in somatic embryogenesis is still largely unknown. Nitric oxide, another gaseous growth regulator, affects gene expression and plays an important role in many types of tissue due to their interactions with ROS and auxin (Beligni and Lamattina, 2001; Correa-Aragunde et al., 2016). Polyamines, polycationic compounds that interact with nucleic acids and proteins, are non-PGR compounds often used as a biochemical marker due to their involvement in many biochemical processes (Shoeb et al., 2001). These diverse biochemicals, with their often-conflicting interactions, are but one dimension of a spider's web of factors. Though their relative abundances or ratios to one another may be indicators of a cell's ability to change its fate, their activities rely on the interplay between other proteins involved in gene expression.

1.2.2 Cell fate determination and dedifferentiation requires vast changes to the epigenetic and genetic state

A cell undergoing dedifferentiation must change is genetic state, effectively 'forgetting' that is a certain cell type so that it may become an embryogenic one (Feher et al., 2002). Though this is often observed as a series of genetic interactions, it may also still be seen; Zeng et al. (2007) observed that *Gossypium hirsutum* somatic embryos underwent two phases of chromatic decondensation: the first was observed during

dedifferentiation, and the second occurred during redifferentation. These physical changes to the nucleosome represent changes to the epigenetic state of the cell as genes are silenced or expressed. Epigenetic regulation occurs through several means: cytosine methylation of key regions of genes; histone binding and the compaction/decompression of specific loci to possibly affect multiple genes; and the interaction of proteins, enzymes, transcription factors, and signaling molecules that modify them. Many such proteins are highly-conserved across multiple kingdoms, and loss-of-function may have severe consequences. For example, DICER-like proteins involved in miRNA formation used for RNA-mediated cytosine methylation were essential to *Arabidopsis* zygotic embryo formation; those that lacked expression ceased to develop due to loss of cell differentiation events mediated by the gene's repression of mRNA from other genes (Nodine and Bartel, 2010). For both ZE and SE, tight epigenetic control is essential during the very first events. Specifically, the right conditions are required to establish bipolarity; the root/shoot meristems must be defined so the embryo can grow properly. Through growth regulators, especially polar auxin transport, this early bipolarity will cause large changes to the epigenetic state and biochemical composition of daughter cells as the embryo develops.

In either ZE or SE, this bipolarity may occur through different mechanisms. After zygote formation after introduction of a sperm cell during ZE, the cell undergoes a transient symmetry phase marked by a fragmented vacuole and prominent nucleus, followed by zygote cell elongation and division. However, these two cells are not equal: one forms the embryo, whereas the other, containing the single vacuole, forms the suspensor, thus forming the basis of bipolarity. Expression of several genes, such as Wuschel-Related Homeobox (*WOX*) transcription factors are preferentially-expressed in the apical side of the two-cell zygote, where its expression will determine shoot development through polar transport of auxin (Ueda and Laux, 2012). In SE, however, somatic cells act as a zygote. Embryogenic cells are marked with similar ultrastructural characteristics, namely a prominent nucleus and a fragmented vacuole (Verdeil et al., 2001). These ultrastructural characteristics also represent the large changes in epigenetic regulation induced in somatic cells.

Despite apparent similarity between zygotic and somatic embryos, they can differ morphologically as a result in differences in epigenetic regulation; however, this might also be related to environment: somatic embryos do not develop in the maternal environment where zygote

growth is fueled by photosynthates and interacts with an endosperm. Rather, somatic embryos are grown in proximity to non-competent somatic cells, other somatic cells, and a tissue culture medium composed principally of basal salts, growth regulators, and sucrose. Additionally, somatic embryos are exposed to more air than zygotic ones. Together, this may lead to greater stress in explants, which is often implicated as being one of the main factors in stimulating SE (reviewed in (Fehér et al., 2003).

After bipolar embryo formation, several 'master regulator' genes are induced due to the effect of auxin response factors. Auxin response factors are proteins released by the auxin-mediated degradation of repressor proteins, which participate in promoting or repressing expression of many genes involved in growth response, often in concert with other growth regulators like ABA, gibberellic acid, and ethylene (Chandler, 2016). Several types of auxin response factors are implicated in regulating PKL-mediated chromatin remodeling (Guilfoyle and Hagen, 2007). PKL genes are thought to perform this by promoting histone H3K27me3 marking, which represses LEC and FUS genes by enhancing PRC2 expression. Loss of PKL and concomitant activity of WUS leads to the activation of LEC genes, which, in turn, modify the expression of other genes involved embryo formation (Fehér, 2015b). In either SE or ZE, many of the same critical genes, such as SERK, BBM, LEC, FUS, and WOX play the same role, making their expression essential (Ikeda et al., 2006). Single and double mutants for LEC and FUS genes in *Arabidopsis* embryos showed decreased embryogenic potential, whereas triple mutants prevented embryogenesis (Gaj et al., 2005). Histone modifications epigenetically regulate the transcription of many of the key transcription factor genes involved in early embryogenesis. Namely, H3K27e3 regulates the expression of BBM1 and LEC1, and H3K9me2 regulates WOX4 expression (Lafos et al., 2011). Trimethylation of the histone H3 lysine 4 (H3K2me3) is implicated in gene activation, but trimethylaton of lysine 27 of the same histone (H3k27me3) represses gene expression. Maintenance of either of these three histone modification are, in turn, affected by expression levels of histone demethylase genes (Chen et al., 2015). Through these mechanisms, a cell with a designated fate may be changed through this summarized and highly simplistic process: its endogenous auxin concentrations were increased through the influence of exogenous auxins; these higher endogenous auxins released auxin response factors; and these auxin response factors interacted with many other proteins to cause a series of changes to the epigenetic state of the cell, leading to an embryogenic state. However, just as biochemical

profile and epigenetic plasticity is important, so, too, is the actual physical location of a particular cell.

1.2.3 Explant type is one of the main factors in determining the success of SE induction

SE protocols are highly dependent on explant source. Though the term 'explant' in a convenient convention used to simplify the origin of a culture to a single unit (e.g. zygotic embryo, meristem thin cell layers, leaf), explants consist of several tissue types, each composed of one or more cell types with their own biochemical and morphological characteristics that may provide the right balance needed to trigger the cascade of changes required to break both cellular programming and apical dominance to reenter the cell cycle. Thus, some tissues are more inherently embryogenic than others (Fehér et al., 2003). Zygotic embryos, the most commonly-used explant in published palm SE protocols, are highly responsive, but have the drawback of possessing an unknown genotype (Ree and Guerra, 2015). Embryo maturity, likewise, is a factor; specialized tissues form, leading to established cell fate that ultimately affects their response to *in vitro* culture (Cangahuala-Inocente et al., 2009b). Immature oil palm zygotic embryos produced more embryogenic tissue than mature zygotic embryos, which tended to germinate or form non-embryogenic tissue (Teixeira et al., 1993). This suggests that the changes in biochemical profile and gene expression in zygotic embryo maturation affected embryogenic potential. Shoot apical meristems, a common explant source in date palms, are responsive, but harvest kills the palm trunk, and the meristem itself is prone to contamination from endophytic bacteria. Yet, both explant sources have vigorous, highly-mitotic well-adapted to SE induction (Gupta et al., 1984; Steinmacher et al., 2007c). Other explant sources, such as immature inflorescences, have been frequently used for SE induction with success (Verdeil et al., 1994). Embryogenic potential in inflorescences is likewise subject to internal mechanics; developing coconut inflorescences contained both the highest endogenous total sugar content and the highest callogenic potential compared to older ones (Fernando et al., 2008). In contrast to highly embryogenic zygotic embryos, apical meristems, and immature inflorescences, palm leaf tissue is considered highly recalcitrant, and the number of published articles are low in comparison to other tissues. However, AL-Mayahi, (2015) succeeded in inducing callogenic growth on white basal sections of date palm leaves, observing that the green leaf tips were completely recalcitrant to growth. Similarly, decreasing IAA concentrations in date palm leaf explants taken increasingly further away

from the excised apical meristems was correlated with decreasing embryogenic potential (Gueye et al., 2009b). The authors also found that cell type might be related to embryogenic potential: lignified sclerenchyma in vascular tissues of leaf distal segments may have had a weak response to exogenous auxin, whereas parenchyma cells perpendicular to the vascular axis acquired the structural features of meristematic cells, thus creating a microenvironment suitable for adjacent perivascular cells to become callogenic. Location, therefore, might be a determining factor in successful SE induction; cells require space to grow, and epidermal tissue or those able to grow within vascular tissue may be particularly suited for growth compared to cells trapped amongst closely-packed neighbors. Similarly, Almeida et al., (2012) found that pre-procambial cells act in different morphological pathways to establish niches of competent cells, which then promote culture growth. Perivascular parenchyma bordering vascular tissue was found to be the first tissue type to begin cellular growth in both juvenile date palm leaves (Sané et al., 2006) and zygotic embryos (Silva et al., 2014), though outlying parenchymal cells on peach palm zygotic embryos (Maciel et al., 2010) or epidermal or subepidermal juçara cotyledonary nodes (Guerra and Handro, 1998) were also observed to the first cell types to reenter the cell cycle. Ultimately, explant source tissues may reflect many of the aspects that determine genotype-based embryogenic potential.

1.2.4 Stress: an inescapable factor in somatic embryogenesis

In tissue culture, abiotic stress may come in many forms: osmotic stress from the salts and sugars in the medium; exposure to oxygen; non-standard atmosphere within a closed vessel; hypoxia; and the severe changed to the tissue itself as it undergoes SE (Karami and Saidi, 2010; Fehér, 2015b). Benson (2000) hypothesized that tissue culture manipulations cause major metabolic and developmental changes which lead to increased ROS formation. If antioxidant protection is compromised, ROS accumulation leads to oxidative stress which cause large scale damage to DNA, proteins, and enzymes, which in turn lead to cellular disruption and culture recalcitrance. Enzymes such as superoxide dismutase, catalase, several types of peroxidases, and the enzymes in the glutathione-ascorbate cycle are able to break down hydrogen peroxide or superoxide ions into water (Potters et al., 2002; Baťková et al., 2008).To prevent tissue death, cells must increase their ability to scavenge ROS by promoting antioxidant pathways (Blokhina et al., 2003). Yet, this too is a rule with many exceptions. Addition of glutathione or ascorbic acid to *Gladiolus hybridus* cultures inhibited the frequency of somatic

embryogenesis but improved organogenesis (Gupta and Datta, 2003). Moreover, organogenic and embryogenic *Gladiolus hybridus* tissues showed different levels of individual antioxidant enzymes: organogenic had more peroxidase and catalase; embryogenic ones had more superoxide dismutase. On the other hand, transcript profiling through RT-PCR of cotton somatic embryogenesis revealed that genes related to stress response correlated with embryo development (Jin et al., 2014). Though a cell may have the means for detoxifying the outcomes of abiotic stress, protocols may be optimized to reduce the amount of stress on a culture. For example, recalcitrance in cotton (*Gossypium hirsutum*) was reduced by halving basal salt macronutrient concentration, which alleviated tissue browning and promoting suspension cell proliferation (Wang et al., 2006). In palms, activated charcoal is required in the vast majority of published articles to prevent necrosis (Ree and Guerra, 2015). Activated charcoal helps regulate tissue oxidation through several proposed mechanisms: darkened environment, absorption of undesirable substances, absorption of excess growth regulators, or release of growth-promoting substances (Weatherhead et al., 1978). However, inclusion often requires increased exogenous auxin concentrations to have the same relative effect on SE induction than media without activated charcoal (Nissen and Sutter, 1990). Addition of AC to Canary Island Date palm cultures resulted in callogenic growth with reduced embryogenic potential compared to cultures grown without activated charcoal (Huong et al., 1999). This change in embryogenic potential may be related to the role of auxin signaling in cell fate; 1 μM concentrations of NAA promoted organogenic roots to form on date palm leaf explants, but 54 μM promoted callus growth (Gueye et al., 2009).

1.3 EARLY EMBRYOGENESIS AND DIFFERENCES BETWEEN SOMATIC AND ZYGOTIC EMBRYOGENESIS

After a cell has gone through dedifferentiation from one stablished fate and undergone redifferentation into an embryo-like state, it must then undergo cellular division. As the embryo develops, its cells undergo various further changes. During early embryogenesis, cells develop several core characteristics: a large, fragmented vacuole, a prominent central nucleus, and visible starch granules (Fehér, 2015b). Additionally, embryogenic coconut cells were found to deposit callose into the intracellular space between cells, breaking symplastic continuity, and closing plasmodesmata, effectively isolating the cell (Verdeil et al., 2001). The resulting cells reenter the cell cycle and effectively form meristematic centers observed during early coconut (Saénz et al., 2006),

date (Sané et al., 2006), rattan (Goh et al., 2001), and oil palm (Kanchanapoom and Domyoas, 1999) SE. Embryogenic palm cells, in general, are characterized by certain traits: a high nucleus:cytoplasm ratio; a central enlarged nucleus with a dense nucleolus; many small, divided vacuoles; and high starch concentration (Kanchanapoom and Domvoas, 1999; Goh et al., 2001; Verdeil et al., 2001; Saenz et al., 2006; Sane et al., 2006; Silva et al., 2014). During early somatic embryo development, the new tissue may form tracheal elements (Maciel et al., 2010) and suspensor-like structures (Guerra and Handro, 1998) connected to the initial explant. Additionally, formation of a net-like extracellular matrix composed of carbohydrates, signaling molecules, arabinogalactan proteins, and other substances (Samaj et al., 1999; Seifert and Blaukopf, 2010), has been a sign of embryogenic competences in several palms: oil (Low et al., 2008), *E. guineensis x E. oleifeira* hybrid (Angelo et al., 2011), and peach palm (Steinmacher et al., 2012). These substances may possibly act as signal molecules (Palanyandy et al., 2013), making them potentially useful to reducing recalcitrance, as suggested by effective stimulation of plant protoplasts to reenter the cell cycle and regrow in *Brassica oleracea* (Chen et al., 2004). These early requirements show similarities between SE and ZE, but later development demonstrates that there are significant differences between the two pathways.

Zygotic and somatic embryos grow in different environments, and this leads to differences in biochemical profiles. *Pinus taeda* zygotic embryos contained significantly less water compared to somatic embryos, and the water content in zygotic embryos decreased during development while water content remained relatively stable in somatic embryos (Pullman et al., 2003). *Larix x eurolepis* zygotic embryos contained more protein than somatic embryos (Teyssier et al., 2013). Additionally, the authors found that global methylation in somatic embryos changed over time: undifferentiated somatic embryos showed a rate of 45.8% methylation; immature somatic embryos were 61.5% methylation; and mature embryos were 53.4% methylated (Teyssier et al., 2013). Date palm (*Phoenix dactylifera*) zygotic embryos contained more total protein compared to somatic embryos, and more proteins were related to storage and stress-related activity than in somatic embryos, which contained more proteins related to glycolysis (Sghaier-Hammami et al., 2009). *Acca sellowiana* somatic and zygotic embryos amino acid proportions were slightly different: zygotic embryos amino acids were predominantly asparagine and glutamine, and somatic embryos produced glutamine, glutamic acid, and GABA (Pescador et al., 2013). Date palm zygotic embryos contained

higher concentrations of ascorbic acid, phenols, flavonoids, and free amino acids (Eldin and Ibrahim, 2015). Among the most consistent differences, especially in palm SE, is that zygotic embryos contain significantly higher concentrations of total proteins (Sane et al., 2006; Aberlenc-Bertossi et al., 2008; Sghaier et al., 2008; Moura et al., 2010; Eldin and Ibrahim, 2015). Embryo protein content was likewise different by type in date palm somatic and zygotic embryos: zygotic embryo proteins were predominantly related to storage and stress-response, but somatic embryos contained more proteins involved with glycolysis (Sghaier-Hammami et al., 2009). These differences can be mitigated, however, by media optimization. Addition of 4-20 μM ABA; 9% sucrose instead of 3% sucrose; or 3mM arginine increased protein content and resulted in a shift in protein composition to favor those related to storage and stress-response (Sghaier et al., 2009). Considering the strict regulation involved in early embryogenesis, these differences may have downstream effects that affect, and possibly change, tissue growth.

1.3.1 Growth of embryogenic and non-embryogenic lines: a case for differences in cell fate, or a matter of mis-regulation?

Multiple types of tissue may emerge from a single explant. In peach palm, there are three distinct categories: embryogenic tissue is composed of somatic embryos, polyembryogenic masses, and embryogenic callus; fibrous, 'popcorn'-like fibrous non-embryogenic tissue; and yellow, fast-growing, and heterogenous callus. The emergence of embryogenic and non-embryogenic tissue has been the focus of much study and speculation, but few stable and reproducible patterns have emerged. Is it a different type of tissue—a different pathway during redifferentation—or is it a case of misregulation in embryogenic cells? Embryogenic *Triticum aestivum* cultures were found to have an accumulation of at least 33 proteins not detected in non-embryogenic proteins; however, technology was not yet developed to fully explore what proteins were differently-expressed (Fellers et al., 1997). Embryogenic *Crocus sativus* cultures showed an up-accumulation of several proteins related to the ascorbate-glutathione cycle, suggesting that embryogenic cultures were able to detoxify ROS more effectively than non-embryogenic ones (Sharifi et al., 2012). Further supporting this result, embryogenic *Saccharum spp* cultures showed a general trend of increased expression of proteins related to antioxidation and NO synthesis compared to non-embryogenic cultures, which expressed a general trend of more proteins related to protein degradation (Heringer et al., 2015). Non-embryogenic grape cultures were found to have significantly more abundant basic

ascorbate peroxidase and catalase, whereas embryogenic had abundant acidic ascorbate peroxidase, leading the authors to suggest that different antioxidation systems were active in either tissue type (Zhang et al 2009). Embryogenic *Cardiospermum halicacabum* cultures contained significantly higher ascorbic acid concentrations, as well as lower phenolic compounds, however the non-embryogenic contained significantly higher polyphenol oxidase and peroxidase (Jeyaseelen and Rao, 2005). Cells from an embryogenic *Araucaria angustifolia* cell line were characterized as containing high levels of ROS and ethylene (Jo et al., 2013). Embryogenic *Hevea barsiliensis* somatic embryos contained higher concentrations of IAA and lower concentrations of ABA compared to non-embryogenic cultures (Etienne et al., 1993). Recently, studies on non-embryogenic and embryogenic peach palm cultures found markedly different biochemical profiles: embryogenic lines contained higher concentrations of IAA, ABA, and total amino acids, whereas non-embryogenic tissue contained higher total polyamines and phenolics (Nascimento-Gavioli et al., 2017). In contrast, however, non-embryogenic *Theobroma cacao* tissue had the highest overall concentration of amino acids, especially GABA (Niemenak et al., 2008). Non-embryogenic *Pinus radiata* (Bravo et al., 2017) and *Eleuterococcus senticosus* cultures contained higher levels of cytosine methylation compared to embryogenic lines. Total sugar concentrations were not significantly different between EC and NEC *Medicago arborea* cultures; however, EC contained a higher proportion of reducing sugars (Martin et al., 2000). A similar result was found in EC and NEC cotton (*Gossypium spp*) cultures with the additional observation that NEC contained overall higher total phenolic compounds (Obembe et al., 2010). Considering these wide-ranging differences, the emergence of non-embryogenic tissue remains a present mystery; however, little work has gone into finding out exactly what tissue these non-embryogenic tissues truly are. Peach palm non-embryogenic tissue left in the light for several weeks turn a deep orange—the same color as peach palm mesocarp in mature fruits. Perhaps this tissue is, indeed, an organogenic development of mesocarp, but there is no evidence for or against this anecdote.

1.4 SOMATIC EMBRYO MATURATION AND CONVERSION

In later embryogenesis, after specialized tissue have developed, there is a pronounced shift in cellular behavior mediated by a steady ABA accumulation. Pérez et al., (2015) found that the onset of maturation in zygotic embryos coincided with a transient increase in ABA content localized to mainly the root and shoot apical meristems. Epigenetic

analysis of larch (*Larix x eurilepis*) somatic embryos found that cytosine methylation was highly dynamic during early and late phases of SE; 45.8%, 61.5%, and 53.4% cytosine methylation were measured in undifferentiated somatic embryos, immature somatic embryo, and mature somatic embryos, respectively (Teyssier et al., 2013). The authors also found that a significant number of proteins differed in expression between each of these stages—most proteins were involved in metabolism, despite onset of maturation—suggesting that changes in gene expression accompanied changes in global methylation. In a related study, eight expression sequence tags corresponding to *Quercus suber* genes involved in epigenetic and genetic regulation found that expression changed throughout embryogenesis. Among the most wide-ranging effects was a change in all seven genes associated with epigenetic regulation over time, suggesting that epigenetic regulation is highly plastic (Pérez et al., 2015b). For example, LEC and BBM remained expressed throughout *Coffea canaphora* zygotic embryo formation, but WOX4 expression peaked early during zygotic embryogenesis before sharply decreasing in expression (Nic-Can et al., 2013). Using chromatic immunoprecipitation assays, the authors found that one of the main epigenetic factors implicated in this difference was that LEC and BBM were epigenetically regulated by H3K27me3, whereas WOX4 was regulated by the repressive mark H3K9me2. Epigenetic shifts toward repressing certain genes and activating others changes cellular behavior from one of constant growth to accumulation of storage reserves and Late Embryogenesis Abundant proteins, which confer a measure of desiccation tolerance (Han et al., 1997).

In general, somatic embryo maturation can be triggered by subculturing embryogenic cultures onto media with less auxin to mimic an auxin pulse (Thomas et al., 2002), and ABA to promote expression of genes associated with maturation (Rai et al., 2011). As *Ocotea catharinensis* zygotic embryos matured and developed specialized tissues, the concentration of ABA increased until full maturity (Santa-Catarina et al., 2006). Exogenous ABA, therefore, often improves somatic embryo maturation and development, as in *Zea mays* (Emons et al., 1993) and *Phoenix dactyilifera* (Sghaier-Hammami et al., 2010). Proteomic analysis of *Cyclamen persicum* somatic embryos found that ABA and 6% sucrose in the medium stimulated the accumulation of storage compounds and decreased the abundance of metabolism-related proteins compared to embryos grown on medium without ABA and with 3% sucrose (Rode et al 2012). As *Acca sellowiana* embryos developed, protein concentration

gradually increased; certain amino acids, such as arginine and asparagine, increased; and total starch decreased (Cangahuala-Inocente et al., 2014). Storage proteins, such as legumin and vicilin, became more prevalent as *Larix x eurolepsis* somatic embryos entered the precotyledonary stage (Teyssier et al., 2013). These storage compounds are essential to fuel the rapid growth of an embryo during conversion into a plantlet.

Somatic embryos usually require no exogenous growth regulators to begin conversion once the embryo has matured, though exogenous GA3 improved embryo conversion in peach palm (Heringer at al., 2013). The tissue culture environment poses one more major obstacle to the plant, however: it's humid compared to the *ex vitro* world. To prevent dehydration, a plant synthesizes a waxy cuticle and regulates water outflow through stomata. The *in vitro* environment, however, may stimulate poor cuticle formation because of the excess amount of moisture present in the medium. Peach palm plantlets germinated in vitro lacked the well-developed cuticle grown by those plantlets germinated under greenhouse conditions (Batagin-Piotto et al., 2012). Acclimatization, to this day, is one of the most significant bottlenecks in tissue culture (see section 2.8.1).

1.5 CULTURE AGING AND STRATEGIES TO CIRCUMVENT IT

The process by which an established peach palm cell line lost its embryogenic potential followed several distinct steps: embryos began to develop higher rates of aberrant structures; cultures began to multiple more slowly; embryos failed to mature despite optimized protocols; cultures became highly-sensitive to wounding and oxidation; and, eventually, they completely lost their ability to regenerate embryos. This was especially problematic, because my first project proposal centered around studying Late Embryogenesis Abundant-type proteins during somatic embryo maturation. Embryos failed to mature, therefore, that project was changed, but the mystery remained: how can cultures be preserved? The simplest is to convert plantlets and keep them *in vitro* or keep them in the field. This, too is a bottleneck, as described above. In the following section, I discuss aspects of two other strategies: restoring embryogenic potential in aging cultures through 5-azacytidine, and preserving embryogenic tissues using cryopreservation.

