Jenny Paola Corredor Prado

ANÁLISE BIOQUÍMICA E PROTEÔMICA DURANTE A INDUÇÃO E REGENERAÇÃO DE CULTURAS NODULARES EM *Vriesea reitzii* Leme & A. Costa

Tese submetida ao Programa de Pós Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutor em Ciências; área de concentração: Recursos Genéticos Vegetais.
Orientador: Profa. Dra. Rosete Pescador
Coorientador: Prof. Dr. Lírio L. Dal Vesco

Florianópolis
2016

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Corredor Prado, Jenny Paola
ANÁLISE BIOQUÍMICA E PROTEÔMICA DURANTE A INDUÇÃO E REGENERAÇÃO DE CULTURAS NODULARES EM Vriesea reitzii Leme & A. Costa / Jenny Paola Corredor Prado ; orientador, Rosete Pescador ; coorientador, Lírio Luiz Dal Vesco. - Florianópolis, SC, 2016.
104 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Agrárias. Programa de Pós Graduação em Recursos Genéticos Vegetais.

Inclui referências

1. Recursos Genéticos Vegetais. 2. Bromeliaceae. 3. Micropropagação. 4. Semente. 5. Base foliar. I. Pescador, Rosete. II. Dal Vesco, Lírio Luiz . III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. IV. Título.

Análise bioquímica e proteômica durante a indução e regeneração de culturas nodulares em *Vriesea reitzii* Leme & A. Costa

por

Jenny Paola Corredor Prado

Tese julgada e aprovada em 18/03/2016, em sua forma final, pelo Orientador e membros da Banca Examinadora, para obtenção do título de Doutor em Ciências. Área de Concentração Recursos Genéticos Vegetais, no Programa de Pós-Graduação em Recursos Genéticos Vegetais, CCA/UFSC.

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Florianópolis, março de 2016

Com amor dedico e agradeço:
À minha mãe e ao meu irmão pelo amor e apoio indondicional
Ao meu namorado pelo amor e incentivo

AGRADECIMENTOS

À Universidade Federal de Santa Catarina, ao Programa de Pós-Graduação em Recursos Genéticos Vegetais e ao seu corpo docente, pela acolhida, pela formação acadêmica e por possibilitar a realização do doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES e à Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina-FAPESC pelo auxílio financeiro.

Ao Laboratório de Fisiologia do Desenvolvimento e Genética vegetal-LFDGV, Laboratório Central de Biologia Molecular Estrutural-CEBIME, Laboratório de Morfogênese e Bioquímica Vegetal e ao Laboratório de Sementes da UFSC, pelo espaço disponibilizado.

Aos meus orientadores Profª. Drª. Rosete Pescador e Prof. Dr. Lírio Dal Vesco pela orientação e colaboração durante a realização deste trabalho.

Ao Prof. Dr. Miguel Pedro Guerra pelos ensinamentos e pela oportunidade de fazer parte do RGV.

À Dra. Gabriela Cangahuala Inocente pela colaboração nas análises proteômicas e por compartilhar seus conhecimentos.

Aos membros da comissão examinadora (Profª. Drª. Lorena Ballod Tavares, Prof. Dr. Rogerio Suzuki, Drª. Gabriela Cangahuala, Profª. Drª. Marisa Santos, Prof. Dr. Miguel Pedro Guerra) pela disponibilidade, e pelo olhar crítico e construtivo do documento da tese.

Agradeço a minha família que de longe sempre me ofereceu seu amor, compreensão e apoio incondicional.

Ao meu namorado, amado, amigo e companheiro nessa caminhada, que a pesar das circunstancias, sempre esteve presente. Obrigada pelas nossas conversas, sempre matando a saudade e com otimismo encarando os nossos desafios.

Aos todos os colegas do laboratório, ao André Knop, à Maíra Tomazzoli e especialmente à Daniela De Conti que compartilharam comigo esta jornada, muito obrigada pelo apoio e ajuda durante minha aprendizagem, e pelos momentos de descontração e companheirismo.

À Marilia Shibata e à Liliana Pila pela amizade e a todas as pessoas que tive a oportunidade de conviver e encontrar nesse período. Conheci através de vocês mais um pouquinho, deste encantador Brasil que levo no meu coração. Recebam todos a minha gratidão!

RESUMO

Além da sua importância ecológica, as bromélias têm reconhecido valor paisagístico na indústria ornamental no mundo inteiro. Técnicas de cultura de tecidos compreendem um conjunto de ferramentas aplicáveis para a conservação e propagação em larga escala. Um padrão de resposta da morfogênese *in vitro* definido como culturas nodulares (CNs), com características diferenciadas a dos sistemas regenerativos tradicionais, tem sido observado nas bromélias. As CNs são aglomerados globulares meristemáticos de coloração verde clara e de textura friável ou levemente compacta, que apresentam um elevado potencial regenerativo. No presente trabalho objetivou-se identificar marcadores associados aos eventos celulares que acontecem durante a aquisição e desenvolvimento das CNs em *Vriesea reitzii*, visando ampliar o conhecimento a respeito da rota morfogenética das CNs nas bromélias. Foram usadas sementes de frutos coletados em plantas localizadas no município de Curitibanos (Santa Catarina-Brasil), bases foliares de brotos jovens e CNs mantidas na coleção *in vitro* do Laboratório de Fisiologia do Desenvolvimento e de Genética Vegetal da Universidade Federal de Santa Catarina. Foram realizadas análises proteômicas mediante a combinação de géis bidimensionais e espectrometria de massas, durante a indução de CNs a partir de bases foliares e sementes, e durante a regeneração de brotos, mediante a avaliação de três estádios de desenvolvimento: CNs, microbroto e broto. Adicionalmente, foram realizadas avaliações do padrão de proteínas, amido, carboidratos e do nível de metilação global, durante a indução de CNs a partir de sementes. Os resultados obtidos indicam que o processo de indução de CNs resulta de uma reprogramação gênica no explante, que leva a expressão significativa de determinadas proteínas, acompanhada de alterações morfológicas, bioquímicas e metabólicas. Concomitantemente, apresenta-se uma dediferenciação e elevada proliferação celular, com elevada demanda energética. Explantes (bases foliares e sementes) em meio de indução apresentam conteúdo de proteína e número de proteínas expressas maior, quando comparados aos explantes em meio controle (sem regulador). Proteínas envolvidas nos processos de resposta ao estresse, metabolismo e divisão celular apresentam significativas alterações na sua expressão durante a indução das CNs. Avaliações bioquímicas e epigenéticas durante a indução de CNs a partir de sementes revelaram uma diminuição significativa no conteúdo de amido, carboidratos e no nível de metilação global de DNA, quando comparados com sementes mantidas em meio sem

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Palavras-chave: Bromeliaceae. Eletroforese bidimensional. Espectrometria de massas. Metilação do DNA global. Micropropagação. Semente.

ABSTRACT

In addition to their ecological role, bromeliads have recognized landscape value in the ornamental industry worldwide. Tissue culture techniques comprise a set of tools for preservation and propagation in large scale. A pattern of morphogenic response defined as nodular cluster cultures (NCs) with different characteristics to the traditional regenerative systems, has been described in bromeliads. NCs are organogenic nodule clusters, from green-yellowish to translucent, with a friable or slightly compact texture, and high regenerative potential. This work aimed to identify markers associated with cellular events that take place during the acquisition and development of NCs in *Vriesea reitzii*, to enhance understanding about the morphogenetic route of NCs in bromeliads. Seeds used were collected from plants located in the Curitibanos city (Santa Catarina, Brazil), leaf bases of young shoots and NCs kept in the *in vitro* collection of Laboratório de Fisiologia do Desenvolvimento e de Genética Vegetal of Universidad Federal de Santa Catarina. Proteomic analyzes were performed by combining two-dimensional gels and mass spectrometry during the induction of NCs from leaf and seeds bases, and during regeneration, by evaluating three development stages: NCs, microshoots, shoots. Additionally, assessments were carried protein, starch, carbohydrates and global methylation level during the induction NCs from seeds. The results indicate that NCs induction process results in reprogramming of gene explant, which leads to significant expression of certain proteins, accompanied by morphological, biochemical and metabolic alterations. Concomitantly, it presents dedifferentiation and high cell proliferation, with high energy demand. Explants (leaf and seeds) in induction medium have protein content and number of expressed proteins greater as compared to the explants in medium control (without regulator). Proteins involved in the response processes to stress, metabolism and cell division show significant changes in expression during induction of NCs. Biochemical and epigenetic assessments during NCs induction from seeds, revealed a significant decrease in starch content, carbohydrates and levels of global DNA methylation, compared with seeds maintained in medium without fitoregulador. During the regeneration of shoots from the NCs, changes in proteins related to cellular metabolic processes, the assembly of the photosynthetic mechanism, the response to the medium culture stressful and the dynamics of the cell cytoskeleton contribute to a high regenerative potential, which characterizes NCs. Proteins related to stress response

and metabolism of amino acids and proteins showed had higher expressions in NCs and microshoots stages, and proteins related to carbohydrate metabolism and ATP synthesis had a higher expression in shoot stage. The results represent a step forward to a better comprehension of NCs morphogenetic route.

Keywords: Bromeliaceae. Two-dimensional electrophoresis. Mass spectrometry. Global DNA methylation. Micropropagation. Seed.

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1 INTRODUÇÃO

A familia Bromeliaceae é um dos grupos morfologicamente e ecologicamente mais diversos, entre as plantas com flores nativas das regiões tropicais e subtropicais do mundo (Givnish et al., 2011). Engloba cerca de 3170 espécies neotropicais pertencentes a 58 gêneros (Luther, 2008), com uma única espécie (*Pitcairnia feliciana*) ocorrendo no oeste da África (Smith e Downs, 1974). A sua distribuição no continente americano vai desde o sul da América do Norte até a Patagônia na América do Sul (Smith e Downs, 1974) e inclui plantas na sua maioria herbáceas, perenes, terrícolas, epífitas ou rupícolas, com caule reduzido, portadoras de folhas longas dispostas em rosetas e densamente imbricadas na base (Souza e Lorenzi, 2005; Wanderley e Martins, 2007). Atualmente são reconhecidas oito subfamílias: Pitcairnioideae, Navioideae, Brocchinioideae, Lindmanioideae, Hechtioideae, Puyoideae, Bromelioideae e Tillandsoideae (Givinish et al., 2011).

A Mata Atlântica, um dos 25 *hotspots* do planeta devido a sua diversidade (Myers et al., 2000), é um dos mais devastados e mais seriamente ameaçados, sendo que restam apenas de 7 a 8% da floresta original (Galindo-Leal e Câmara, 2005). As bromélias são importantes componentes desse bioma, por estarem associadas às mais variadas formas de vida (Reitz, 1983). A família Bromeliaceae é a quarta mais diversa na Mata Atlântica (Stehmann et al., 2009), registrando-se um total de 31 gêneros e 803 espécies, das quais 653 são endêmicas (Martinelli et al., 2008). No entanto, cerca de 40% das espécies se enquadram em alguma categoria de ameaça (Martinelli et al., 2008).

O gênero *Vriesea* Lindl. apresenta a maior riqueza de espécies registradas na Mata Atlântica, com um total de 166 espécies, das quais 145 são endêmicas. Entre elas, a bromélia *Vriesea reitzii* Leme & Costa, uma espécie endêmica epífita, com uma distribuição geográfica restrita ao Sul do Brasil, nos estados de Paraná, Santa Catarina e Rio Grande do Sul (Forzza et al., 2015) (Figura 1a). Ocupa altitudes que variam de 750-1200 m predominando nos ecossistemas da Floresta Ombrófila Mista (Leme e Costa, 1991) (Figura 1a). Além da extração de seu habitat natural e a progressiva devastação de seu bioma, a *V. reitzii* é considerada como vulnerável pelo fato de seu habitat epifítico primário (*Araucaria angustifolia*), ter suas populações naturais extremamente reduzidas (Klein, 1990; Leme e Costa, 1991). No entanto, segundo Bonnet (2006), *V. reitzii* pode ser utilizada como uma bromélia indicadora de florestas com elevado grau de conservação.

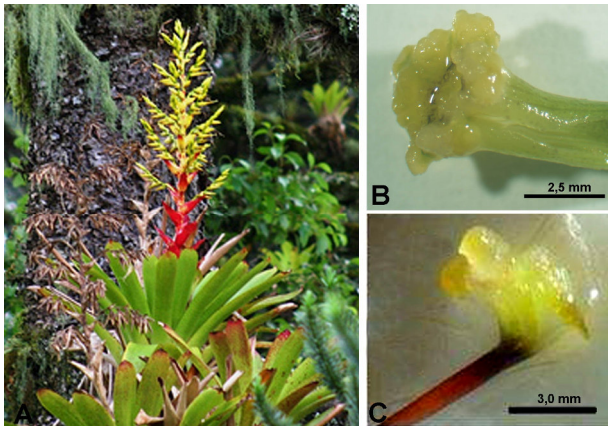

Figura 1 – Bromélia *Vriesea reitzii*. No ambiente natural (A) e nas condições *in vitro*, induzindo CNs a partir de base foliar (B) e a partir da semente (C). Fonte: A: http://www.flickr.com/photos/luizfilipevarella/; B: Corredor-Prado et al, 2015; C: Dal Vesco et al, 2014b.

Algumas das características que determinaram o sucesso de colonização das bromélias em diversos ambientes estão relacionadas com a presença de tricomas para absorção foliar, um ambiente fitotélmico, adaptações metabólicas (fotossíntese via CAM) e suculência das folhas (Benzing, 2000). O tanque formado pela disposição das folhas permite o acúmulo de água e nutrientes que favorecem o desenvolvimento de vários organismos, oferecendo uma fonte de água, alimento, proteção ou sítio de acasalamento (Rocha et al., 1997; Benzing, 2000; Frank e Lounibos, 2009). Isto permite diferentes tipos de interações bióticas que conferem a estas plantas um importante papel ecológico (Rocha et al., 1997). Por outro lado, algumas espécies são apreciadas pelas propriedades medicinais (por exemplo, *Bromelia antiacantha*) ou são cultivadas como frutas tropicais (por exemplo, abacaxizeiro: *Ananas comosus*) (Zanella et al., 2011). Outros usos etnobotânicos associados a estas plantas incluem ser fonte de fibras e forragem, uso como cerca viva e uso místico em cerimônias (Benzing, 2000; Hornung-Leoni, 2011). Porém, nas últimas décadas as belas formas e cores e a fácil adaptação a pequenos jardins aumentaram seu uso como plantas ornamentais, sendo reconhecidas na indústria ornamental no mundo inteiro (Guerra e Dal Vesco, 2010; Negrelle et al., 2012; Zanella et al., 2011).

1.1 CULTURA DE TECIDOS VEGETAIS

Programas de conservação de germoplasma vegetal envolvem diferentes estratégias incluindo estudos de conservação *ex situ*. Segundo Iriondo (2001), na conservação *ex situ* de espécies ameaçadas, podem ser consideradas estratégias de preservação do germoplasma e do desenvolvimento de métodos que possibilitem sua propagação. Desta forma, técnicas de cultura de tecidos, têm se apresentado como uma estratégia de conservação e propagação em larga escala para as espécies de bromélias (Guerra e Dal Vesco, 2010).

A cultura de tecidos vegetais baseia-se no princípio da totipotência das células vegetais, ou seja, na potencialidade de uma única célula se diferenciar e regenerar uma planta completa (Kerbauy, 1998). Este complexo processo morfogenético ocorre com um importante papel dos fatores extrínsecos e intrínsecos nos tecidos (Duclercq et al., 2011). Por conseguinte, a cultura *in vitro* é um sistema útil para as investigações de processos morfológicos, bioquímicos e moleculares relacionados com o desenvolvimento inicial das plantas (Mitrović et al., 2012).

Através da morfogênese *in vitro* tem sido possível a formação de brotos ou raízes (organogênese) e embriões (embriogênese somática) (George, 2008). Assim, duas vias principais levam a regeneração *in vitro* de uma planta: embriogênese somática e organogênese (Iliev et al., 2010). A organogênese é caracterizada pela produção de estruturas unipolares em conexão vascular com o tecido vascular pré-existente e ocorre com o subsequente desenvolvimento de um primórdio de gema vegetativa ou raiz (Brown e Thorpe, 1986). Por outro lado, a embriogênese somática é um processo pelo qual as células somáticas se diferenciam em embriões, os quais são estruturas bipolares que contêm ápice caulinar e radicular e se desenvolvem de uma forma análoga à de embriões zigóticos (Phillips, 2004; Von Arnold, 2008). Segundo Guerra et al. (1999) a formação de uma estrutura bipolar integrada em um único eixo, sem ligações vasculares com o tecido matriz, torna a embriogênese somática diferente do padrão organogênico.

Basicamente, a morfogênese *in vitro* consiste de três fases: desdiferenciação, que é a perda da especialização e a reversão da célula diferenciada, onde o tecido adquire competência para responder ao processo morfogênico; indução, quando as células se tornam determinadas a desenvolver uma estrutura (monopolar ou bipolar); e desenvolvimento, com crescimento de órgãos ou embrião (De Klerk et al., 1997; Kerbauy, 1998).

