Daniela De Conti

CARACTERIZAÇÃO FISIOLÓGICA E BIOQUÍMICA DO PADRÃO DE DESENVOLVIMENTO DE ESTRUTURAS SEMELHANTES À PROTOCORMOS DE *Cattleya tigrina* A. Richard

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.

Co-Orientador: Prof. Dr. Miguel Pedro Guerra

Florianópolis
2016

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DE CONTI, DANIELA
CARACTERIZAÇÃO FISIOLÓGICA E BIOQUÍMICA DO PADRÃO DE DESENVOLVIMENTO DE ESTRUTURAS SEMELHANTES À PROTOCORMOS DE Cattleya tigrina A. Richard / DANIELA DE CONTI ; orientadora, ROSETE PESCADOR ; coorientador, MIGUEL PEDRO GUERRA. - Florianópolis, SC, 2016.
136 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. Propagação in vitro. 3. Orquídeas. 4. Proteômica. 5. Hormônios. I. PESCADOR, ROSETE . II. GUERRA, MIGUEL PEDRO . III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. IV. Título.

Caracterização fisiológica e bioquímica do padrão de desenvolvimento de estruturas semelhantes à protocormos de *Cattleya tigrina* A. Richad

por

Daniela De Conti

Tese julgada e aprovada em 17/03/2016, 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

Aos meus amados pais Anselmo e Marilene,
aos meus queridos irmãos Evandro, Marilei e Cristiane,
ao meu namorado Wagner
por vossa confiança e carinho.
Dedico e Ofereço.

AGRADECIMENTOS

Agradeço acima de tudo à Deus, por me dar tantas bênçãos, inclusive a gratificação de mais uma realização da minha vida. Por me dar saúde, esperança e força para superar batalhas e desafios.

Aos meus queridos pais, Anselmo e Marilene, que não mediram esforços e amor, sempre acreditando no meu potencial, os quais mesmo distantes souberam dosar de maneira ímpar saudade e compreensão para que eu pudesse chegar até aqui. Amo vocês.

Ao meu namorado Wagner por estar sempre ao meu lado me apoiando, incentivando e acreditando no meu trabalho. Obrigada pelo amor, carinho e compreensão que sempre tivestes comigo. Sou muito grata por tudo.

Aos meus queridos irmãos Evandro, Marilei e Cristiane pelo apoio e amizade, sempre me incentivando e vibrando a cada conquista. O meu muito obrigado.

À minha orientadora Profª Rosete Pescador pela oportunidade, confiança e ensinamentos durante a realização deste trabalho.

Ao meu co-orientador Prof. Miguel Pedro Guerra pelo apoio na realização desse trabalho.

Aos professores do Programa de Pós-Graduação em Recursos Genéticos Vegetais, pelo grande aprendizado e ensinamentos transmitidos.

À Jenny Paola Corredor Prado pela amizade, apoio, trabalho conjunto na obtenção de muitos resultados. Obrigada por tudo.

À querida Marília Shibata, sempre disponível para ajudar quando necessário. Minha amiga que o doutorado proporcionou que tenho imenso carinho e respeito.

Aos ICs Jessica Bueno e Djalma Roecker que apoiaram na execução deste trabalho, sempre dispostos a aprender e ajudar no que fosse preciso. Muito obrigada.

À Jessica Bueno mais uma vez, pela pessoa maravilhosa que és e pela linda amizade que construímos.

Às minhas queridas manas Diana Cánova e Pâmela Vieira. Obrigada pelos agradáveis momentos ao longo desses quatro anos de convivência.

À Gabriela Cangahuala-Inocente pela amizade e por toda ajuda e suporte nas análises proteômicas e por compartilhar seus conhecimentos.

Ao Daniel Rosa, grande amigo que fiz neste período, o qual tenho enorme admiração.

À Morgana Lopes que me recebeu muito bem quando vim morar em Florianópolis. Obrigada pela amizade sempre.

À querida "Vila do Gerson" o qual vivi durante quatro anos. Obrigada pelos momentos de descontração e amizade.

Aos demais colegas e amigos do laboratório Julia Zappelini, Lara Carva, Rafaela De Liz, Rubens Zimmermann, Márcia Rossarola, Ramon Scherer, Fernando Sanchez, Luciano Saifert, Edison Cardona, Luiz Mamona, Vivian Almeida, Ana Paula Lando. Obrigado pelos momentos de descontração e pelo companheirismo.

Aos demais colegas do Laboratório de Fisiologia do Metabolismo e do Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, obrigada pela agradável convivência e auxílio.

À Martina Blank do CEBIME/UFSC, pela ajuda nas análises proteômicas. Muito obrigada.

Ao Yohan Fritsche e a Francis Dias pela disponibilidade de material vegetal para a realização do trabalho. Muito obrigada.

Ao laboratório de Fisiologia Vegetal do Instituto de Biociências da USP que abriram as portas para me receber para a realização das análises hormonais, em especial a professora Helenice Mercier, Aline Cruz e ao Paulo Rayner pela grande ajuda.