1.6 CULTURE AGING AND HYPOMETHYLATION

Successive subcultures are frequently blamed as the source of the decreased vigor noticed in a number of species over time. Aberrant

structures developed with increasing frequency over several years of areca palm somatic embryos (Wang et al., 2010). Bradaï et al. (2016) found that older olive (*Olea europaea*) somatic embryo cultures cultivated for eight years showed decreased ability to form mature embryos, increased time for culture line proliferation, and plantlets converted from older cultures grew slower than those converted from younger cultures. Similarly, *Pinus pinaster* cultures began to lose embryogenic ability over numerous subcultures, grew aberrant structures at increasing rates, and embryos failed to mature (Breton et al., 2006). *Picea glauca* cultures likewise showed decreased proliferation and conversion rates as cultures aged, and aberrant structures became more prevalent over time (Dunstan and Bethune, 1996). *Kalopanax septemlobus* cultures 2.5 years and 6 months in age showed several differences: the younger tissue was composed of cells with dense cytoplasm with a prominent nucleus; higher mitotic activity, higher protein content, and produced more embryos compared to the older culture (Park et al., 2011). The authors identified the most prominent difference between the two cultures as the ratio of cytokinins to ABA; younger cultures contained a higher proportion of cytokinin:ABA than older cultures. Several theories suggest different factors that may be at the heart of these observations. A disruption to auxin signaling—often terms as 'habituation'—may suggest that formerly-competent tissues no longer react to auxin like they did in their younger stages. Endogenous IAA content was 7.5 times greater in a recalcitrant *Picea morrisonicola* genotype that had lost its ability to form mature somatic embryos; however, treatment with either anti-auxin, 3,5-triiodobenzoic acid or 2-(4-chlorophenoxy)-2-methylpropionic acid (PCIB) induced the recalcitrant line to produce mature somatic embryos at the cost of reduced embryogenic capacity of the embryogenic line (Liao et al., 2008). However, another plausible theory suggests that cytosine methylation can gather over time until the DNA becomes hypermethylated, much to the tissue's detriment. Rival et al. (2013) analyzed the effects of culture age on global methylation rates and embryogenic capacity using several cell lines from five oil palm genotypes; methylation rates increased over time in all but one clonal line, which was also unable to form somatic embryos even when it was younger. Similarly, older *Pinus pinaster* (Klimaszewska et al., 2009) and cacao (*Theobroma cacao*) cultures (Quinga et al 2017) tended to have higher overall methylation rates compared to younger cultures. In the latter study, higher methylation rates were observed along with overall decreased embryo production. This observation is not limited only to *in vitro* cultures; younger *Pinus radiata* shoot meristems showed

both higher growth rates and overall decreased methylation compared to more mature trees (Fraga et al., 2002). In addition to this methylation, other forms of epigenetic regulation may be affected by age. Coffee cultures maintained for over two years showed an increase in the number of somatic plants displaying somaclonal variation compared to those derived from younger cultures. Though methylation was not significantly different, the total number of chromosomes was often different between somaclonal plants displaying aberrant phenotypes than normal ones (Landey et al., 2015). Genotype may also have a significant effect on culture aging. Of 20 oil palm clones maintained on multiplication for over 20 years, six still proliferated readily, although the resulting plantlets has overall decreased vigor (Konan et al., 2010). The authors observed that 'mantled' phenotype plantlets, which develop an aberrant phenotype during fruit formation, became far more prevalent over years of culture. Pea (*Pisum sativum*) cultures maintained for 24 years, however, remained vigorous without significant difference in DNA methylation rates between young and old cultures and little difference in DNA mutations detected by AFLP, but plants derived from old cultures displayed some signs of decreased fertility and senescence (Smykal et al., 2007).

The compound 5-azacytidine inhibits cytosine methylation, thus leading to changes to the epigenetic state of the cell (Tokuji et al 2011). Specifically, it may undo age-related methylation, but, by its nature, 5-azacytidine's demethylation is random. Plants have several mechanisms that control the balance of both cytosine and histone methylation in the form of both cytosine methylases and demethylases. Demethylase enzymes, such as DEMETER (DME), may reactivate transposon expression, causing both their expression and, often, subsequent remethylation via RNA-dependent DNA methylation (RdDM) using the RNAi pathway (Calarco et al., 2012). In this pathway, transposons may be digested by DICER-like (DCL3) proteins, which create siRNA. This siRNA, in turn, binds to the conserved nucleic acid binding motif on ARGONAUTE-like proteins (AGO), which then guides DNA sequence-specific cytosine methylation (Fang and Qi, 2016). The importance of this regulation pathway is exemplified by the epigenetic state of pollen and the gametophyte. In pollen, CHH-type methylation is lost, leading to transposon expression and generating the formation of 24 nt siRNA, and AGO9 proteins remained undetected in the unfertilized female gametophyte (Calarco et al., 2012). After fertilization, these siRNA may reinforce transposon silencing through the AGO-RdDM pathway, in effect 'resetting' the repression of transposable elements (Feng et al.,

2010). An unintentional effect of demethylation may be the expression of transposable elements. Transposons can affect gene expression after insertion near genes, which may affect their transcription (Lanciano and Mirouze, 2018). This may occur through several means, including histone modifications near transposable inserts near the coding regions of genes, or methylation caused by DCL-dependent siRNA which might guide cytosine methylation to nearby regions (Cui and Cao, 2014). Strange et al., (2011) found that a transposon inserted itself into *Arabidopsis thaliana* FLOWERING LOCUS C, where its effect on gene expression depended on whether or not the plant was vernalized; it either promoted upregulation of the FLC gene before vernalization, or silenced it during vernalization, leading to earlier (non-vernalized) or later (vernalized) flowering times compared to plants without the transposon. This change in expression, however, occurs because the transposon is present in the first intron of the gene; insertion of transposable elements into the coding region of a gene generally results in a loss-of-function for that specific allele (Hirsch and Springer, 2017). Demethylation affects not only cytosine methylation in promoter and coding regions of DNA, but also histone modifications responsible for binding and silencing transposable elements (Komashko and Farnham, 2010). In this study, human cancer cells treated with 5-azacytidine showed large differences in gene expression, and subsequent analysis using chromatin immunoprecipitation (ChIP) found that epigenetic repression marks had shifted after treatment with 5-azacytidine (histone modifications switching from H3K27me3 to H3K9me3 and vice versa). The authors found that most changes in gene expression were not expressly mediated by histone modification or methylation, but several were. This property makes this compound valuable for studying the effect of changes in methylation on living tissue. The addition of 5-azacytidine was found to restore embryogenic potential to old cacao cultures (Quinga et al., 2017), but it was also found to disrupt *Arabidopsis* embryo formation (Yamamoto et al., 2005). In both *Brassica napus* and *Hordeum vulgare*, 5-azacytidine was found to promote embryogenesis initiation and decrease overall methylation, but successive embryo formation was hindered (Solís et al., 2015). 5-azacytidine might have different effects on different cell types; a human multiple myeloma cell line suffered decreased viability on exposure to 5-azacytidine than other human cell lines, such as peripheral blood mononuclear cells or bone marrow stromal cells (Kiziltepe et al. (2007).

One important factor for determining the use of 5-azac is that, just as naturally-occurring epigenetic states can be passed down to progeny, so too can those changes be induced by 5-azac exposure. For example, flax (*Linum usitatissimum*) seedlings exposed to 5-azac flowered earlier and displayed hypomethylation compared to control seedlings. The subsequent nine generations displayed the same tendency between the distinct lines, although the hypomethylation rates in the 5-azac-treated line became more variable over time (Fieldes et al., 2005). Considering these wide-ranging effects, I sought to validate the past reports of 5-azac restoring vigor to aging peach palm cultures while observing the possible deleterious effects it might have on embryogenic and non-embryogenic peach palm tissues.

1.7 CRYOPRESERVATION

1.7.1 Cryopreservation and the physics of cellular freezing

Cryopreservation is the freezing of tissues, usually with liquid nitrogen, at sub-freezing temperatures and revivifying them at a later date. This technology is complex; the same biological aspects that govern embryo growth are involved, but there are also the physical aspects of water undergoing phase transition both within and without the cell that govern whether a given tissue will return to life. Cellular death during cryopreservation is a major obstacle to the technology. Peach palm cells, without exception, die if not properly treated before being submerged in liquid nitrogen (Heringer et al., 2013b). Considerable work has gone into understanding how cells die when they are frozen: what kills them, where it occurs, and when it occurs. Ice formation inside of bovine cells, filmed at 8000 frames sec^{-1}, began at a single point within the cell and formed a unified front as it moved; in most cases this was located in the periphery of the cell (Stott and Karlsson, 2009). In this study, the authors were able to see that the ice front changed conformity as it met organelles. Extracellular water is likewise a major concern: high-speed cryophotography showed that water trapped in the paracellular space between the cell and the glass substrate into which it was embedded formed ice crystals and penetrated the cell (Stott and Karlsson, 2009). Han and Bischof (2003) reviewed two methods of cell injury: the formation of ice crystals in intracellular water and a form of osmotic pressure created by 'dehydration' of the cell as it froze (Han and Bischof, 2004). As water froze, the electrolyte and solute components of the cell were forced into the unfreezable water fraction, thus creating a large imbalance in osmotic pressure between water and the 'brine' is unfreeable water and solutes; upon warming, osmotic pressure was equalized with a

physical force that ruptures the cellular membrane. Muldrew and McGann, (1994) presented a mathematical model based on a literature review that showed that, during freezing, there is a water flux driven by an osmotic pressure gradient that could be a physical, pressure-driven force imparted to the cellular membrane during dehydration as the source of cell damage.

Crystal formation, likewise, is a source of cellular death through causing membrane punctures as a form of mechanical damage. As a liquid cools below its melting point, molecules can arrange themselves into an ordered structure. This initial arrangement is called a nucleation center, which is lattice of hydrogen-bound molecules onto which other molecules can be incorporated, eventually creating a crystal (Akyurt et al., 2002; Matsumoto et al., 2002). Nucleation centers form and are broken at high rates close to the melting temperatures because molecules are still relatively active in a liquid state; however, should a stable nucleation center form, crystal growth is high. Consequently, nucleation center formation is highest at very low temperatures, but, by extension, the consequent low mobility of water molecules results in slow crystal formation (Akyurt et al., 2002; Matsumoto et al., 2002). A liquid can exist in a supercooled state below its melting point, especially if cooling is rapid and the liquid's viscosity is high, leading to molecules unable to rearrange themselves into an ordered structure as they lose energy; effectively, they maintain the same disordered distribution of a liquid state without the ability to significantly rearrange themselves into a crystalline lattice (Wok, 2009). At this stage, solution viscosity plays an essential role: it delays the intermolecular rearrangements of water molecules into thermodynamically-favored crystals, effectively holding molecules together long enough for the glass transition to be passed (Fahy and Wowk, 2015). At this point, water takes on the characterization of 'solidified water.' NMR analysis of water and heavy water mixed with corn starch found that much of the water remained mobile even after the solution reached a glass state in temperatures as low as -23°C, however mobility decreased with temperature (Li et al., 1998). Thus, at temperatures near freezing, water molecules are still quite active and able to rearrange themselves into crystals able to perform mechanical damage to cells.

After a sample is frozen, it must be then thawed. Due to the nature of the water molecule, thawing can be just as destructive to cells as the initial freeze, if not more so. Warming a frozen solution shows that this mobility

creates an environment ultimately dangerous to cellular life because ice nucleation readily occurs at lower temperatures, leading to the formation of a multitude of nucleation centers able to incorporate warming water molecules to make countless small crystals (Wowk, 2010). Such a phenomenon is a bottleneck for preserving organs for transplants (Ruggera and Fahy, 1990). As rat cells were warmed at a rate of 5°C min^{-1}; ice formation occurred as temperatures warmed from -40 to -38°C, a result which was reversed by a spike of heat flow as the very same eutectic ice melted. At about -6°C, there was a spike of ice crystal growth. This likely contributed to a large portion of cellular damage. An analysis using neutron-scattering found that the highest rates of crystallization happens during warming when water in the cryopreserved carp (*Cyprinus carpo*) embryo tissue melted enough to leave the glass state. However, this process was significantly repressed in samples cryopreserved with 50% D2O, 23% 1,2-propanediol, 27% methanol, and 20% DMSO (Kirichek et al., 2015). These cryoprotectants—chemicals that aid in protecting tissue from ice and osmotic-related damage—affected the rate at which ice formed, how it formed, and how it behaved when it melted.

1.7.2 Cryoprotectants and cryopreservation methods

Pre-treatments are often required to prepare tissue for cryopreservation through either removing water from tissue or by acting as stabilizing molecules to protect molecular integrity and increase viscosity. As mentioned above, carp embryos treated with a cryoprotectant solution were found to survive freezing much more than non-treated embryos (Kirichek et al., 2016). The authors used neutron scattering analysis to determine how their cryoprotectant solution worked; it encapsulated water in a matrix of nanoparticles that formed clusters, which then formed into amorphous ice, but lacked the structural relaxation needed to crystalize during rapid freezing, thus preventing damage to cells. In such a situation, ice crystal formation becomes impossible within the rapid cooling period. Slow-cooling without liquid nitrogen is, likewise, a viable option with cryoprotectant; olive somatic embryos survived controlled cooling cryopreservation after three days incubation in a 0.75M sucrose solution (Lynch et al., 2011).

Cryoprotectants are divided into two overarching groups: permeating and non-permeating. Permeating cryoprotectants include DMSO, ethylene glycol, glycerol, and propylene glycol; non-permeating include polyvinylpyrrolidone, polyethylene glycol, sucrose, trehalose, and other larger molecules (Fahy and Wowk, 2015). Each cryoprotectant has its

own effectiveness, as well as its own drawbacks. Propylene glycol, for example, decreased the temperature at which water freezes, but also limited the rate at which ice formed due to the molecular interactions between propylene glycol and water (Rhys et al., 2016). Increased cryoprotectant concentration; however, may interfere with the health of the cell. High concentrations of cryoprotectants, even without freezing, can be harmful for both animal and plant cells (Fahy and Wowk, 2015). Human ovarian tissue was infiltrated using ethylene glycol or DMSO, with the latter conveying more protection to cryopreservation (Newton et al., 1998); however, infiltration at warmer temperatures caused significantly more damage than colder temperatures. Hagedorn et al. (2009) observed that algal cells exposed to cryoprotectants for more than 20 min suffered damage. Cryoprotectants increase viscosity, which reduce diffusion of water molecules on nucleation centers or ice crystals (Fahy and Wowk, 2015). Cryoprotectants may also serve to reduce damage upon sample warming. Poly(vinyl alcohol) prevented recrystallization of ice in a concentration-dependent manner; the higher the concentration of poly(vinyl alcohol), the less ice crystals grew upon warming (Inada and Lu, 2003). Both the physics of water and the biological reaction to these cryoprotectants ultimately govern revivification post cryopreservation

Past work with peach palm somatic embryogenesis first achieved success with vitrification. Somatic embryos were incubated in a PVS3 (50% glycerol *w/v*, 50% sucrose *w/v*) for 240 min and then submerged in liquid nitrogen (Heringer et al., 2013a). A later experiment employed a variation of vitrification called droplet vitrification, so called because samples are placed onto droplets of cryoprotectant on aluminum strips, which, due to the thermoconductive properties of the metal, decrease the time needed to freeze the samples (Heringer et al., 2013b). In this experiment, somatic embryos were incubated in PVS3 for certain durations and then placed onto aluminum strips and then submerged into LN.

Standard and widely-used PVSs, such as PVS2 and PVS3, have achieved wide success; however, possible new compositions could improve on their successes by providing compounds that stabilize water. Ionic compounds not only provide an osmotic equalizing agent, but they freely transfer across cell membranes. Additionally, dicationic compounds such as magnesium and calcium form hydration spheres, in which the negative pole of the water molecule forms a transitionary bond with the positively-

charged ion, which might potentially interfere with the free arrangement of unfreezable water into crystalline lattices.

1.7.3 Frontiers in cryopreservation methods

Heavy water, also called deuterium oxide or D_2O, is simply water with its two bonded hydrogens replaced with deuterium molecules. This appears to be a rather simple change, and, indeed, in most respects water and D_2O are equal for such parameters as surface tension, boiling point, heat of fusion, heat of vaporization, and refractive index (Jackson and Rabideau, 1964). Heavy water and normal water crystals are identical in size and shape under equal environmental conditions (Megaw, 1934). There is one essential characteristic that does set heavy water over regular water for the purpose of cryopreservation: the hydrogen bonds between D_2O molecules are stronger (Soper and Benmore, 2008). These stronger hydrogen bonds result in only a few select, though important differences: D_2O freezes at a higher temperature and it is nearly 25% more viscous than water at 20°C (Fernandez-Prini et al., 2004). This viscosity reflects stronger bonds between molecules, and this extends to non-water molecules, such as ionic compounds or polymers like polyethylene glycol, which creates a solution in which molecules are not as free to move about as they would be in a water-based solution. As the temperature of solutions plummet below freezing, this becomes critical for the formation of ice. Specifically, D_2O might serve as the solvent for vitrification solutions. D_2O has already seen great applicability as a stabilizer of vaccines (Sen et al., 2009). Ice penetration into poliovirus capsids were reduced when the vaccines were stored in 87% D_2O with 1 M $MgCl_2$ compared to water, D_2O, or $MgCl_2$ alone. Similarly, D_2O and 0.2% *w/w* Tween 20 were found to improve BSA protein stability; their hydration and native conformation in a frozen state during freezing and later thawing remained unchanged (Jena et al., 2017). $MgCl_2$ and D_2O together improved vaccine stability D_2O alone in several studies, suggesting that $MgCl_2$ could be an important additive in cryoprotectant solutions (Wu et al., 1995; Chen et al., 1997). D_2O readily permeates across cellular borders. Hagedorn et al. (2009) tested the uptake of D_2O in coral alga symbiotes by placing the cells in a vessel with D_2O (Hagedorn et al., 2010). Initially, the alga cells floated, but, over time as D_2O was taken in, they sank to the bottom of the vessel, a phenomenon easily replicated by placing heavy water ice on liquid H_2O: the heavy water ice sinks to the bottom of the vessel unlike normal ice. D_2O has only been applied several times to cryopreserving animal tissue, such as a component of a ‘stabilizing’ buffer for rat oocytes prior to

cryopreservation (Egerszegi et al., 2013); however I've been unable to uncover widespread use in plants. Curiously, deuterium oxide seems to have a stabilizing effect in both extreme cold and warm; *Bacillus cereus* bacteria grown in deuterium oxide showed greater stability at culture in 40°C medium than those grown in water-based media (Lu et al., 2013). Considering the possibility of increased damage through thawing, rather than freezing due to the behavior of water to form nucleation centers, stability of water structure may be essential for preserving tissue.

Considering that this 'stabilizing' effect has been shown to improve survivability at cold and temperatures in proteins, viruses, bacteria, fungi, and animals, it stands to reason that plants cells, too, might be stabilized for cryogenics, however, D_2O negatively-affected the growth of growing plants in many cases (Giovanni, 1961; Stein and Forrester, 1963; Bhatia and Smith, 1968; Waber and Sakai, 1974). Mg2+ might function act as a stabilizer in the following way: the dicationic charge forms a 'hydration sphere'—the negative pole of water molecules is attracted to the positive ion (Pavlov et al., 1998; Kiriukhin and Collins, 2002), creating a force that may prevent molecule movement during cooling or thawing, provided either process is fast. This phenomenon is said to 'increase' water structure through stabilizing hydrogen bonds, ultimately making the solution more viscous (Marcus, 2010). Consequently, water structure can be decreased through other types of ions; large-sized ions, such as I^- and Cs^+. These ions are too large to fit inside holes between networks of hydrogen-bonded water molecules, requiring them to destroy some hydrogen bonds to create a cavity large enough to move into, which accelerates the flow of molecules within the solution (Marcus, 2010). The structure-making an structure-breaking effect of a given ion can be measured (Table 1 of Marcus, 2010). Increased viscosity in salt-containing solutions followed the following trend: the more positive the structure-making value is after accounting each ion, the higher the viscosity (i.e. LiCl solutions were more viscous than NaI) (Goldsack and Franchetto, 1977). Curiously, solutions containing net negative structure-breaking ions (KI) did not show the opposite trend; initially, viscosity decreased until salt concentrations increased to a certain point, where after viscosity increased, but never returned to the viscosity of pure water. *Stychnos gerrardii* embryonic axes treated with high pH solution containing cathodic water made by electrolyzing a solution of calcium and magnesium chloride suffered less damage from ROS (Berjak et al., 2011). ROS was still produced within tissue subjected to cryopreservation, but the authors postulate that the cathodic water either

stimulated internal antioxidant systems. However, this may also reflect the structure-building capabilities of either magnesium or calcium ions in solution. This is a possible means of reducing the damage caused by high cryoprotectant solutions; viscosity can be increased using ions, such as magnesium and calcium, which can passively enter and leave the cell easier than larger molecules, like glycerol. Considering the damaging nature of most PVS3, these properties may be applied to reduce PVS's overall toxicity without losing its effect on promoting vitrification survival.

1.8 GAS EXCHANGE, EMBRYO CONVERSION, AND PLANTLET ACCLIMATIZATION

1.8.1 Ethylene, its effects on plant growth, and the benefits of gas exchange

Closed environments do not allow plants to exchange gases; this is disruptive to physiologies that evolved over millions of years toward certain atmospheric conditions. The *in vitro* environment creates conditions unlike those found in a normal environment. For example, a closed contained with active cell cultures may accumulate ethylene and other hydrocarbons and have different concentrations of oxygen and carbon dioxide compared to *ex vitro* conditions (Buddendorf-Joosten and Woltering 1994). Ethylene accumulation, either through stress or ripening, has an overall negative effect on cell growth, depending on the developmental age of the plant (reviewed in Dubois et al 2018). Accumulation can cause drastic changes in gene expression and cell behavior, possibly limiting plant growth. Though ethylene stimulated early *Heavea brasiliensis* mitotic activity, cultures without ventilation soon began to suffer necrosis (Auboiron et al 1990). Ethylene and *Scrophularia yoshimurae* stem hyperhydricity were correlated, but increased gas exchange caused by removing the parafilm sealing the containers gradually reduced hyperhydricity and increased shoot quality, though at the expense of decreased shoot proliferation (Lai et al., 2004). However, it might be simpler and cheaper to simply use ventilated vessels, which has the additional benefit of potentially reducing hyperhydricity and acclimatization efficiency. *Capsicum annuum* plantlets grown in ventilated vessels had significantly more total chlorophyll content, lower stomatal density, higher dry mass, and an overall improved regeneration rate compared to plants grown in non-ventilated vessels (Mohamed and Alsadon, 2011). *Scrophularia yoshimurae* plantlets grown in ventilated vessels had increased survival compared to those grown in non-ventilated vessels (Chen et al 2006).

However, *Picea mariana* somatic embryo conversion was not significantly different between those grown in sealed or ventilated vessels (Meskaoui and Tremblay, 1999). Even if embryo conversion is not different, the decreased humidity might be beneficial regardless. Aloe (*Aloe polyphylla*) plants grown in ventilated vessels showed greater deposition of cuticle and greater chlorophyll content in shoots, but at the expense of decreased regeneration, likely caused by the gradual evaporation of water from medium and the increased concentration of gelling agents and solutes (Ivanova and Staden, 2010). Peach palm *in vitro* plantlets lacked cuticle (Batagin-Piotto et al., 2012), and, relatedly, acclimatization rate is still the major bottleneck to introducing regenerated plantlets into field conditions. Controlling this relationship with water is likely the means of overcoming this obstacle. Partial desiccation of mature date palm somatic embryos from 90% to 75% water led to increased conversion rates (Fki et al., 2003). Ventilation led to greater *Tagetes erecta* plantlet survival upon transfer to field conditions compared to those grown in medium supplemented with ABA; both had greater survival than plantlets grown in sealed vessels on medium without ABA (Aguilar et al., 2000). ABA-treatment is already an established practice in peach palm, but ventilation is not. Ventilation will likely lead to significant difference in both biochemical profiles and morphological characteristics.

1.9 GENERAL OBJECTIVES

The goal of this work was to elaborate several of the developing technologies related to impermanence in tissue culture by studying the long-term effects of *in vitro* culture using an older and younger culture line of the same genotype; test means of reversing aging; and improve existing means of maintaining a culture line either with mixed developmental stages for re-induction of SE or cryopreservation of existing cultures

1.9.1 Specific objectives:

Chapter 1: Study the biochemistry of eight-year-old and two-year-old peach palm cultures and evaluate the effect of 5-azacytidine on reversing age-related methylation

Sub-objectives

- Evaluate the effect of 5-azacytidine on the growth of eight-year-old and two-year-old peach palm cultures

- Compare the protein profiles of two-year-old embryogenic cultures, embryogenic cultures derived from 5-azacytidine-treated eight-year-old cultures, and eight-year-old cultures
- Compare the total global methylation between embryogenic and non-embryogenic two-year-old cultures and eight-year-old cultures
- Compare carbohydrate content between embryogenic and non-embryogenic two-year-old cultures and eight-year-old cultures

Chapter 2: Improve peach palm somatic embryo conversion from embryos to functioning plantlets in closed and ventilated vessels.