1.2 CULTURAS NODULARES (CNs) EM BROMÉLIAS

Durante o estabelecimento do cultivo *in vitro* nas bromélias observa-se um padrão de resposta morfogênico que apresenta características diferentes dos sistemas regenerativos tradicionais baseados na organogênese e embriogênese somática, sendo definido como culturas nodulares (CNs) (Guerra e Dal Vesco, 2010). Segundo George (1993), esta rota morfogenética pode ser classificada como uma terceira via da morfogênese *in vitro* das plantas. As CNs são aglomerados de nódulos organogênicos com coloração verde-amarelada a translúcida, com textura friável ou levemente compacta (Dal Vesco e Guerra, 2010; Dal Vesco et al., 2011) (Fig. 1). Esta rota morfogenética foi descrita em *V. reitzii* por vários autores (Alves et al., 2006; Rech Filho et al., 2009; Dal Vesco e Guerra 2010; Dal Vesco et al., 2014a e Dal Vesco et al., 2014b); e em outras bromélias como *V. friburgensis* var. *paludosa* (Alves e Guerra, 2001), *Bilbergia zebrina* (Dal Vesco et al., 2011) e *Ananas comosus* (Scherer et al., 2013). As CNs mantidas em condições de cultura adequadas apresentam alto potencial regenerativo, culminando na produção múltipla de brotos adventícios (Guerra e Dal Vesco, 2010). O meio de cultura suplementado com auxinas como ANA (ácido α-naftalenoacético), AIA (ácido indolil-3-acético) em combinação com citocininas como BAP (6-benzilaminopurina) e 2ip (2-isopentenil adenina), tem sido usado para otimizar a indução e multiplicação das CNs (Rech Filho et al., 2009; Alves et al., 2006; Scherer et al., 2013; Dal Vesco et al., 2010, 2011, 2014a, 2014b; Corredor et al., 2015). Na bromélia *V. reitzii*, Dal Vesco e Guerra (2010) obtiveram a maior eficiência regenerativa e a proliferação de microbrotos em grande escala no meio de cultura constituído por sais minerais (Murashige e Skoog, 1962), vitamina de Morel (Morel e Wetmore, 1951) e 30 $g.L^{-1}$ de sacarose, suplementado com ANA e 2-iP (2 μM cada). Os autores conseguiram uma eficiência regenerativa de 12,4 g/g de CN inoculada, e estimaram uma regeneração de mais de 5.300 novos microbrotos e na fase de alongamento, uma taxa efetiva de 75% destes microbrotos que podem ser convertidos em brotos completos a cada 10 semanas de cultivo.

Estudos indicam que o processo de indução das CNs está relacionado com as células meristemáticas presentes nos explantes. Assim, a indução está associada ao meristema radicular e caulinar do embrião quando as CNs são originadas a partir de sementes (Corredor-Prado et al., 2015) ou associada ao meristema intercalar quando

originadas a partir de bases foliares (Hosoki e Asahira, 1980; Firoozabady e Moy, 2004; Corredor-Prado et al., 2015) (Figura 1b-c). Posteriormente, o processo regenerativo ocorre a partir da proliferação dos grupos de células meristemáticas resultando no desenvolvimento de múltiplos brotos (Dal Vesco e Guerra, 2010).

A maior parte dos trabalhos tem visado a otimização da composição do meio de cultura para o desenvolvimento das CNs (Rech Filho et al., 2009; Alves et al., 2006;. Dal Vesco e Guerra, 2010; Dal Vesco et al., 2011; Dal Vesco et al., 2014a;. 2014b; Scherer et al., 2013; Corredor et al., 2015). Enquanto que estudos moleculares abordaram a fidelidade genética de plantas micropropagadas por meio de marcadores moleculares AFLP (*Amplified Fragment Length Polymorphism*) (Dal Vesco et al., 2012; Scherer et al., 2015) e avaliações da dinâmica dos níveis de metilação do DNA global durante a multiplicacao das CNs em diferentes sistemas de imersão temporária (Scherer et al., 2015). Não obstante, abordagens proteômicas e avaliação das alterações bioquímicas e epigenéticas durante o processo de indução e regeneração de brotos das CNs ainda não foram estudadas.

A falta de conhecimentos fisiológicos específicos é um dos fatores que determina o sucesso dos protocolos de conservação *in vitro* (Pilatti et al., 2011). Nesse contexto, considerando o alto potencial regenerativo das CNs nas bromélias, se faz necessário uma caracterização detalhada dessa rota morfogênica, que contribua sobre o conhecimento das diferentes formas de desenvolvimento *in vitro* das plantas. Estas informações poderiam auxiliar no estudo dos processos de desenvolvimento de outros grupos de espécies com limitado sucesso na micropropagação.

1.3 ALTERAÇÕES FISIOLÓGICAS E BIOQUÍMICAS DURANTE A MORFOGÊNESE *IN VITRO*

Estudos durante a morfogênese *in vitro* em outros grupos de plantas revelam a dinâmica das alterações fisiológicas e bioquímicas, entre elas aquelas associadas ao metabolismo de carboidratos e proteínas ao longo dos processos organogênicos em *Humulus lupulus* (Fortes et al., 2008), *Crocus sativus* (Sharma et al., 2009) e *Vanilla planifolia* (Palama et al., 2010); e durante o desenvolvimento da embriogênese somática em *Musa spp.* (Wang et al., 2013); *Elaeis guineensis* (Silva et al., 2014); *Acca sellowiana* (Cangahuala-Inocente et al., 2014) e *Carica papaya* (Vale et al., 2014) entre outras.

Nas plantas, os carboidratos desempenham inúmeras funções essenciais, sendo substratos para respiração e representando um importante papel na rota biosintética de diversos compostos (Calamar e De Klerk, 2002). Também atuam como moléculas sinalizadoras, alterando a expressão gênica e influenciando nos processos de desenvolvimento e rotas metabólicas (Smeekens, 2000; Halford, 2010). Alguns dos efeitos sobre o crescimento e desenvolvimento das plantas sugerem interação dos sinais dos açúcares com a regulação hormonal (Rolland et al., 2006). A transdução de sinais induzidos pelos açúcares controla a expressão de genes, através de diversos mecanismos, que incluem transcrição, tradução, modificação de mRNA e estabilidade de proteínas (Rolland et al., 2006). Segundo Millam (1994), os carboidratos apresentam uma interação significativa com as auxinas nas fases iniciais na cultura *in vitro*.

As alterações nos teores de carboidratos e proteínas requerem a expressão de genes específicos para a síntese ou a mobilização destes compostos. Esta regulação gênica pode ser afetada por mecanismos epigenéticos, os quais desempenham um papel importante no desenvolvimento da planta (Valledor et al., 2007). A metilação da citosina no DNA, é um dos eventos epigenéticos que se refere à metilação pós-síntese de deoxicitosinas na posição 5' do anel de pirimidina da citosina para formar a metildeoxicitosina (Finnegan, 2010). O padrão de metilação do DNA pode desencadear variações genéticas tais como alterações cromossômicas, inserções, deleções e outros (Wang e Wang, 2012). Estudos têm mostrado que a metilação em determinadas regiões impede a expressão de genes alvo enquanto que eventos de demetilação são acompanhados da ativação gênica (Meng et al., 2012;. Shan et al., 2013). O crescimento e o desenvolvimento são regulados por hormônios vegetais específicos, sendo a modulação da metilação do DNA um dos mecanismos moleculares de ação hormonal na planta (Vanyushin et al., 2004). Segundo Noceda et al. (2009) é possível relacionar o estado de metilação com a competência morfogenética *in vitro* específica. De acordo com isso, células embrionárias, geralmente exibem níveis mais baixos de metilação do que as não-embrionárias (Miguel e Marum 2011). Assim mesmo, níveis baixos de metilação de DNA estão correlacionados com o aumento na capacidade da organogênica (Valledor et al., 2010).

Por outro lado, as proteínas desempenham um papel crucial nos organismos, devido a sua participação na maioria dos processos e funções celulares. Elas estão envolvidas na regulação da expansão celular e no estabelecimento das características biofísicas requeridas

para a morfogênese (Jiménez, 2001). Segundo Catusse et al. (2008) o uso de tecnologias pós genômicas, como a proteômica, permite uma melhor compreensão das bases moleculares dos processos de desenvolvimento, pois é uma abordagem mais direta para definir as funções dos genes associados. A proteômica tem um papel central nos sistemas biológicos porque complementa a análise genômica, transcriptômica e metabolômica (Baginsky, 2008). O desenvolvimento de diversas ferramentas para a integração da proteômica com outras "ômicas" e o levantamento de dados fisiológicos tem possibilitado estudos de sinalização, regulação e redes metabólicas fundamentais para o fenótipo da planta (Kitano, 2002).

Enquanto as análises de transcriptoma fornecem informações importantes sobre a expressão de genes de um organismo num determinado estado, isso não reflete a expressão de proteínas deste organismo (Chen e Harmon, 2006), uma vez que vários mecanismos estão envolvidos na regulação da síntese protéica (Figura 2). Modificações pós-transcricional e pós-traducionais, podem regular a expressão espacial e temporal e a conformação das proteínas, modulando classes de proteínas distintas que, bioquimicamente e estruturalmente, podem desempenhar papéis diferentes nas vias metabólicas de um organismo (Balbuena et al., 2011).

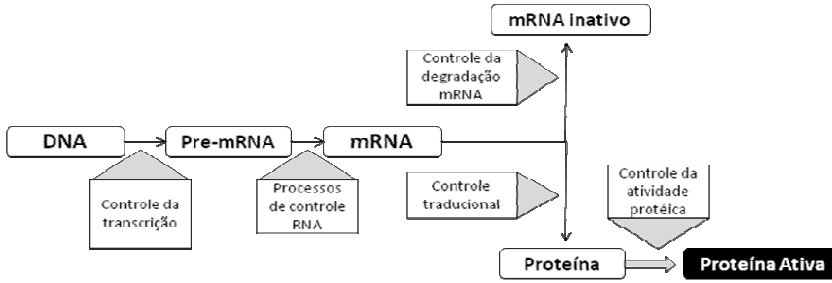

Figura 2 – Mecanismos de controle que agem na síntese de proteínas em que um único gene dá origem à conformação de proteínas com funções diferentes. Fonte: Balbuena et al., 2011.

Análises proteômicas permitem a identificação de proteínas diferencialmente expressas em determinado momento durante o cultivo *in vitro*. Assim, oferece-se a oportunidade de examinar e classificar padrões temporais de acumulação de proteínas em diferentes estádios de desenvolvimento e / ou condições de crescimento (Balbuena et al., 2011). Segundo Jorrin et al. (2006), os estudos em proteômica

apresentam quatro justificativas importantes: 1) a função gênica é levada à "ação" pelas proteínas; 2) a maior parte dos genes identificados (em torno de 85%) não tem nenhuma função conhecida; 3) a informação obtida usando a investigação pelo transcriptoma é incompleta, reflexo das modificações pós-traducionais e as interações proteína-proteína e; 4) a correlação entre mRNA e níveis de proteína é baixa.

Na proteômica comparativa, o objetivo é identificar diferenças quantitativas e qualitativas entre amostras de proteínas (Cánovas et al., 2004). Para isso, é utilizada a interface que consiste principalmente na separação das proteínas através de eletroforese bidimensional (2-DE) em gel de poliacrilamida com posterior identificação da proteína por espectrometria de massas (Dias et al., 2007). No entanto, espécies tropicais apresentam um maior desafio nas análises proteômicas, tendo em conta a falta de sequências completas do genoma (Balbuena et al., 2011).

A identificação de proteínas diferencialmente expressas durante o desenvolvimento vegetal configura-se como um potente marcador molecular do metabolismo em plantas, podendo fornecer informações importantes sobre a competência e grau de evolução da morfogênese *in vitro* (Dias et al., 2007). Os dados gerados a partir desse tipo de análise contribuem significativamente na compreensão e monitoramento dos eventos fisiológicos vegetais e no desenvolvimento de estratégias biotecnológicas (Balbuena et al., 2011). A maioria dos estudos proteômicos tem focado em investigar as mudanças nos perfis de proteínas durante a embriogênese somática (Marsoni et al., 2008; Correia et al., 2012; Sharifi et al., 2012; Vale et al., 2014), com poucos estudos relacionados a outras vias de morfogênese *in vitro*.

A presente tese foi estruturada em três capítulos. No capítulo primeiro, análises proteômicas foram realizadas durante a indução de CNs a partir de bases foliares e sementes da bromélia *Vriesea reitzii*, identificando proteínas que apresentaram alterações significativas no nível de expressão. No segundo capítulo, o estudo proteômico durante a regeneração das CNs permitiu identificar as proteínas diferencialmente expressas em distintos estados de desenvolvimento: CNs, microbrotos e brotos. No terceiro capítulo, o nível da metilação do DNA global e a avaliação do padrão dos carboidratos, amido e proteínas, durante a indução de CNs a partir de sementes de *V. reitzii*, permitiu avaliar a dinâmica celular que conduz a aquisição de competência para essa rota morfogenética. Ao final, são apresentadas as considerações finais e perspectivas futuras do trabalho.

1.4 OBJETIVOS

1.1.1 Objetivo Geral

Identificar marcadores associados aos eventos celulares que acontecem durante a aquisição e desenvolvimento das CNs em *Vriesea reitzii*, visando ampliar o conhecimento a respeito da rota morfogenética das CNs nas bromélias.

1.1.2 Objetivos Específicos

Obter um perfil protéico e identificar as proteínas diferencialmente expressas durante a indução de CNs.

Obter um perfil protéico e identificar as proteínas diferencialmente expressas durante o processo de regeneração de brotos a partir das CNs.

Avaliar o padrão das proteínas, amido e açúcares, assim como a dinâmica da metilação do DNA global durante a indução das CNs.

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2 CAPITULO I. PROTEOMIC ANALYSIS IN THE INDUCTION OF NODULAR CLUSTER CULTURES IN THE BROMELIAD *Vriesea reitzii* LEME AND COSTA

2.1 ABSTRACT

The nodular cluster cultures (NCs) are globular organogenic clumps with high regenerative potential. In the present work we identified differentially expressed proteins involved in the induction of NCs from seeds and leaf explants of the Brazilian native bromeliad *Vriesea reitzii*. Those explants were inoculated into Murashige and Skoog (MS) liquid medium free of plant growth regulators (PGR). To promote the induction of NCs, the seeds were grown in MS medium supplemented with 4μM α-naphthaleneacetic acid (NAA), and the leaf segments in MS medium supplemented with 4μM NAA and 2μM 6-benzylaminopurine (BAP). After 21 days in culture, samples of each type of explant were collected for histological analysis and protein extraction. Proteomic analysis was performed by two-dimensional electrophoresis and protein identification by MALDI-TOF-TOF mass spectrometry. Enhanced protein content and spots number on cultures supplemented with PGR were observed as compared to the cultures maintained in PGR-free MS culture medium. Five differentially expressed proteins were identified during the induction of NCs: Heat shock 22 kDa, Chaperone protein dnaJ 50, S-adenosylmethionine synthase 3, UDP-arabinopyranose mutase 1 e 14-3-3-like protein E. Such proteins are involved in stress response, cell metabolism and cell division. The ability to regulate the effects of stress conditions in which the explants were subjected evidenciates the presence of competent tissues for the acquisition of the morphogenic route associated to the induction of NCs.

Keywords: Bromeliaceae. Growth regulators. Mass spectrometry. Two-dimensional.

2.2 INTRODUCTION

Bromeliaceae is one of the most diverse groups of plants of the Atlantic Forest Biome (Reitz, 1983; Stehmann et al., 2009). The beautiful shapes and colors and the easy adaptation to small gardens increased their use as ornamental plants worldwide, generating a trade that may pose a significant threat to the maintenance of its biodiversity

(Negrelle et al., 2012). In recent decades, growers have benefited from the development of tissue culture techniques to develop new mass propagation strategies in bromeliads (Guerra and Dal Vesco, 2010). These techniques include tools that can be employed for the conservation of these species, especially those endangered (Guerra and Dal Vesco, 2010).

During the establishment of *in vitro* culture, a pattern of morphogenic response defined as nodular cluster cultures (NCs) has been described for several bromeliads (Alves et al., 2006; Guerra and Dal Vesco, 2010; Dal Vesco et al., 2011; Scherer et al., 2013). The NCs are defined as globular organogenic nodules groups, with yellow-greenish to translucent coloration, friable to slightly compact texture (Dal Vesco and Guerra, 2010; Dal Vesco et al., 2011), with a high regenerative potential, culminating in the production of multiple adventitious buds (Guerra and Dal Vesco, 2010). Its induction has been reported starting from the leaf basal region in *Vriesea reitzii* (Dal Vesco and Guerra, 2010; Dal Vesco et al., 2014b; Corredor-Prado et al., 2015) and *Ananas comosus* (Scherer et al., 2013), from seeds in *Vriesea reitzii* (Dal Vesco et al., 2014a) and *Vriesea friburgensis* (Corredor-Prado et al., 2015) or from nodal segments in *Bilbergia zebrina* (Dal Vesco et al., 2011). The bromeliad *V. reitzii*, endemic to the Atlantic Forest is geographically restricted to Southern Brazil (Forzza et al., 2015), and considered as a model for the study of this morphogenetic route. Some works with this species aimed at optimizing the composition of the culture medium for the NCs development (Alves et al., 2006; Dal Vesco and Guerra, 2010; Dal Vesco et al., 2014a; 2014b) and at characterizing the process of induction in terms of morphological and anatomical changes (Corredor-Prado et al., 2015). Notwithstanding, it was not yet studied the induction of NCs at molecular level, using proteomic approaches. Molecular studies on NCs of other species are related with the use of amplified fragment length polymorphism (AFLP) markers and with global DNA methylation levels (Dal Vesco et al 2012; Scherer et al 2015).

Proteomics constitutes an important tool to study the responses of plants to various biotic and abiotic stresses as well as the biochemical changes associated with development (Job et al., 2011). Most of proteomic studies on plant tissue culture focused on investigating the changes in protein profiles during somatic embryogenesis (Marsoni et al., 2008; Correia et al., 2012; Sharifi et al., 2012; Vale et al., 2014). Nonetheless, there are just few studies related to other pathways of *in vitro* morphogenesis. In addition, most proteomic studies use species of

plants of economic importance or native species of temperate regions, while tropical species remain poorly studied (Balbuena et al., 2011).

Comparative proteomic analysis can evaluate changes in protein expression of an organism under different conditions. This tool provides information on the molecular changes in response to external agents. Thus, the use of proteomics in tissue culture can assist in the detection of proteins regulated by phytohormones (Takáč et al., 2011) and that in our case allow the induction of NCs. Accordingly, in the present study cultures of *V. reitzii* were established in culture media supplemented with PGR (induction medium) and PGR-free culture medium (control) and were then subjected to comparative proteomic. Therefore, this study aimed at to identify differentially expressed proteins involved in the induction of NCs from seeds and leaf segments of the bromeliad *Vriesea reitzii*.

2.3 MATERIAL AND METHODS

2.3.1 Plant materials

Seeds from mature fruits of *V. reitzii* were collected from plants kept in Curitibanos, altitude 990 m (Santa Catarina State, Brazil 27º16'58"S - 50º35'04"W), and used as explants source. Sterilization was carried out according to the procedures described by Alves et al. (2006). For NCs induction, seeds were inoculated into Murashige and Skoog (MS) liquid medium supplemented with 4 μM NAA, previously established by Dal Vesco et al. (2014a).

Basal segments of leaves (0.5 ± 0.1 cm length) were excised from young *in vitro*-grown shoots (2.0 ± 0.5 cm high) that arose from successive sub-cultures in MS medium. Such explants were inoculated in MS liquid medium supplemented with 4 μM NAA and 2 μM BAP for NCs induction (Dal Vesco et al., 2014b). All explants were inoculated over filter paper bridges, into test-tubes (22 mm × 150 mm) containing 12 ml of medium. Cultures were maintained in a growth room at 25 ± 2°C and 16 h photoperiod, with a light intensity of 50–60 μmol m^{-2} s^{-1}. Explants inoculated in induction medium supplemented with plant growth regulators (PGR) were identified as seeds forming NCs (SeNCs) and leaf segments forming NCs (LeNCs). Explants inoculated in PGR-free MS medium were identified as seeds in MS medium (SeMS) and leaf segments in MS medium (LeMS).

2.3.2 Histological analysis

After 21 days samples were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) sodium phosphate buffer overnight. The samples were then dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were infiltrated with Historesin (Leica® Historesin, Heidelberg, Germany). Sections (5 µm) were obtained using a manual rotation microtome (Slee Technik®) and were stained with Toluidine Blue O (TB-O) 0.5% aqueous solution, pH 3.0. Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

2.3.3 Proteomic analysis

Protein extraction and quantification

After 21 days of culture, five replicates of each sample were collected and fast-frozen in liquid nitrogen before being kept at −80 °C until use. Each replicate (500 mg) was prepared from approximately either 300 seeds or 80 leaf segments. The extraction of total proteins was performed following the method of Carpentier et al. (2005) with modifications. In brief, tissue collected was ground to powder with the aid of liquid nitrogen. The macerated material was homogenized with 5.0 ml of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 5.0 ml of buffer-saturated phenol (pH 8,0) by vortexing for 30 min. The homogenates were centrifuged for 30 min at 10,000 g, 4 ºC. The phenolic phase was recovered and homogenized with 5.0 ml of extraction buffer by vortexing for 30 min. The homogenate was centrifuged for 30 min at 10,000 g, 4 ºC. The phenol phase was collected. After precipitation with 100mM of ammonium acetate in methanol (1:5 v/v), proteins were maintained 12h at -20°C. The tube was centrifuged for 30 min at 10,000 g at 4 ºC. The pellet was washed three times with 1.0 ml of pure methanol and three times with 1.0 ml of acetone. Finally, the proteins were solubilized in 0.3 ml of solubilization buffer (7M urea, 2M thiourea, 3% CHAPS, 2% IPG-buffer, 1,5% DTT), by mild vortexing and stored at -20°C. Protein quantification was determined by means of the copper-based method 2-D Quant Kit® (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden).

Two-dimensional gel electrophoresis (2DE)

Isoelectric focusing was carried out in strips of 13 cm, pH 3-10 at linear scale (GE Healthcare). The strips were rehydrated in 250 µl of rehydration buffer (7M Urea, 2M Thiourea, 3% CHAPS, 2% IPG-buffer, 0.002% Bromophenol blue, and 0,2% DTT) containing 500 mg of protein for 12-14 h at room temperature. The strips were focused on an Ettan-IPGphor 3 isoelectric focusing unit (GE Healthcare). After IEF, strips were equilibrated for 15 min in equilibrium solution (50 mM Tris-HCl pH 8.8, 6M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS, and 0.002% (w/v) Bromophenol Blue) containing 1% (w/v) DTT, followed by 15 min in equilibration buffer containing 2.5% (w/v) Iodoacetamide. Second dimension SDS-PAGE gels were run in 12% (w/v) acrylamide gels using the Hoefer SE 600 Ruby System (GE Healthcare) at 10 mA/gel for 1 h, 20 mA/gel for 1 h and 30 mA/gel using Precision Plus Protein Standards 10 e 250 kD (Bio-Rad). For each sample analyzed, five 2-D gels were made from five independent protein extractions. Gels were stained with Coomassie blue (1% Coomassie Blue G-250, 2% H3PO4, 8% (NH4) 2SO4, and 20% methanol) and stored in 20% ammonium sulfate at 4 ºC. The gels were scanned in ImageScanner® (GE Healthcare) and analyzed using ImageMaster 2D Platinum® software v. 7.0. Spot detection was performed with the parameters smooth, minimum area and saliency set to 4, 15 and 60, respectively. This was done automated by the software used, followed by manual spot editing, such as artificial spot deletion, spot merging and splitting. Individual protein spots were quantified using the percentage volume parameter (%Vol). Only spots that were reproducibly found in at least three biological replicates were considered to be present in a sample.

A comparative analysis of the proteome of explants inoculated in NCs induction medium and explants inoculated in PGR-free medium were performed: SeNCs vs. SeMS and LeNCs vs. LeMS. The proteins were accepted as having been differentially expressed between samples when they displayed a fold change of ±2 and were significant in Student's t-test at a significance level of 95%. The spots with %Vol >0.2 and differentially expressed in explants in induction medium were selected for further characterization using mass spectrometry.

Trypsin digestion and identification of proteins

Selected protein spots were manually excised from the gels, and in-gel digested by trypsin was achieved according to the protocol of

Westermeier and Naven (2002). Briefly, protein spots were destained with a solution of 50% acetonitrile and 25 mM ammonium bicarbonate, pH 8, for 1 h at room temperature. Subsequently, were dehydrated by adding 100% acetonitrile for 5 min and then drying in a Speedvac (ThermoSavant, Milford, USA) for 15 min. Gel plugs were re-hydrated in 10 µL of a solution containing 10 µg ml^{-1} of Trypsin (Promega, Madison, USA), prepared in 25 mM ammonium bicarbonate and digested overnight at 37°C. Peptides were extracted three times with a solution of 50% acenotrile and 5% trifluoroacetic acid (TFA), and then vortexed for 30 min. Samples were dried in SpeedVac 1 h at room temperature, and re-suspended in 2 µl 0.1% TFA. 1 µl of peptide solution was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid) solution, deposited on a 384-MPT AnchorChip plate (Bruker Daltonics), and air-dried at room temperature.

Mass spectra measurements were obtained with an Auto- flex/MS matrix-assisted laser desorption ionization time- of-flight mass spectrometer (MALDI-TOF/MS, Bruker Daltonics). External calibration was performed using the standard proteins Angiotensin II [M+H]+ mono 1046.5418, Angiotensin I [M+H]+ mono 1296.6848, Substance P [M+H]+ mono 1347.7354, Bombesin [M+H]+ mono 1619.8223 and ACTH clip (18–39) [M+H]+ mono 2465.1983. The data were submitted for identification using MASCOT search engine (Matrix Science, London; http://www.matrixscience.com) against SwissProt database. The parameters used for the acceptance of identification were: taxonomy restrictions to Viridiplantae, one missed cleavage, 100 ppm mass tolerance in MS, and 0.5 Da for MS/MS data, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. The confidence in the peptide mass fingerprinting matches ($p<0.05$) was based on the MOWSE score. Functional categorization and cellular component of proteins was performed according to the GO (Gene Ontology) by searching the protein knowledgebase (UniProtKB).

2.4 RESULTS AND DISCUSSION

2.4.1 Histological analysis

The SeMS germinated and formed normal seedlings after 21 days in culture (Figure 1a and 1b). However, in SeNCs, there was a proliferation of cell cultures of yellowish coloring (Fig. 1c), with the formation of protuberances with intense cell division (Fig. 1d); in

histological sections it was observed groups of meristematic cells (Fig. 1e). Similarly in the leaf segments, LeMS showed no proliferation of meristematic cells, at 21 days in culture (Fig.1 f-g). While in LeNCs it was observed the formation of protuberances (Fig. 1h). In histological sections it was observed intense cell proliferation, containing cells with meristematic characteristics, with dense cytoplasm and prominent nuclei, giving birth to the formation of NCs (Fig. 1i and 1j).

The PGR NAA and BAP altered the biochemical and molecular processes, leading to NCs induction in *V. reitzii*. The proliferation of meristematic cells during the induction of NCs from seeds and leaf bases in *Vriesea* bromeliads was previously described by Corredor-Prado et al. (2015), and such meristematic zones give rise to multiple shoots (Dal Vesco and Guerra, 2010; Dal Vesco et al., 2011). According to Alves et al. (2006), the NCs are associated with the development of meristematic tissues, with structural features similar to those seen in the somatic embryogenesis pathway.

2.4.2 Protein quantification and two-dimensional electrophoresis

Seeds normally show higher protein content than leaves, and this is related to the reserve compounds accumulated in the endosperm of the seeds of bromeliads (Magalhaes and Mariath, 2012; Corredor-Prado et al., 2014). Nevertheless both explant sources had a significant increase in the protein content when maintained in the NCs induction medium (Table 1). Two-dimensional electrophoresis enabled the correct separation of proteins according to their pI and molecular masses (Figure 2). In SeNCs, 183 spots were detected, and in SeMS, 174 spots. Likewise, in LeNCs 322 spots were detected and, in LeMS, 239 spots (Table 1). The significant increase in the protein content and the higher number of spots detected in explants in induction medium (SeNCs e LeNCs) indicates an increase in protein synthesis and cell metabolism associated with the high mitotic activity during cell proliferation (Fig. 1; Table 1). According to Fehér et al. (2003), the adaptation of plant cells to environmental conditions is usually accompanied by changes in the gene expression and reorganization of the metabolic pathways and physiological processes.

In the comparative analysis between the seeds (SeNCs vs. SeMS), explants that induced NCs presented 47 exclusive spots and 6 spots with a significant increase ($p<0.05$) in the level of expression. In the analysis between the leaf (LeNCs vs. LeMS), 110 spots were unique and 9 spots had a significantly increased expression ($p<0.05$) in explants that formed

NCs (Table 1). Accordingly, the induction of NCs involves a cellular reprogramming leading to alterations in the expression level of genes and proteomics.

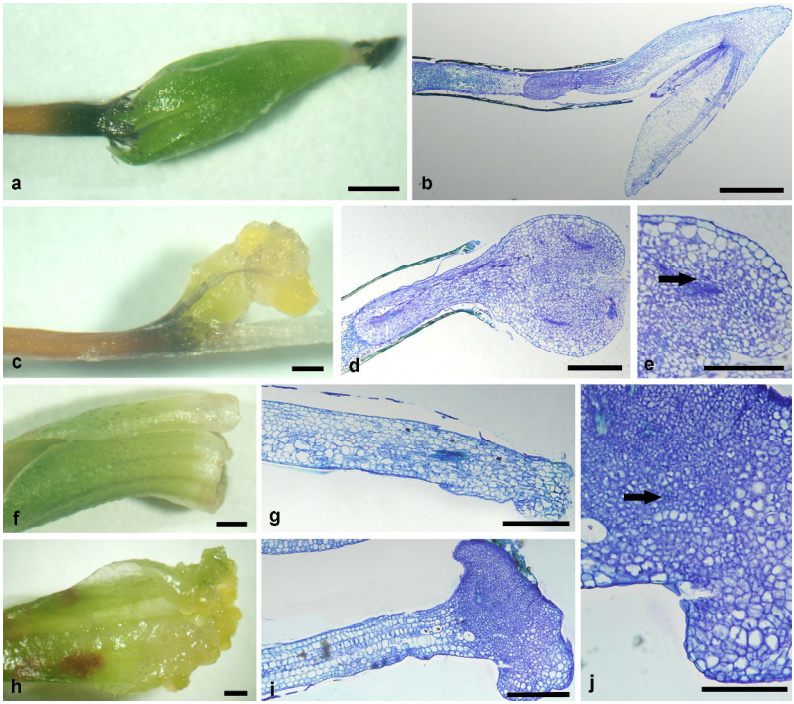

Figure 1 – *Vriesea reitzii in vitro* cultures after 21 days. Seeds: a) cultured in PGR-free MS medium; b) Section stained with TB-O; c) cultured in induction medium (MS with NAA 4 µM); d) Section stained with TB-O; e) detail of meristematic cells (arrow). Leaf segments: f) cultured in PGR-free MS medium; g) Section stained with TB-O; h) cultured in induction medium (MS with NAA 4 µM and BAP 2 µM); i) Section stained with TB-O; j) detail of meristematic cells (arrow). Bars: a-d, f-i: 500 µm; e, j: 250 µm

2.4.3 Identification of proteins

A total of 19 spots (Vol% >0.2) was collected and prepared for subsequent analyses in MALDI-TOF-TOF. Based on the significant score values ($p<0.05$) calculated by MASCOT, five spots were identified (26% of collected spots). Most spots analyzed showed low coverage of protein sequences, providing only non-statistically

significant scores. It is noteworthy that *V. reitzii* genome has not been sequenced and there are few sequences of the genome of other members of the Bromeliaceae family that are available in the databases. This contributes to the low percentage of identification in the proteomic analyses of this species.

Table 1 – Protein content (mg g^{-1} FM) and comparative 2-DE analysis during the induction of NCs from seeds and leaf segments of *Vriesea reitzii* cultured *in vitro*.

	Sample	Protein content (mg g^{-1} FM)[a]	No. of spots detected	No. exclusive spots[b]	No. differentially expressed spots[c]	No. collected spots[d]	Identified spots (Spot ID)
Seeds	NCs induction medium (SeNCs)	8.95 ± 0.43*	183	47	6	3	88; 221
	PGR-free MS medium (SeMS)	7.51 ± 0.16	174	38	1	-	-
Leaf segments	NCs induction medium (LeNCs)	5.55 ± 0.13*	322	110	9	16	101; 76; 331
	PGR-free MS medium (LeMS)	2.66 ± 0.20	239	27	11	-	-

[a] Values are means of five replicates ± standard deviation. * Significantly different by the Student's t-test at a significance level of 95%.
[b] Detected only in a type of sample.
[c] Spots were considered differentially expressed when they displayed a fold change of ±2 and were significant in Student's t-test at a significance level of 95%.
[d] Spots with %Vol >0.2 and differentially expressed in explants in induction medium.

Table 2 provides the functional categorization, access number to the *Swiss-Prot* database, plant organism from which it was identified, protein name, score value, coverage percentage, cellular localization, volume percentage and theoretical and experimental values of the molecular weight and *pI*. Some proteins showed deviations in the theoretical and experimental values of the *pI*, probably caused by unknown post-translational modifications. According to Balbuena et al. (2011), modifications such as phosphorylation, methylation, acetylation, and proteolytic cleavage, among others, make the prediction of final structure and function of proteins a challenge for the proteomic research.

Table 2 – Differentially expressed proteins during the induction of NCs from seeds and leaf segments of *Vriesea reitzii* after 21 days in induction medium.