Sub-objectives

- Evaluate the effect of ventilated or sealed vessels on the growth and morphology of maturing peach palm embryos
- Determine if there is a difference in the methylation states between peach palm somatic embryos grown in ventilated or sealed vessels
- Compare the carbohydrate profiles of either tissue to determine potential differences in metabolism

Chapter 3: Improve existing peach palm vitrification cryopreservation using novel or little-studied approaches

Sub-objectives

- Determine the role of heating rate, vitrification strip material, and vitrification method on post-vitrification peach palm cluster growth
- Evaluate the effects of heavy water and dissolved ions on the effectiveness of a modified plant vitrification 3 solution
- Analyze the feasibility of including a partial dehydration step before vitrification on post-vitrification peach palm cluster regrowth

2 5-AZACYTIDINE RESTORES EMBRYOGENIC POTENTIAL TO EIGHT-YEAR-OLD PEACH PALM (*BACTRIS GASIPAES* KUNTH) CULTURES AND CAUSES CHANGES TO GLOBAL METHYLATION AND PROTEIN ACCUMULATION

2.1 ABSTRACT

Formerly-embryogenic eight-year-old and two-year-old embryogenic peach palm (*Bactris gasipaes* Kunth.) cultures were treated with 0, 4, 16, or 64 μM 5-azacytidine, an inhibitor of cytosine methylation, to evaluate its ability to restore embryogenic potential to old cultures and observe its effects on younger embryogenic tissue. Either 16 or 64 μM 5-azacytidine restored the ability of a small proportion of older cultures to produce somatic embryos, but the same concentration also caused somatic embryos from the two-year-old embryogenic culture line to lose regulation, resulting in non-embryogenic cultures and fast-growing yellow callus. Two-year-old embryogenic tissue had slightly lower global methylation, but only 4 μM 5-azacytidine caused a drop in global methylation. Two-year-old embryogenic cultures, eight-year-old cultures, and embryogenic cultures from 16 μM 5-azacytidine-treated eight-year-old cultures were analyzed using shotgun proteomics. Both embryogenic cultures were similar compared to one another, and both shared the same increased accumulation of several sets of proteins involved in a number of cellular functions: cellular redox control, anaerobic fermentation, protein degradation, NO-related synthesis and storage, and several forms of epigenetic regulation, including the highly-conserved Argonaute 4 protein; and older cultures showed increased amounts of proteins involved in cell wall formation and rearrangement, defense-related peroxidases associated with the cell wall, and phospholipidase D. All three tissues showed proteins involved in hypoxia response. HPLC analysis of eight-year-old cultures with embryogenic and non-embryogenic two-year-old cultures revealed that eight-year-old cultures lacked any detectable carbohydrates, whereas the younger ones contained measurable amounts of arabinose, ribose, and sucrose.

2.2 RESUMO

Uma linha de culturas de pupuna de oito anos de idade que perdeu sua capacidade de formar embriões somáticos foi comparada com uma cultura de dois anos de idade que poderia produzir embriões. Ambas as linhas de cultura foram tratadas com 0, 4, 16 e 64 μM 5-azacitidina, um inibidor da metilação da citosina, para avaliar sua capacidade de restaurar o potencial embriogênico em culturas mais velhas e observar seus efeitos

em culturas jovens. A 5-azacitidina nas concentrações de 16 ou 64 µM restauraram a capacidade de uma pequena proporção de culturas mais antigas de produzir embriões somáticos (8,80% e 4,80%, respectivamente), porém as mesmas concentrações também causaram embriões somáticos da linha de culturas de dois anos de idade a perder regulação, resultando em tecido não embriogênico e calo amarelo. A linha de culturas embriogênicas de dois anos de idade apresentou menor metilação global quando comparado com culturas de oito anos de idade. Em seguida, comparamos o perfil proteico de três tecidos destintos usando *shotgun proteomics*: (i) culturas embriogênicas de dois anos de idade; (ii) culturas embriogenicas derivadas de culturas de oito anos tratadas com 16 µM 5-azacitidina; e (iii) culturas de oito anos sem tratamento. As culturas embriogênicas (i) e (ii) obtiveram resultados semelhantes, e ambas compartilhavam a mesma *up accumulation* de proteínas envolvidas em várias funções celulares: controle do estado redox celular; fermentação anaeróbica; degradação protéica; síntese e armazenamento de óxido nítrico e várias formas de regulação epigenética - incluindo a proteína altamente conservada Argonaute 4. Culturas mais antigas mostraram maiores quantidades de proteínas envolvidas na formação da parede celular, peroxidases relacionadas à defesa associadas com a parede celular, e fosfolipidase D. Todas as três culturas de tecidos mostraram proteínas envolvidas em resposta à hipóxia. A análise por HPLC de culturas de oito anos de idade com culturas embriogênicas e não embriogênicas de dois anos de idade revelou que as culturas de oito anos de idade não tinham carboidratos detectáveis, enquanto as mais jovens continham quantidades mensuráveis de arabinose, ribose e sacarose.

2.3 INTRODUCTION

Plant cells are totipotent; they are able to change their underlying genetic, epigenetic, biochemical, and morphological profiles from that of cell in a specialized tissue to that of an undifferentiated cell, from which it may redifferentiate into an embryogenic state, reenter the cell cycle, and gradually grow specialized tissues through steps similar to zygotic embryogenesis (Jiménez, 2001; Verdeil et al., 2007; Fehér, 2015b). This process, called somatic embryogenesis (SE), is a means of producing thousands of identical plants from a single explant. SE induction, however, requires several main conditions in peach palm (*Bactris gasipaes*): exogenous synthetic auxins, such as 2,4-D or picloram; exogenous activated charcoal to serve as an antioxidant; exogenous sucrose and basal salts; semi-solid medium; and months of undisturbed growth (Steinmacher, 2010; Heringer et al., 2013b; Ree and Guerra, 2015;

Nascimento-Gavioli et al., 2017). Resulting somatic embryos can be further multiplied through secondary somatic embryogenesis by subculture month-after-month onto fresh medium. Although certain culture lines can are able to grow consistently over several years (Konan et al., 2010), many others show severe signs of aging: decreased vigor (Breton et al., 2006), aberrant structure formation (Dunstan and Bethune, 1996), increased somaclonal variation (Landey et al., 2015), and infertility in resulting plantlets (Smýkal et al., 2007). There are several main possibilities long thought to be responsible: loss of genetic regulation, possibly as a result of hypermethylation (Phillips et al., 1994), or as a result of changes in gene expression brought on by habituation—the loss of the requirement for exogenous auxins or other plant growth regulators—and a subsequent change in cell behavior (Smulders and De Klerk, 2011). Over time, peach palm embryogenic cultures began to slow in growth and develop certain characteristics: first, an increasing number of embryos became hyperhydritic or had slight malformations; second, many of these aberrant somatic embryos 'aborted' to form fibrous tissue similar to non-embryogenic tissue; third, cultures became hypersensitive to wounding and tended to develop necrotic tissue; fourth, previously-established optimized protocols for somatic embryo maturation (Heringer et al., 2014) became completely ineffective—embryos remained in the globular state regardless of maturation medium they were placed upon; and, finally, they eventually lost the ability to form new somatic embryos entirely. As this was occurring, we restored the same genotype *in vitro* through using a previously-established method (Steinmacher et al., 2007c) of SE induction using thin cell layers (TCLs) taken from the single apical meristems of somatic embryo-derived plantlets converted before age-related problems began to emerge in large numbers, and the resulting cultures were vigorous, free of aberrant structures, and able to serve as a model for examining the effects of age through differentially-accumulated proteins using shotgun proteomics. Further, the compound 5-azacytidine (5azac), a potent blocker of cytosine methylation (Tokuji et al., 2011), has recently been shown in our lab to restore embryogenic potential to aging *Theobroma cacao* (Quinga et al., 2017) cultures, therefore, we wished to explore the use of 5azac on restoring embryogenic potential in peach palm cultures as well. Our objective was to compare older and younger cultures using shotgun proteomics to analyze the differences in relative protein abundance for key metabolic and redox pathways, observe the effects of 5azac on tissue response to both old and young cultures, attempt to restore embryogenic potential to older cultures,

examine total global methylation among all tissues, and analyze the comparative abundances of carbohydrates using HPLC.

2.4 METHODS

2.4.1 Cell line induction and maintenance

Older cultures were first induced from apical meristem TCLs ~1mm thick were extracted from *in vitro*-germinated peach palm zygotic embryos. TCLs were placed on plant culture medium consisting of Murashige and Skoog salts (Murashige and Skoog, 1962), Morel and Wetmore vitamins (Morel and Wetmore, 1951), 30 g L^{-1} sucrose, 1 g L^{-1} glutamine, 2.3 g L^{-1} phytagel, 2.5 g L^{-1} activated charcoal, and 450 µM picloram (Heringer et al., 2013a). After embryogenic callus formed, cultures were transferred to medium containing the same basal media without activated charcoal and with the picloram concentration reduced to 10 µM and subcultured monthly onto the same medium for eight years. After about four years, somatic embryos were matured on culture medium consisting of the basal medium without activated charcoal and picloram, but with the addition of 5 µM abscisic acid (ABA) for two weeks. Afterwards, cultures were then placed on conversion medium consisting of the basal medium without activated charcoal and growth regulators. Embryos were placed in a 25 $^{+}$/- 2°C growth chamber with a 16 hr photoperiod. To establish a younger line of the same genotype, plantlets from converted somatic embryos were removed and subjected to the same SE induction protocol. Resulting embryogenic cultures were multiplied over two years. All tissue culture medium was adjusted to pH 5.8 prior to being autoclaved at 121°C and 1.5 atm for 15 min.

2.4.2 5-azac treatment

Three types of tissue were selected for testing: eight-year-old cultures consisting of hyperhydritic, malformed somatic embryos (OC); two-year-old highly-embryogenic cultures consisting of somatic embryos and polyembryogenic clusters (EC); and two-year-old fibrous non-embryogenic callus (NEC) (Figure 3-1). Twenty-five cultures from each of the three main culture lines were placed on multiplication medium with either 0, 4, 16, or 64 µM 5azac for two weeks, and then subcultured onto 5azac-free multiplication medium for four weeks. Cultures were then evaluated for what type of tissue grew—i.e. if EC grew EC, or if it

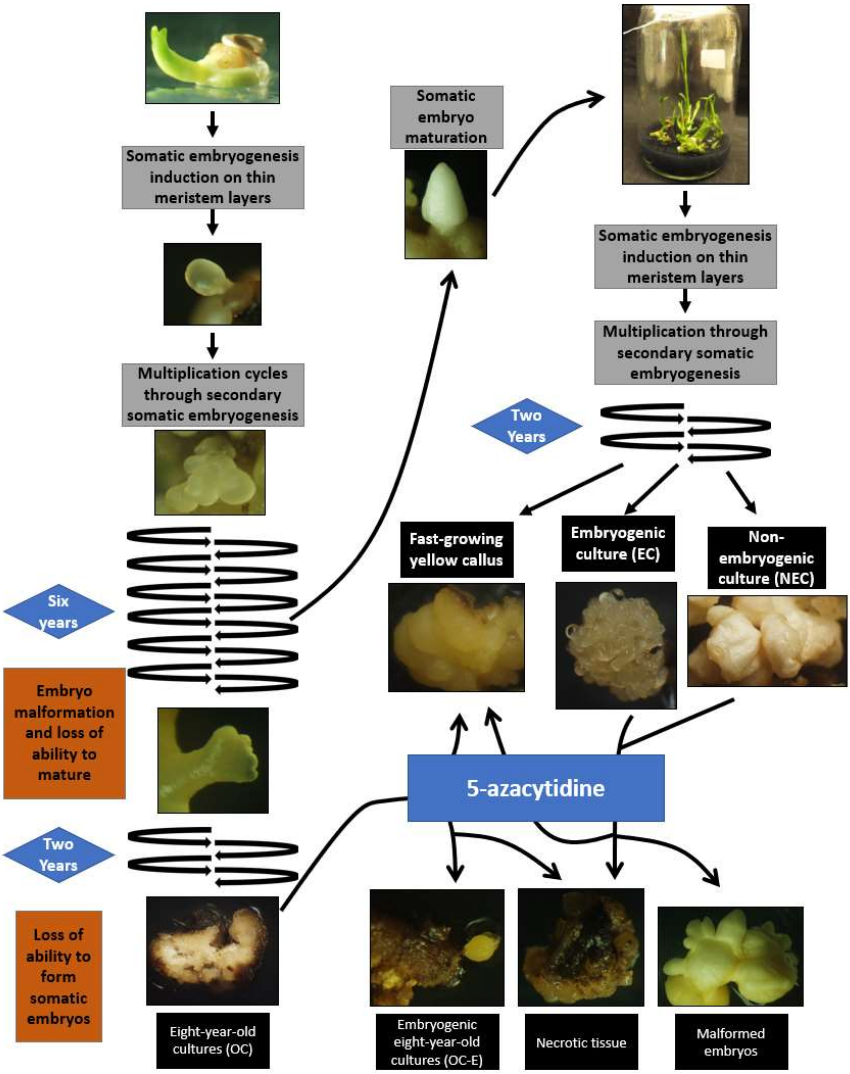


Figure 2-1:Timeline of the development of eight-year-old peach palm (Bactris gasipaes *Kunth*) cultures, establishment of two-year-old cultures, and the tissue types encountered under various culturing conditions.

produced a different tissue type, such as yellow callus or NEC—and what proportion of cultures developed necrotic tissue.

2.4.3 Shotgun proteomics

Three tissues were compared: EC, OC, and EC derived from OC (OC-E). To generate enough tissue for proteomic analysis, OC were placed on multiplication medium containing 16 µM 5azac and left to grow for two weeks and then subcultured onto multiplication medium without 5azac to generate EC. Three biological replicates consisting 500 mg each of culture type were harvested and macerated under liquid nitrogen. Samples were lyophilized overnight at -50°C to preserve protein integrity.

Lyophilized samples were solubilized in protein extraction buffer consisted of 7 M urea, 2 M thiourea, 2% triton X-100, 1% dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were vortexed for 30 min at 8 °C and centrifuged at 16,000 g for 20 min at 4 °C. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology to remove any interferent from samples (Nanjo et al., 2012). After protein precipitation, samples were resuspended in urea 7 M:thiourea 2 M solution for proper resuspension. Protein digestion was performed using the filter-aided sample preparation (FASP) methodology as described by Wiśniewski et al., (2009), with modifications.

Before starting the digestion procedure, an integrity test was made to check for damaged filter units (Hernandez-Valladares et al., 2016), thus only the working units were used. After that, 100 µg protein aliquots were added to the Microcon-30 kDa filter units (Millipore) (Lipecka et al., 2016), washed with 200 µL of 50 mM ammonium bicarbonate (solution A), and centrifuged at 10,000 g for 15 min at 25 °C (unless otherwise stated, all centrifugation steps were performed at this condition). This step was repeated once for complete removal of urea before reduction of proteins. Next, 100 µL of 50 mM DTT (GE Healthcare) freshly made in solution A were added, gently vortexed, and incubated for 20 min at 60 °C (1 min agitation and 4 min resting, 650 rpm). Then, 200 µL of 8 M Urea in 50 mM ammonium bicarbonate (solution B) were added, and

centrifuged for 15 min. For protein alkylation, 100 µL of 50 mM iodoacetamide (GE Healthcare) freshly prepared in solution B were added, gently vortexed, and incubated for 20 min at 25 °C in the dark (1 min agitation and 19 min resting, 650 rpm). Next, 200 µL of solution B were added and centrifuged for 15 minutes. This step was repeated once. Then, 200 µL of solution A were added and centrifuged for 15 min. This step was repeated twice. Approximately 50 µL of sample remained after the last wash. For protein digestion, 25 µL of 0.2% (v/v) RapiGest (Waters, Milford, CT, USA) and 25 µL of trypsin solution (1:100 enzyme:protein final ratio, V5111, Promega, Madison, WI, USA) were added, gently vortexed, and incubated for 18 h at 37 °C (4 min resting and 1 min agitation at 650 rpm). For peptide elution, the filter units were transferred for new microtubes and centrifuged for 10 min. Then, 50 µL of solution A were added and centrifuged for 15 min. This step was repeated once. For RapiGest precipitation and trypsin inhibition, 5 µL of 15% trifluoroacetic acid (TFA, Sigma-Aldrich) were added, gently vortexed, and incubated for 30 min at 37 °C. Then, samples were centrifuged for 15 min, the supernatants collected, and vacuum dried. Peptides were resuspended in 100 µL solution of 95% 50 mM ammonium bicarbonate, 5% acetonitrile and 0,1% formic acid. The resulting peptides were quantified by the A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

MS analysis of each samples was performed in a nanoAcquity ultraperformance liquid chromatograph (UPLC) connected to a Synapt G2-Si HDMS mass spectrometer (Waters) for electrospray—liquid chromatography—tandem mass spectrometry analysis. To separate the proteins, 1 µg of each sample of digested proteins were loaded onto a nanoAcquity UPLC 5 µM C18 trap column (180 x 20 mm) at 5uL min^{-1} for3 min and then onto the nanoAcquity HSS T3 1.8 µM analytical reverse-phase column (75 µm x 150 mm) at 400 nL min^{-1}. Column temperature was maintained at 45°C. Peptides were eluted using a binary gradient: mobile phase A consisted of water and 0.1% formic acid (Sigma-Aldrich); and mobile phase B consisted of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. The gradient elution program was as follows: 7% B for 3 min; from 7% to 40% B until 90.09 min; from 40% to 85% B until 94.09 min; constant 85% until 98.09 min; decrease to 7% B until 100.09 min; and constant 7% B until 108.09 min. Mass spectrometry was performed in positive and resolution mode (V mode) at 35000 fwhm resolution with ion mobility and in the data-independent acquisition mode. The ion mobility wave velocity was 600 m s-1; transfer

collision energy ranged from 19 to 45 V in high energy mode; cone can capillary voltages were set at 30 and 2800 V, respectively; and the source temperature was maintained at 70°C. The nano flow gas pressure was 0.50 bar, and the purge gas flow ranged from 145 to 150 L h^{-1}. TOF parameters consisted of a scan time of 0.5 s in the continuum mode and a mass range of 50 to 2000 Da. Human [GLU1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol uL^{-1} was used for external calibration, and lock mass acquisition occurred every 30 s.

ProteinLynx Global Server (PLGS, version 3.0.2) (Waters) and ISOQuant workflow(Distler et al., 2014, 2016) were used for spectral processing and database searching. The PLGS data was processed using a low-energy threshold of 150 counts, an elevated energy of 50, and an intensity threshold of 750. The analysis was performed with the following parameters: two missed cleavage; minimum fragment ion per peptide equal to 3; minimum fragment ion per protein equal to 7; minimum peptide per protein equal to 2; fixed modifications of carbamidomethyl; and variable modifications of oxidation and phosphoryl. False discovery rate for peptide and protein identification was set to a maximum of 1% with a minimum length of 6 amino acids. The proteomics data was compared to *Phoenix dactyilifera* protein databank from the UniProtKB (www.uniprot.org) for protein identification because it is the largest databank with proximity to peach palm. Comparative label-free quantification analysis was performed with ISOQuant software using previously-described settings and algorithms (Distler et al., 2014, 2016) with an analysis that included retention time alignment; exact mass retention time; and ion-mobility spectrometry clustering, as well as data normalization and protein homology filtering. ISOQuant annotated the resulting feature clusters by evaluating consensus peptide identification and identification probabilities. Protein identification false discovery rate in ISOQuant was set to 1%; a peptide score greater than 6; a minimum peptide length of 6 amino acids; and at least two peptides per protein. Label-free quantifications were estimated using the TOP3 quantification approach (Silva et al., 2006), followed by multidimensional normalization implemented within ISOQuant.

After ISOQUant data analysis, only proteins that were present or absent for unique proteins in all three biological replicates of a given tissue type were considered for differential abundance analysis. Data were analyzed using a two-tailed Student's t test. Proteins with P-values less than 0.05 were considered up-accumulated if the log2 of the fold change was

greater than 0.5, and down-accumulated if the fold change was less than -0.5. Proteins were blasted against Nonredundant Plants/Viridplantae_Protein_Sequences database using the Blast2GO software (www.blast2go.com) (Conesa et al., 2005) for protein description.

2.4.4 Global methylation Analysis

2.4.4.1 DNA Extraction

DNA was extracted from 300 mg fresh matter using the protocol of Doyle (1987). Tissue was macerated under liquid nitrogen and then transferred to a tube containing 700 µL of extraction buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 2 % polyvinylpyrrolidone, and 0.2% 2-mercaptoethanol). The slurry was incubated in a 60°C water bath for 1 h. Afterwards, the samples were centrifuged at 6000 *x g* for 5 min. The aqueous phase was transferred to a fresh tube, and an equal volume of 24:1 chloroform:isoamyl alcohol was subsequently added to each tube. The tubes were mixed by inversion for 1 min before they were centrifuged at 15,000 *x g* for 8 min. The upper aqueous phase was removed and placed into a fresh tube, and 2/3 volumes of chilled isopropanol were added. Samples were then incubated at -20°C for 2 h to precipitate the nucleic acids. Afterwards, the tubes were centrifuged at 4°C at 15,000 *x g*. The aqueous layer was removed and the pellet rinsed twice with chilled 70% ethanol before the samples were dry for 2 h. The pellet was resuspended in 100 µL MilliQ water, and the quantity and quality of the DNA assessed using a NanoDrop® 1000 (NanoDrop Technologies, USA).

The nucleic acid was further purified and prepared for HPLC analysis using a modified form of the protocol described in Johnston et al. (2005) and Heringer et al. (2013a). Samples of 100 µL of 1 µg μL^{-1} nucleic acid were denatured at 65°C for 5 min, cooled to 4°C for 10 min, and then 10 µL of 1 µg μL^{-1} RNase A and 10 µL of 20 µg μL^{-1} RNase T1 were added. Samples were vortexed and incubated at 37°C for 17 h. After digestion, 20 µL of sodium acetate (3M, pH 5.4) were added and mixed by vortexing. DNA and RNase-resistant RNA were precipitated with one volume of cold isopropanol for 30 min at -20°C. The samples were then centrifuged at 6000 *x g* for 12 min. The supernatant was discarded, and the pellet was washed twice with 70% (*v/v*) ethanol. Samples were dried at room temperature and then resuspended in 50 µL of deionized water. The concentration of nucleic acids was determined using Nanodrop®, and the concentration adjusted to 0.25 µg μL^{-1}. 100 µL of each sample were then separated and denatured by heating to 100°C for 2 min and then

cooled to 4°C for 5 min. 5 µL of 10 mM $ZnSO_4$ and 10 µL of nuclease P1 (Sigma Aldrich) (1.0 U ml^{-1} in 30 mM NaOAc, pH 5.4) were added to each sample, vortexed, and then incubated at 37°C for 15 hr. Afterwards, 10 µL of Tris-HCl (0.5M, pH 8.3) and 10 µL of alkaline phosphatase (10 U ml^{-1} in 2.5M $(NH_4)_2SO_4$) were added, mixed, and incubated at 37°C for 2 h. Samples were then centrifuged at 10,000 *x g* for 5 min and the supernatant was removed and stored at -20°C until HPLC analysis.

2.4.4.2 HPLC methylation analysis

HPLC analysis was performed using a modified protocol from Johnston et al. (2005) and Heringer et al., (2013a). A HyperClone™ 5 µm ODS (C18) 120 A LC Column 250 x 4.6 mm (Phenomenex®), guard column 4.0 x 3.0 mm (Phenomenex®), and UV detector (280 nm) were used to analyze the samples. Aliquots of 20 µL were injected into the HPLC and subjected to a flow rate of 1 ml min^{-1} under the following gradient program: 3 min at 100% buffer A (0.5% (*v/v*) methanol in 10 mM KH_2PO_4, pH 3.7 adjusted with phosphoric acid, 0.22 µm pore-filtered), linear increase over 3 to 20 min to 100% buffer B (10% (*v/v*) methanol in 10mM KH_2PO_4, pH adjusted to 3.7 with phosphoric acid, 0.22 µm pore-filtered); and from 20 to 25 min with 100% buffer B. All samples were repeated in triplicates. The standards consisted of all nucleotides, including methylated cytosine. A standardized curve was created using samples consisting of known concentrations of each nucleosides. 5mdC quantification was determined by 5mdC concentration divided by the 5mdC plus the dC concentration multiplied by 100. Concentrations were determined by the peak area using the LC Solution software (Shimadzu®, Kyoto, Japan).

2.4.5 HPLC carbohydrate analysis

Carbohydrate analysis was performed based on the protocol by Filson and Dawson-Andoh (2009) with modifications. Approximately 500 mg of fresh matter from EC, NEC, or OC—taken with extreme care to prevent tissue medium contamination—were macerated under liquid nitrogen and in the presence of 0.5 g PVPP and 0.015 g ascorbic acid. The slurry was then added to a centrifuge tube, and 0.75 mL of 80% ethanol was added. The sample was vortexed and then incubated in a 70°C water bath for 90 min. The sample was then vortexed at 15,000 x g for ten min. The liquid phase was removed to a new tube. The extraction was then repeated, resulting in a sample totaling 1.5 mL, which was then filtered through a 0.2 µm filter and stored at -20°C until analysis. Samples aliquots of 5 µl were injected into the HPLC with an evaporative light scattering detector

(ELSD-LT II, Shimadzu) at a flow rate of 1 ml min^{-1}. Carbohydrates were fractionated using two separate columns: a Prevail Carbohydrate ES 5 µm (250 x 4.6 mm) and a Prevail Carbohydrate ES 5 µm (7.5 x 4.6 mm) kept at 25°C. Carbohydrates were separated under the following gradient program: 20% Solution A (MilliQ water) and 80% Solution B (100% acetonitrile, filtered) for 16 min, from 80 to 70% Solution B for 16 to 16.5 min, and then 16:30 to 23 min. The ELSD detector had a drift tube temperature of 40°C and a nitrogen pressure of 350 mPa. Standard concentrations of ribose, arabinose, xylose, fructose, mannose, glucose, sucrose, and maltose were used to construct the model used to compare peaks and determine the concentration of carbohydrates.