Spot ID[a]	Functional categorization	Accesion number/ Species[b]	Protein name[b]	Mascot score (MS/ MS-MS)	% Coverage	Cellular component[c]	% Vol	Theoretical mass (kDa)/*pI*	Experimental massa (kDa)/*pI*
76	Cell división	RGP1_ORYSJ / *Oryza sativa Japonica Group*	UDP-arabinopyranose mutase 1	64/-	27	Golgi apparatus	0.33	41.8/5.8	37.3/6.0
88	Cell metabolism	DNJ50_ARATH / *Arabidopsis thaliana*	Chaperone protein dnaJ 50	60/-	13	Endoplasmic reticulum	0.48	35.1/8.5	50.8/9.1
101	Cell metabolism	METK3_SOLLC / *Solanum lycopersicum*	S-adenosylmethionine synthase 3	59/-	33	Cytoplasm	0.39	43.1/5.8	45.1/6.2
221	Stress	HS22M_SOLLC / *Solanum lycopersicum*	Heat shock 22 kDa protein, mitochondrial	/7	21	Mitochondrion	0.20	6.4/6.4	22/8.2
331	Unknown	1433E_TOBAC / *Nicotiana tabacum*	14-3-3-like protein E (*psi isoform*)	/73	6	Unknown	0.35	30.7/5.0	28.5/4.7

[a] Spot ID from gel (Fig. 2).
[b] According to the best hit of MASCOT search against *SwissProt* database
[c] According to the Gene Ontology (GO) database

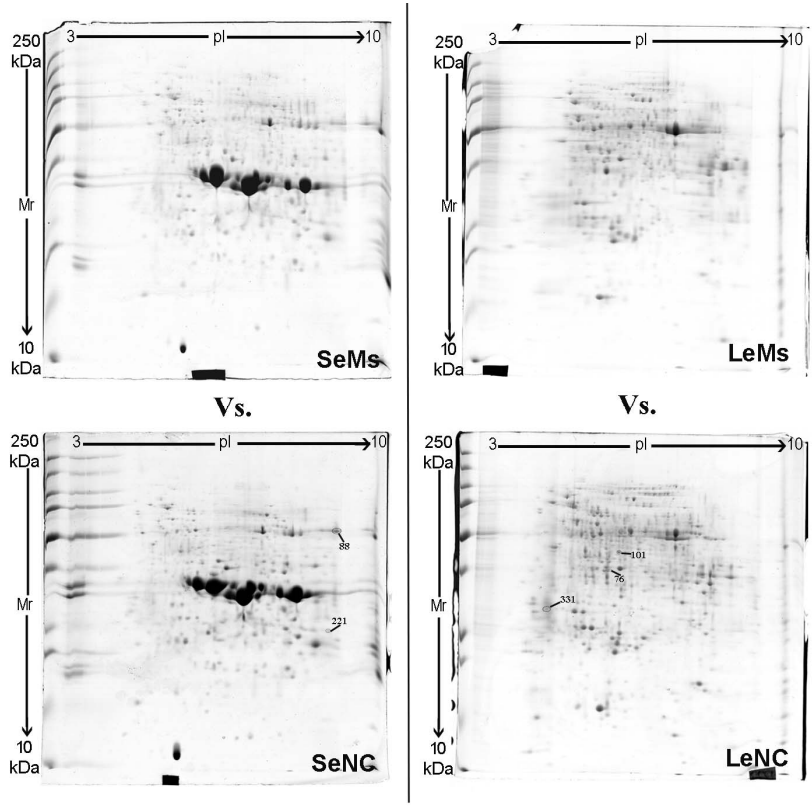

Figure 2 – Representative two-dimensional gel of the proteome of seeds (SeMS and SeNC) and leaf segments (LeMS and LeNC) of *Vriesea reitzii* after 21 days in cultive. Delimited spots correspond to differentially expressed proteins identified for mass spectrometry. Approximately 500 μg of proteins were resolved in IPG strips of pH 3–10 and acrylamide gels 12% stained with Colloidal Coomassie Blue.

The results showed that the morphogenetic route of NCs is accompanied by alterations in the expression of proteins involved in three functional classes: stress response, cell division and metabolism. During the induction of NCs from seeds, it were identified proteins related to protein metabolism (spot 88: Chaperone protein dnaJ 50) and stress (spot 221: Heat shock 22 kDa). From the leaf bases were identified proteins related to amino acid metabolism (spot 101: S-adenosylmethionine synthase 3), to cell division (spot 76: UDP-arabinopyranose mutase 1) and a protein with no function assigned in

the literature (spot 331: 14-3-3-like protein E) (Table 2). The identified proteins correspond to the numbered spots on the two dimensional gels of Figure 2.

Stress-related protein

The occurrence of different proteins related to stress during the morphogenic processes *in vitro* has been reported previously (Sharifi et al., 2012; Correia et al., 2012; Vale et al., 2014). It has been recognized that, stress plays an important role as interruptor of a morphogenic response (Fehér et al., 2003). Therefore, it is expected that stress-related proteins are actively synthesized during the induction of NCs. The protein "Heat shock 22 kDa protein, mitochondrial" (spot 221), involved in protection against oxidative stress (Banzet et al., 1998), was found in the seeds that formed NCs. This protein belongs to the family of the small heat shock proteins (sHSPs). Most of the sHSPs respond to a wide range of environmental stresses like heat, UV-B exposure, oxidative stress, and osmotic stress (Waters, 2013). Under stress conditions, sHSPs act as chaperones protecting proteins from denaturation and maintaining the protein homeostasis of the cells (Sarkar et al., 2009).

The induction of HSP proteins may confer tolerance to the stress phenomena encountered as problems in establishing plant tissue cultures (Cassells and Curry, 2001). Therefore, the fact that the protein "Heat shock 22 kDa protein, mitochondrial" (Spot 221) has been found only in the seeds that induced NCs indicates that it presents itself in response to the stress caused by the hormone present in the culture medium. The addition of auxin in the culture medium is an important factor for high rates of induction of NCs from seeds in bromeliads (Dal Vesco et al., 2014a; Corredor-Prado et al., 2015). Notwithstanding, the auxins can cause oxidative stress due to increased ROS (Reactive Oxygen Species), a process previously reported in *in vitro* conditions involving morphogenetic processes (Libik-Konieczny et al., 2012). It has been suggested that the induction of sHSP localized in the mitochondria is used for protection against oxidative stress (Banzet et al., 1998; Waters, 2013). Mitochondrial sHSPs containing a "Met-rich" domain may function as anti-oxidants, protecting electron transport through a mechanism that involves ROS scavenging by sulphoxidation of Met residues (Hamilton and Heckathorn, 2001). The sHSP found in the seeds that formed NCs may be acting this way, protecting cells against the oxidative stress generated by NAA present in the induction medium, and allowing the continuity of the NCs induction process. The oxidative

stress can improve a process driven by auxin leading to cell division and the formation of agglomerates of morphogenetic cells (Fortes et al., 2008). Other studies show that the HSPs were differentially regulated during morphogenesis *in vitro*, in response to auxins (Kitamiya et al., 2000; Sharifi et al., 2012).

Proteins related to cell metabolism

The induction of NCs involves an intense cell division, as observed in figure 1d and 1i. This demands a high level of energy that would explain the higher expression of proteins related to metabolism. Proteomic analyses revealed increases in the expression of proteins related mainly to the cellular metabolism during somatic embryogenesis (Marsoni et al., 2008; Correia et al., 2012; Vale et al., 2014), in the development of calluses (Tan et al., 2013) and in the organogenesis of shoots (Ghosh and Pal., 2013).

Cell reprogramming and differentiation require the synthesis, assembly and stabilization of proteins (Marsoni et al., 2008), therefore it is not unexpected to find increases in the expression of the "Chaperone protein dnaJ 50" (spot 88) during the induction of NCs. Located in the endoplasmic reticulum, this protein is involved in protein folding (Yamamoto et al., 2008). Over-expression of chaperone proteins plays an important role during early *in vitro* development (Marsoni et al., 2008; Correia et al., 2012; Ghosh and Pal., 2013; Tan et al., 2013). The heat shock proteins of the 70 kDa family (Hsp70) are chaperones that perform various essential cellular functions, including the translocation of proteins across membranes, the folding of newly synthesized proteins and refolding of misfolded and aggregated proteins (Bukau et al., 2006). DnaJ belongs to the family of J-protein molecular chaperones, and regulates the Hsp70s activity. The DnaJ binds to the substrate, and then interacts with Hsp70, stimulating the ATPase activity of Hsp70 and stabilizing the HSP70-substrate interaction (Kampinga et al., 2010; Walsh et al., 2004). The DnaJ also associates with unfolded polypeptide chains and prevents their aggregation (Han and Christen et al., 2004).

In the present work the enhanced expression of chaperone dnaJ 50 (spot 88) in seeds suggests that this protein is required for the initiation of NCs, possibly relieving the stress associated with cell reprogramming. The enhanced expression of this protein can be interpreted as a mechanism of metabolic shift, related to various processes in which the HSP70 are involved in response to development stimuli or changes in the environmental conditions.

In leaf bases that induced NCs, it was identified S-adenosylmethionine synthetase 3 (spot 101), related to amino acid metabolism. Fortes et al. (2008) reported an increased transcription of genes related to the S-adenosylmethionine cycle during the development of organogenic nodules in *Humulus lupulus* L. The S-adenosylmethionine synthetase catalyzes the synthesis of S-adenosylmethionine (SAM) from adenosine triphosphate (ATP) and methionine (Horikama et al., 1990), which is essential for cellular methylation, transcription and proliferation (Yoon et al., 2012). SAM is a major donor of methyl groups in the transmethylation of proteins, nucleic acids, polysaccharides and lipids (Tabor and Tabor, 1984). It acts as a precursor in the biosynthesis of the polyamines spermidine and spermine (Roje, 2006), which are implicated in various metabolic processes such as development, cell division, morphogenesis and response to various types of stress, including oxidative stress (Kaur-Sawhney et al., 2003; Rider et al., 2007). Guo et al (2014) found that, in Medicago sativa, S-adenosylmethionine synthetase overexpression promotes the synthesis and oxidation of polyamines, which improves the antioxidant protection induced by ROS. Therefore, the increased S-adenosylmethionine synthetase during the NCs induction process at the leaf base is probably involved in the protection against the oxidative stress through its contribution in the synthesis of polyamines, which would favor the response of the morphogenetic route of NCs.

Protein related to cell division

The cell cycle is dependent upon the synthesis of new proteins that cause morphological and biochemical changes associated with the mitotic activity (Dewitte and Murray, 2003; Silveira et al., 2004). It has been reported that high rates of cell division require proteins involved in the degradation, loosening or biosynthesis of the cell wall (Ghosh and Pal, 2013). Accordingly, leaf bases that induced NCs showed significantly increased expression of the UDP-arabinopyranose mutase 1 (spot 76), a protein related to the biogenesis of the cell wall (Konishi et al., 2007; 2010).

Konishi et al. (2007) demonstrated that the UDP-arabinopyranose mutase catalyzes the interconversion of UDP-arabinopyranose (UDP-Arap) and UDP-L-arabinofuranose (UDP-Araf) on *Oryza sativa*. UDP-Araf is a donor substrate for the biosynthesis of polysaccharides, proteoglycans and glycoproteins containing Arabinofuranosyl (Araf) (Konish et al., 2010), thereby, Araf residues are an important constituent

of plant primary and secondary cell walls (O'Neill and York, 2003). However, during the induction of NCs, it was observed proliferation of cells with meristematic characteristics. These cells types are surrounded by a semi-rigid and polysaccharide-rich matrix (0.1-1 μm thick), referred as the primary wall (Cosgrove, 1999).

Sugar biosynthesis and availability is critical to provide substrate for cell wall polysaccharide biosynthesis throughout plant growth and development (Rancour et al., 2015). In *Oryza sativa*, the low regulation of the expression of UDP-arabinopyranose mutase reduced the amounts of Araf in the cell walls, affecting their growth (Konishi et al., 2011). Similar results were found in Arabidopsis suggesting that Araf residues are required for normal plant growth and development (Rautengarten et al., 2011). Thus, increased expression of the UDP-arabinopyranose mutase protein indicates that the changes during the induction of NCs involve providing substrates for the synthesis of cell wall components that contribute in increasing the cell division rate during cell proliferation.

Protein related to other biological functions

The 14-3-3-like protein E (spot 331) identified in the leaf bases that induced NCs presents specific function still unknown in literature. According to Piotrowski and Oecking (1998) is a psi isoform of the 14-3-3 protein. The 14-3-3 proteins family is implicated in a number of essential physiological processes, in particular, metabolism, abiotic and biotic stress responses, as well as various aspects of plant growth and development (Deninson, 2011). Like stress, hormones also affect the levels of transcription and expression of the 14-3-3 genes (Yao et al., 2007; Kumar et al., 2015). Thus, the explants of leaf when cultivated in culture medium in the presence of the phytoregulators BAP and NAA, can be subjected to stressful conditions that would be causing the gene expression of 14-3-3.

The high degree of conservation between the different isoforms of 14-3-3 suggests that these proteins show an overlap of functions in a single organism (Aitken, 2011). In this sense, and considering that many aspects related to the functional mechanisms of 14-3-3 are still not completely elucidated (Obsil and Obsilova, 2011), possible role of 14-3-3-like protein E during the induction of NCs remains unclear.

2.5 CONCLUSION

To the best of our knowledge this is the first study using proteomic analysis to explain the possible molecular mechanisms associated to the process of nodular cultures induction in bromeliads. Different protein profiles observed in explants that induced nodular cultures allow us to suggest candidate proteins to act as markers of the induction process of this morphogenetic route, among them: heat shock 22 kDa, chaperone protein dnaJ 50, S-adenosylmethionine synthase 3 and UDP-arabinopyranose mutase 1.

Proteins identified in the induction phase of nodular cultures process are related to the regulation of the effects of stress conditions in the culture medium, the adjustment of the metabolism and the increase in cell division. This reveals the presence of tissues with high competence in the acquisition of the morphogenic response, culminating in the development of nodular cultures. Notwithstanding, the fact of having found proteins with low expression or no specific function involved in the induction process suggests the need for further studies using new and complementary proteomic approaches.

ACKNOWLEDGEMENTS

The authors thank Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC-PRONEX 2780/2012-4) for fellowships, research grants, and financial support. The authors also thank Centro de Biologia Molecular Estrutural da Universidade Federal de Santa Catarina (CEBIME/UFSC) where part of the work was developed.

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3 CAPÍTULO II. PROTEOMIC IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS DURING THE REGENERATION FROM NODULAR CLUSTER CULTURES IN *Vriesea reitzii* (BROMELIACEAE)

3.1 ABSTRACT

This study aimed to identify the proteins expressed differentially during the regeneration of shoots from nodular cluster cultures (NCs) of the bromeliad *Vriesea reitzii*. It was selected three development stages: NCs, microshoots and shoots. Proteomic analysis was performed by two-dimensional electrophoresis and by MALDI-TOF mass spectrometry. Images analyzed using ImageMaster 2D Platinum v. 7.0 software from NCs, microshoots and shoots resulted in detection of 345, 482 and 209 protein spots, respectively. 23 proteins differentially expressed through evaluated stages were identified. Proteins related to stress response (Catalase, Probable L-ascorbate peroxidase 3 and Heat shock protein 81-1) and metabolism of amino acids (Glutamine synthetase, 4-hydroxy-tetrahydrodipicolinate reductase 1, Serine hydroxymethyltransferase 1 and 14-3-3-like protein GF14-F) and proteins (Elongation factor Ts and Probable signal peptidase complex subunit 2) showed had higher expressions in the early stages of development. On the other hand, proteins related to carbohydrate metabolism (Ribulose bisphosphate carboxylase/oxygenase activase, Ribulose bisphosphate carboxylase and Phosphoglycerate kinase 1) and ATP synthesis (ATP synthase subunit alpha and ATP synthase subunit beta) had a higher expression in later stages. Changes in proteins related to cellular metabolic processes, the assembly of the photosynthetic mechanism, the response to the medium culture stressful and the dynamics of the cell cytoskeleton contribute to a high regenerative potential, which characterizes NCs. To our knowledge, this is the first proteomic analysis performed during the regeneration process from NCs in bromeliads. It may provide important insights into proteins and cellular events involved in the micropropagation of this group of plants.

Keywords: Bromeliad. Micropropagation. Protein expression analysis. Regeneration.

3.2 INTRODUCTION

Most applications in plant biotechnology are based on the potential of a single somatic cell to develop into an entire organism (totipotency). This complex morphogenetic process occurs under *in vitro* conditions, with an important role of extrinsic and intrinsic factors (Duclercq et al. 2011). Therefore, *in vitro* culture is a useful model system for the investigation of morphological, biochemical and molecular processes linked with early development of plants (Mitrović et al. 2012).

In vitro plant regeneration occurs via two major pathways: de novo organogenesis and somatic embryogenesis (Davey and Anthony 2010). However, in contrast to embryogenesis, organogenesis is more frequently used in biotechnology for the micropropagation of plants and genetic transformation (Zhang and Lemaux 2004; Duclercq et al. 2011). A large amount of information is available on the adjustment of tissue culture conditions in the induction of organogenic shoots. However, there are few works focusing the molecular aspects of this process.

Bromeliads, a group of tropical plants with an important ecological role due to biotic interactions, are also one of the bases of the ornamental industry worldwide (Guerra and Dal Vesco 2010). Protocols for their micropropagation have been described by a system defined as nodular cluster cultures (NCs), which have a high regenerative potential (Guerra and Dal Vesco 2010; Dal Vesco and Guerra 2010; Dal Vesco et al. 2011; Scherer et al. 2013; Dal Vesco et al. 2014a; Dal Vesco et al. 2014b). NCs are defined as organogenic nodule clusters, from green-yellowish to translucent, with a friable or slightly compact texture (Dal Vesco and Guerra 2010; Dal Vesco et al. 2011). The development of NCs leads to the formation of monopolar structures called microshoots, which further evolve to elongated shoots (Dal Vesco and Guerra 2010).

The culture medium supplemented with auxins such as NAA, IAA combined with cytokinins, such as BAP and 2ip, has been used to optimize the induction and proliferation of NCs (Rech Filho et al. 2005; Alves et al. 2006; Dal Vesco and Guerra 2010; Dal Vesco et al. 2011; Scherer et al. 2013; Dal Vesco et al. 2014a; Dal Vesco et al. 2014b; Corredor-Prado et al. 2015). The NCs of the bromeliad *Vriesea reitzii* originated in medium supplemented with 2 μM NAA plus 4 μM BAP had on average 140 microshoots.g^{-1} of NCs after 13 weeks in culture medium without growth regulators (Dal Vesco et al. 2014b). When NCs were subcultured in medium with IAA (4 μM), they had a higher average number of microshoots (1478 microshoots.g^{-1} of NCs) after 9

weeks of cultivation (Dal Vesco et al. 2014a). However, Dal Vesco and Guerra (2010) estimated a production of more than 5000 microshoots.g^{-1} of NCs, which can be expected after 10 weeks of culture in medium supplemented with NAA and 2ip (2 μM each). Subsequently, the inoculation in a growth culture free of growth regulators results in the elongation of microshoots (Alves et al. 2006; Rech Filho et al. 2009; Dal Vesco and Guerra 2010; Dal Vesco et al. 2014b), while a medium supplemented with 10 μM promotes their synchronic development (Dal Vesco and Guerra 2010). Because of this high regenerative capacity of NCs, Scherer et al. (2013) established a protocol based on NCs of Ananas comosus associated with temporary immersion systems for a large-scale micropropagation with low production costs.

The growing interest in plant proteomics results in a great number of developmental studies on plant cell division, elongation, differentiation and formation of various organs (Takáč et al. 2011). Thus, these analyses have greatly improved our understanding of metabolic pathways and specific regulatory mechanisms of tissues in plants (Ahsan and Komatsu 2009). Although the mechanisms related to the multiplication of the NCs and its microshoot differentiation have been analyzed regarding the dynamics of global methylation levels of DNA (Scherer et al. 2015) and polyamine content (Scherer et al. 2013), there is yet no knowledge about the changes in proteome levels during the regeneration process. According to Takáč et al. (2011), molecular, genetic and biochemical methods, complemented by proteomic approaches, bring novel systemic and functional information on plant development.

Proteomic studies during *in vitro* organogenesis or the formation of shoots have been conducted with other plant groups. In rice, as a model plant in monocots, proteins mainly related with carbohydrate metabolism and stress/defense are mostly analyzed. They have significantly different expressions from the start of differentiation until the regeneration of shoots (Yin et al. 2007). Ghosh and Pal (2013) reported an increase in proteins associated with the folding process during the organogenesis of shoots in Vigna radiata (Fabaceae), inferring that these proteins mediate cell reprogramming and contribute to a fast regeneration. Palama et al. (2010), found significant expressions of proteins involved in the metabolism of amino acids, energetic metabolism pathways and photosynthetic activity during the early shoot differentiation from callus of *Vanilla planifolia* (Orchidaceae). Considering that proteomic techniques facilitate the understanding of protein qualitative and quantitative changes in different

development stages (Ghosh and Pal 2013), this study aimed at to identify the proteins expressed differentially during the regeneration of shoots of *V. reitzii* NCs, by evaluating three different stages of development.

3.3 MATERIAL AND METHODS

3.3.1 Plant materials

NCs were originated from the leave basal region in basal culture medium (BMS) composed of MS basal salts (Murashige and Skoog 1962), plus Morel vitamins (Morel and Wetmore 1951) and 30 g l^{-1} sucrose. The BMS was supplemented with 4 µM NAA and 2 µM BAP (Dal Vesco et al. 2014b). NCs were maintained in successive subcultures into glass flasks, each containing 20 ml of BMS supplemented with 2 µM of NAA plus 2-iP (Dal Vesco and Guerra 2010; Dal Vesco et al. 2014a). Microshoots derived were subcultured in glass flasks containing 18 ml of PGR-free BMS to shoots formation. Cultures were maintained in a growth room at 25 ± 2°C and 16 h photoperiod, with a light intensity of 50–60 µmol m^{-2} s^{-1}. Were collected three stages of development: NCs, microshoots of 4 ± 1 mm in height and shoots 20 ± 5 mm in height (Fig. 1a-c).

3.3.2 Histological analysis

The material was fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) sodium phosphate buffer overnight. The samples were dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were infiltrated with Historesin (Leica® Historesin, Heidelberg, Germany). Sections (5 µm) were obtained using a manual rotation microtome (Slee Technik®) and were stained with Toluidine Blue O (TB-O) 0.5% aqueous solution, pH 3.0. Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

3.3.3 Proteomic analysis

Protein extraction

Five replicates (500 mg per each replicate) of each stages of development were collected and fast-frozen in liquid nitrogen. The

extraction of proteins was performed following the method of Carpentier et al. (2005) with modifications. In brief, tissue collected was ground to powder with the aid of liquid nitrogen. The macerated material was homogenized with 5.0 ml of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 5.0 ml of buffer-saturated phenol (pH 8,0) by vortexing for 30 min. The homogenates were centrifuged for 30 min at 10,000 g, 4 ºC. The phenolic phase was recovered and homogenized with 5.0 ml of extraction buffer by vortexing for 30 min. The homogenate was centrifuged for 30 min at 10,000 g, 4 ºC. The phenol phase was collected. After precipitation with 100mM of ammonium acetate in methanol (1:5 v/v), proteins were maintained 12h at -20°C. The tube was centrifuged for 30 min at 10,000 g at 4 ºC. The pellet was washed three times with 1.0 ml of pure methanol and three times with 1.0 ml of acetone. Finally, the proteins were solubilized in 0.3 ml of solubilization buffer (7M urea, 2M thiourea, 3% CHAPS, 2% IPG-buffer, 1,5% DTT), by mild vortexing and stored at -20°C. Protein concentration was estimated by 2-D Quant Kit® (GEHealthcare).

Two-dimensional gel electrophoresis (2DE)

Isoelectric focusing was carried out in strips of 13 cm, pH 3-10 at linear scale (GE Healthcare). The strips were rehydrated in 250 µl of rehydration buffer (7M Urea, 2M Thiourea, 3% CHAPS, 2% IPG-buffer, 0.002% Bromophenol blue, and 0,2% DTT) containing 500 mg of protein for 12-14 h at room temperature. The strips were focused on an Ettan-IPGphor 3 isoelectric focusing unit (GE Healthcare). After, strips were equilibrated for 15 min in equilibrium solution (50 mM Tris-HCl pH 8.8, 6M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS, and 0.002% (w/v) Bromophenol Blue) containing 1% (w/v) DTT, followed by 15 min in equilibration buffer containing 2.5% (w/v) Iodoacetamide. Second dimension SDS-PAGE gels were run in 12% (w/v) acrylamide gels using the Hoefer SE 600 Ruby System (GE Healthcare) at 10 mA/gel for 1 h, 20 mA/gel for 1 h and 30 mA/gel using Precision Plus Protein Standards 10 e 250 kD (Bio-Rad). For each sample analyzed, five 2-D gels were made from five independent protein extractions. Gels were stained with Coomassie blue (1% Coomassie Blue G-250, 2% H3PO4, 8% (NH4) 2SO4, and 20% methanol) and stored in 20% ammonium sulfate at 4 ºC. The gels were scanned in ImageScanner® (GE Healthcare) and analyzed using ImageMaster 2D Platinum® software v. 7.0. Individual protein spots were quantified using the

percentage volume parameter (%Vol). Only spots that were reproducibly found in at least three biological replicates were considered to be present in a sample.

A comparative analysis of the proteome of the three stages of development was performed. The proteins were accepted as having been differentially expressed between samples when they displayed a fold change of ±2 and were significant in Student's t-test at a significance level of 95%. The spots with %Vol >0.2 and differentially expressed were selected for further characterization using mass spectrometry.

Trypsin digestion and identification of proteins

Selected protein spots were manually excised from the gels, and in-gel digested by trypsin was achieved according to the protocol of Westermeier and Naven (2002). Briefly, protein spots were destained with a solution of 50% acetonitrile and 25 mM ammonium bicarbonate, pH 8, for 1 h at room temperature. Subsequently, were dehydrated by adding 100% acetonitrile for 5 min and then drying in a Speedvac (ThermoSavant, Milford, USA) for 15 min. Gel plugs were re-hydrated in 10 µl of a solution containing 10 µg ml-1 of Trypsin (Promega, Madison, USA), prepared in 25 mM ammonium bicarbonate and digested overnight at 37°C. Peptides were extracted three times with a solution of 50% acenotrile and 5% trifluoroacetic acid (TFA), and then vortexed for 30 min. Samples were dried in SpeedVac 1 h at room temperature, and re-suspended in 2 µl 0.1% TFA. 1 µl of peptide solution was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid) solution, deposited on a 384-MPT AnchorChip plate (Bruker Daltonics), and air-dried at room temperature.

Mass spectra measurements were obtained with an Auto- flex/MS matrix-assisted laser desorption ionization time- of-flight mass spectrometer (MALDI-TOF, Bruker Daltonics). External calibration was performed using the standard proteins Angiotensin II [M+H]+ mono 1046.5418, Angiotensin I [M+H]+ mono 1296.6848, Substance P [M+H]+ mono 1347.7354, Bombesin [M+H]+ mono 1619.8223 and ACTH clip (18–39) [M+H]+ mono 2465.1983. The data were submitted for identification using MASCOT search engine (Matrix Science, London; http://www.matrixscience.com) against SwissProt database. The parameters used for the acceptance of identification were: taxonomy restrictions to Viridiplantae, one missed cleavage, 100 ppm mass tolerance in MS, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification.

Positive identification was based on the Mascot score ($p<0.05$), and the additional experimental confirmation of observed molecular mass (Mr) and isoelectric focusing point (pI) of each protein. Gene ontology analysis for individual proteins was performed by searching the protein knowledge base (UniProtKB) in order to reveal the cellular component, molecular function, and the associated biological processes.

3.4 RESULTS

3.4.1 Histological analysis

Histological sections of NCs allowed observing nodules and meristematic zones composed of isodiametrical cells with a dense cytoplasm and a voluminous nucleus (Fig. 1d). In microshoots, apical meristem and leaf primordia were observed. It was possible to identify trichomes and vascularized tissues composed of elongated cells (Fig. 1e). Successive cell divisions and differentiation led to the formation of the laminar structure. Young leaves of shoots have larger cells and a greater number of trichomes, which already reached a mature state (Fig. 1f).

3.4.2 Two-dimensional gel electrophoresis

The proteomic maps of the developmental stages, revealing that 345, 482 and 209 protein spots were detected for NC, microshoots and shoots, respectively (Fig. 2- Fig. 3). The shoot stage had the lowest diversity of protein spots and more than 160 spots were common in the three studied stages. All 2-DE profiles had a similar distribution pattern of spots and the microshoots had the highest number of spots common with other stages, indicating that it is a transitional stage from NCs to shoots (Fig. 3). In the comparative analysis between NCs and microshoots, 47 spots were differentially expressed ($p<0.05$). In the analysis between microshoots and shoots, there were 39 spots and between shoots and NCs there were 34 spots differentially expressed.

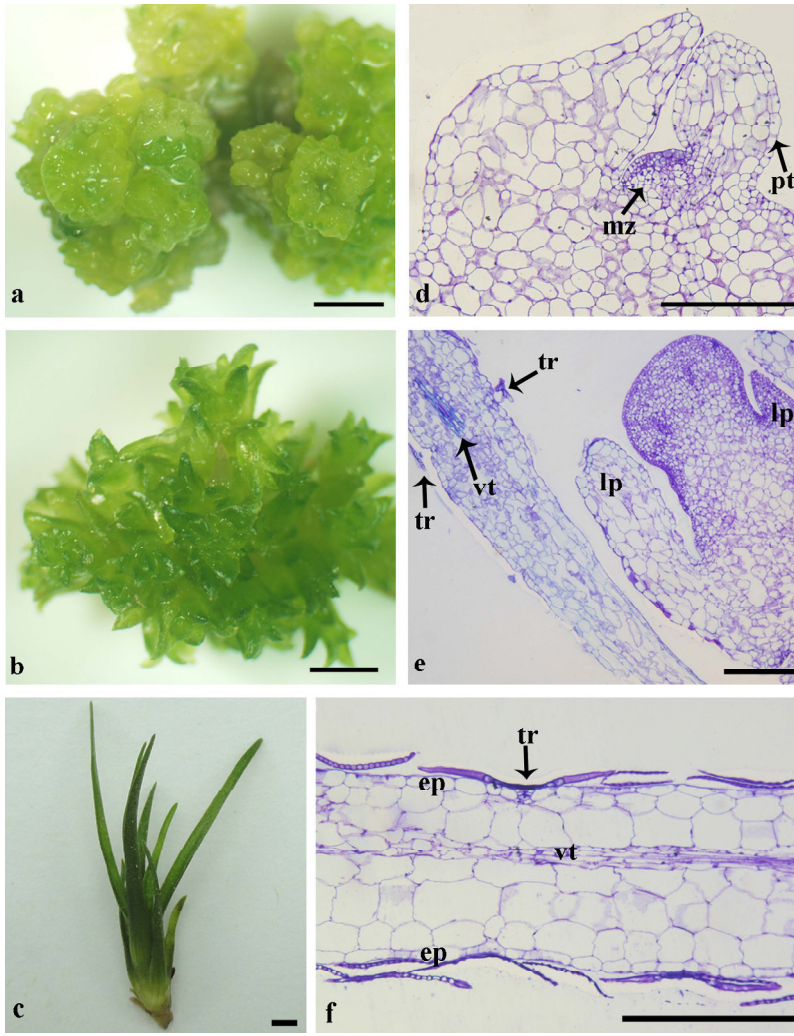


Figure 1 – Developmental stages during the regeneration from nodular cluster cultures in *Vriesea reitzii*. a) nodular cluster cultures; b) microshoots; c) shoots. Sections subjected to TB-O. d) nodular cluster cultures; e) microshoots; f) shoots. Abbreviations: ep: epidermis; lp: leave primordia; mz: meristematic zone; pt: protoderm; tr: trichome; vt: vascular tissue. Bars: a-c 2 mm; d-f 200 µm.

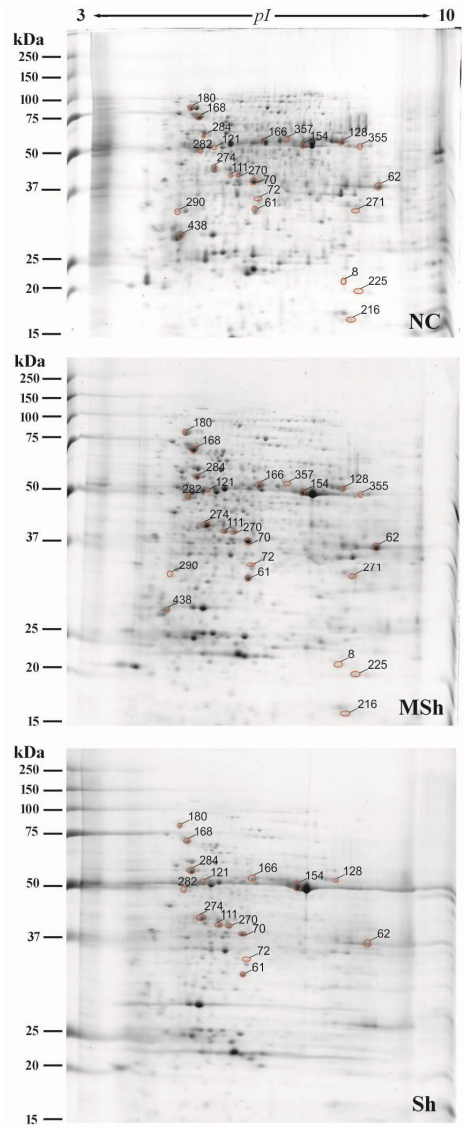


Figure 2 – Two-dimensional electrophoresis profiles in different developmental stages *in vitro* of *Vriesea reitzii*. a) nodular cluster cultures (NCs); b) microshoots (MSh); c) shoots (Sh). The gels shown were stained with Coomassie blue. Numbered spots, correspond to identified proteins using MALDI-TOF. The molecular weight of protein standard is indicated on the left of each panel.

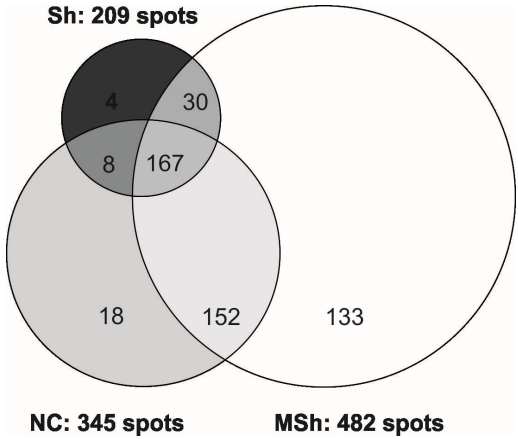


Figure 3 – Distribution of all protein spots detected by Image Master 2D Platinum (GE Healthcare) during the regeneration from nodular cluster cultures in *Vriesea reitzii*. Number of exclusive and common spots to the different developmental stages: nodular cluster cultures (NCs), microshoots (MSh) and shoots (Sh).

3.4.3 Protein identification

More than 50 protein spots were collected for mass spectrometry analyses, obtaining an identification rate of approximately 45%. 23 proteins were identified, which, according to GO annotations, are involved in broad variety of regulatory events, including biological processes, molecular functions and cellular components. The biological process associated with a great proportion of identified proteins was cellular metabolism followed by stress response. Moreover, most proteins are located in the cytoplasm and the chloroplast, and present mainly a binding or catalytic activity molecular function. Due to the lack of information on GO annotations in the UniProtKB database, three proteins were cataloged in the unknown associated biological processes category and two proteins were cataloged with an unknown molecular function (Fig. 4).