2.4.6 Statistical analysis

Growth response and global methylation analysis were arranged in a randomized design using three culture types (EC, OC, and NEC). Growth responses analysis consisted of five replicates, each consisting of 25 independent cultures on a petri dish for a total of 125 cultures per treatment. Global methylation and carbohydrate analysis was performed using three separate replicates. Growth response, global methylation, and carbohydrate data was analyzed with ANOVA ($P < 0.05$) using R software version 3.5.1 (R Development Core Team, 2014) with a Tukey post hoc test when a statistical significance was found.

2.5 RESULTS

2.5.1 Azacytidine treatment significantly affected culture growth in a dose-dependent manner

Growth response depended on 5azac concentration and tissue type; as 5azac concentration increased, so did the emergence of tissue types different than the original culture (i.e. compact yellow callus growing from embryogenic tissues) and the appearance of necrotic tissues (Figure 3-2). In EC, this was detrimental due to the loss of embryogenic potential; however, a small percent of the OC did show increases in embryogenic potential, as determined by the emergence of yellow globular embryos from yellowish-brown, fibrous, amorphous tissue (shown in Figure 3-1). Only OC treated with either 16 or 64 µM 5azac resumed production of somatic embryos (8.80% and 4.80% of cultures, respectively) by the 42nd day post treatment. OC also formed an increasing amount of fast-growing yellow compact callus as 5azac concentration increased (8.80, 23.2, and 48.8% of cultures for 4, 16, and 64 µM 5azac, respectively). There was a significant interaction between 5azac treatment and culture type ($p<0.05$). EC began to form a significantly-increased amount of non-EC tissue in

the form of either NEC or fast-growing yellow callus after treatment with 16 or 64 μM 5azac. NEC, unlike the other two tissues, showed no significant differences in emergence of other tissue types regardless of 5azac treatment. Tissue type and 5azac treatment had a significant interaction in determining culture necrosis. Higher 5azac concentrations correlated with increased rates of tissue necrosis in both EC and NEC; however, OC showed significantly high rates of necrosis even when not exposed to 5azac. The slight decrease in mean necrosis as 5azac concentration increased in OC might be explained by the emergence of both fast-growing yellow compact callus and embryogenic cultures.

2.5.2 Two-year-old EC and EC derived from OC are similar in protein profiles; both differ greatly from OC

Shotgun proteomic analysis identified 398 proteins in total among EC, OC, and OC-derived EC cell lines (OC-E) (Table S1 for complete list, Table 2 for proteins of high interest). Among those, 98 were up-accumulated in EC and OC-E lines compared to the OC line. Eleven proteins were found exclusively in both EC and OC-E, but not in OC, and four were unique to the OC line. Curiously, three proteins were found exclusive to both the OC line and the OC-E line, but not the EC line. 15 amino acids were detected in the EC line, but not in either the OC-E or OC lines. Based on the results of comparing the proteins to those of known proteins, the represented differentially-accumulated proteins fell within several main categories: metabolism; structural proteins; protein degradation; redox-state maintenance; epigenetic factors; and cell-cycle related proteins. Both EC and OC-E contained 23 up-accumulated metabolism-related proteins, whereas OC had two up-accumulated proteins. 13 proteasome protein subunits were up-accumulated in both EC and OC-E, whereas the only protein-degradation protein that was up-accumulated in non-embryogenic tissue was an aminopeptidase. Redox-related proteins were differently-accumulated between EC and OC-E

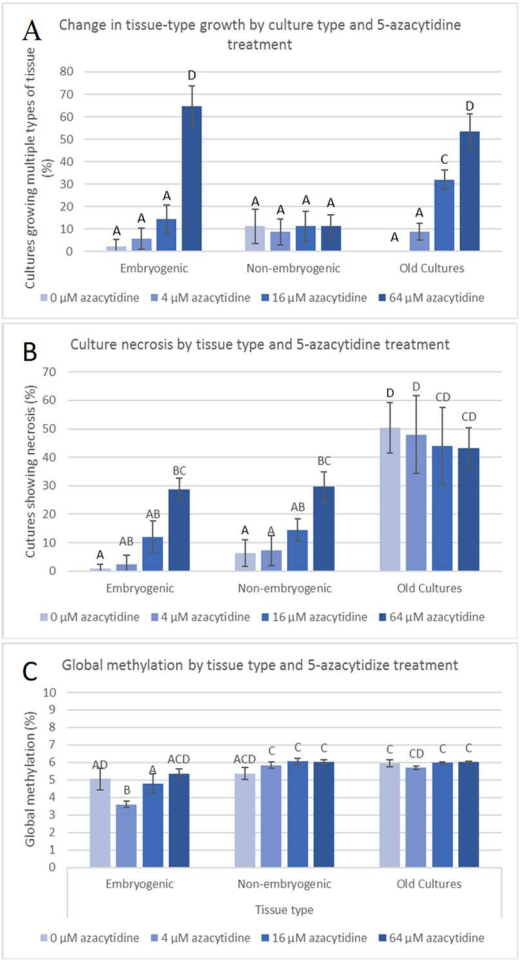


Figure 2-2:Effects of 5-azacytidine on peach palm (Bactris gasipaes *Kunth*) tissue types and its influence on stimulating the formation of tissue types other than the original tissue (A); inducing necrosis (B); and its effect on global methylation (C)

compared to OC: six proteins related to redox state were up-accumulated in EC and OC-E compared to seven in OC, four of which were peroxidases. The four epigenetic state-related proteins that were differentially-accumulated were found in both EC and OC-E. Five amino acid synthesis proteins were up-accumulated in EC and OC-E compared to two for OC. Similar to redox state-related proteins, structural molecule-related proteins were somewhat balanced, with both EC and OC-E lines containing eight up-accumulated proteins for tubulin subunits or actin, but OC had five up-accumulated proteins each with distinct roles in either cell wall or cell membrane maintenance.

2.5.3 Embryogenic, non-embryogenic, and old cultures differed little in global methylation profiles

NEC and OC showed no significant differences in global methylation profiles regardless of 5azac treatment (Figure 3-2) according to a Tukey post hoc test, though there was a significant interaction between tissue type and 5azac concentration ($p<0.05$). The global methylation of EC on medium without 5azac was significantly less than OC on 5azac-less medium. Only 4 μM 5azac seemed to cause a significant decrease in global methylation compared to non-treated EC. Elsewise, 5azac had little apparent effect on influencing global methylation within culture types.

2.5.4 HPLC carbohydrate analysis suggests OC is poor in available energy reserves, but EC and NEC differ little

Of eight carbohydrates, we detected measurable amounts of three—ribose, arabinose, and sucrose—among all three tested tissue types, but several other peaks frequently appeared, but either their placement or shape prevented their inclusion in the results (Figure 3-3). OC, however, lacked measurable amounts of all three of these carbohydrates, whereas all three were detected in both EC and NEC (Table 3-2). When abundances were compared between EC and NEC, we found that sucrose was not significantly different ($p=0.786$), and neither was ribose ($p=0.194$). Arabinose, however, was more abundant in EC than in NEC ($p=0.0498$).

Table 2-1:Select differentially-accumulated proteins in peach palm (Bactris gasipaes *Kunth*) two-year-old embryogenic cultures (EC), eight-year-old cultures (OC), and embryogenic cultures derived from 6-azacytidine-treated OC (OC-E)

Accession	Reported Peptides	Max Score	Description	EC/OC	EC-O/OC	EC-O/EC
A0A2H3X3S9_PHODC	13	6997.09	Pyruvate kinase	Up	Up	Unchanged
A0A2H3YPN9_PHODC	14	4514.85	monodehydroascorbate reductase 5, mitochondrial isoform X2	Up	Up	Unchanged
A0A2H3X7A1_PHODC	14	22891.95	Glyceraldehyde-3-phosphate dehydrogenase	Up	Up	Unchanged
A0A2H4A0T6_PHODC	2	678.63	S-(hydroxymethyl)glutathione dehydrogenase	Up	Up	Unchanged
A0A2H3XB86_PHODC	6	8413.34	1-Cys peroxiredoxin	Up	Up	Unchanged
A0A2H3XSB9_PHODC	20	13134.35	actin-3-like	Up	Up	Unchanged
A0A2H3ZGE4_PHODC	18	7687.39	Tubulin alpha chain	Up	Up	Unchanged
A0A2H3YG00_PHODC	4	1093.35	gamma-aminobutyrate transaminase 1, mitochondrial	Up	Up	Unchanged
A0A2H3YY07_PHODC	15	3688.60	protein argonaute 4B-like isoform X1	Up	Up	Unchanged
A0A2H3Y3E1_PHODC	10	1720.18	26S protease regulatory subunit S10B homolog B	Up	Up	Unchanged
A0A2H3Z7Q3_PHODC	3	913.07	prohibitin-3, mitochondrial-like	Up	Up	Unchanged
A0A2H3Z0J6_PHODC	10	3088.84	DEAD-box ATP-dependent RNA helicase 56 isoform X1	Up	Up	Down
A0A2H3ZC38_PHODC	5	5414.24	ubiquitin-conjugating enzyme E2 36-like	Up	Up	Down
A0A2H3Y9U5_PHODC	5	1076.37	WD-40 repeat-containing protein MSI4-like	Up	Up	Down
A0A2H3YLB4_PHODC	16	4385.03	Sucrose synthase	Up	Up	Down
A0A2H3YXF8_PHODC	8	4224.95	pyruvate decarboxylase 1-like	Up	Up	Down
A0A2H3YRJ8_PHODC	3	8920.43	heat shock cognate 70 kDa protein 2-like	unique EC	Unique EC	Down
A0A2H3X5K5_PHODC	7	574.32	protein argonaute 4B-like	unique EC	Unique EC	Up
A0A2H3XJ36_PHODC	6	6460.13	Peroxidase	Down	Down	Up
A0A2H3YFG0_PHODC	5	1492.34	alpha-xylosidase 1	Down	Down	Unchanged
A0A2H3ZDE3_PHODC	9	5153.61	UDP-arabinopyranose mutase 1	Down	Down	Unchanged
A0A2H3XDA3_PHODC	9	1417.93	aldehyde dehydrogenase family 2 member B7, mitochondrial	Down	Down	Unchanged
A0A2H3YZI3_PHODC	10	984.09	Phospholipase D	Down	Down	unchanged
A0A2H3XMT5_PHODC	5	1096.88	UDP-glucose 6-dehydrogenase	Down	Down	Down

Table 2-2:Abundances of detected carbohydrates in peach palm (Bactris gasipaes *Kunth*) embryogenic cultures (EC), non-embryogenic cultures (NEC), and eight-year-old cultures (OC)

Carbohydrate type	Tissue line	mg per 500 mg of fresh tissue
	EC	5.99 ± 1.48
Ribose	NEC	4.79 ± 0.318
	OC	Not detected
	EC	11.4 ± 1.62
Arabinose	NEC	8.83 ± 0.742
	OC	Not detected
	EC	2.24 ± 0.342
Sucrose	NEC	2.11 ± 0.829
	OC	Not detected

2.6 DISCUSSION

2.6.1 Cell line growth response to 5-azac treatment

Over the course of several years, we observed that peach palm cultures lost vigor; grew increasing numbers of malformed and hyperhydric embryos; failed to undergo embryo maturation despite previously-established methodologies (Heringer et al., 2014); and often aborted, leading to the formation of fibrous cultures with a tendency to oxidize and turn necrotic. Loss in embryogenic capacity over time is not a new observation: long-term *Areca catechu* cultures developed higher rates of aberrant structures (Wang et al., 2010a); six-months-old *Kalopanax septemlobus* cultures had overall different cellular morphology, higher mitotic activity, higher protein content, produced more somatic embryos, and had a higher ratio of cytokinin:ABA than 2.5 year-old cultures (Park et al., 2011); and both *Picea glauca* (Dunstan and Bethune, 1996) and *Pinus pinaster* (Breton et al., 2006) cultures developed aberrant structures and a reduced capacity for somatic embryo proliferation and maturation over time. We tested the effects of 5azac, a potent inhibitor of cytosine methylation (Fieldes and Amyot, 2000), on reversing the age-related hypermethylation. 5azac treatment, however, had wide-ranging effects among all culture lines (Figure 3-2). As concentration increased, so, too, did the rates of both necrosis and the formation of tissues different than the original culture types, with some cultures forming multiple types of

tissue on the same culture. This effect of 5azac can be both beneficial and detrimental; young EC seemed to suffer a breakdown in regulation, leading to the formation of heterogenous, fast-growing yellow compact callus and NEC, but some of the old cultures began to form new embryos in support of the previous research found in *Theobroma cacao* cultures (Quinga et al., 2017). The wide-ranging effects of 5azac as an inhibitor of cytosine methylation, therefore, might be tissue-specific in its practical application. 5azac promoted embryogenesis initiation in *Brassica napus* and *Hordeum vulgare*, however, subsequent embryo development was hindered (Solís et al., 2015). Exposure of *Arabidopsis* somatic embryo to 5azac led to a decrease in *DcLEC1c* expression in treated cells, leading to a disruption in the signaling pathway required for embryo formation, thus causing malformations (Yamamoto et al., 2005). A similar result may have occurred in peach palm EC, where 5azac may have disrupted the strict gene regulation required for establishing and maintaining root:shoot bipolarity and caused loss of apical dominance. Further, disruption to cell cycle regulation might have led to the loss of order in highly-mitotic meristematic cells, leading to the rapid growth of fast-growing yellow compact callus. These results agree with those found in *Brachypodium distachyon*, which found that as 5azac concentration increased, so did the disruption to culture growth with the highest used concentration (50 µM) completely inhibiting embryogenic mass formation (Betekhtin et al., 2018). The authors deduced that 5azac caused DNA double-stranded breaks, leading to signal disruption and changes to gene expression. In the same study, 5azac stimulated increased expression of glutathione S-transferase, a protein involved in ROS detoxification, which may have been in response to stress (Ogawa, 2005). Another possible result of demethylation is the liberation of methylated transposable elements, which, under normal circumstances, are often tightly regulated. Demethylase enzymes, such as DEMETER (DME), may reactivate transposon expression, causing both their expression and, often, subsequent remethylation via RNA-dependent DNA methylation (RdDM) using the RNAi pathway (Calarco et al., 2012). In this pathway, transposons may be digested by DICER-like (DCL3) proteins, which create siRNA. This siRNA, in turn, binds to the conserved nucleic acid binding motif on ARGONAUTE-like proteins (AGO), which then guides DNA sequence-specific cytosine methylation (Fang and Qi, 2016). Transposons can affect gene expression after insertion near genes, which may affect their transcription (Lanciano and Mirouze, 2018). This may occur through several means, including histone modifications near transposable inserts near the coding regions of genes, or methylation

caused by DCL-dependent siRNA which might guide cytosine methylation to nearby regions (Cui and Cao, 2014). Strange et al., (2011) found that a transposon inserted itself into *Arabidopsis thaliana* FLOWERING LOCUS C, where its effect on gene expression depended on whether or not the plant was vernalized; it either promoted upregulation of the FLC gene before vernalization, or silenced it during vernalization, leading to earlier (non-vernalized) or later (vernalized) flowering times compared to plants without the transposon. This change in expression, however, occurs because the transposon is present in the first intron of the gene; insertion of transposable elements into the coding region of a gene generally results in a loss-of-function for that specific allele (Hirsch and Springer, 2017). Currently, however, it is too early to say if the loss-of-regulation observed in 5azac-treated EC was due to change in gene expression, changes to histone modifications, reactivation of formerly-silenced transposable elements, a combination of some or all of these, or a completely other factor.

Necrosis, too, increased with 5azac concentration in both healthy young tissue lines, suggesting that 5azac has a toxic effect due to both the disruption to signaling and possibly by direct DNA damage. OC necrosis appeared to slightly decrease as 5azac concentration increased, but this was likely the result of the emergence of healthy tissue. Curiously, the wide-ranging, and often toxic, effects of 5azac is not unique to the plant kingdom: human multiple melanoma cells were significantly more sensitive to 5azac, as reflected in decreased proliferation and increased mortality, than non-cancerous bone marrow stromal cells (Kiziltepe et al., 2007). Another important consideration is the long-term effects of 5azac. *Linum usitatissimum* seedling treated with 5azac flowered earlier and had overall less methylation than non-treated cells, and this remained generally true over the nine successive generations, although methylation rates became more variable over time (Fieldes et al., 2005). We cannot yet say what the effects of 5azac are on long-term survival and proliferation of regenerated EC, however, the effect of 5azac on growing cultures was quite severe by both disrupting cellular growth and inducing necrosis.

2.6.2 Differences in protein amounts among cultures

2.6.2.1 Proteins involved in energy generation are, in general, up-accumulated in young EC and EC regenerated from older tissues, and are likely the result of hyperhydricity-related hypoxia

OC, unlike young EC and the EC derived from 5azac-treated older cultures, grows slowly, and this is partly reflected in the overall greater abundance of proteins involved in the TCA cycle, glycolysis, or other means of energy generation in either EC (Table 3-1). More telling about the behavior of all three tissues, however, was the presence of proteins involved in anaerobic fermentation. Pyruvate decarboxylase, one of the main enzymes involved in the breakdown of pyruvate to ethanol (Mithran et al., 2014), was found in all three tissues; however, it was significantly more up-accumulated in EC and OC-E compared to OC. Alcohol dehydrogenase, the second enzyme involved in the fermentation pathway, was also found in all three tissues. This suggests that all tissues were subjected to hypoxia. Hypoxia could be induced *in vitro* under several possible conditions: oxygen concentrations can be depleted from the headspace over time (Linden et al., 2001), and, even under normal atmospheric conditions, oxygen diffusion into plant cells is a slow, passive process (Geigenberger, 2003), especially when tissues are waterlogged (Fukao and Bailey-Serres, 2004; Rojas-Martínez et al., 2010). We, therefore, hypothesize that all three examined tissues were undergoing hypoxia based on several reasons: 1), pyruvate decarboxylase and alcohol dehydrogenase were detected in all tissues; 2), EC, NEC, and yellow compact callus were foud to be ~90% water by mass (88.8.1 ± 2.47, 89.8 ± 2.94, and 89.9 ± 2.33%, respectively), however, not enough OC samples were available to determine hyperhydricity in those cultures; and, 3) the three tissues showed dramatic losses in volume during sample lyophilization. Higher pyruvate carboxylase accumulation in EC and OC-E is, therefore, a critical factor in analyzing cellular behavior, because it may suggest that those tissues were able to produce more energy under the same stressful conditions. As oxygen becomes less plentiful, the cell changes much of its biochemistry, leading to the accumulation of specific TCA cycle and sucrose degradation intermediates (Obata and Fernie, 2012). For example, waterlogged *Lotus japonicus* showed severely-impaired glycolysis and TCA cycle activities, leading to the accumulatio of GABA, alanine, succinate, and a definite shift toward the fermentation pathway for energy generation (Rocha et al., 2010). Plants respond to low oxygen as a means of survival and adaptation; even if overall ATP

generation is reduced because of decreased respiration from low available oxygen, plants are able to switch to other pathways to produce as much energy as possible while consuming the least amount of oxygen (van Dongen et al., 2011), however, the increased reliance on fermentation also results in cytosolic acidification, ROS accumulation, and lipid peroxidation (Kosová et al., 2011). To this end, pyruvate decarboxylase serves an additional purpose beyond creating ATP through fermentation: controlling the amount of pyruvate inside a cell to prevent its use in oxygen-consuming respiration (Zabalza et al., 2009). For example, *Pisum sativum* roots treated with pyruvate and succinate—an electron-donating substrate for complex II of the oxygen-consuming electron transport chain (Bhagavan and Ha, 2015)—showed severely-reduced oxygen content to the point of anoxia, significantly lower even than roots cultivated under hypoxic conditions (Zabalza et al., 2009). Alcohol dehydrogenase activity is correlated with increased hydrogen peroxide concentrations under hypoxia (Fukao and Bailey-Serres, 2004). Overexpression of either pyruvate decarboxylase or alcohol dehydrogenase, however, significantly increased *Arabidopsis* hairy root survival and growth, suggesting that the benefits of energy generation in an environment unfavorable to respiration can be more beneficial than cytoplasmic acidosis is detrimental (Shiao et al., 2002), provided that the cells have the means of detoxifying fermentation products. Gamma-amino butyrate transaminase 1, an enzyme that catabolized GABA to succinate semialdehydate as the second enzyme involved in the GABA shunt (Koike et al., 2013), was up-accumulated in EC compared to OC. Reduced cytosolic pH stimulates the activity of glutamate decarboxylase, yielding GABA, which can act as signaling molecules that affect gene expression (Shelp et al., 1999). GABA transaminase catabolizes GABA, suggesting that GABA is either being produced at higher levels, or is being consumed at faster rates in EC compared to OC. Considering that GABA accumulated under low-pH conditions, either is possible; however, more analysis would be required to confirm the relative amounts of GABA in either tissue. GABA application to *Helianthus annuus* promoted ethylene accumulation (Kathiresan et al., 1997), which, because of its volatile nature, can either travel from cell-to-cell, or be released into the atmosphere inside a closed container, where it can cause large changes to cell behavior (Biddington, 1992). *Brassica napus* seedlings treated with a GABA transaminase inhibitor responded with decreased root growth, but a significant increase in all amino acids, especially alanine, which increased 19-fold compared to untreated seedlings (Deleu et al., 2013). This led the authors to hypothesize that

GABA catabolism was intrinsically linked to metabolism, which was also supported by the severe dwarfism and infertility in tomato mutants lacking an active GABA transaminase (Koike et al., 2013). Succinate, which accumulates under hypoxic conditions due to decreased activity of the electron transport chain, is also created by the oxidation of succinic semialdehyde, a byproduct of GABA catabolism (Eprintsev et al., 2017). Considering this common link between stress response, metabolism, hypoxia, and cytosolic acidosis, the amount of GABA present in *in vitro* cultures is of great interest for future research. Both EC and OC-E showed an increase in a decaboxylating malate dehydrogenase malic enzyme, which acts as a means of limiting cytosolic acidosis under hypoxic conditions (Roberts et al., 1992). The redox state of the cell also depends on the NADH:NAD^+ ratio (Moller, 2001). The transport of glyceraldehyde-3-phosphate dehydrogenase (G3PD)—also up-accumulated in EC—to the mitochondrial matrix bypasses the ROS-producing complex I of the electron transport chain to donate electrons to a mitochondrial FAD:G3PD complex, which then donates electrons to the ubiquinone pool instead of the transport of electrons from NADH to complex I, producing NAD^+ (Moller, 2001; Shen et al., 2006) . *Arabidopsis* mutants lacking G3PD activity had an increased NADH/NAD^+ ratio and overall increased concentrations of ROS under normal growing conditions and significantly more so under stress conditions (Shen et al., 2006). Higher concentrations of G3PD in EC cultures suggest that they have an active means of maintaining their redox state despite the increased acidosis that would result from hypoxia-induced fermentation. In addition to fermentation-related enzymes, sucrose synthase was found in all cultures, which was expected because sucrose is the only carbohydrate source available to the cultures. Sucrose synthase is energy-saving under hypoxic conditions; instead of the two ATPs consumed by invertase to breakdown sucrose, sucrose synthase uses only one (Geigenberger, 2003; van Dongen et al., 2011). Indeed, as shall be discussed in a later section, OC proved to be bankrupt of energy reserves—not even sucrose, the most prevalent carbohydrate in EC and NEC, was detected in any amount. Together, these results suggest that all three tissues were growing under hypoxic conditions, and either EC was better equipped to survive and grow under this condition due to greater abundance of fermentation enzymes and the means of reducing the oxygen-consuming activity of the mitochondrial electron transport chain, however, all of these processes require an active means of detoxifying stress and fermentation-induced ROS generation and cytoplasmic acidosis.