Table 1 shows protein spot number, access number to the Swiss-Prot database and plant species from which was identified, protein name, score value, matched peptides number, percentage of coverage, theoretical and experimental values of molecular weight and isoelectric point (pl), and graphs representative of the average volume percentage (%Vol.) for each evaluated stage of development. The identified proteins correspond to the spots numbered on two-dimensional gels of

Fig. 2. Most were identified due to sequence similarity with the species *Oryza sativa*, followed by *Arabidopsis thaliana*. However, most were identified with monocots species of the taxonomic Order Poales. During the regeneration process, through all three stages evaluated, the identified proteins showed differential changes in the level of expression.

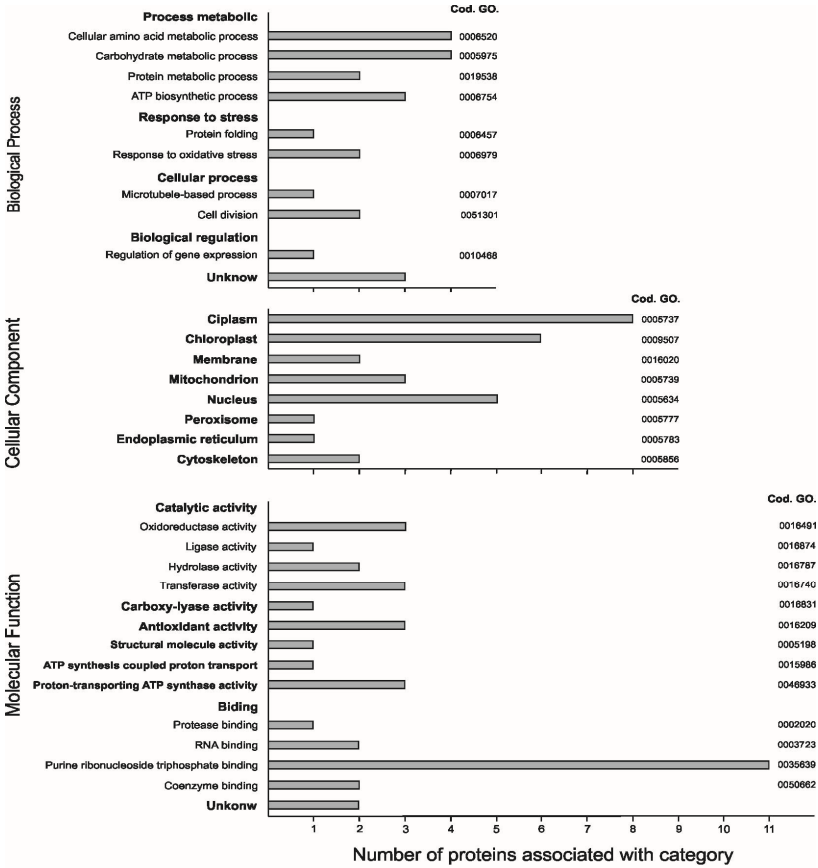


Figure 4 – Number of proteins identified associated with different GO categories during the regeneration from nodular cluster cultures in *Vriesea reitzii*.

Proteins related to stress (Catalase, Probable L-ascorbate peroxidase 3 and Heat shock protein 81-1) and metabolism of amino acids (Glutamine synthetase, 4-hydroxy-tetrahydrodipicolinate reductase

1, Serine hydroxymethyltransferase 1 and 14-3-3-like protein GF14-F) and proteins (Elongation factor Ts and Probable signal peptidase complex subunit 2) showed higher expressions in the early stages of development (NCs and microshoots), while other proteins, such as those related to carbohydrate metabolism (Ribulose bisphosphate carboxylase/oxygenase activase, Ribulose bisphosphate carboxylase and Phosphoglycerate kinase 1) and ATP synthesis (ATP synthase subunit alpha and ATP synthase subunit beta) showed a higher expression in stage of shoot (Table 1).

3.5 DISCUSSION

The regeneration of NCs is a key process for the micropropagation of bromeliads. Cell proliferation in the meristematic zones of NCs provides the further development and elongation of multiple adventitious shoots. In fact, previous studies reported the presence of meristematic regions composed of isodiametrical cells (Alves et al. 2006; Dal Vesco and Guerra 2010; Dal Vesco et al. 2011; Scherer et al. 2013) and the formation of nodules with protoderm (Dal Vesco and Guerra 2010). During the induction of NCs from seed and leaf bases, the proliferation of these meristematic cells was observed during the second week after the inoculation in the medium (Corredor-Prado et al. 2015). Microshoots cross sections of *Ananas comosus*, originated from NCs in temporary immersion systems, showed similar general features to those observed in this work, specifically leaf primordia cells with large vacuoles and trichomes (Scherer et al. 2013).

In this study, proteomic analyses were performed in order to elucidate the molecular mechanism of the efficient regenerative potential of NCs in the protein level. 23 differentially expressed proteins were identified during the regeneration of shoots. Some protein spots could not be identified due to the unavailability of specific protein sequences in the databases included in MASCOT. This is common especially with organisms with incomplete genomic information (Ghosh and Pal 2013). Indeed, the dependency on a sequenced genome or cDNA library may often be restrictive in the scope of studies, particularly for non-model organisms (Gupta et al. 2015). The identified proteins are discussed below based on their classification regarding the associated biological process (Fig. 4; Table 1).

3.5.1 Proteins related to cell metabolism

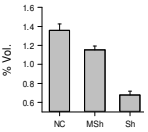
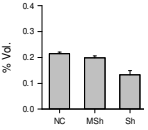
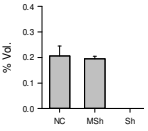
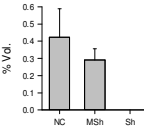
Cell division for the development of shoot apical meristems requires energy, which is supplied by an increase in the rate of metabolism within the cell (Ghosh and Pal 2013). Accordingly, most proteins identified during the regeneration from NCs were related to the cellular metabolism in order to contribute to the process of division and differentiation, resulting in the development of shoots.

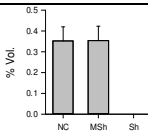
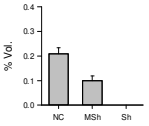
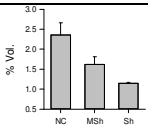
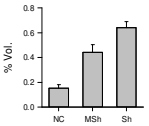
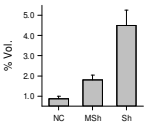
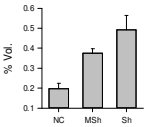
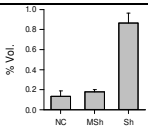
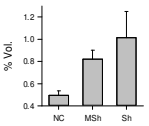
Proteins involved in the metabolism of amino acids (spots 70, 72, 355 and 438) had the highest expression in the NCs stage decreasing subsequently. Glutamine synthase is known to play a central role in the metabolism of nitrogen, increasing the synthesis of amino acids necessary for protein synthesis (Miflin and Habash 2002). It aids in the assimilation of ammonia generated by numerous plant processes and produces glutamine from glutamate for transport purposes (Teixeira et al. 2006). Ghsoh and Pal (2013) found several isoforms of glutamine synthase with differential expressions during the differentiation of shoots on cotyledons explants of *Vigna radiata*. The authors consider that this provides a larger pool of transportable amino acids during the differentiation process. On the other hand, the "serine hydroxymethyl transferase" (spot 355) catalyzes the reversible interconversion of serine and glycine, a central pathway of the metabolism of amino acids and the largest source of carbon groups for a variety of pathways (Matthews et al. 1998). The expression of this protein was highly increased during the somatic embryogenesis of *Cyclamen persicum* (Rode et al. 2011).

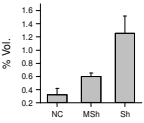
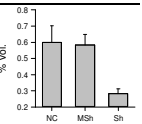
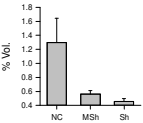
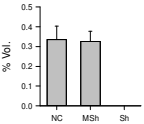
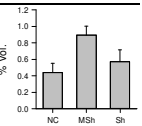
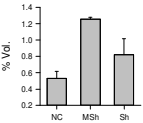
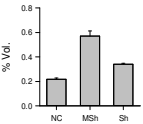
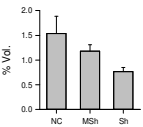
According to Bian et al. (2010), physiological and metabolic changes during cellular reprogramming require the assembly and stabilization of newly synthesized proteins, as well as the modification and removal of peptides. The "Probable signal peptidase complex" (spot 216) and "Elongation factor" in translation (spot 290), involved in the metabolism of proteins, also had the highest expression in the NCs and the microshoot stage. They were not detected in the shoot stage. The higher expression of these proteins in the early stages suggests that protein synthesis is more active at this stage of development. This is related to an increase in metabolic rate and cell proliferation and differentiation. According to Sharifi et al. (2012), the synthesis of new proteins and the removal of old and unnecessary proteins are a prerequisite for the establishment of a new cell phenotype. Proteins involved in the metabolism of amino acids and proteins were significantly regulated in the early stages of differentiation of shoots from calluses in *Vanilla planifolia* (Palama et al. 2010).

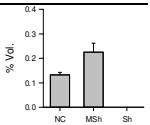
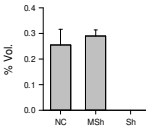
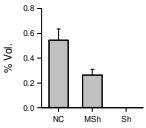
Sucrose is the source of carbon and energy for the growth of plant tissue in culture medium (Ovono et al. 2009). Accordingly, the greater abundance of Glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis (spot 62) allows a greater availability of carbohydrates in the NCs stage, suggesting an increase in cellular respiration. This enzyme is often involved in organogenesis *in vitro* (Fortes et al. 2008). Ghosh and Pal (2013) found an increase in enzymes related to glycolysis, which was associated with the fast growth and organization of meristematic tissues that subsequently formed shoots.

Table 1 – Differentially expressed proteins during the regeneration from nodular cluster cultures in *Vriesea reitzii.*

Spot ID[a]	Accesion number/ Species[b]	Protein name[b]	score	Mat. pept.[c]	% Coverage	Theo./Exp.[d] Mass (kDa) *pI*	% Vol[e]
Metabolic process-amino acids							
70	GLNA1_MEDSA/ *Medicago sativa*	Glutamine synthetase cytosolic isozyme	63	6	19	39.3/37.6 5.5/6.2	
72	DAPB1_ARATH/ *Arabidopsis thaliana*	4-hydroxy-tetrahydrodipicolinate reductase 1, chloroplastic	58	6	25	37.8/34 6.0/6.3	
355	GLYM1_ARATH/ *Arabidopsis thaliana*	Serine hydroxymethyltransferase 1, mitochondrial	58	12	20	57.5/49.8 8.1/8.4	
438	14336_ORYSJ/ *Oryza sativa Japonica Group*	14-3-3-like protein GF14-F	96	10	27	29.3/28 4.8/4.7	

Metabolic process-proteins							
216	SPCS2_ARATH/ *Arabidopsis thaliana*	Probable signal peptidase complex subunit 2	58	4	25	21.7/18 9.0/8.2	
290	EFTS_ORYSI/ *Oryza sativa Indica Group*	Elongation factor Ts, mitochondrial	54	7	21	42.6/32.5 9.1/4.7	
Metabolic process-carbohydrates							
62	G3PC3_ORYSJ/ *Oryza sativa Japonica Group*	Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic	63	7	18	36.7/36.4 7.6/8.8	
111	RCA_ORYSJ/ *Oryza sativa Japonica Group*	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	122	1	3	51.8/40.1 5.4/5.8	
154	RBL_NEUTE/ *Neurachne tenuifolia*	Ribulose bisphosphate carboxylase large chain	149	18	39	53.5/49.8 6.2/7.3	
270	PGKH1_ARATH/ *Arabidopsis thaliana*	Phosphoglycerate kinase 1, chloroplastic	65	6	19	50.2/40.2 5.9/5.9	
Metabolic process-ATP							
121	ATPB_COCNU/ *Cocos nucifera*	ATP synthase subunit beta, chloroplastic	83	10	26	53.8/50.5 5.3/5.5	
166	ATPAM_MAIZE/ *Zea mays*	ATP synthase subunit alpha, mitochondrial	136	19	41	55.4/56.2 5.9/6.4	

284	ATPA_WHEAT/ *Triticum aestivum*	ATP synthase subunit alpha, chloroplastic	82	11	19	55.3/57.5 6.1/5.4	
Response to stress							
128	CATA_SECCE/ *Secale cereale*	Catalase	72	10	22	57.1/52.5 6.7/8.1	
180	HSP81_ORYSI/ *Oryza sativa Indica Group*	Heat shock protein 81-1	82	15	18	80.4/81.4 5.0/5.0	
271	APX3_ORYSJ/ *Oryza sativa Japonica Group*	Probable L-ascorbate peroxidase 3	55	7	32	32.2/32.8 8.3/8.3	
Cellular process							
61	GIG1_ARATH/ *Arabidopsis thaliana*	Protein GIGAS CELL1	58	7	32	27.1/32 10.3/6.4	
274	ACT2_ORYSI/ *Oryza sativa Indica Group*	Actin-2	67	9	30	41.9/41 5.3/5.4	
282	TBA1_ORYSJ/ *Oryza sativa Japonica Group*	Tubulin alpha-1 chain	73	12	32	50.3/48.2 4.9/5.1	
Biological regulation							
168	MD37E_ARATH/ *Arabidopsis thaliana*	Probable mediator of RNA polymerase II transcription subunit 37e	81	12	27	71.7/70.5 5.0/5.2	

Unknown							
8	MGN_ORYSJ/ *Oryza sativa Japonica Group*	Protein mago nashi homolog	58	5	38	18.5/21.2 5.8/7.9	
225	RUB3_ORYSJ/ *Oryza sativa Japonica Group*	Ubiquitin-like protein-NEDD8-like protein RUB3	51	5	24	17.3/20.5 6.3/8.2	
357	CDPKN_ARATH/ *Arabidopsis thaliana*	Calcium-dependent protein kinase 23	53	8	20	59.1/54 6.1/6.8	

[a] Spot ID from gel (Fig. 2).
[b] According to the best hit of MASCOT search against *SwissProt* database
[c] Mat. pept.: Matched peptides
[d] Theo: Theoretical; Exp: Experimental
[e] %Vol: percent volumes, in nodular cluster cultures (NCs), microshoots (MSh) and shoots (Sh)

Proteins involved in the metabolism of carbohydrates that showed an increased expression in the last stage (shoot) are enzymes involved in the Calvin cycle (Ribulose bisphosphatecarboxylase/oxygenase activase-spot 111; Ribulose bisphosphatecarboxylase-spot 154; Phosphoglycerate kinase-spot 270). Despite the availability of sucrose in the culture medium, proteins related to photosynthesis have been differentially expressed in *in vitro* tissues (Yin et al. 2007; Fortes et al. 2008; Yin et al. 2008; Duby et al. 2010; Palama et al. 2010) found an increase of glycolysis-related proteins in rice calluses. However, when they differentiated into early shoots, such proteins decreased and an increase in proteins related to the Calvin cycle was observed. Previous studies have related early organogenesis of *in vitro* shoots to the establishment of a photosynthetic system as another source of energy (Yin et al. 2007; Palama et al. 2010). *In vivo* studies show that proteins related to photosynthesis were less abundant in the earlier stages of leaves and slowly accumulated during its development (Ahsan and Komatsu 2009).

The activity of RuBisCO is functionally regulated by RuBisCO activase (Portis et al. 2008). According to Komatsu et al. (2003), RuBisCO Activase is a receiver in the GA signal transduction pathway and may have a role the regulation of leaf sheath elongation in rice. This could be related to the fact that this protein (spot 111) significantly increased its expression until the shoot stage. Proteomic and transcriptomic analyses also revealed increases of this protein in early shoots emerging from rice calluses (Yin et al. 2007).

Proteins related to the synthesis of ATP (spot 166, 284 and 121) showed an increase in expression during the shoot stage. This may be related with the increase of the carbon source from sucrose of the culture medium and from a photosynthetic system that allowed a more active energetic metabolism with a greater production of ATP. According to Rolletschek et al. (2003), during the embryogenesis process the embryos are limited regarding ATP. However, during the differentiation of the photosynthetic apparatus, expression of glycolytic enzymes and TCA, the levels of ATP increase. The lower expression of ATP synthase in the early stages of development (NCs and microshoots) of *V. reitzii* could also be related to the development status of cellular organelles. A low accumulation of ATP synthase in the early stages of biogenesis of corn plastids has also been demonstrated by a proteomic approach (Lonosky et al. 2004). Several ATP synthase proteins were highly expressed in tissues with regenerative capacity via embryogenesis (Lyngved et al. 2008; Sghaier-Hammami et al. 2009).

3.5.2 Proteins related to stress

Proteins related to stress also participated in the changes in proteomic profiles during the formation of shoots of *V. reitzii*. Previous studies have shown that reactive oxygen species (ROS) and their scavenging enzymes participate in processes related to the development, differentiation and growth of plant cells during *in vitro* morphogenesis (Libik-Konieczny et al. 2012; Mitrović et al. 2012). Miltrovic et al. (2012) examined the correlation between the activities of antioxidant enzymes during the *in vitro* organogenesis of shoots of Tacitus bellus, finding an increase in catalase and peroxidase activity in early organogenic stages. According to Sujatha et al. (2000), the peaks of peroxidase activity are associated with the formation of meristematic centers during the regeneration of shoots in *Jatropha integerrima*.

Other proteomic studies have shown that enzymes related to oxidative stress, including ascorbate peroxidase, are linked to an

increased cell division activity (Holmes et al. 2006; Takáč et al. 2011). Accordingly, the present study found a greater expression of catalase (spot 128) and probable L-ascorbate peroxidase (spot 271) in NCs and microshoot stages, and a decrease in the shoot stage. According to Vatankhah et al. (2010), a high catalase and peroxidase activity can be correlated with the differentiation process during organogenesis, and the decrease in its activity is observed in shoots formed later.

The Heat shock protein 81-1 (spot 180), also involved in mediating stress, was differentially expressed during the formation of shoots. It acts as a molecular chaperone involved in the processing of proteins and it was identified in all regeneration stages, with a higher intensity in the NCs stage. According to Holmes et al. (2006), meristematic tissues accumulate proteins involved in stress mediation induced by ROS such as the Heat shock protein. Accordingly, proteomic studies have shown that the heat shock protein plays an important role during the early plant development *in vitro* (Yin et al. 2007; Guzmán-García et al. 2013; Tan et al. 2013; Silva et al. 2014).

The increase in the expression of proteins related to stress (spots 128, 271 and 180) during the early stages of development evaluated in the present study indicates its correlation with the differentiation process and cell proliferation, as well as cellular protection against the deleterious effects of the quantity of ROS to continue the regenerative process *in vitro*.

3.5.3 Proteins related to other cellular processes

The protein GIGAS CELL1 (spot 61) had the highest expression in the microshoot stage. This protein is mainly expressed in rapidly dividing tissues such as shoot apical meristems and young leaves. It is required for a proper progression of cell division and the determination of cell destination (Iwata et al. 2011). Furthermore, proteins involved in cytoskeletal dynamics are also related to cell division and cell elongation (Takáč et al. 2011). Cell expansion is determined primarily by actin and microtubules (Dong et al. 2001; Chan et al. 2007), which allows the cytoskeletal to play a central role in cell growth control and its spatial regulation (Smith and Oppenheimer 2005). This has reflected on proteomic studies (Zhao et al. 2010). Accordingly, in this study, the greatest expression of actin (spot 274) and tubulin (spot 282) found in microshoots and shoots compared to NCs probably contributes to the cell elongation and differentiation observed in anatomical sections in these stages (Fig. 1). There is evidence of the involvement of tubulins

and actin in the regulation of plant cell morphogenesis (Sampathkumar et al. 2011; Sambade et al. 2014). This type of protein has been shown to be differentially regulated during the somatic embryogenesis of Citrus sinensis (Pan et al. 2009) and organogenesis of shoots of *Vigna radiata* (Ghosh and Pal 2013). Differently, the spot 168 had a significantly decreased expression only at the last stage of development evaluated, while in NCs and microshoots it was stable. The spot 168 was identified as "Probable mediator of RNA polymerase II" and would be related to the regulation of gene expression in the establishment of shoots.

3.5.4 Proteins related to unknown biological processes

The proteins included in the unknown associated biological processes category were Protein mago nashi homolog (spot 8), Ubiquitin-like protein-NEDD8-like protein RUB3 (spot 225) and Calcium-dependent protein kinase 23 (spot 357), which are expressed during the NCs and microshoots stages, and were not detected in the shoot stage. Although biological processes related to these proteins are unknown, from their expression patterns we speculated that they might be relative to the differentiation of shoots.

3.6 CONCLUSION

Cell reprogramming associated with morphogenesis is achieved through changes in protein expression during developmental processes. To our knowledge, this is the first work using two-dimensional electrophoresis in combination with mass spectrometry to evaluate the proteomic changes during shoot regeneration from NCs in bromeliads. The ability to alter metabolic pathways, to allow assembling the photosynthetic mechanism, to control the stress of the medium and to change the dynamics of the cytoskeleton in the cell appear to be a key component for a successful NCs regeneration process.

The identification of the level of expression of proteins in the different developmental stages evaluated will help to better understand the biochemical and molecular processes underlying the formation of shoots from NCs of *V. reitzii*. The results here described deepen on the molecular basis of this *in vitro* morphogenetic route.

ACKNOWLEDGEMENTS

The authors thank Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC-PRONEX 2780/2012-4) for fellowships, research grants, and financial support. The authors also thank Centro de Biologia Molecular Estrutural da Universidade Federal de Santa Catarina (CEBIME/UFSC) where part of the work was developed.

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4 CAPÍTULO III. DYNAMIC OF PROTEINS, CARBOHYDRATES AND GLOBAL DNA METHYLATION PATTERNS DURING INDUCTION OF NODULAR CLUSTER CULTURES FROM SEEDS IN *Vriesea reitzii* (BROMELIACEAE)

4.1 ABSTRACT

Tissue culture techniques have been employed for bromeliad mass propagation by means of the morphogenetic route of nodular cultures (NCs). This study aimed to assess protein, carbohydrate and global DNA methylation (GDM) levels dynamics during NCs induction from *Vriesea reitzii* seeds. Seeds were inoculated into Murashige and Skoog (MS) liquid medium supplemented with 4 µM α-naphthaleneacetic acid (NAA) to induce NCs, and in culture medium without growth regulators to form normal seedlings. Samples collected at 0, 3, 7, 10, 14 and 21 days of culture were analysed. All parameters assessed showed the same variation pattern. However, seeds inducing NCs showed significantly lower starch (6.0 mg g^{-1} FM) carbohydrate (10.7 mg g^{-1} FM) and GDM (11.0%) levels as compared to seeds forming normal seedlings, after 21 days in culture. On the other hand, protein content (9.1 mg g^{-1} FM) was significantly higher during induction. NCs induction process through seeds is the result of gene reprogramming in the explant, which leads to morphological, biochemical and metabolic alterations. This involves dedifferentiation, high cell proliferation, high energy demand and protein synthesis, which is related to elevated metabolic activity.

Keywords: Bromeliad. Germination. Micropropagation. Seedling.

4.2 INTRODUCTION

Bromeliads are complex ecological systems, which contribute to the maintenance of the forest ecosystems stability due to their high levels of specialization (Benzing 2000). In addition to their ecological role, bromeliads have a high landscape value, which is recognized by the ornamental industry worldwide (Guerra and Dal Vesco 2010; Negrelle et al. 2012). Among the bromeliads, *Vriesea reitzii* is an epiphytic plant that occurs in southern Brazil at altitudes ranging from 750 – 1,200 m (Leme and Costa 1991). As observed in most of bromeliads, the beautiful shape and colors of *Vriesea reitzii* enables its ornamental use (Negrelle et al. 2012). In recent decades, the

development of tissue culture techniques has promoted new strategies for mass propagation bromeliads (Guerra and Dal Vesco 2010). Previous work on bromeliad micropropagation reported *in vitro* morphogenetic pathway based on nodular cluster cultures (NCs). NCs are defined as groups or conglomerates of organogenic nodules with high regenerative efficiency (Dal Vesco and Guerra 2010; Dal Vesco et al. 2011). Strategies for induction of NCs in bromeliads include nodal segments (Dal Vesco et al. 2011), leaf segments (Alves et al. 2006; Dal Vesco and Guerra 2010; Scherer et al. 2013; Dal Vesco et al. 2014b) and seeds (Dal Vesco et al. 2014a; Corredor-Prado et al. 2015). These works have established a system of NCs induction and regeneration of shoots in response to different types and combinations of plant growth regulators (PGR). Other studies regarding NCs multiplication and differentiation to microshoots are related to GDM levels (Scherer et al. 2015) and genetic analysis using amplified fragment length polymorphism (AFLP) markers (Dal Vesco et al. 2012; Scherer et al. 2015). On the other hand, characterization of the NCs induction process has been focused on morphological and anatomical alterations (Corredor-Prado et al. 2015). Biochemical and epigenetic alterations have not been evaluated.

The development of morphogenetic pathways is accompanied by changes in cellular components such as proteins and polysaccharides (Martin et al. 2000; Cangahuala-Inocente et al. 2014; Morel et al. 2014). These changes require the expression of genes necessary to synthesis or mobilization of those compounds. Therefore, epigenetic mechanisms play an important role in development plant (Valledor et al. 2007). One of the epigenetic changes is methylation of cytosine in DNA, which refers to post-synthesis methylation of deoxycytosines at the 5' position of the pyrimidine ring of cytosine, forming methyldeoxycytosine, which interferes with gene translation and expression (Finnegan 2010). Previous findings have shown that methylation in coding or regulatory regions prevents the expression of the target genes while demethylation events are accompanied with gene activation (Meng et al. 2012; Shan et al. 2013). Furthermore, the action of sugars as signalling molecules affects the expression of genes and the activity of enzymes in many metabolic pathways (Halford 2010).

Considering that biochemical and epigenetic changes must be accomplished in order to induce NCs, the present work aimed to evaluate the dynamic changes in proteins, carbohydrates, and global DNA methylation (GDM) levels from seeds during NCs induction.

4.3 MATERIAL AND METHODS

4.3.1 Plant materials

Seeds from mature fruits of *V. reitzii* were collected from plants kept in Curitibanos, altitude 990 m (Santa Catarina State, Brazil 27º16'58"S - 50º35'04"W). Sterilization was carried out according to the procedures described by Alves et al. (2006). For NCs induction, seeds were inoculated into Murashige and Skoog (MS) liquid medium supplemented with 4 μM NAA, previously established by Dal Vesco et al. (2014a) (hereinafter termed MS-NAA). Seeds inoculated in PGR-free MS medium (hereinafter termed MS) were considered as control. All explants were inoculated over filter paper bridges, into test tubes (22 mm × 150 mm) containing 12 ml of culture medium. Cultures were maintained in a growth room at 25 ± 2 °C and 16 h photoperiod, with a light intensity of 50–60 μmol m^{-2} s^{-1}. Samples were collected at 0, 3, 7, 10, 14 and 21 days of culture.

4.3.2 Protein content

Each replicate (500 mg) was prepared from approximately either 350 seeds. The extraction of proteins was performed following the method of Carpentier et al. (2005) with modifications. In brief, tissue collected was ground to powder with the aid of liquid nitrogen. The macerated material was homogenized with 5.0 ml of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 5.0 ml of buffer-saturated phenol (pH 8,0) by vortexing for 30 min. The homogenates were centrifuged for 30 min at 10,000 g, 4 ºC. The phenolic phase was recovered, homogenized with 5.0 ml of extraction buffer and vortexed and centrifuged again. The phenol phase was collected and precipitated with 100mM of ammonium acetate in methanol (1:5 v/v) during 12h at -20 °C. After centrifugation, the pellet was washed with pure methanol and acetone. The proteins were solubilized in 0.3 ml of solubilization buffer (7M urea, 2M thiourea, 3 % CHAPS, 2 % IPG-buffer, 1,5 % DTT). Protein quantification was determined by means of the copper-based method 2-D Quant Kit® (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The data were submitted to analysis de varianza (ANOVA) and presented as means of three biological replicates.

4.3.3 Total soluble carbohydrates

Samples of 500 mg were ground to powder with the aid of liquid nitrogen and subsequently submitted to an 80% ethanol extraction at 70 °C for 5 min. The extracts were centrifuged at 3,000rpm, at 20 °C, for 10 min and filtered through fiberglass. The extraction was repeated three times and the final volume adjusted to 5 ml with ethanol (80%). The total soluble carbohydrates content were determined using phenol-sulfuric method (Dubois et al. 1956), using glucose as standard. The absorbance was measured at 490 nm. The data were submitted to ANOVA and presented as means of three biological replicates.

4.3.4 Thin Layer Chromatography

Analysis for the qualitative detection of carbohydrates was carried out on the extracts described previously. Chromatographic conditions: stationary phase: Silica Gel 60 TLC aluminum plate (Alugram®); mobile phase: 2-propanol:ethyl acetate:nitroethane:acetic acid:water (45:25:10:1:19); detection reagent: orcinol-sulfuric acid-ethanol reagent; volume of sample: 2 µl. Rf values were calculated for samples and compared with standard (fructose, glucose, xylose, maltose and sucrose).

4.3.5 Starch content

The pellets used in the total soluble carbohydrates extraction received the addition of 1 ml of cold distilled water and 1.3 ml of 52 % perchloric acid and was maintained in an ice bath with occasional agitation. Subsequently, 2.0 ml of water was added, and the material was centrifuged at 3,000 rpm for 15 min. The extraction was repeated and the final volume adjusted to 10 ml with distilled water. The starch content was estimated by the phenol-sulfuric method (Dubois et al. 1956), using glucose as a standard, according to the method proposed by McCready et al. (1950). The absorbance was measured at 490 nm. The data were submitted to ANOVA and presented as means of three biological replicates.

4.3.6 Global DNA Methylation (GDM)

DNA isolation was based on CTAB methodology (Doyle and Doyle 1990). Digestion procedures, purification, and analysis by high

performance liquid chromatography (HPLC) were based on the method described by Fraga and contributors (2012). Samples containing nucleosides, including 5-methyl-2'-deoxycytidine (5mdC) and deoxycytidine (dC), were filtered (0.22 µm pore size) and injected into HPLC. A Hyperclone 5 µm C18 column (250 x 4.6 mm) (Phenomenex®, Torrance, CA, USA), a pre-column (4.0 x 3.0 mm) (Phenomenex®), and UV detector at 280 nm were used. Two eluents were used: A (0.5 % v/v methanol in 10 mM KH2PO4 buffer, pH adjusted to 3.7 with H3PO4) and B (10 %v/v methanol in 10 mM KH2PO4 buffer, pH adjusted to 3.7 with H3PO4). The column was eluted with 100 % eluent A for 3 min, followed by a linear gradient of 0–100 % eluent B over 17 min and then with 100 % eluent B for 5 min. Nucleoside separation was performed at flow rate of 1 ml min-1, with 20 µl of each sample injected. Deoxynucleotides, dNTPs (Fermentas®, Vilnius, Lithuania), were digested for 2 h with alkaline phosphatase (10 U ml-1) and Tris–HCl (0.5 M, pH 8.3) to form the nucleoside standards (dA, dT, dC, 5mdC, and dG) (5–50 mM). The identification of each nucleoside was made by comparison with the external standards according to the peak area formed. 5mdC quantification (%) was calculated according to 5mdC concentration divided by 5mdC concentration plus dC concentration and multiplied by 100. The data were submitted to ANOVA and presented as means of three biological replicates.

4.3.7 Histological analysis

Samples were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) sodium phosphate buffer overnight. The samples were then dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were infiltrated with Historesin (Leica® Historesin, Heidelberg, Germany). Sections (5 µm) were obtained using a manual rotation microtome (Slee Technik®) and were stained with Periodic Acid-Schiff (PAS) to identify neutral polysaccharides and Coomassie Brilliant Blue (CBB) 0.4% in Clarke's solution to identify proteins. Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

4.4 RESULTS AND DISCUSSION

Vriesea reitzii bromeliad seeds germinated in the 3th day of *in vitro* culture. Subsequently, seeds on MS culture medium resulted in the

development of normal seedlings (Figure 1a-c), while seeds inoculated on MS-NAA supplemented culture medium showed enhanced cell division and proliferation of cells with meristematic characteristics (Figure 1d-f). In this context, NAA in the culture medium altered the seedling development for NCs induction. Other studies have reported the effect of auxins combined with cytokinins, which also caused NCs induction from *Vriesea* seeds, but in lower percentages (Dal Vesco et al. 2014a; Corredor-Prado et al. 2015). In morphogenetic routes, such as somatic embryogenesis, several steps including dedifferentiation and somatic cells reprogramming, are necessary for the initiation and progression of development programs (Zhang and Ogas 2009). In this respect, auxins are important signaling molecules related to division, elongation and differentiation regulation in plants (Perrot-Rechenmann 2010). In the present study, carbohydrate, starch, protein and GDM levels showed significant differences between *V. reitzii* seeds on MS and those cultured on MS-NAA medium.

4.4.1 Protein and starch content

Gradual decrease in protein and starch levels was observed until the last measured time (Figure 2a-b). Previous studies have shown that *Vriesea* genus seeds store starch and protein in the endosperm (Magalhaes and Mariath 2012; Corredor-Prado et al. 2014). According to Bewley (1997), reserves stored in seeds are predominantly mobilized during and after radicle protrusion. Hydrolyzed amino acids release large ammonium amounts during protein degradation, which are assimilated again, synthesizing new nitrogenous molecules (Cantón et al. 2005), while starch degradation is related to triglyceride release and subsequent carbohydrate metabolism (Stone and Gifford 1999).

In the present work, dry seeds (0 days of culture) with the highest protein and starch content (35.4 and 17.8 mg g^{-1} FM, respectively) showed marked decrease in 3th day of culture, when seeds germinated. In barley seeds, Sreenivasulu et al. (2008) demonstrated that genes involved in reserves catabolism are expressed much earlier during seed germination, i.e. already 24 hours after imbibition. According to the authors, reserve mobilization activation is one of the first events that occur before radicle protrusion.

Cultures on MS-NAA medium that developed NCs had higher protein (9.1 mg g^{-1} FM) and lower starch (6.0 mg g^{-1} FM) levels as compared to seedlings after 21 days in culture (Figure 2a-b). Silveira et

al. (2004) also related protein levels increase with high mitotic activity during exponential growth stages in *in vitro* cultures of *Pinus taeda*.