2.6.2.2 EC from old and young tissue show different means of coping with stress compared to old cultures

In addition to the ROS generated by fermentation and respiration, plant cells produce ROS in response to stress (Baťková et al., 2008). *In vitro* plants, in particular, are subjected to several constant stresses: a head space containing high concentrations of water and quantities of carbon dioxide, oxygen, and nitrogen different than normal earth atmosphere (Linden et al., 2001), as well as the accumulation of ethylene, aldehyde, and other metabolic byproducts (Buddendorf-Joosten and Woltering, 1994); salt-related stress (Gupta and Huang, 2014); osmotic stress (Zavattieri et al., 2010); and hyperhydricity-related hypoxia (Rojas-Martínez et al., 2010). Either EC and OC-E differed with OC in their strategies to adapt to these stresses. Both EC and OC-E contained higher accumulation of amino acids corresponding to monohydroascorbate reductase 5, which catalyzes the conversion of monodehydroascorbate to ascorbate as an integral part of the glutathione-ascorbate cycle by oxidizing NADH (Rodziewicz et al., 2014). 1-cys peroxiredoxin, another antioxidant, was also found up-accumulated in ECs. 1-cys peroxiredoxins have dual purposes: first, they act as a mild antioxidant by detoxifying hydrogen peroxide and degrading alkyl hydroperoxides to their alcohol form, and, second, the enzyme functions as a chaperone under overoxidized conditions, thus preventing protein denaturation under unfavorable conditions (Kim et al., 2011). Additionally, 1-cys peroxiredoxin gene *FePer1* was found to be expressed solely in the developing seeds of *Fagopyrum esculentum*, but it was absent in leaves or stems (Lewis et al., 2000). This suggests that the old cultures, despite becoming malformed and nearly amorphous, did not necessarily change cell fate. S-hydrocymethyl glutathione dehydrogenase, despite its name, is not part of the ROS-scavenging ascorbate-glutathione cycle; rather, it is an enzyme that catalyzes the NADH-dependent reduction of S-nitroglutathione, a potential reservoir of nitric oxide (NO) (Kubienová et al., 2013). However, a byproduct of the reaction is GSSG—the oxidized form of glutathione, which may then enter the ascorbate-glutathione cycle. This is a potential link between both hydrogen peroxide detoxification and NO. NO is essential for proper growth; *Arabidopsis* mutants lacking the S-hydrocymethyl glutathione dehydrogenase gene had malformed leaves, sensitivity to 2,4-D, reduced hypocotyl elongation, proliferation of secondary shoots, and loss of apical dominance (Kwon et al., 2012). The first signs of malformation in older cultures as they lost embryogenic potential was similar: embryos formed, aborted, and grew into amorphous, fibrous masses devoid of apparent

bipolarity or apical dominance. Prohibitin-3 proteins involved in cell cycle regulation, mitochondrial electron transport, apoptosis signal-induction, and NO-mediated stress response (Aken et al., 2007; Wang et al., 2010b), were detected in all three tissues, however, several isoforms were found more prevalently in either EC or OC-E. *Arabidopsis* loss-of-function mutants for the prohibitin-3 gene *AtPHB3* showed malformed mitochondria, decreased meristematic cell production, decreased cell division rates, decreased cell expansion rates, and overall retarded growth, and double knockouts were completely non-viable (Aken et al., 2007). Wang et al., (2010b) found that mutants deficient for the *Arabidopsis* prohibitin-3 gene *AtPHB3* did not accumulate NO in response to hydrogen peroxide under abiotic stress, possibly as a result of disruption to mitochondrial function from decreased electron flux, resulting in less NO synthesis or NO degradation. These results suggest that OC likely underwent a breakdown in NO-related polarity due to reduced capacity for NO storage and transport, possibly as a result of cytoplasmic acidosis caused by hypoxia, resulting in tissue malformation and a reduced capacity to maintain an active metabolism. In addition, both EC and OC-E showed higher accumulation of amino acids corresponding to Universal Stress proteins PHO S32, a relatively-unstudied protein whose exact function remains unknown, however, similar proteins were found to be up-accumulated in *Oryza sativa* under glyphosate-induced stress (Ahsan et al., 2008) and in *Arabidopsis* under drought conditions (Sakuma et al., 2006). Thiamine thiazole synthase catalyzes the formation of the thiazole ring in thiamine, a cofactor for several metabolic processes in glycolysis and the citric acid cycle. Biotic and abiotic stress induces thiamine thiazole synthase activity as a means of defense response (reviewed in Goyer, 2010).

OC displayed several differently-accumulated types of ROS-related proteins, including several types of peroxidases. There are many types of peroxidases; *Arabidopsis*, for example, has at least 74 peroxidase genes, each expressed in a tissue-specific manner and located in many different potential locations within the cell (Tognolli et al., 2002; Blokhina and Fagerstedt, 2010). Without a detailed analysis of each peroxide, it isn't possible to know the specific roles of each detected peroxide; however, several of the peroxides up-accumulated in OC had high similarity to peroxides confined to the cell wall and extracellular regions of the plant cells, suggesting that these peroxides might function either a means of detoxifying the paracellular space or serving to modulate ROS-mediated signaling (Kawano, 2003). These plant cell wall-related peroxidases are

potentially involved in a direct defense against invading organisms by producing large volumes of hydrogen peroxide; however, they are also involved in the process of lignin synthesis and deposition, as well as cell wall loosening and reformation (Kawano, 2003). Additionally, OC showed up-accumulation of amino acids corresponding to hypersensitive-induced response protein 1, which was involved in stimulating a response closely associated with cell death in *Arabidopsis* (Jung and Hwang, 2007). Various forms of catalase were identified in all tissues, with some forms up-accumulated in ECs and others in OC, which suggests that all tissues had means of detoxifying hydrogen peroxide, but a more detailed study would need to confirm the activity of catalase. Additionally, a study of cellular pH and the NADH/NAD^+ would shed much light on the relative redox state of each tissue type. Based on these results, it is possible that OC did not have sufficient means of detoxifying ROS, considering that all tissues were subject to the same hypoxia-inducing environment. This is partly reflected in the high rates of tissue necrosis under all treatments.

2.6.2.3 EC and OC-E showed up-accumulated cytoskeleton-related proteins; OC showed several up-accumulated proteins related to cell wall growth and modification

Actin and tubulin were, in general, up-accumulated in both ECs. Both make up the two main networks of polymers that comprise the cytoskeleton; they form the shape of the cell, maintain cytoplasmic integrity, respond to cell division, and establish routes for inter and intracellular transport (Petrášek and Schwarzerová, 2009). There are various types of either actin or tubulin, and their expression may be tissue-specific: β-tubulin was expressed more in embryogenic *Vitis vinifera* cultures; however, one form of actin was up-accumulated in embryogenic and a different one was up-accumulated in non-embryogenic cultures (Zhang et al., 2009). Similarly, actin was up-accumulated in non-embryogenic *Zea mays* cultures, but tubulin was up-accumulated in embryogenic ones (Sun et al., 2013). Actin was expressed more in non-embryogenic *Cyclamen persicum* tissue, but tubulin was inconsistent: one form was up-accumulated in embryogenic, and another form was up-accumulated in the other (Lyngved et al., 2008). Considering these wide-ranging results, relative actin and tubulin expression is likely not a consistent marker of embryogenic potential. An explanation for the relative increase in actin and tubulin might be simpler: both EC and OC-E grow much more quickly than OC, necessitating changes to cytoskeletal formation to accommodate cell division. The up-accumulated expression

of T-complex proteins, which may serve as chaperonins for macromolecule assembly (Sternlicht et al., 1993), further suggests that the roles of either actin or tubulin were likely related to changes in the cytoskeleton, though it remains to be verified if this was as a result of either higher mitotic activity or a more-active system of molecule trafficking inside the cell.

OC, by contrast, displayed a much different set of up-accumulated proteins related to cell structure. Whereas EC and OC-E had higher accumulation of cytoskeletal-related proteins, OC accumulated several proteins related to cell wall formation and modification. As discussed in the previous section, several of the peroxidases up-accumulated in OC had similarity to certain peroxidases involved in either cellular defense; ROS-based cell signaling; lignin synthesis or deposition; or loosening of the cell wall (Kawano, 2003). OC produced more of an aldehyde dehydrogenase involved in the phenylpropanoid pathway, where it catalyzes the oxidation of sinapaldehyde to sinapic acid and coniferaldehyde to ferulic acid (Skibbe et al., 2002). Both compounds are hydrocinnamic acids found in cell walls and used in cross-linking cell wall polysaccharides to lignin (Nair et al., 2004). Similarly, α-xylosidase was up-accumulated in OC cells, where it catalyzes the hydrolysis that releases α-D-xylose residues from the xyloglucan oligosaccharides that make up the heteropolymeric hemicellulose of plant cell walls. *Arabidopsis* mutants lacking this enzyme showed that this gene is essential for growth: loss-of-function mutants had hemicellulase with weak connections to the rest of the cell wall, but, on the other hand, mutants overexpressing the enzyme showed little overall different phenotypes other than shortened siliques and overall reduced fitness (Günl and Pauly, 2011). In a different study, Sampedro et al. (2010) found that α-xylosidase activity was tissue-dependent according to a GUS promoter reporter system, which showed that the enzyme was expressed more in the meristems and root tips, vasculature, guard cells, trichomes of leaves, and within various tissues of the silique. Relatedly, α-xylosidase activity was high in young *Arabidopsis* leaves, but this activity decreased with age (Sampedro et al., 2001). Another cell wall-related enzyme up-accumulated in OC was UDP-arabinopyranose mutase 1, a highly-conserved enzyme found in many plants, where it catalyzes the interconversion of UDP-L-arabinopyranose and UDP-L-arabinofuranose, which are non-cellulose polysaccharidic components of the plant cell wall (Konishi et al., 2007). Knock-out *Arabidopsis* mutants that lacked UDP-arabinopyranose mutase showed decreased survivability (Rautengarten et

al., 2011), and rice mutants with decreased enzyme activity showed that this resulted in reduced amounts of ester-linkage between ferulic acid and p-coumaric acid, which bind to arabinofuranose, in their cell walls, leading to dwarfism and infertility (Konishi et al., 2011). Lastly, OC contained higher amounts of UDP-glucose-6-dehydrogenase, which is involved in the synthesis of glucuronic acid, one of the nucleotide sugars used in hemicellulose and a central intermediate of UDP-arabinose, UDP-xylose, UDP-apiose, and UDP-galacturonic acid (Seitz et al., 2000). *Arabidopsis* knock-out mutants deficient for UDP-glucose 6-dehydroganse synthesized less sugar for cell wall biosynthesis, leading to developmental defects and changes to pectin deposition due to the reduction of cell wall sugars, especially the pectin-binding arabinose side chains (Reboul et al., 2011). This is especially curious, because OC, as well as NEC, both have a similar fibrous texture, which may reflect differences in cell wall composition compared to EC. However, both tissues have a different origin: NEC arise either directly from explants or emerge from either compact yellow callus or EC, whereas older cultures arose solely from formerly-embryogenic cultures. Further, considering the central role of the carbohydrate arabinose in the aforementioned proteins, it is strange that no measurable amounts of arabinose were detected in OC, but it was detected in both NEC and EC. Further, the presence of 1-cys peroxiredoxin in all tissues suggests that older cultures are still expressing seed-specific genes, but they have changed either due to a breakdown in gene regulation or a non-universal change in cell fate. In addition, OC contained higher concentrations of phospholipase D, which has a wide-range of roles within the cell: it catalyzes the hydrolysis of phospholipids into phosphatidic acid and a head group; it serves as a biochemical 'tether' linking membranes and proteins; it degrades membrane lipids; and it aids in cytoskeletal rearrangements, membrane remodeling, and vesicle formation (Zhang et al., 2005; Bargmann and Munnik, 2006). Importantly, considering the roll of peroxidases, phospholipase D indirectly promotes the release of ROS by increasing the concentration of phosphatidic acid, which activates membrane-bound NADPH oxidases to create ROS as a potential ABA-mediated cellular response to stress or as signal molecules (Zhang et al., 2005).

2.6.2.4 Embryogenic tissues contain higher numbers of proteins involved in maintaining the epigenetic state of the cell

Differences in epigenetic state are associated with the gene expression programs associated with cell fate (Costa and Shaw, 2007). Several proteins involved with multiple methods of epigenetic regulation

were up-accumulated in both EC and OC-E. Either contained higher accumulations of a putative heat shock cognate 70, a member of the heat shock protein family found to be up-accumulated during most types of stress, including heat, cold, heavy metal, salt, osmotic, and hypoxia stresses (Timperio et al., 2008). Argonaute 4B-like isoform X1 is one of several highly-conserved Argonaute proteins; isoforms are found in bacteria, archaea, and eukaryotes alike due to their conserved role in mediating RNA-directed DNA methylation, although the number of *AGO* genes differs greatly from organism to organism (Fang and Qi, 2016). Argonaute proteins contain four conserved domains with specific functions: a variable amino terminal with possible sRNA duplex unwinding or cleavage activity; an oligonucleotide/oligosaccharide binding site for binding to single-stranded nucleic acid; a looped domain for recognizing the 5' nucleotide of sRNAs in a sequence-specific manner; and an RNase H-like fold that allows certain AGO proteins to cleave RNA to form siRNA (Fang and Qi, 2016). These domains allow AGO4 to function as effectors for regulating genome organization and transcriptional gene silencing through a series of RNA and protein interactions (Qi et al., 2006). First, a target loci is transcribed by RNA polymerase IV; the transcripts are then made into double-stranded RNA by RNA-dependent RNA polymerase 2; processed further by *DICER*-like 3 into 24-nt siRNA; loaded onto *AGO4* to form an *AGO*/siRNA complex; imported into the nucleus, where it targets a specific loci via base-pairing with transcripts produced by RNA polymerase V; and the resulting complex recruits the Domain Rearranged Methyltransferase 2 protein complex to methylate the target DNA (Qi et al., 2006; Fang and Qi, 2016; Lahmy et al., 2016; Ye et al., 2016) . Like many regulatory-related proteins, AGO4 may be tissue specific; it was expressed in *Arabidopsis* leaves, embryos, and vascular tissue, but undetectable in many other cell types (Havecker et al., 2010). Two putative *AGO4*-like proteins were detected in peach palm: one form was unique to either EC and OC-E, and the other was found in all three tissues, though it was up-accumulated in EC and OC-E compared to OC. Considering its conserved role in guiding gene expression, AGO4 expression in EC indicates that the cultures were maintaining a stricter control of gene expression. This may be reflected in the slower, ordered growth of embryos compared to the faster, but non-ordered growth of compact yellow callus, but further research is necessary.

A high-mobility group (*HMG*) Y-related protein A-like was also up-accumulated in EC and OC-E. *HMG* proteins are characterized by

containing multiple DNA-binding peptide motifs, referred to as the 'AT hook,' that bind to AT-rich DNA sequences and possess a degree of flexibility because the molecule does not have a defined structure that directs its function, rather, it can change conformation under a wide range of conditions (Grasser et al., 2000; Reeves and Beckerbauer, 2001). *HMG* proteins participate in a number of processes involved in regulating gene expression by affecting DNA structure through nucleosome remodeling. Specifically, they bind to DNA, including transcription factors, and thus change DNA conformation, leading to change to the heterochromatic state of DNA, leading to large changes to gene expression (Reeves and Beckerbauer, 2001). *HMG-Y* proteins were found in all examined *Arabidopsis* tissue types (Gupta et al., 1997), just as it was in all three examined peach palm tissues, however, due to its conserved-nature and role in epigenetic regulation, differences in abundance between the tissues is likely related to large changes in heterochromatic formation and maintenance. EC also showed increased accumulation of a WD-40 repeat-containing protein MS14-like, which is a histone chaperone involved in chromatic structure maintenance as a putative subunit of a histone deacetylase complex (Ausín et al., 2004). There are many types of MSI1-like proteins conserved amongst all eukaryotes, demonstrating that this family's role in epigenetic regulation is likely essential (Hennig et al., 2005). Their expression is often tissue and time-dependent; a member of the MSI family involved in binding acetylated histones H3 and H4 were abundant in early *Zea* mays endosperm development, but decreased over time, remaining only abundant in the apical meristems and lead primordia of the embryo (Rossi et al., 2001). The histone HS2B was up-accumulated in EC and OC-E. Histone HS2B transcription was found to require certain conditions, most notably a favorable NADH:NAD^+ ratio, signaling that expression was dependent on the redox state of the cell (Dai et al., 2008). Disruption to the redox state of the cell, therefore, can have a direct impact on the epigenetic state of the cell independent of DNA methylation by changing the level of heterochromatic regulation. Cell division cycle protein 48 was found to be more prevalent in both EC and OC-E; the protein contains a highly-conserved nucleotide-binding domain called an ATPase module found in plants, animals, and yeasts (Feiler et al., 1995). Not much is known about the specific action of the protein; however, proliferating *Arabidopsis* tissues expressed the CDC48 gene more than other tissues (Feiler et al., 1995). Interestingly, Rientes et al. (2005) found that *CDC48* was found to interact with numerous proteins, including the much-studied marker of embryogenic competence, Somatic Embryogenesis Receptor Kinase (*SERK*) (Hecht et al., 2001; Rienties et

al., 2005). However, we did not detect *SERK* proteins in any of the tissues. A DEAD-box RNA helicase, so called for a conserved Asp-Glu-Ala-Asp (D-E-A-D) motif, was found to be up-accumulated in EC and OC-E. These RNA helicases mediate RNA transcription and degradation by unwinding RNA duplexes and allowing the opportunity for RNA to bind to protein complexes or displace proteins from other RNA (Linder and Jankowsky, 2011). Curiously, both ECs showed up-accumulation of amino acids corresponding to several 26S proteasome subunits and a ubiquitin-conjugating enzyme E2 36-like protein. The ubiquitin-26S proteasome pathway directly affects cellular behavior by controlling the abundance, trafficking, and localization of proteins, making an active pathway for protein degradation and amino acid recycling indispensable for proper cell behavior (Jamet et al., 1990; Sullivan et al., 2003; Stone, 2014). In the ubiquitin-26S proteasome pathway, there are two main steps: attachment of polyubiquitin chains to the substrate of a protein, and then the protein is degraded by the 26s proteasome (Jamet et al., 1990; Sullivan et al., 2003; Stone, 2014). There are three types of ubiquitin enzymes: E1, E2, and E3, each with a growing order of possible encoding genes, e.g. there are several E1 genes, tens of E2 genes, and hundreds of E3 encoding genes, suggesting that these genes are intrinsically linked to cellular regulation by tagging and degrading certain proteins (Stone, 2014). This is especially true for abiotic stress, where proteomic plasticity is indispensable for responding to stress and other external stimuli (Sullivan et al., 2003). Together, these results show that both EC and OC-E displayed greater epigenetic control compared to OC, and this likely led to greater ability to maintain cellular health and energy generation under hypoxia and ROS-accumulation.

2.6.3 Global methylation changes over time and 5-azac concentration

In general, methylation rates were similar over all 5-azac treatments and tissue types with one exception: young EC cultures treated with 4 μM 5azac was decreased by a small, but significant, amount. Otherwise, methylation rates were similar within tissue types. Yet, the large changes in cultural composition only became more prevalent in higher 5azac concentrations. This suggests that the effect of 5azac can be transitory, and may only serve to reduce age-dependent hypermethylation that may inhibit certain essential genes that may show reduced expression over time. Global methylation of non-5azac-treated EC was slightly, but significantly, lower than non-5azac-treated OC, which suggests that hypermethylation may have occurred over time, which agrees with the

gradual accumulation of hypermethylation observed in *Elaeis guineensis* (Rival et al., 2013), cacao (Quinga et al., 2017), *Pinus pinaster* (Klimaszewska et al., 2009), and *Olea europaea* (Bradaï et al., 2016). Relatively few OC regained the ability to form new embryos, and these embryos grew from one or, at best a few locations on each culture, suggesting that this reversal in age-related loss of embryogenic potential was likely rare, and dependent on certain competent cells. Curiously, methylation profiles for EC was not significantly different from NEC, which disagrees with the higher methylation rates found in non-embryogenic *Eleuterococcus senticocus* callus (Chakrabarty et al., 2003) and *Pinus radiata*(Bravo et al., 2017). Similarly, *Larix x eurolepsis somatic* embryo global methylation depended on growth stage: undifferentiated somatic embryos showed a methylation of 45.8%; immature embryos had 61.5%; and mature embryos 53.4% (Teyssier et al., 2013). Global methylation may not be a good marker of plant cell behavior because some genes might be expressed, and others silenced depending on tissue type, environment, and, importantly for tissue culture, time between subcultures. Rather than global analysis as a tool of observing methylation-related epigenetic regulation, *in situ* immunolocalization of key regions of the chromatin over several time periods might reveal much more (De-la-Peña et al., 2015). Additionally, even though early embryos lack many of the developed meristems and vasculature of mature embryos, they are still composed of several cell types, each with their own programming. Determining the change in methylation between specific cell types would lead to breakthroughs in our understanding of embryo development. Differences in gene expression and protein abundance, however, seems to be more descriptive of cellular behavior because it is more focused on specific differences over a global profile.

2.6.4 Arabinose abundance in EC: a possible reinforcement of the role of arabinogalactan proteins in embryogenic capacity?

As previously discussed, both EC and NEC had detectable amounts of ribose, sucrose, and arabinose, but OC had no detectable amounts of any of these three, nor indeed any carbohydrate at all. This suggests that OC may have lacked a functioning respiratory pathway, though it may be possible that any carbohydrate generated by the breakdown of sucrose was then immediately expended without the chance to accumulate any reserves. However, the greater emphasis on accumulation of proteins involved in the cell wall may also suggest that OC cells were expending much energy in wall formation or reformation. A microscopic analysis

may reveal much about the relative states of EC, NEC, and OC. Both EC and NEC contained similar amounts of ribose and sucrose, but EC contained significantly more arabinose. Previous research in peach palm found that arabinogalactan proteins—a family of proteins with connected arabinogalactan carbohydrate moieties consisting of arabinose and galactan—were found in the cell walls of embryogenic cells and closely associated with the extracellular matrix of globular embryos and especially with where new embryos form through secondary somatic embryogenesis (Steinmacher et al., 2012). At this point, however, there is no clear link between these previous results and the higher concentration of arabinose found in EC; more research would be required. In addition, decreased carbohydrate reserves over time may be a relatively simple marker of aging; if reserves are declining over time, the tissue may be either expending more to maintain itself or its respiratory biochemical pathway is slowly becoming impaired due to constant hypoxia. A summary of the morphological and biochemical changes associated with gaining peach palm cultures is displayed in Figure 2-3.

2.7 CONCLUSION

Age-related hypermethylation was reversed in a small proportion of OC using 5azac, but 5azac also caused disruption to somatic embryo formation EC in a dose-dependent manner. Necrosis, too, increased in either EC or NEC in a dose-dependent manner. Yet, global methylation was only decreased in EC treated with 4 μM 5azac. The complex interactions between 5azac and DNA and its role in reducing age-related hypermethylation remains to be studied in depth. However, the resumption of somatic embryo growth in OC suggests that an epigenetic

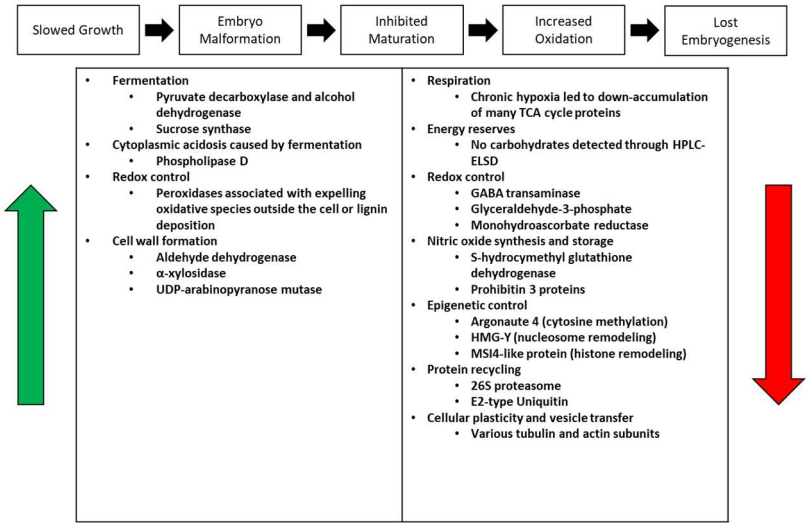


Figure 2-3: Summary of the physiological and biochemical changes associated with the aging of peach palm (*Bactris gasipaes* Kunth) embryogenic cultures *in vitro* for eight years. The column next to the green arrow contains processes that increased and proteins that were up-accumulated in eight-year-old cultures compared to two-year-old embryogenic cultures; and the column next to the red arrow contains processes that decreased and proteins that were down-accumulated.

rearrangement took place sufficient to cause a change in the biochemistry of the cells, allowing them to return to their formerly-embryogenic state, even if it was only a few cells capable of producing new somatic embryos. The similarity between the protein profiles of EC and OC-E suggests that accumulation of certain proteins is essential to embryo growth. Specifically, the mechanisms that led to the up-accumulation of highly-conserved and universal proteins involved in epigenetic regulation are of great interest to our understanding of aging in tissue culture and the possibility of undoing the detrimental effects of aging in a hypoxic environment. Further, NO storage and transport was implicated as being especially important to maintaining somatic embryo form and growth in EC. Lastly, the lack of detectable carbohydrates of any kind in OC suggests that, over time, the breakdown of cellular mechanisms and chronic hypoxia-related cytoplasmic acidosis resulted in the dramatic slow-down of culture metabolism, as evidenced by the reduced growth speed over several years of culture. These results shed light on the biochemical effects of *in vitro* cultures, as well as the detrimental effects of age on cultures.

2.8 FUTURE PROSPECTS

Hypoxia appears to the be the main driving factor in *in vitro* aging. The dependence on fermentation over more efficient, but oxygen-demanding, respiration likely led to a loss in normal cell functions due to runaway cytoplasmic acidosis, which led to the inability of these cells to generate enough energy to produce the critical proteins they required for growth under stressful conditions. Further research would require years to generate more of the tissue—the large demand for tissue during my experiments and the slow-growing and oxidation-prone behavior of the OC cell line suggests that what few cultures survived will not endure to grow into a sufficiently-large population for further study. However, further research may be particularly interesting. Specifically, content of reduced and oxidized forms of glutathione and ascorbate; NAD+/NADH ratio; ethylene evolution; and a microscopic analysis may provide more information which may refine the analysis in this dissertation. Further, the use of partially ventilated vessels may provide a means of potentially reversing the onset of culture aging by providing more oxygen to the cultures, but this would need to be tested.

3 VENTILATED GROWING VESSELS STIMULATE BIOCHEMICAL CHANGES IN PEACH PALM (*BACTRIS GASIPAES* KUNTH) SOMATIC EMBRYOS

3.1 ABSTRACT

Somatic embryos placed in a sealed vessel are subjected to atmospheric conditions different than those of their natural environment; some gases are consumed, and others may accumulate. This environment may hinder growth and delay the transfer of regenerated plants from *in vitro* conditions to greenhouse conditions. Peach palm (*Bactris gasipaes*) mature somatic embryos placed in sealed or ventilated vessels showed great differences in growth response: somatic embryos in sealed vessels accumulated greater fresh weight, though somatic embryos in ventilated vessels had overall greater dry mass. Further, a greater proportion of somatic embryos in ventilated vessels showed a greater rate of the onset of photosynthesis, marked as the appearance of green pigments. Putrescine was present in significantly-greater concentrations in somatic embryos from ventilated vessels compared to both somatic embryos in sealed vessels and immature somatic embryos undergoing multiplication. In contrast, both cultures in sealed vessels and immature embryos contained greater concentrations of spermine than somatic embryos in ventilated vessels, suggesting a major difference in cellular behavior. Global methylation was not significantly different between mature somatic embryos in either vessel, though either was significantly higher than immature embryos. Concentrations of sucrose, ribose, and arabinose were not significantly-different between either mature embryos, but both glucose ($p=0.0624$) and fructose ($p=0.0587$) were greater, though not significantly so, in concentration in embryos in sealed vessels. Together, these results suggest that the underlying biochemical changes associated with the response of somatic embryos to a ventilated or sealed environment may be major factors in determining the conversion of somatic embryos to plantlets.

3.2 RESUMO

Embriões somáticos colocados em um vaso selado foram submetidos a condições atmosféricas diferentes do ambiente natural; mostrando que alguns gases podem ser consumidos e outros podem ser acumulados. Este ambiente pode reduzir o crescimento das plantas e impedir a transferência de plantas regeneradas de condições *in vitro* para condições do campo. Embriões somáticos maduros de pupunha (*Bactris gasipaes* Kunth) colocados em vasos selados ou ventilados mostraram diferenças

significativas na resposta de crescimento: embriões somáticos selados acumularam maior peso fresco, embora embriões somáticos ventilados tenham massa seca total maior. Além disso, foi observado uma taxa de início de crescimento da clorofila em maior proporção nos embriões somáticos de vasos ventilados. A putrescina estava presente em concentrações significativamente maiores em embriões somáticos de vasos ventilados, em comparação com embriões somáticos em vasos selados e embriões somáticos imaturos em meio de multiplicação. Em contraste, ambas as culturas em vasos selados e embriões imaturos continham maiores concentrações de espermina do que embriões somáticos em vasos ventilados, sugerindo uma diferença significativa no comportamento celular. A metilação global não foi significativamente diferente entre os embriões somáticos maduros em qualquer tipo de vaso, embora sejam significativamente maiores do que os embriões imaturos. Concentrações de sacarose, ribose e arabinose não foram significativamente diferentes entre as culturas em vasos ventilados ou selados, mas as concentrações de glicose ($p = 0,0624$) e frutose ($p = 0,0587$) foram maiores em embriões em vasos selados. Juntos, esses resultados sugerem que as alterações bioquímicas associadas à resposta de embriões somáticos maduros a um ambiente ventilado ou selado podem ser fatores importantes na determinação da conversão de embriões somáticos em plântulas.

3.3 INTRODUCTION

Somatic embryos – embryos induced to form from somatic tissue and developed through morphological steps similar to zygotic embryogenesis (Jiménez, 2005) – require the correct culture conditions to convert to plantlets. Unlike somatic embryo induction, in which individual cells must change their genetic and biochemical state in order to re-enter the cell cycle (Fehér, 2015b), somatic embryo maturation and conversion involves many specialized tissues working together to produce a healthy plantlet (Liao et al., 2008). This is often difficult; somatic and zygotic embryos often differ in their biochemical profiles (Pullman et al., 2003; Lara-Chavez et al., 2012; Jin et al., 2014), potentially leading to greatly different efficiencies in plantlet production. Indeed, though somatic embryogenesis (SE) and zygotic embryogenesis (ZE) are quite similar, the environments they proceed in are drastically different; SE occurs in an artificial environment far removed from the seed zygotic embryos develop in. The *in vitro* environment is quite humid; converted plantlets often develop characteristics that make acclimatization difficult, such as

hyperhydricity (Ivanova and Staden, 2010) or a lack of cuticle (Batagin-Piotto et al., 2012). One other factor is the role of air composition on somatic embryo behavior; as they grow, respire, and produce secondary metabolites, the atmospheric composition of closed vessels changes: ethylene and aldehyde can accumulate; and oxygen can be depleted by growing cultures (Linden et al., 2001). Ventilation, therefore, might be beneficial for two reasons: improved air quality for the growing plants and decreased humidity (Lai et al., 2005; Mohamed and Alsadon, 2011). Previous research in peach palm (*Bactris gasipaes*), a palm (Arecacea) native to the Amazon, suggested that air exchange might be a significant factor that led to improved somatic embryo conversion in a temporary immersion system over traditional semi-solid medium culture (Heringer et al., 2014). Despite this advancement in improving culture multiplication, embryo conversion and subsequent plantlet acclimatization remain as major bottlenecks to peach palm micropropagation. In order to investigate the role of gas exchange, we analyzed the differences in growth, biochemistry, and morphology in peach palm cultures grown in either sealed (SV) or ventilated vessels (VV).

3.4 METHODS

3.4.1 Culture induction

Peach palm somatic embryos were induced from thin cell layers (TCLs) ~1mm thick were harvested from the apical meristems of previously-existing *in vitro* plantlets created from previous studies (Heringer et al., 2014). TCLs were placed on culture medium containing Murashige and Skoog basal medium (Murashige and Skoog, 1962), Morel and Wetmore vitamins (Morel and Wetmore, 1951), 30 g L^{-1} sucrose, 1 g L^{-1} glutamine, 2.5 g L^{-1} activated charcoal, 2.3 g L^{-1} Phytagel (Sigma), 450 µM picloram. All culture medium was autoclaved for 20 min. at 1.2 atm and 120°C. TCLs were left on culture medium for several months until embryogenic callus formed. After embryogenic callus formed, they were placed onto multiplication medium consisting of MS basal salts, MW vitamins, 30 g L^{-1} sucrose, 1 g L^{-1} glutamine, 2.3 g L^{-1} Phytagel, and 10 µM picloram. Every month, embryogenic tissue was divided and placed onto fresh medium. Embryogenic cultures were placed onto basal medium without picloram and with 5 µM abscisic acid for two weeks. Equal amounts of these cultures were afterwards placed onto basal medium without plant growth regulators or activated charcoal in Microbox plastic containers

closed with lids with or without ventilation. Containers were placed in a growth room with 16 hr of daylight and 8 hr of darkness.

3.4.2 Polyamine analysis

Polyamine determination was based on the procedures described in Silveira et al. (2004) with modifications by Cangahuala-Inocente et al. (2014). Samples consisting of 200 mg fresh mass of cultures grown in SV or VV were harvested and then ground in 3 ml of 5% (*v/v*) perchloric acid and left to incubate for one hr at 4°C. Samples were then centrifuged at 15,000 *x g* at 0°C for 20 min. The supernatant was saved and the pellet was re-extracted. The supernatants from both extractions were combined. Forty-microliter aliquots of these samples were removed and added to a tube containing 100 µL dansyl chloride (5mg mL^{-1} dissolved in acetone), 10 µl of 0.05 mM diaminoheptane, and 50 µL of a saturated sodium carbonate solution. Samples were incubated in the dark for 50 min at 70°C. Afterwards, the dansylated PAs were extracted with 200 µL of toluene and incubated for a further 30 min. The toluene phase was collected and then dried under liquid nitrogen. The dried PAs were then solubilized in 200 µL acetonitrile. Aliquots of 20 µL were then injected into a reverse-phase HPLC containing a C-18 reverse-phase column (Shimadzu SHIM-PACK CLC ODS, 5 µm particle size, L x I.D. 25 cm x 4.6 mm). PAs were separated using gradients of absolute acetonitrile at a flow rate of 1 mL min^{-1} at 40°C. The changes in gradients were programmed as follows: to 65% within the first ten minutes; from 65% to 100% from minute 10 to minute 13; and 100% acetonitrile from minute 13 to minute 21. A fluorescence detector set to 340 nm (excitation) and 510 nm (emission) compared polyamine type and concentration in the samples to standard solutions containing a known concentration of putrescine (Put), spermidine (Spd), and spermine (Spm).

3.4.3 Global methylation analysis

DNA was isolated from 200 mg fresh mass according to (Doyle, 1987). Tissue was ground in liquid nitrogen and incubated for one hr in 700 µL of extraction buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100mM Tris-HCl, pH 8.0, 2% PVP, and 0.2% 2-mercaptoethanol) at 60°C. Afterwards, the samples were centrifuged at 8,000 *x g* for eight minutes. The supernatant was removed and placed into a new centrifuge tube. An equal volume of 24:1 chloroform-isoamyl alcohol was added and mixed through inversion for five minutes. The samples were then centrifuged at 15,000 *x g* for eight minutes. The aqueous phase was removed and placed into a new tube. A 2/3 volume of isopropanol was added to each tube,

mixed by inversion, and then left in a -20°C for at least two hr. Afterwards, the samples were then centrifuged at 15,000 *x g* for 15 min. The supernatant was carefully removed from the pellet. The pellet containing the nucleic acid was then washed twice with 70% ethanol and then dried until no residual ethanol was detected. The nucleic acid was then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Nucleic integrity and quantity were checked using a Nanodrop 1000 (NanoDrop Technologies, USA).

DNA was further purified by removing the RNA based on procedures described in (Johnston et al., 2005) and Heringer et al (2013). Total nucleic acid concentration was adjusted to 1 μg μL^{-1} with TE buffer. One-hundred microliter aliquots of each sample were heated to 65°C for five min before 10 μL of 1 μg μL^{-1} RNase A (Sigma-Aldrich, St. Louis, USA) and 10 μL of 20 μg μl^{-1} RNase T1 (Sigma-Aldrich) were added to each sample. Samples were mixed and incubated at 37°C for 14 hrs. Afterwards, 20 μL of sodium acetate (3M, pH 5.6) were added to each tube and each tube was vortexed. Two-thirds volumes of cold isopropanol were added to each tube, mixed, and left to incubate at -20°C for at least one hr. Samples were then centrifuged at 5,000 *x g* for ten minutes. The supernatant was removed and the pellet was washed once with 70% ethanol. After the pellet dried at room temperature, it was resuspended in 100 ul of autoclaved deionized water. The concentration of nucleic acids was determined with a Nanodrop and then adjusted to 0.25 μg μL^{-1}.

Aliquots of 100 μL of the nucleic acid samples were denatured at 100°C for two min and then chilled at to 4°C for five minutes. Five microliters of 10 mM $ZnSO_4$ and 10 μL of a nuclease P1 solution (1.0 U ml^{-1}, 30 mM NaOAc, pH 5.4) were added to each sample, vortexed, and then incubated at 37°C for 17 h. Afterwards, 10 μL of 0.5 M Tris-HCl (pH 8.3) and 10 μL of alkaline phosphatase (10 U $ml^{-1,}$ 2.5M $(NH_4)_2SO_4$) were added, mixed, and then incubated for two h at 37°C. Samples were then centrifuged at 10,000 *x g* for eight minutes. The supernatant was removed and stored at -20°C.

The supernatant was analyzed using an HPLC system with a HyperClone™ 5 μm ODS (C18) 120 A, LC Column 250 x 4.6 mm (Phenomenex®, Torrance, SA), guard column (4.0 x 3.0 mm) (Phenomenex®), and UV detector (280 nm). Twenty microliters of each sample were used for each injection, and each sample was tested in

triplicate. The flow rate was 1 ml min-1, and the gradient program was as follows: 3 min of 100% buffer A (0.5% *v/v* methanol, 10 mM KH_2PO_4, pH 3.7 adjusted with phosphoric acid, filter sterilized); a linear gradient change from minute 3 to minute 20 to 100% buffer B (10% *v/v* methanol, 10mM KH_2PO_4, pH 3.7 adjusted with phosphoric acid, filter-sterilized); from minute 20 to minute 25 with 100% buffer B. To create a standard curve, dNTPs (dA, dT, dG, dC, and 5mdC) (Fermentas®, Hanover, MD, USA) were digested with alkaline phosphatase (10 U ml^{-1}) and Tris-HCl (0.5 M, pH 8.3) for two h at 37°C to obtain nucleotides. Standardized samples were prepared in deionized water.

3.4.4 HPLC analysis of carbohydrate abundance

Carbohydrate analysis was performed based on the protocol by Filson and Dawson-Andoh (2009) with modifications. Approximately 500 mg of fresh matter from EC, NEC, or OC—taken with extreme care to prevent tissue medium contamination—were macerated under liquid nitrogen and in the presence of 0.5 g PVPP and 0.015 g ascorbic acid. The slurry was then added to a centrifuge tube, and 0.75 mL of 80% ethanol was added. The sample was vortexed and then incubated in a 70°C water bath for 90 min. The sample was then vortexed at 15,000 x g for ten min. The liquid phase was removed to a new tube. The extraction was then repeated, resulting in a sample totaling 1.5 mL, which was then filtered through a 0.2 µm filter and stored at -20°C until analysis. Samples aliquots of 5 µl were injected into the HPLC with an evaporative light scattering detector (ELSD-LT II, Shimadzu) at a flow rate of 1 ml min^{-1}. Carbohydrates were fractionated using two separate columns: a Prevail Carbohydrate ES 5 µm (250 x 4.6 mm) and a Prevail Carbohydrate ES 5 µm (7.5 x 4.6 mm) kept at 25°C. Carbohydrates were separated under the following gradient program: 20% Solution A (MilliQ water) and 80% Solution B (100% acetonitrile, filtered) for 16 min, from 80 to 70% Solution B for 16 to 16.5 min, and then 16:30 to 23 min. The ELSD detector had a drift tube temperature of 40°C and a nitrogen pressure of 350 mPa. Standard concentrations of ribose, arabinose, xylose, fructose, mannose, glucose, sucrose, and maltose were used to construct the model used to compare peaks and determine the concentration of carbohydrates.

3.4.5 Dry mass

Ten 200 mg samples from each tissue were harvested and placed onto petri dishes lined with autoclaved filter paper. The samples were then placed in a 60°C oven for two hr or until volume loss ceased. The samples were then weighed again, and the ratios of dry mass were compared.

3.4.6 Statistical analysis

Data were analyzed using ANOVA with Tukey post hoc tests were applicable (alpha=0.05). All data was analyzed with R statistical software (R Development Core Team, 2014).

3.5 RESULTS AND DISCUSSION

3.5.1 Culture induction

After 42 days, cultures grown in either SV or VV became distinct from the other; cultures in SV grew in size but remained white masses of seemingly-fused somatic embryos, and embryos in VV showed signs of chloroplast development and displayed the early signs of conversion (Table 4-1). Embryos grown in VV showed a significantly higher ($p<0.05$) proportion of embryos showing chloroplast, with an average of 63.6 ± 9.66 green somatic embryos compared to only 19.8 ± 5.15 green embryos in SV. Conversely, SV contained a higher number of white somatic embryos, with 170 ± 19.2 white embryos compared to 58.8 ± 10.2 in VV. Proportionally, 52.0% of all distinguishable embryos in VV were green, compared to 10.4% in SV (Figure 4-1).

Table 3-1: Growth response of peach palm (*Bactris gasipaes* Kunth) cultures in sealed and ventilated containers

	Starting Mass (mg)	Sample Mass at Day 21 (mg)	Sample Mass at Day 42 (g)	White Embryos	Green Embryos	Culture dry mass (%)
Sealed	200	763 ± 7.23	4.720 ± 646	169 ± 19.3	19.8 ± 5.15	6.56 ± 0,494
Ventilated	200	572 ± 57.5	3.71 ± 0.578	58.8 ± 10.2	63.6 ± 9.66	8.38 ± 0.503

Cultures in SV acquired significantly greater fresh mass by the 21st day than cultures in VV ($p<0.05$). By the 21st day, the initial 200 mg of fresh mass increased to an average of 763 ± 7.23 mg in SV, and 572 ± 57.5 mg in VV. This trend continued until the 42nd day, on which fresh mass in SV reached 4.72 ± 0.646 g, and VV reached 3.71 ± 0.578 g. Cultures grown in VV, however, had significantly higher ($p<0.05$) dry matter (8.38 ± 0.503 %) compared to cultures grown in SV (6.56 ± 0.494 %). The increased dry mass in cultures from VV and the increased emergence of green embryos, signaling the growth of chlorophyll and the start of conversion, may be related. *Aloe polyphylla* plants grown in SV had a high rate of hyperhydricity; those grown in VV showed no hyperhydricity at all (Ivanova and Staden, 2010). Further, aloe plants grown in VV had

greater chlorophyll content and greater deposition of epicuticular wax. Partial desiccation of mature date palm somatic embryos from 90% to 75% water led to increased conversion rates (Fki et al., 2003). This relation suggests that water content affects conversion, though determining the exact reason why this is would require further research. Likely, the hyperhydricity may lead to stressful conditions in which oxygen is not immediately available to fuel respiration, requiring the tissue to use fermentation to supply much of its energy needs. *Pinus taeda* zygotic embryos contained less water than somatic ones, and though zygotic embryo water content decreased during germination, somatic embryos remained fairly stable (Pullman et al., 2005). *Capsicum annuum* plantlets grown in VV had significantly more total chlorophyll content, lower stomatal density, higher dry mass, and an overall improved regeneration rate compared to plants grown in SV (Mohamed and Alsadon, 2011). However, these results may be species-specific. The rate of *Picea mariana* somatic embryo conversion into plantlets was not significantly different between those grown in SV or VV (Meskaoui and Tremblay, 1999). We found that VV improved peach palm somatic embryo conversion through either, or a combination of, desiccation of hyperhydric cultures and/or removing excess gases, such as ethylene and allowing the entrance of oxygen to prevent hypoxic conditions. Additionally, a morphological analysis may be beneficial because, though chlorophyll began to develop in many VV somatic embryos and several SV ones, they may not be able to supply other areas of the embryo if they have not yet developed functioning phloem to transport photosynthates to interior tissues, and, similarly, tissue may remain hyperhydrated without xylem to transpire it out. A detailed microscopic analysis may reveal if these tissues when and if vascular developed, but it might be difficult to determine if they are functioning, and, if so, to what extent.

3.5.2 Polyamine analysis

Cultures grown in VV showed several significant differences between either those grown in SV or multiplication cultures. Putrescine was the most abundant polyamine in all three tissue types, though it was significantly higher in cultures grown in VV compared to either SV cultures or multiplication cultures ($p<0.05$), which were not significantly different from one another (Figure 4-2). VV cultures, however, has significantly less SPD than the other two cultures, and SPM was significantly higher in multiplication cultures and nearly undetectable in

the other two. Polyamine concentrations can be highly dependent on tissue type and activity; in general, total polyamines increase during

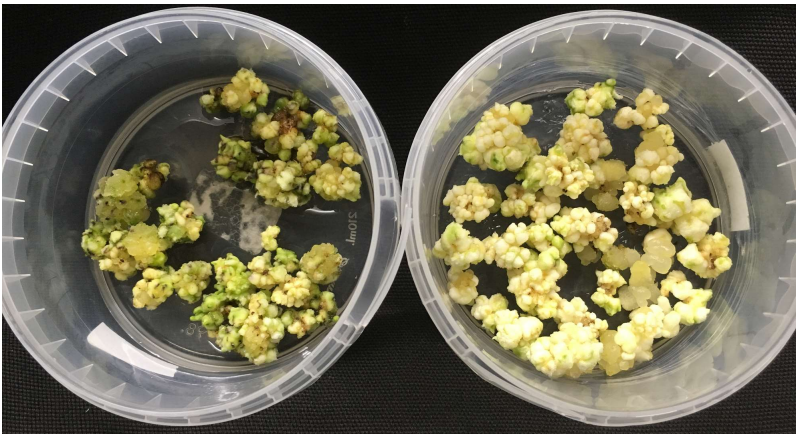


Figure 3-1: Peach palm (*Bactris gasipaes* Kunth) cultures grown for 42 days in either ventilated (left) or sealed (right) vessels

periods of rapid cell division and drop with reduced metabolic activity (Bais and Ravishankar, 2002). High putrescine levels were associated with low cellular growth in *Pinus taeda* suspension cells (Silveira et al. 2004b); however, addition of exogenous Put stimulated increases in endogenous IAA and ABA in *Araucaria angustifolia* embryogenic cultures (Steiner et al., 2007), suggesting that Put plays a role in guiding PGR regulation. Moreover, PAs may act as act as secondary messengers in mediating the effects of PGRs such as GA and ABA during seed germination (Palavan and Galston, 1982). Though the exact role of PAs is not well understood, they may be related to hormone metabolism and stimulate the mobilization of reserve compounds for cellular division and elongation (Krasuska et al., 2014). Krasuska et al. (2017) found that addition of Put or Spd to dormant apple (*Malus domestica*) embryos stimulated dormancy breakage and germination, leading to the stimulation of the urea cycle, reserves of free arginine, and formation of NO. The authors found that this NO formation led to changes to overall protein profiles and thus directly stimulated embryo germination. In addition to its roles in mediating the effects of PGRs, PAs have a role in adapting the plant to abiotic stress (Igarashu and Kashiwagi, 2000; Saha

et al., 2015). The overall greater PA concentrations, and especially that of Put, in cultures placed in VV may suggests that PAs could have a role in both mediating the hormone-driven changed as somatic embryos convert, develop chlorophyll, and grow into plantlets and adapt to an overall less-humid environment compared to the growth in sealed containers. However, PA concentration may be developmental stage-specific; PA content changed during *Pinus taeda* embryo development (Silveira et al., 2004). The ratio of PUT:SPD+SPM ratio, often considered a marker of embryogenic competence between embryogenic (low PUT:SPD+SPM ratio) to non-embryogenic (high ratio) (Shoeb et al., 2001; Santa-Catarina et al., 2004; Nascimento-Gavioli et al., 2017), demonstrated a curious trend between multiplying, VV, and SV cultures: the lowest ratio belonged to multiplication (ratio = 16.4), then SV (48.6), and, finally, VV (73.1). The role of polyamines during conversion is mostly unknown, however, accumulation of putrescine, and, by extension, increase in the ratio, may suggest that large biochemical changes are occurring within each type as cultures transition from embryos to plantlets. *Pinus sylvestris* embryogenic cultures gradually accumulated Put during the exponential phase of tissue growth due to decreased biosynthesis of Spd and Spm, resulting in a high ratio of Put: Spd+Spm (Vuosku et al 2012). Similarly, VV and SV embryos showed significantly less Spm and Spd than

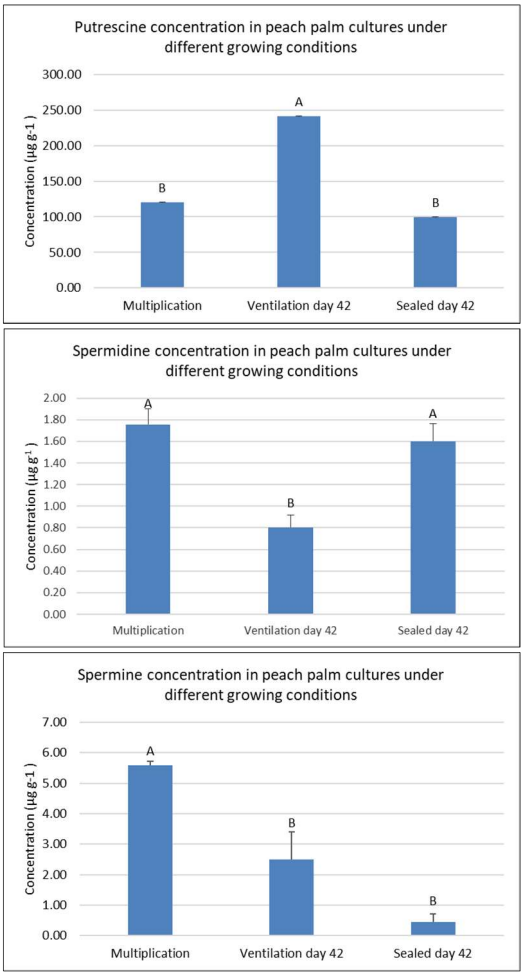
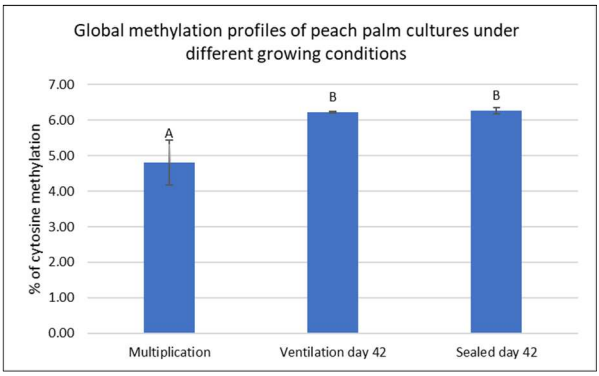


Figure 3-2: Concentrations of putrescine, spermine, spermidine, and global methylation in multiplying peach palm (*Bactris gasipaes* Kunth) cultures compared to cultures placed on conversion media in either ventilated or sealed vessels. Letters signify a statistical difference as determined by a Tukey post hoc test (α=0.05).

multiplication medium; both were undergoing transformation from embryos to plantlets, with VV cultures advancing more rapidly.