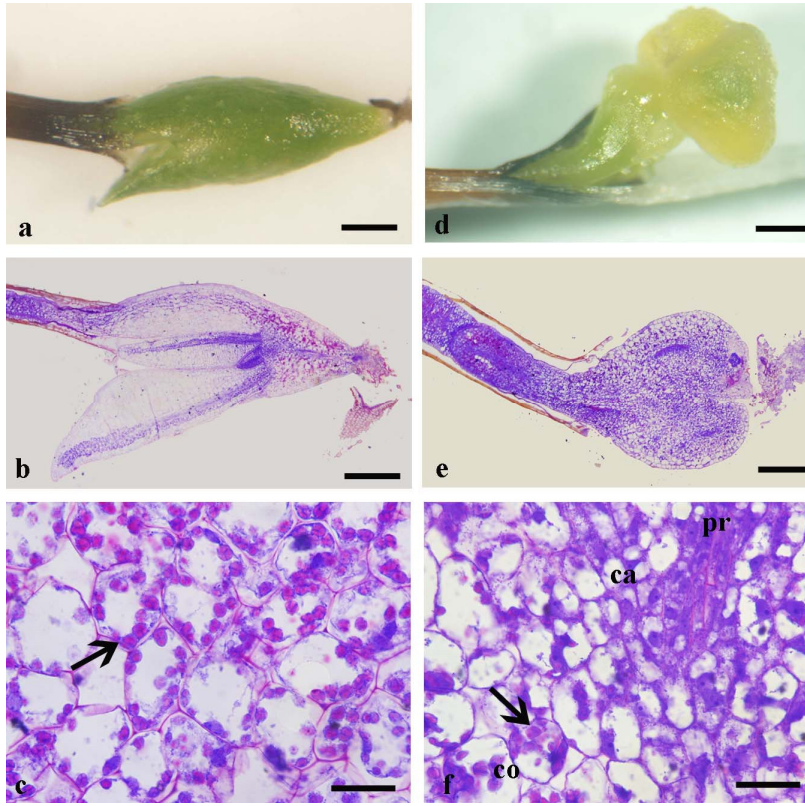

Figure 1 – *Vriesea reitzii* seeds after 21 days of *in vitro* culture. Seeds on MS medium (a-c) and NCs induction medium supplemented with NAA 4 µM (d-f). Section stained with PAS-CBB (b,c,e,f). Starch grains (arrow); cambial tissue (ca); cortex (co) provascular tissue (pr). Bars a,b,d,e 500 µm; c,f 250 µm

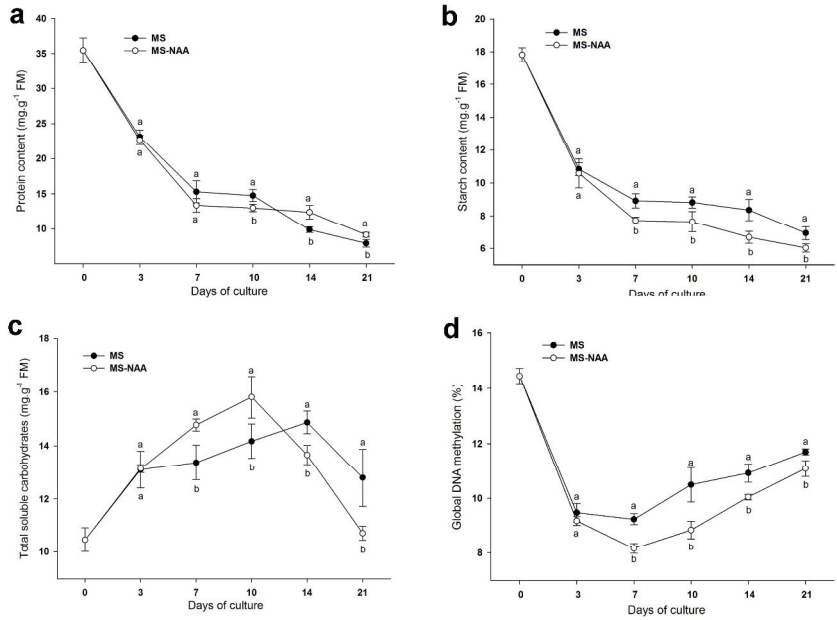

Figure 2 – Content of proteins (a), starch (b), carbohydrates (c) and global DNA methylation (f) in *Vriesea reitzii* seeds cultivated on NCs induction medium (MS-NAA) and PGR-free MS medium (MS). Data are means ± standard errors.

In histological sections with double staining (PAS-CBB), starch granules were observed in lower amount in NCs-inducing seeds (Figure 1f) than in seedlings (Figure 1c). This event may be related to the increased metabolism of sugars, during the induction of NCs, generating a higher consumption of starch. Figure 1f, shows cells with starch, close to others with high mitotic activity, forming a cambial and provascular tissue. Thus, cortex with starch and cambial tissue surrounding the bundle would be the beginning of the NCs. Starch presence in plant cells has been reported during early *in vitro* development, acting as an energy source or as an osmotic agent that is essential for development (Martin et al. 2000). It has been suggested that starch accumulation might be a prerequisite of morphogenesis. For instance, in tobacco, starch acts as a direct cellular reserve of the energy, that it is rapidly converted to hexoses for the development of meristenoids (Thorpe et al. 2008).

4.4.2 Total soluble carbohydrates

Quantification of the carbohydrates (mono- and disaccharides), reveled the lowest value (10.4 mg g^{-1} FM) for dry seed (0 days of culture). Carbohydrates reached the highest level at the 10th day of culture in seeds inducing NCs (15.8 mg g^{-1} FM) and in seeds that formed seedlings (14.2 mg g^{-1} FM) (Figure 2c). Through thin layer chromatography qualitative method, glucose monosaccharide (Rf: 0.58) and sucrose disaccharide (Rf: 0.50) were identified in seeds on MS and MS-NAA medium (Figure 3, Table 1). Fructose, xylose and maltose were not identified using this technique. However, at later stages of rice seed germination, Howell et al. (2009) detected several carbohydrate types, with increases primarily in fructose, glucose, and maltose. Before radicle protrusion in germination of barley seeds, activation of transcription factors for mobilization of starch reserves supplies sucrose and hexoses, which provide energy for development (Sreenivasulu et al. 2008).

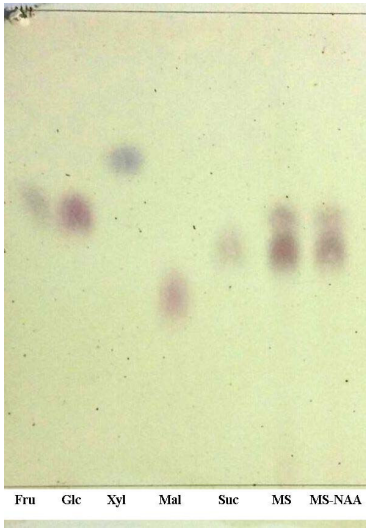

Figure 3 – Thin Layer Chromatogram of *Vriesea reitzii* seeds cultivated 21 days in PGR-free medium (MS) and in NCs induction medium (MS-NAA) against standard (fructose-Fru, glucose-Glc, xylose-Xyl, maltose-Mal and sucrose-Suc).

Table 1 – Retention factor (Rf) of detected spots in thin layer chromatography (TLC) from *Vriesea reitzii* seeds. Standard used: fructose (Fru), glucose (Glc), xylose (Xyl), maltose (Mal) and sucrose (Suc). PGR-free medium (MS) and in NCs induction medium (MS-NAA).

Days of culture	Retention factor (Rf) of detected spots			
	MS		MS-NAA	
	Glc (0.58)	Suc (0.50)	Glc (0.58)	Suc (0.50)
0	0.57	-	0.57	-
3	0.57	0.49	0.58	0.51
7	0.57	0.50	0.57	0.50
10	0.58	0.49	0.58	0.50
14	0.57	0.50	0.58	0.51
21	0.57	0.49	0.57	0.51

Besides having the role of substrates for cell component synthesis, sugars also act as signaling molecules in the expression regulation of various genes (Marsch-Martínez and Pereira 2010). There is a strong hexose sugar increase in seeds after imbibition, followed by large changes in gene transcription (Fait et al. 2006; Howell et al. 2009). From this, significant difference in carbohydrate content in 10th day of culture on MS-NAA and MS medium, could be related to gene expression programming variation for NCs induction or for seedling development. According to Weber et al. (1997), sugars may interfere with cell cycle regulation by changing morphogenetic responses.

In the last day assessed (21 days of culture), carbohydrates content decreased in *V. reitzii* seeds. Significant differences between seeds on MS and MS-NAA medium were found (Figure 2c). Probably, the presence of structures with different differentiation levels, cause different energy levels necessary for support their metabolism. Lower values found in seeds that induced NCs (Figure 2c), indicating that this morphogenetic route has high energy demand. These results suggest that glycolytic intermediates formed by starch degradation are mainly catabolized to provide the necessary ATP to the metabolism during the cell proliferation that leads to NCs induction. On the other hand, higher carbohydrate content in seeds on MS medium allowed higher storage in starch (Figure 1c, 2b). In *Aechmea blanchetiana* bromeliad seedlings cultured *in vitro* with different auxin concentrations, Chu et al. (2010) found the highest starch and soluble carbohydrate content in plants that

were in the auxin-free medium. These results support the relation of auxin effect in carbohydrate metabolism.

4.4.3 Global DNA Methylation (GDM)

GDM levels decreased during germination until the 7th day of culture, reaching 9.2% in seeds on MS medium and 8.1% in seeds on MS-NAA medium (Figure 2d). Our results corroborate those previously reported, in which methylation level was higher in dry seeds than germinating seeds in *Capsicum annuum* L. (Portis et al. 2004), Brassica napus (Lu et al. 2006), *Triticum aestivum* L. (Meng et al. 2012) and *Phelipanche ramosa* L. (Lechat et al. 2015). DNA hypomethylation during seed germination seems to be a necessary step for transcription activation on gene expression, contributing to development gene regulation (Lu et al. 2006). During wheat seeds germination, Meng et al. (2012) found that the number of demethylation events was three times higher than methylation events, indicating demethylation predominance. According to Zluvova et al. (2001), seed global hypomethylation during Silene latifolia germination and post-germination periods reflects transition from the metabolically quiescent seed to the actively growing and developing seedling.

In the last days evaluated (10, 14, and 21 days of culture), methylation level increase was observed, reaching up to 11.7% in seeds that formed seedlings and 11.0% in seeds that induced NCs (Figure 2d). Quercus Suber L. somatic embryos germination coincides with significant 5- MDC content decrease. However, when somatic embryos developed in seedling with roots and initial leaves, strong DNA methylation increase was observed (15%) (Pérez et al. 2015). Generally, differentiation coincides with increased DNA methylation, which is associated with normal ontogenic development (for review see Valledor et al. 2007).

Results in the last days also show significant GDM differences between seeds on MS and MS-NAA medium (Figure 2d). Methylation level in seeds on MS medium could be associated with the differentiation process during normal seedling development. Therefore, the significantly lower level found in seeds that induced NCs is associated with tissue dedifferentiation. It has been reported during *in vitro* morphogenetic route induction, significant GDM decreases related to cell dedifferentiation and undifferentiated state cell maintenance (Fraga et al. 2016). According to Valledor et al. (2007), demethylation normally precedes the onset of new differentiation programmes.

However, even if GDM remains lower in seeds inducing NCs (associated with cellular dedifferentiation), the percentage keeps rising in the last days evaluated, which may be related to three factors, as follows: 1) some cells could be continuing the differentiation process, causing GDM level increases; 2) high cell proliferation, which is associated with high methylation levels (Wang et al. 2012); and 3) new meristematic zone formation in explants, which is in accordance with Scherer et al. (2015), who observed that high methylation levels were associated with new apical meristems formation in NCs of pineapple. Neelakandan and Wang (2012) conducted studies involving Arabidopsis thaliana epigenetic mutant characterization, and observed that WUSCHEL (WUS) gene expression and signaling derived from auxin, which are crucial for meristem formation, are regulated, in part, by DNA methylation.

It is possible to relate methylation status with specific *in vitro* morphogenetic competence (Noceda et al. 2009). Accordingly, during somatic embryogenesis, embryogenic cell generally display lower GDM levels than non-embryogenic cell (Miguel and Marum 2011). Similarly, lower GDM levels has been correlated with organogenic capability increase (Valledor et al. 2010). The results of this study suggest that low GDM levels are related to explant capacity for NCs induction. This indicates that NAA supplemented in induction medium, result in alterations of gene expression. According to Zhang et al. (2012) there is a functional interplay between environmentally induced epigenetic modifications, response to PGRs and phenotypic plasticity. Auxin used in PGR-supplemented treatment, have been frequently linked to compromise genomic stability through the promotion of DNA methylation deregulation coupled with gene expression modifications in embryogenic cultures of *Daucus carota* (LoSchiavo et al. 1989) and *Araucaria angustifolia* (Fraga et al. 2016).

4.5 CONCLUSION

The findings of this study suggest that NCs induction from *V. reitzii* seeds using the culture medium supplemented with NAA results in explant gene reprogramming, which leads to morphological, biochemical and metabolic changes. NCs induction is a complex process in which epigenetic control (related to GDM levels decrease), seems to play an important role to initiate morphogenic response. Concomitantly, dedifferentiation followed by cell proliferation, high energy demand

given by starch degradation present in the tissues, and higher protein content is related to elevated metabolic activity.

However, more detailed studies should be conducted to determine specific relations between the different signaling networks that regulate changes associated with the acquisition of skills to develop this morphogenic route.

ACKNOWLEDGEMENTS

The authors thank Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC-PRONEX 2780/2012-4) for fellowships, research grants, and financial support.

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5 CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

O presente estudo contribuiu para a ampliação dos conhecimentos sobre o desenvolvimento da rota morfogenética das CNs em bromélias, até então pouco compreendida. As abordagens proteômicas, bioquímicas e epigenéticas, foram fundamentais para descrever os processos de indução e regeneração das CNs na bromélia *V. reitzii*.

De modo geral, os resultados do capítulo 1, indicam que explantes em meio de indução apresentam conteúdo de proteína e número de proteínas expressas maior, quando comparados aos explantes em meio controle (sem regulador). Proteínas envolvidas nos processos de resposta ao estresse, metabolismo e divisão celular apresentam significativas alterações na sua expressão durante a indução das CNs. O capítulo 2 apresenta proteínas identificadas durante a regeneração de brotos, as quais aquelas relacionadas à resposta ao estresse e ao metabolismo de aminoácidos e proteínas, apresentaram maiores expressões nos estádios de CNs e microbroto, e as proteínas relacionadas com o metabolismo de carboidratos e síntese de ATP têm maior expressão no estádio de broto. Por fim, as avaliações bioquímicas e epigenéticas no capítulo 3, indicaram que sementes após 21 dias de indução de CNs apresentaram uma diminuição significativa no conteúdo de amido, carboidratos e no nível de metilação global de DNA, quando comparados com sementes mantidas em meio sem fitoregulador.

Os resultados sugerem que o processo de indução de CNs em *V. reitzii*, resulta de uma reprogramação gênica no explante, que leva a alterações morfológicas, bioquímicas e metabólicas. A análise proteômica realizada nesta tese levanta algumas limitações quanto à pouca disponibilidade de sequências da família Bromeliácea nas bases de dados, que intervém na identificação das proteínas. Isto, considerando que algumas proteínas poderiam ser únicas das bromeliáceas ou apresentar modificações específicas. Este cenário revela a importância de estudos gênicos nas bromélias que contribuam com esse tipo de informação. Adicionalmente o fato de ter encontrado algumas proteínas com baixo nível de expressão, sugere a necessidade de aprofundar estudos utilizando novas e complementares abordagens proteômicas.

Durante a indução das CNs, foi observado o aumento na expressão da proteína "S-adenosylmethionine synthase" a qual catalisa a síntese de S-adenosylmethionine, um precursor na biossíntese de poliaminas, e também doador de grupos metil para várias moléculas, entre elas os ácidos nucléicos. A diminuição no nível da metilação do

DNA global observada durante a indução das CNs sugere que a S-adenosylmethionine synthase, provavelmente esteja envolvida principalmente com a proteção contra o estresse oxidativo através da sua contribuição na síntese de poliaminas. Com isso, seria interessante avaliar as mudanças temporais no conteúdo de poliaminas, as quais segundo a literatura têm sido implicadas em muitos processos celulares fundamentais, incluindo a regulação da expressão do gene, a proliferação de células e modulação de sinalização celular, entre outros.

Assim mesmo, o envolvimento dos processos metabólicos dos aminoácidos, carboidratos e proteínas durante a indução e regeneração das CNs, expõe a necessidade de avaliações qualitativas e quantitativas de aminoácidos e carboidratos solúveis, mediante o uso da Cromatografia líquida de alto desempenho (HPLC) e cromatografia gasosa acoplada à espectrometria de massa (GC/MS). Por fim, a determinação dos níveis de hormônios endógenos como auxinas e citocininas, também pode revelar informações importantes sobre a regulação gênica ocasionada pelo meio de indução das CNs.

O desenvolvimento da rota das CNs em bromélias é um processo complexo, uma vez que uma série de fatores diferentes age concomitantemente. Alguns componentes importantes foram abordados no presente trabalho, contudo, a continuidade dos estudos permitirá elucidar as relações específicas entre as diferentes redes de sinalização que regulam as mudanças associadas à aquisição de competências para este desenvolvimento. Essas informações contribuíram para a delimitação das CNs como outra rota da morfogênese *in vitro* ocorrente nas plantas, com alto potencial regenerativo. Assim como poderia auxiliar nos estudos de outros grupos de espécies que apresentam limitações nas técnicas de micropropagação.