Figure 3-3: Percent of global cytosine methylation in peach palm (Bactris Gasipaes



Kunth) cultures in either multiplication medium compared to cultures placed for 42 days in ventilated or sealed vessels. Different letters signify a statistical difference using a Tukey post hoc test (α=0.05)

3.5.3 Global methylation analysis

The total global methylation between SV (6.27 ±0.090%) and VV (6.23 ± 0.0178%) were no significantly different; however, multiplication cultures had significantly less global methylation (4.81 ± 0.704%) ($p<0.05$) (Figure 4-2). This suggests that, although large changes in embryo morphology were occurring, this did not necessarily mean that a large net shift in global methylation occurred. Rather, certain genes may have been repressed, and others expressed, without a large net change in global methylation. Gene-specific or changed to chromatic conformation may reveal much more (De-la-Peña et al., 2015), because it is visually obvious that large morphological changes are occurring due to the formation of chlorophyll and the overall appearance of photosynthetic coleoptiles (Figure 3-1).

3.5.4 Comparative carbohydrate amounts

Ribose, arabinose, and sucrose concentrations were not significantly different between cultures grown in VV or SV; however, fructose and glucose were at the borderline of significance (α=0.05) ($p=0.0587$ and $p=0.024$, respectively) and more prevalent in SV than VV. This may suggest that cultures in VV may have used some of their immediate reserves to fuel their growth. However, this behavior contrasts greatly

with the carbohydrate profiles of germinating zygotic embryos; the concentration of both fructose and glucose increased within hours of the start of maize (*Zea mays*) (Leprince et al., 1992) and bean (*Phaseolus vulgaris*) (Sfaxi-Bousbih et al., 2010) germination. In this latter report, the concentration of starch declined as the concentration of glucose and fructose increased, though sucrose remained relatively stable. Future research should analyze the concentration of starch in either SV or VV cultures; it is possible that the decline in either monosaccharide occurred because there was little available starch to use as a means of replenishing them after they had been used to fuel tissue growth. If so, then this protocol must be further improved by stimulating the accumulation of greater starch content during embryo maturation. Sghaier et al. (2009) found the increased sucrose, ABA, and arginine increased the accumulation of protein content of maturing date palm (*Phoenix dactyilifera*) somatic embryos. Such an approach may be useful if little starch is found in either SV or VV cultures.

Table 3-2: Amount of detected carbohydrates in peach palm (*Bactris gasipaes* Kunth) cultures grown for 42 days in ventilated or sealed vessels

Sugar type	Vessel type	mg per 500 mg of fresh tissue
Ribose	Ventilated	11.8 ± 3.08
	Sealed	12.6 ± 1.56
Arabinose	Ventilated	5.26 ± 4.59
	Sealed	7.08 ± 2.44
Fructose	Ventilated	0.282 ± 0.0709
	Sealed	0.967 ± 0.507
Glucose	Ventilated	0.279 ± 0.0847
	Sealed	0.815 ± 0.401
Sucrose	Ventilated	1.81 ± 0.429
	Sealed	1.43 ± 0.526

3.6 CONCLUSION

Gas exchange played a role in a greater number of mature peach palm somatic embryos into beginning the conversion process into plantlets, as marked by the greater accumulation of chloroplasts. Cultures in SV,

though some also formed chloroplasts, accumulated significantly greater fresh mass, which may have signaled a continuation of the secondary somatic embryogenesis process used to mass produce cultures. Additionally, VV cultures were marked by greater dry mass, higher overall polyamine, greater putrescine, and lesser spermidine compared to SV and multiplication cultures. Though global methylation rates and three of five carbohydrate concentrations were not significantly different between VV and SV embryos, the decrease in mean fructose and glucose in VV cultures suggests that they expended a portion of their internal reserves to fuel their greater development.

3.7 FUTURE PROSPECTS

The effectiveness of any given somatic embryogenesis protocol is ultimately judged by how many plants are converted from somatic embryos and how many of those survive the transition to greenhouse conditions. Cultures in VV accumulated greater dry mass, began to show earlier signs of photosynthesis, and showed signs of expending carbohydrate reserves, but these results serve only as the foundation for a greater study. Starch content should be the first factor tested; if there is not enough energy to supply a converting somatic embryo, then the effects of SV or VV are diminished. Greater development is nonetheless delayed if there is not enough energy to fuel the transition to photosynthesis. The decrease in glucose and fructose, despite literature in zygotic embryos suggesting that it should be otherwise, in converting somatic embryos indicates that though embryos may be converted more efficiently in VV, it may not be the complete solution. If so, then steps need to be made to address it. Additional sucrose, ABA, or exogenous amino acids (see Sghaier et al., 2009) may provide a relatively easy means of increasing starch content to fuel greater plantlet development. Among the most informative data yet to be taken is the growth of cuticle—the waxy covering plants use to shield themselves from the effects of unregulated water loss—as well as the differences in atmospheric conditions, notably the partial pressure of ethylene. A greater production of cuticle, as determined by microscopy and histological stains, would suggest that those plants would more effectively make the transition to greenhouse conditions. Ethylene accumulation may delay growth by affecting plantlet conversion—a process naturally done under normal atmospheric conditions where excess ethylene is diffused away into the surrounding environment. Beyond this, these incipient plantlets must be grown further. There are many strategies to improve the current protocol.

During my Masters research, I performed some rudimentary work with artificial seeds using açaí (*Euterpe oleracea*). The preliminary tests identified that a combination of encapsulation in alginate with medium containing activated charcoal and gibberellic acid (GA) (10 μM) seemed to have an improvement, but these tests were small and soon I returned to working solely on peach palm. In brief, alginate encapsulation uses the following steps: the alginate solution (1-2% sodium alginate, MS salts without Ca-containing compounds, autoclavable PGRs, and carbohydrates) is mixed with embryos; embryos surrounded with alginate are pipetted into a beaker containing $CaCl_2$ to polymerize the alginate; the synthetic seeds are left to harden for several minutes; and then the synthetic seeds are ready for culture in the environment of your choosing. Now that there is an embryogenic peach palm line, alginate encapsulation becomes important again for several reasons: surrounding tissues in GA may stimulate conversion more than laying cultures on GA-containing medium because only the tissue on the side in contact with medium will be affected; encapsulation in a dark medium in a well-lit growth room may stimulate elongation and greater growth; a more uniform source of carbohydrates may give the embryo the energy it requires because the embryos are greater than 90% water, and therefore their internal carbohydrate reserves may be dilute, though this is untested; and, if cultures grown in VV have developed sufficient cuticle, they may be potentially encapsulated and grown in autoclaved soil in a ventilated vessel, which may potentially combine the traditionally-separated steps of conversion and acclimatization. As a brief aside, there are fundamental complications in typical encapsulation protocols: using a common food dye, I found that any dissolved components in the alginate solution will leach into the $CaCl_2$ solution and this solution, too, enters into the synthetic seeds. In effect, any protocol that does not account for this nutrient loss effectively cultures synthetic seeds with very dilute salts and sugars, but likely high in $CaCl_2$. To counteract this, synthetic seeds may be left to incubate in a culture medium after they have hardened, and thus replace its encapsulated solution. I used food dye to test this: after placing undyed solid synthetic seeds in a solution with food dye, the seeds became dyed thoroughly. This may allow a more-uniform of synthetic seed compared to those in published literature. Or, perhaps this may be a straightforward case of 'over-engineering'—I've never seen a report of direct sowing of palm somatic embryos onto autoclaved soil or sand in SV or VV. Perhaps a combination of these potential protocols may solve the problems of plantlet conversion and acclimatization. At this point,

there is comparatively little need to improve peach palm culture multiplication, but there is immense need to improve the later steps.

4 FASTER THAW RATES, PARTIAL DEHYDRATION, AND A LESS-TOXIC FORM OF PVS3 IMPROVE PEACH PALM (BACTRIS GASIPAES KUNTH) EMBRYOGENIC CLUSTER POST-VITRIFICATION REGROWTH

4.1 ABSTRACT

Droplet vitrification is a means of cryopreserving plant cultures, but it is far from a perfect methodology. Culture mortality—even for hardy, vigorous cultures—can be high. We found that, of the factors we tested, that the speed of devitrification had the single highest impact on peach palm (*Bactris gasipaes*) embryogenic cluster regrowth: the highest tested temperature—70°C—had the highest overall regrowth, with diminishing regrowth as thaw temperature decreased, regardless of thaw duration. When compared to conventional vitrification in cryotubes, droplet vitrification led to slightly, though statistically-significant, greater regrowth. Larger somatic embryo clusters showed greater regrowth compared to smaller clusters, suggesting that increased manipulation may be the source of much damage even prior to vitrification. The use of heavy water compared to regular water as the solvent for PVS3 found a slight, though significant increase in regrowth in heavy water-based PVS, but the overall impact likely does not justify the increased cost. Reducing PVS3 to 80% of its original concentration significantly reduced its effectiveness in post-vitrification regrowth. However, the addition of 0.1M KCl, $MgCl_2$, $MgSO_4$, or $K_2H(PO_4)$ to 80%PVS3 solutions significantly increased regrowth regardless of salt type. Partial dehydration for 1-3 hr increased survival for embryogenic clusters incubated in PVS3 for 60 minutes, but longer incubation times led to increased mortality.

4.2 RESUMO

A vitrificação *droplet* é um método de criopreservar culturas de plantas, porém pode ser ineficiente. Até culturas de plantas mais vigorosas e resistentes podem sofrer alta mortalidade. Dentre os fatores que testamos, a velocidade de desvitrificação teve o maior impacto sobre o crescimento de *clusters* embriogênicos de pupunha (*Bactris gasipaes* Kunth): 70°C foi a temperatura mais alta testada e obteve a maior regeneração; já as temperaturas mais baixas obtiveram menor regeneração, independentemente da duração do descongelamento. Ao comparar a vitrificação *droplet* com a convencional em criotubos, a vitrificação *droplet* levou a um recrescimento ligeiramente maior, porém estatisticamente significativo. *Clusters* maiores de embriões somáticos

mostraram maior regeneração em comparação com *clusters* menores, sugerindo que o aumento da manipulação pode causar fontes danos, mesmo antes da vitrificação. O uso de água pesada em comparação com a água regular como solvente para o PVS3 resultou em um aumento leve, embora significativo, na regeneração de *clusters*, mas o impacto geral provavelmente não justifica o aumento do custo. A redução do PVS3 para 80% de sua concentração original reduziu significativamente sua eficácia na regeneração pós-vitrificação. No entanto, a adição de 0.1 M KCl, $MgCl_2$, $MgSO_4$ ou $K_2H(PO_4)$ a 80% PVS3 aumentou significativamente o recrescimento independentemente do tipo de sal. Desidratação parcial de 1 a 3 horas aumentaram a sobrevivência para os clusters embriogénicos incubadas em PVS3 durante 60 minutos, mas os tempos de incubação mais longos levaram a um aumento da mortalidade.

4.3 INTRODUCTION

Tissue cryopreservation is complex; unless prepared correctly, most tissue will be irreparably damaged by the transition of water from a liquid to a solid state (Heringer et al., 2013a). Freezing causes damage through at least two methods: ice crystals can form and puncture a cell; and the solutes inside a cell can become concentrated as ice forms until it forms a 'brine' of solutes, which, when warmed, imparts a physical force on the cell as the osmotic pressure equalizes (Muldrew and McGann, 1994; Han and Bischof, 2004). If tissue is frozen fast enough, water forms a 'solidified liquid'—also called the glass state, where molecules freeze before they can become organized into a crystal, and, by extension, trap solutes between glass-state water molecules before they can be concentrated (Fahy and Wowk, 2015). As water cools, molecules may form a nucleation center composed of organized molecules, from which crystals will form through the diffusion of water molecules into the crystalline lattice (Wowk, 2010). Crystal growth and formation of nucleation centers depend on temperature: nucleation centers form rapidly under very low temperatures, but the diffusion of water is hindered, thus slowing crystal growth (Kirichek et al., 2015). As temperatures approach the melting point, molecules move more quickly, leading to rapid crystal growth onto nucleation centers. Warming, therefore, can be just as deadly as freezing, because as water leaves the glass state, many nucleation centers form, from which many more crystals can grow. All of these processes can be affected with cryoprotectants (Cañavate and Lubian, 1995). Most forms of cryoprotectants have several main uses: passive removal of water from cells through diffusion from

the cytoplasm to high-osmotic cryoprotectant; molecule stabilization through interactions between proteins and cryoprotectants; and increase water viscosity to slow water molecule movement (Elliott et al., 2017). Viscosity may aid in reducing freezing/thawing-related damage by reducing the flow of water to form either nucleation centers or reducing water incorporation into crystals. Peach palm (*Bactris gasipaes*) has already achieved a relatively high rate of post-vitrification survival using the cryoprotectant solution PVS3 (50% *w/v* sucrose, 50% *w/v* glycerol); however, both cryoprotectant and cryopreservation decreased culture survival (Heringer et al., 2013a, 2013b). Many aspects of cryopreservation are well documented: various forms of PVS2 or PVS3 led to successful cryopreservation of several species (Ruggera and Fahy, 1990; Newton et al., 1998; Antony et al., 2013; Heringer et al., 2013a). One central idea tends to prevail: cryoprotectants are necessary, but they are also toxic. Here, we sought to shed light on such factors from the perspective of both a physics-oriented understanding of water undergoing phase transitions and a biology-oriented understanding of reducing tissue mortality from either freezing/thawing-related damage or cryoprotectant toxicity.

4.4 METHODS

4.4.1 Plant material

Thin cell layers (TCL) were harvested from the apical meristems of converted peach palm somatic embryos and placed on culture medium containing Murashige and Skoog basal medium (Murashige and Skoog, 1962), Morel and Wetmore vitamins (Morel and Wetmore, 1951), 30 g L^{-1} sucrose, 1 g L^{-1} glutamine, 2.5 g L^{-1} activated charcoal, 2.3 g L^{-1} Phytagel (Sigma), and 450 µM picloram. The genotype used here is different than those used in Heringer et al. (2013a, 2013b). The pH of all medium was adjusted to 5.8 before being autoclaved for 20 min at 1.2 atm and 120°C. TCL sections were left on culture medium for several months until embryogenic callus formed. After embryogenic callus formed, they were placed onto multiplication medium consisting of the basal medium without activated charcoal and with picloram concentration reduced to 10 µM. This medium was also used for post-vitrification or post-PVS3-treated control embryogenic clusters (EC). ECs selected for experiment were composed of a mass approximately 4-6 mm in width and containing several somatic embryos. EC 2-3 mm and 7-9 mm cultures were selected for a separate experiment comparing the size of EC to regrowth rates, but were otherwise discarded for other experiments. EC were then sorted

evenly and randomly into experimental treatments. In another experiment, the role of partial dehydration prior to vitrification was performed by opening Petri dishes in a laminar flow hood for 0, 1, 2, or 3 hrs.

4.4.2 PVS Mixing

All plant vitrification solutions (PVS) were prepared by heating several mL of deionized water to boiling. All solid reagents—50% w/v sucrose and 4.33 g L^{-1} MS salts—were added to the boiling water and swirled until dissolved. Glycerol (50% w/v) was added once all solids were dissolved, and the solution's final volume was adjusted to its final amount with water. Unless otherwise stated, PVS3 (50% w/v sucrose, 50% w/v glycerol, 4.33 g L^{-1} MS salts) was used as the vitrification solution for all experiments, except those that focused on PVS3 modifications: regular water and heavy water (D_2O) as the PVS3 solvent with 0, 0.01, or 0.1 M $MgCl_2$; and 80%PVS3 (40% w/v sucrose, 40% w/v glycerol, 4.33 g L^{-1} MS salts) with 0 or 0.1 M KCl, $MgCl_2$, $MgSO_4$, or $K_2H(PO_4)$.

4.4.3 Vitrification

Peach palm EC were preconditioned for 60 min in a liquid MS culture medium supplemented with 0.3 M sucrose, and then for another hr in liquid MS medium with 0.6 M sucrose. Afterward, EC were incubated in PVS for 60, 120, 180, or 240 min. Droplets of PVS were placed on aluminum strips small enough to fit inside a cryotube, and ECs were placed into each droplet. The Al strips holding the ECs were then submerged into liquid nitrogen and held until frozen. Two strips containing EC were then placed into a cryotube and stored in a cryotank containing liquid nitrogen. To test the effect of vitrification strip material based on thermal conductance, the standard Al strips (~150 µm thick) were compared to strips made from silver (~260 µm thick), stainless steel (~108 µm thick), Al foil (~28 µm thick), standard Al strips without a droplet of PVS3, polystyrene (PS) (~100 µm thick), and expanded polystyrene (EPS) (~ 2 mm thick). Non-cryopreserved controls underwent the same round of incubations, but not cryopreserved. For conventional vitrification, EC were incubated in PVS3 within cryovials for 60, 120, 180, 240 min and then the tube was submerged into liquid nitrogen for one minute before it was removed and stored in a storage cryotank containing liquid nitrogen for at least 24 h.

4.4.4 Thaw and unloading buffer

Cryovials were removed from the liquid nitrogen-containing storage drum and submerged in a 45°C water bath for 2 min. Afterward, cryopreserved tissue, as well as non-cryopreserved controls, were incubated in an unloading buffer consisting of MS salts and 1.2 M sucrose for 20 min. Thereafter, EC were transferred to multiplication medium and placed in the dark at 25°C. Samples were checked for regrowth after 12 weeks (Figure 5-1).

4.4.5 Statistical analysis

A biological replicate consisted of a cryovial loaded with two strips containing between 10 and 16 EC, depending on experiment, with five replicates per treatment, totaling a total of between 50 to 80 EC per treatment per experiment, with equal numbers of non-vitrified control where relevant. Regrowth was calculated as a proportion, and these proportions were analyzed using regression in R software (R Development Core Team, 2014) using the 'readx1,' 'psych,' 'betareg,' emmeans,' lmtest,' multcompView,' and 'ggplot2' packages. A Dunnett post hoc test was used when a statistical difference was calculated.

4.5 RESULTS

4.5.1 Thawing rate, droplet vitrification strip material, and a direct comparison between droplet and conventional vitrification

Increased thaw temperature significantly affected post-vitrification peach palm EC regrowth ($p<0.05$); however, the time in which they were thawed did not ($p=0.533$) (Figure 5-2A). In general, the hotter the thaw bath, the greater the recovery: the lowest recovery occurred when EC were thawed at 30°C, and the greatest at 75°C. However, three min. of incubation at 75°C may have had a detrimental effect on the tissue, as noted by the sharp decrease in regrowth. Despite this sudden drop, the interaction between thaw temperature and incubation time were not significant ($p=0.69$).

The strip used for vitrification likewise showed a significant effect of material on EC regrowth ($p<0.05$) (Figure 5-2B). EPS and PS showed decreased regrowth compared to the standard Al strips. Steel, likewise, showed a very minor drop deemed non-significant by a Dunnett post-hoc test. Silver, despite having greater thermal conductance compared to Al, was not significantly-different. Al strips loaded only with cultures and no

fresh PVS likewise showed little difference compared to standard Al. Thin Al foil promoted the largest regrowth rates, but these, too, were not significantly greater than the standard Al.

A direct comparison between droplet vitrification and conventional vitrification using immersion of cryovials instead of cultures on strips into liquid nitrogen showed a significant difference between either technique ($p<0.05$), as well as PVS3 incubation duration ($p<0.05$), but there was no significant interaction between neither technique nor PVS3 incubation time ($p=0.1936$) (Figure 5-2C). Though droplet vitrification achieved the overall greatest recovery of 61.4 ± 7.29 % after a 180 min incubation in PVS3, it was comparable 120 min and 240 min. in conventional vitrification.

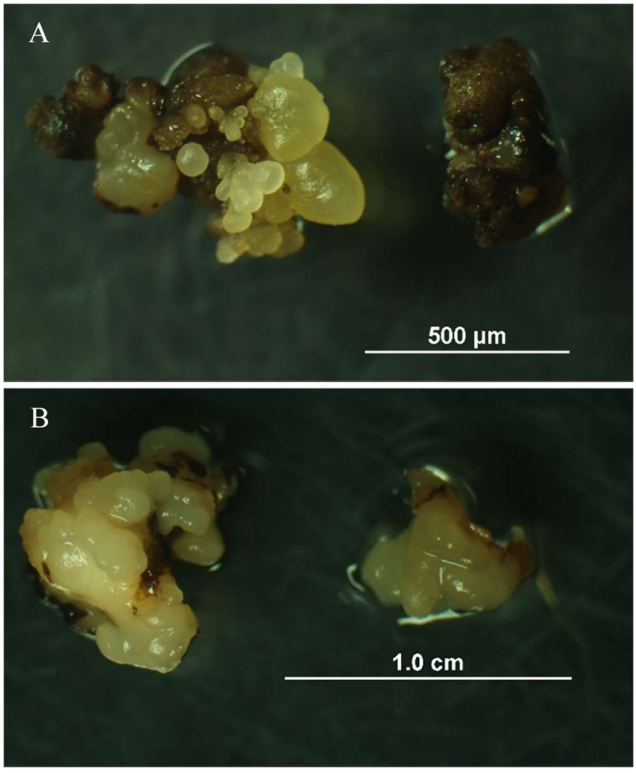


Figure 4-1: A) Non-vitrified peach palm (Bactris gasipaes *Kunth*) embryogenic cluster regrowth compared to a dead, completely necrotic one. B) Vitrified embryogenic cluster regrowth compared to one that didn't regrow after 12 weeks. The tissue in this damaged tissue is soft, watery, and dark—but not necessarily necrotic.

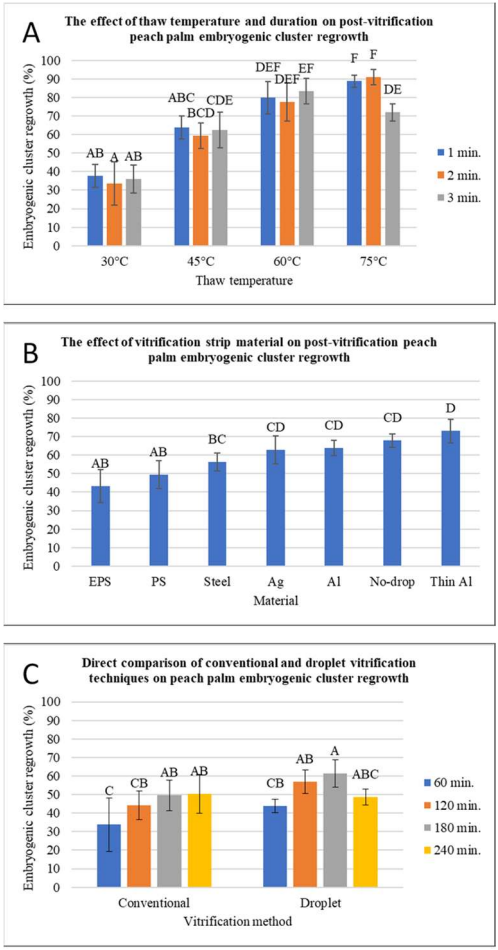


Figure 4-2:Regrowth response of peach palm (Bactris gasipaes *Kunth*) embryogenic clusters incubated for 120 min. in PVS3 and vitrified. Letters signify a statistical difference according to a Dunnett post hoc test (α=0.05)

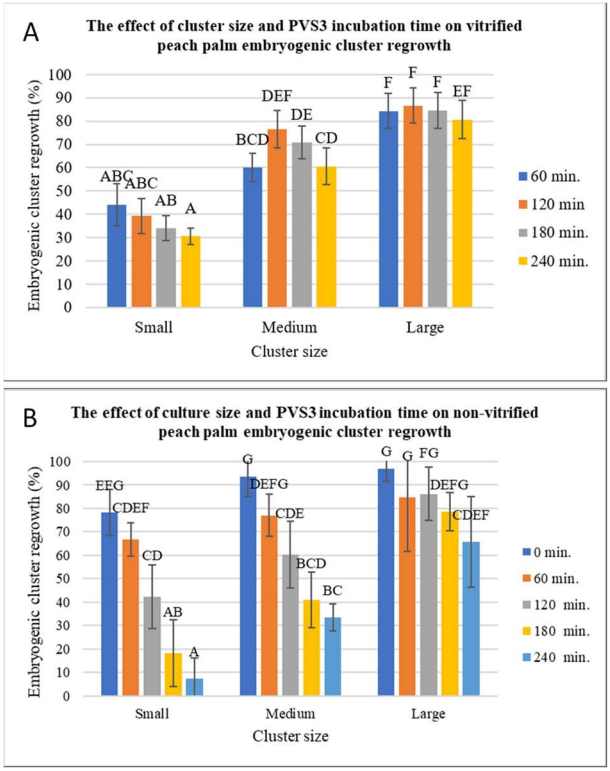


Figure 4-3:The effects of cluster size on (A) vitrified and (B) non-vitrified peach palm (Bactris gasipaes *Kunth*) embryogenic cluster regrowth by PVS3 incubation duration. Letters signify a statistical difference according to a Dunnett post hoc test (a=0.05)

4.5.2 EC size significantly affected regrowth rates

In general, EC post-vitrification rates increased with larger culture sizes (Figure 5-3), where both size ($p<0.05$), and PVS3 incubation time ($p<0.05$) were significantly different, but there was no significant interaction between the two ($p=0.111$) (Figure 5-3A). The standard medium-sized EC used in all other experiments showed the typical regrowth curve across all three incubation times; however, this was not repeated in either small or large-sized cultures: large-sized EC displayed

an attenuated curve with no significant difference between all four incubation times; and small-sized EC showed its highest regrowth rates within the first incubation time and then a decrease thereafter. Non-vitrified control cultures submitted to the same sample processing and PVS3 incubation duration showed much of the same results: the standard medium-sized cultures showed a typical gradual decrease in regrowth over time; the small-sized cultures showed a more-extreme version of this; and the large-sized showed an attenuated version in contrast (figure

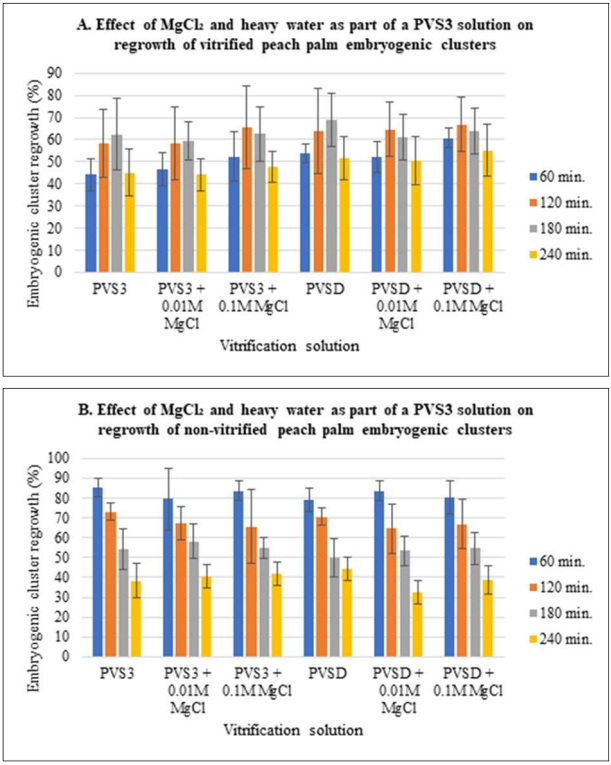


Figure 4-4:The effects of water or heavy water-based PVS3 with 0, 0.01, or 0.1 $MgCl_2$ on vitrified (A) or non-vitrified (B) peach palm (Bactris gasipaes *Kunth*) embryogenic cluster regrowth

5-3B). Both size ($p<0.05$) and PVS3 incubation duration ($p<0.05$) were shown to be statistically different amongst all non-vitrified treatment, as was the interaction between either factor ($p<0.05$). Though not different according to a Dunnett post-hoc test, the small-sized EC showed a decreased mean regrowth rate even when not exposed to PVS3.

4.5.3 Heavy water may increase post-vitrification survival slightly if used as the solvent for PVS

The effect of D_2O and $MgCl_2$ as part of a modified PVS3 mixture on post-vitrification regrowth is not immediately apparent: though PVS3 incubation duration was a significant indicator of EC regrowth ($p<0.05$), mixture type was not ($p=0.0756$), and neither was any interaction between the two ($p=0.992$) (Figure 5-4A). Due to the low, but non-significant, p-value, we analyzed which of two additions to the PVS3 mixture—$MgCl_2$ or D_2O—had the greater effect by pooling data together and running another analysis. When $MgCl_2$ treatments were pooled and the solvent type (H_2O or D_2O) were ignored, the treatments were non-significant ($p=0.20$); but, when $MgCl_2$ concentration was ignored and the two solvents were compared, EC incubated with D_2O-based PVS3 had slightly greater regrowth rates than those incubated in H_2O-based PVS3 ($p=0.016$). These impacts are, nonetheless, small in scope compared to other tested factors. Neither D_2O nor $MgCl_2$ showed a toxic effect on non-vitrified EC: though incubation duration was, once again, a significant factor ($p<0.05$), neither mixture type ($p=0.8264$) nor an interaction between incubation duration or mixture type ($p=0.9386$) were significant (Figure 5-4B).

4.5.4 Dissolved salts in 80% PVS3 solutions conveyed greater post-vitrification regrowth than PVS3 or 80% PVS3 alone

Full PVS3 and 80%-concentration PVS3—with or without dissolved salts—showed markedly-different regrowth patters: PVS3 showed a curved arch of regrowth which peaked at 120 and 180 min incubation and then decreased, whereas 80% PVS3 mixtures tended to reach their peak regrowth at the latter two tested incubation times (Figure 5-5A). Mixture type ($p<0.05$) and time ($p<0.05$) had significant effect on regrowth, but

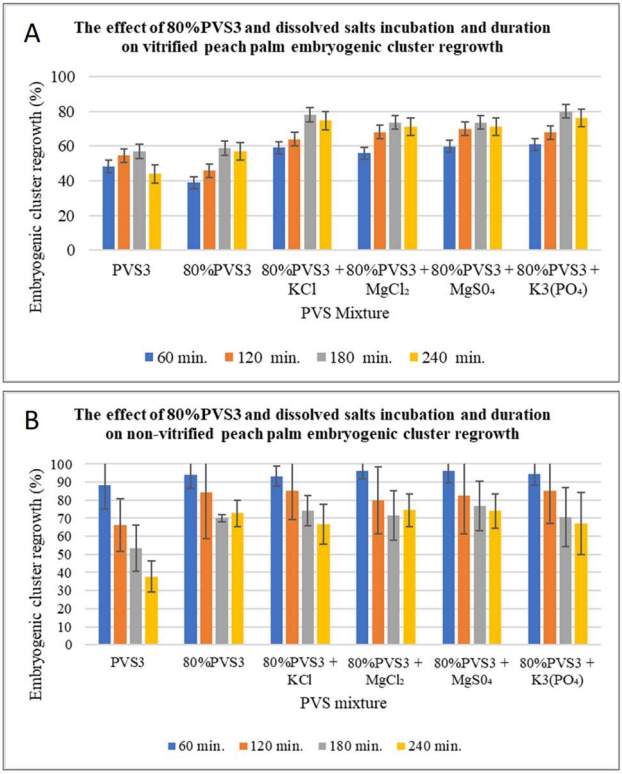


Figure 4-5:The effects of PVS3 on vitrified (A) or non-vitrified (B) peach palm (Bactris gasipaes *Kunth*) embryogenic clusters compared to 80%PVS3 with or without dissolved salts by incubation duration.

there was no apparent interaction between the two (p=0.30). All four of the 80% PVS3 mixtures with dissolved salts achieved mean regrowth

rates above either PVS3 or 80% PVS3 without dissolved salts within the four PVS incubation times, though the type of salt did not appear to have any apparent effect. Non-vitrified EC exposed to the same vitrification solutions showed a distinct trend: EC incubated in full-strength PVS3 showed a decrease in regrowth rate as incubation time increased, whereas this decrease was either attenuated or seemed to come to a stop in 80%

PVS3, with or without dissolved salts. Like in vitrified EC, non-vitrified EC were significantly affected by both PVS3 mixture type ($p<0.05$) and time ($p<0.05$), but there was no significant interaction between either ($p=0.152$). Those EC incubated in 80%PVS3 suffered less loss in regrowth compared to those incubated in PVS3.

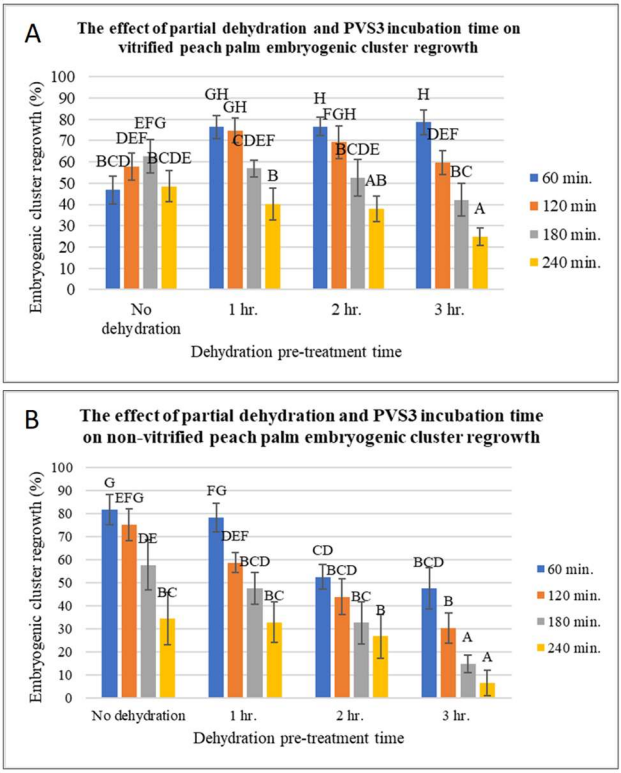


Figure 4-6:The effects of partial dehydration on vitrified (A) or non-vitrified (B) peach palm (Bactris gasipaes *Kunth*) embryogenic clusters by PVS3 incubation duration. Letters signify a statistical difference according to a Dunnett post hoc test ($\alpha=0.05$)

4.5.5 Dehydration of peach palm EC affects post-vitrification regrowth in a time-dependent manner

Partial incubation and PVS3 incubation time significantly-affected peach palm EC post-vitrification regrowth (both $p<0.05$), and these factors had a significant interaction ($p<0.05$). One, two, or three hr of partial dehydration before PVS3 incubation showed an immediate increase in post-vitrification regrowth with 60 min. of PVS3 incubation (Figure 5-6A). However, as partial dehydration times increased along with PVS3 incubation times, regrowth decreased severely. Among non-vitrified EC, dehydration had an overall negative effect on regrowth ($p<0.05$). Though one hr of dehydration pre-treatment had little negative effect on regrowth, longer dehydration times resulted in decreased regrowth to the point where the mean regrowth for three hr of pre-treatment and 240 min. of PVS3 incubation resulted in a mean regrowth of 6.34 ± 6.22% of EC—the lowest mean regrowth rate of all experiments in this article (Figure 5-6B).

4.6 DISCUSSION

4.6.1 Speed of freezing and thawing are major factors in determining post-vitrification regrowth

One of the main obstacles to successful regrowth after cryopreservation is the behavior of water molecules at sub-freezing temperatures. For example, at 0°C, water molecules retain enough energy to prevent the formation of nucleation centers; they may form, but have too much energy to remain as such and are soon broken, but, as temperatures decrease further, more nucleation centers may form and remain intact because water molecules lack the energy to break free, and soon nearby molecules are drawn into an expanding crystal (Akyurt et al., 2002; Matsumoto et al., 2002). These crystals may puncture living cells, causing damage that may lead to cellular death (Han and Bischof, 2004). Freezing with liquid nitrogen bypasses much—though likely not all—of this window of opportunity for these nascent ice crystals to form. Rather than the formation of an organized ice crystal lattice, water molecules remain in a glass state—or, in other words, a 'solidified liquid' state—where molecules remain in place with minimal diffusion (Wowk, 2010). Under these conditions, the impact of two forms of direct cellular damage are mitigated: mechanical damage from cell puncturing by extracellular ice (Stott and Karlsson, 2009) is reduced through the decreased formation of ice crystals; and the lack of crystal ice likewise cannot push solutes into an unfreezable 'brine' and thus prevent osmotic-related damage

(Muldrew and McGann, 1994; Han and Bischof, 2004). Yet, despite this, some nucleation centers may still form—though it is not clear if more are formed during freezing or thawing.

Freezing rate isn't the sole determining factor in determining culture regrowth; thaw speed, too, was overwhelmingly important. Warming a frozen solution shows that increased water molecule mobility after water leaves the glass state creates an environment ultimately dangerous to cellular life because ice nucleation readily occurs at lower temperatures, leading to the formation of a multitude of nucleation centers able to incorporate warming water molecules to make countless small crystals (Rall, 1987; Wowk, 2010). An analysis using neutron-scattering found that the highest rates of crystallization happens during warming when water in cryopreserved carp (*Cyprinus carpo*) embryo tissue melted enough to leave the glass state (Kirichek et al., 2015). However, the authors process was significantly repressed in samples cryopreserved with 50% D2O, 23% 1,2-propanediol, 27% methanol, and 20% DMSO. In order to prevent these nascent nucleation centers from giving rise to crystals, the temperature of water within a vitrified sample must be increased as fast as possible. Indeed, peach palm EC warmed at 30, 45, 60, or 75°C showed a direct increase in regrowth as thaw temperature increased. This is not the first observation of the impact of thaw speed on post-vitrification recovery. Mouse oocytes recovered from vitrification when heated at the fastest tested rate; however, the authors found that as cooling rate increased, so too did the required warming temperature needed to be increased (Mazur and Seki, 2011). Fahy and Wowk (2015) reviewed the thermodynamics of cryopreservation, finding that heating rate directly increased cryopreservation survival in a number of studies focused on both animal and plant cells and tissues. Further, this effect seems to be the most important within the first minute of the thaw; all tested thaw durations—save for the 3 min. at 75°C—were not significantly different from the other ones within that temperature. The most critical events that decided the extend of thaw-related damage likely take place in the first minute. A quick incubation at higher temperatures—perhaps even as high as boiling—may lead to even further increases in regrowth rates, but this remains untested.

Our results suggest that the faster EC are vitrified, as determined by the type of droplet vitrification strip material, the more likely that it will regrow. All metals—except the relatively-low thermal conducting stainless steel (Ashby, 1989)—had significantly greater regrowth rates

than polystyrene. Of the metals, the greatest regrowth occurred in EC placed on Al foil (~28 µm thick), but this was not significantly higher than cultures places on 'standard' Al strips (150 µm in width) or cultures placed on the same strips without a droplet of cryoprotectant. Further, Al foil was difficult to manipulate. Ag did not have a significantly-higher regrowth rate, but the Ag strips used in this protocol measured an estimated ~260 µm in width—far thicker than standard Al. Further tests with thinner Ag strips may show greater regeneration, but this remains to be tested, and its high cost may be prohibitive to standard use. Copper; however, might be more cost-effective, being overall cheaper than Ag and more thermal conductive than Al (Ashby, 1989). EC vitrified on strips made from stainless steel (~108 µm thick) showed a 'bridge' between the highly-conductive metals Al and Ag and either form of the non-conductive polystyrene. Both PS (~100 µm thick) and EPS (~ 2 mm thick) showed comparable mean regrowth rates, which may suggest that there might be a 'limit' on the effect of vitrification strip material on reducing peach palm EC regrowth. Low energy transfer rates from the room temperature EC through the polystyrene, and into the liquid nitrogen may have delayed the glass state transition sufficiently to cause damage, but not enough to prevent regeneration; rather, EC tissue close to the PS or EPS might have transferred energy to the EC tissues cooled by closer proximity to liquid nitrogen, and were thus delayed in reaching vitrification, though an exact study would be needed to confirm this. The material used for vitrification, therefore, is important for optimizing protocols; however, even using an insulator will not completely prevent regeneration in vigorous peach palm EC. A vitrification strip should have the following properties based on this data: composed of a metal with a high thermal conductance rate; thin enough to allow rapid thermal transfer through it but thick enough for ease-of-manipulation; and cost-effective for the available budget.

We also directly compared droplet vitrification using Al strips and 'conventional' vitrification using direct immersion of cryovials filled with PVS3 and cultures as an offshoot of the role of thermal conductance and its role in affecting EC regeneration. In general, droplet vitrification was more effective than conventional vitrification over all PVS3 incubation durations. Curiously, rather than follow the wave-like pattern of droplet vitrification, in which the 120 and 180 min. incubations are superior to either the 60 or 240 min. incubation periods, regeneration for conventional vitrification reached a peak at 120 min. and then remained

constant for the further two incubation periods. This may be explained by the workflow of either method: in droplet vitrification, EC were removed from an incubation medium, in which water was osmotically removed from cultures and thus gradually reduce the overall concentration of solute in the solution, and then placed into fresh PVS3 before being submerged into LN; in conventional vitrification, this reduced-concentration PVS3 remains. We observed that a hazy gradient formed around cultures suspended in PVS3 in cryovials as water leeched from them into the surrounding solution, thus changing the osmotic gradient. After four hr, this osmotic gradient might reduce deleterious loss of water from cells. The 240 min. incubation periods for either droplet or conventional vitrification were not significantly different. Though these data suggest that droplet vitrification is overall more effective, it is not overwhelmingly so for peach palm EC. Further, both have advantages: droplet vitrification for its greater regrowth rate; conventional vitrification for its comparatively simpler workflow. However, comparisons between these methods have shown different results in other species. Leunufna and Keller (2003) found that yam (*Dioscorea* spp.) apical buds or shoots had far greater survival in droplet or a modified droplet protocol compared to conventional vitrification in cryovials.

4.6.2 Greater PVS3 infiltration in smaller EC may not be worth the added damage from cutting and manipulation

Several studies propose that smaller cultures more readily absorb cryoprotectants and are therefore more likely to survive vitrification. Smaller *Musa* corms had the greatest post-thaw survival compared to larger corms (Panis et al., 2005), and, similarly, so did smaller *Allium sativum* apices show greater regeneration rates after cryopreservation than larger ones (Baek et al., 2003). These studies used individuals, whereas peach palm EC is composed of several somatic embryos, each of which may give rise to more through secondary somatic embryogenesis. Rather than test the size of these embryos themselves, we tested the effect of making these clusters smaller than their standard size or leave them even larger with less manipulation. Smaller EC were less likely to regenerate for all given incubation periods compared to medium (control) EC and larger cultures in either vitrified or non-vitrified conditions even without exposure to any cryoprotectant, suggesting that the mere act of cutting tissues into smaller pieces may lead to a loss of regrowth potential even before vitrification. Smaller pieces, however, seemed to absorb cryoprotectants more rapidly: the greatest survival rate occurred after 60

min. incubation and then declined in subsequent incubation times. Medium EC followed the general trend for all PVS3 cultures: greatest at 120 and 180min and lower in 60 or 140 min. times. Large cultures followed no clear trend; their regrowth rates remained relatively high for all time periods. We merely counted regrowth rate on a culture-by-culture basis; we did not count it embryo-by-embryo due to the sheer number of embryos and the added ambiguity of tracking each embryo before and after vitrification to see which were new and which were already there. Obvious regrowth was easier to see due to color contrasts and size, and therefore easier to measure with a higher degree of assurance. Medium EC in either 120 or 180 min. incubation times were comparable with all four of the large EC incubation times, yet these samples require extensive time to prepare. In this case, leaving somatic embryo clusters in larger size not only makes workflow easier, but it may also prevent tissue damage.

4.6.3 PVS3 can be improved through several means

For most species, successful post-vitrification regrowth requires cryoprotectants. Peach palm EC is no exception—no untreated culture survived vitrification (data not shown). Considering that peach palm EC is ~90% water (non-published data), large-scale ice formation was expected—upon vitrification ice visibly erupted from non-PVS-treated EC, which expanded, turned white and, upon thawing, resulted in pale, spongy tissue incapable of even turning necrotic. PVS is, therefore, indispensable for peach palm EC. Among the most important factors in PVS's function, beyond water removal, is conveying greater viscosity. Viscosity reduces diffusion of water molecules into nucleation centers or onto established crystals (Fahy and Wowk, 2015), which, if frozen quickly enough, may also reduce later damage upon warming by also reducing the chance for ice nucleation centers to form upon thawing. In PVS3, viscosity is supplied by high concentrations of sucrose (a non-cell-permeating cryoprotectant) and glycerol (a cell-permeating cryoprotectant) dissolved in H_2O (Elliott et al., 2017). D_2O, though in many respects similar to the physical properties of H_2O, is more viscous (Fernandez-Prini et al., 2004; Soper and Benmore, 2008) and has shown to act as stabilizers for vaccines (Sen et al., 2009), proteins (Jena et al., 2017), and even bacteria (Lu et al., 2013). In the first study, vaccine stabilization improved with additional $MgCl_2$. We investigated the use of either as part of an improved PVS3 mixture. After removing MgCl2 concentrations as one of the factors, D_2O seemed to improve EC regrowth

slightly, though significantly. To the best of our knowledge, this is the first attempt of using D_2O as a solvent for a vitrification solution; however, the cost of D_2O may not be cost-effective for cultures with already-high post-vitrification regrowth rates.

Another aspect to PVS effectiveness is its effect on tissue health. All non-vitrified controls in all experiments suffered increasing damage—usually in the form of large-scale necrosis—with increasing PVS3 incubation times. The same observations were made in a different peach palm genotype (Heringer et al., 2013; Heringer et al., 2013b). This was not restricted to just this species; algae cells exposed to cryoprotectants for more than 20 min. suffered damage (Hagedorn et al., 2010). We were faced with a difficult question: to what extent is failure to regrow attributed to cryoprotectant-related toxicity or freezing-related damage? Rather than test different types or concentrations of standard cryoprotectant agents as means of removing water, we explored the use of dissolved ions on water structure. Certain ions—mostly dications—may stabilize hydrogen bonds and, ultimately, make water more viscous (Marcus, 2010). Consequently, other charged ions—such as I- or Cs+—may reduce water structure by virtue of their very size; they are too large to fit inside holes between networks of hydrogen bonds, requiring them to break those bonds and accelerate the flow of molecules in a solution. We included several types of salts based on their net structure-reinforcing potential based on the models of Goldsack and Franchetto (1977) with the expectation that regrowth would follow this trend. No such relation appeared in EC regrowth data, however. Though all five of the 80%PVS3 mixtures were less toxic to non-vitrified controls, only the four 80%PVS3 mixtures containing dissolved salts conveyed greater post-vitrification regrowth than PVS3. This is not the first report of added ions affecting the recovery of plant tissue after cryopreservation. Berjak et al., (2011) treated *Stychnos gerrardii* embryonic axes with a high-pH solution containing cathodic water made by electrolyzing a solution of calcium and magnesium chloride, resulting in less cellular damage by what the authors believed to be a mild antioxidant activity to council out radicle oxidative species. The role of ions in increasing post-vitrification regrowth of peach palm EC remains unknown. However, there are many possibilities that could be investigated: similar to the role of magnesium chloride in vaccine and protein stability (Jena et al., 2017; Sen et al., 2009), ions may act as stabilizing molecules for proteins and structures within the cell; dissolved ions may be diffused out of the cytoplasm with

water through passive channels during PVS3 incubation, and thus deprive cells of many types of essential macro and micronutrients; or the salts may depress the freezing point of water or interfere with the formation of nucleation centers. A more in-depth study would need to be conducted.

4.6.4 Tissue dehydration removes water and improves post-vitrification regrowth, but its application is time-sensitive

Partial dehydration greatly increased peach palm EC regrowth under specific circumstances. When partial dehydration was conducted for one, two, or three hr and then followed by a 60 min. incubation in PVS3, regrowth was significantly greater than any of the four incubation times in non-dehydrated peach palm EC. However, only partial dehydration for one or two hr followed by a 120 min. incubation remained higher than the control, with three-hour dehydration and 120 min. incubation matching it. Later incubation times tended to decrease regrowth rapidly—especially in the three-hour dehydration and 240 min incubation reaching regrowth values significantly lower than the non-dehydrated control. In negative controls, this was even more severe, with the same treatment reaching a mean regrowth rate of less than 10%. Dehydration overall decreased regrowth in non-vitrified controls compared to the non-dehydrated control. Partial dehydration of oil palm (*Elaeis guineensis*) somatic embryos likewise improved cryopreservation survival (Dumet et al., 1993). Wen et al., (2010) found that *Citrus grandes* zygotic embryos could be partially dehydrated in order to increase their cryopreservation survival, but loss of too much water resulted in seed non-viability. If applied correctly, partial dehydration may be an integral part of a vitrification protocol; however, its use should be optimized.

4.7 CONCLUSION

Peach palm EC post-vitrification regrowth could be improved through several means: faster thawing speed; use of thinner, more conductive metal for droplet vitrification; less manipulation of EC size; use of an 80%PVS3 solution with 0.1M KCl, $MgCl_2$, $MgSO_4$, or $K_2H(PO_4)$, and partial dehydration prior to cryopreservation. Many of these changes are simple and easy to adopt for standard use. Droplet vitrification promoted greater EC regrowth compared to conventional vitrification; however, the decreased workflow required by conventional vitrification, when combined with factors such as increased thaw speed, might be more suitable for larger-scale cryopreservation projects.

4.8 FUTURE PROSPECTS

Several potential ways of improving the regrowth of peach palm EC after vitrification proved to be superior to the current methodology. The question now becomes: what if these methods were combined? Could we achieve consistent regrowth of over 90% with two or more of these strategies? Do the same strategies apply to multiple genotypes? What about other species? My personal hypothesis is that EC partially-dehydrated for 1 hr and then incubated in an 80% PVS3 solution with 0.1 M KCl for 60 min and then loaded onto a thin copper strip, frozen, and then thawed in a 70°C water bath for 1 min would lead to high survival in multiple species, but this would need to be substantiated. If so, it would provide a powerful took for conservationists. Consequentially, the same treatments with droplet vitrification substituted for conventional vitrification may likewise yield high regrowth rates with much less of the physical work. Ultimately, cryopreservation remains a powerful, but under-studied field with commercial and conservational importance. As we lose genetic diversity, it becomes more imperative that we store this rich resource before it is extinguished. Cryopreservation is a means of saving plant genetic resources, and it is more important than ever.

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