Ao Prof. Marcelo Maraschin e a querida Maira Tomazzoli pela ajuda nas análises dos açúcares.

Ao laboratorista André Knop pela grande ajuda com equipamentos, reagentes e técnicas laboratoriais.

À secretária Bernadete Ribas por ajudar nos documentos, solicitações e esclarecimentos.

Aos membros da banca examinadora – Profa. Lorena Benathar Ballod Tavares, Dr. Rogério Mamoru Suzuki, Drª Gabriela Claudia Cangahuala-Inocente, Profa. Neusa Steiner e Prof. Lírio Luiz Dal Vesco. Agradeço pelo olhar crítico e construtivo e pela disponibilidade em participar deste momento.

À Universidade Federal de Santa Catarina pelo ensino público gratuito e de qualidade.

À CAPES pelo apoio financeiro através da bolsa de estudos, e ao CNPq e FAPESC pelos recursos financeiros que permitiram a realização de todo esse trabalho.

À todos que de alguma forma colaboraram para a concretização desse trabalho e cujos nomes não foram citados aqui. Nunca me esquecerei de vocês.

“Determinação, coragem e autoconfiança são fatores decisivos para o sucesso. Se estamos possuídos por uma inabalável determinação, conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho”.

Dalai Lama

RESUMO

As orquídeas compreendem espécies frequentes do bioma Mata Atlântica, as quais têm sofrido ameaça continua devido à destruição e fragmentação dos seus habitats naturais. Devido às características ornamentais e paisagísticas, elas têm sido extraídas desordenadamente de seus habitats e comercializadas em todo o país. Técnicas de cultura de tecidos abrangem ferramentas que podem ser aplicadas à propagação massal e a conservação de orquídeas em extinção. Durante o estabelecimento *in vitro*, um padrão de resposta morfogênico definido como estruturas semelhantes à protocormos (ESPs) tem sido proposto para várias espécies de orquídeas como a espécie em estudo. Neste contexto, o objetivo desse trabalho foi avaliar o padrão de desenvolvimento de ESPs em *Cattleya tigrina*, visando o avanço no conhecimento desta rota da morfogênese e dos fatores que modulam, bem como, suas aplicações para a conservação e propagação de germoplasma. Explantes foliares, obtidos de plantas jovens, micropropagadas *in vitro* foram inoculados em meio de cultura MS/2 suplementado com 9µM de TDZ, para a indução e desenvolvimento das ESPs. As culturas foram coletadas aos zero, dois, sete, 14, 30, 60 e 100 dias de cultivo para posteriores análises. Realizou-se análises proteômicas mediante a combinação de géis bidimensionais e espectrometria de massas. Adicionalmente, foram realizadas avaliações do padrão de carboidratos solúveis totais e amido, qualificação dos açúcares e quantificação do hormônio AIA (ácido indol-3-acético) durante a indução e desenvolvimento das ESPs. Através dos resultados foi possível verificar uma reprogramação gênica durante a indução e desenvolvimento dessas estruturas, acompanhada de alterações anatômicas, bioquímicas e metabólicas. Na indução das ESPs foi possível verificar um número significativo de proteínas expressas ao zero dia de cultivo, quando comparado aos demais tempos de cultivo. Proteínas relacionadas ao metabolismo energético e carboidratos; defesa e resposta ao estresse e síntese de proteínas apresentaram significativas alterações na sua expressão durante a indução das ESPs. Durante o desenvolvimento das ESPs alterações na expressão de proteínas associadas aos processos metabólicos; metabolismo energético e carboidratos; ciclo celular e metabolismo secundário e fitohormônios, contribuíram no elevado potencial regenerativo dessas estruturas. Aos 100 dias de cultivo houve maior expressão de proteínas em relação aos demais tempos, o que pode estar relacionado com a ativação do metabolismo em relação ao desenvolvimento das ESPs. Em relação as avalições bioquímicas, foi possível observar que a indução e o desenvolvimento das ESPs foram

claramente acompanhados por um aumento nos teores de AIA e diminuição nos teores de açúcares, sendo que este sinal parece ter sido importante para que as células se diferenciassem e adquirissem a necessária competência. Os resultados aqui obtidos ampliam a base científica para aprofundar a compreensão dos fatores que modulam o processo de indução e desenvolvimento das ESPs em *C. tigrina.*

Palavras-chaves: Estruturas Semelhantes à Protocormos, micropropagação, orquídeas, hormônio, proteômica.

ABSTRACT

Orchids are species of the Atlantic Forest biome, which are under continuing threat due to the destruction and fragmentation of their natural habitats. Because of its ornamental features, they have been inordinately taken from their habitats and marketed throughout the country. Tissue culture techniques include tools that can be applied to mass propagation and conservation of endangered orchids. During establishment of *in vitro* cultures of this species, a pattern of morphogenic response defined as protocorm-like bodies (PLBs) have been proposed for various orchid species as the species under study. In this context, the aim of this study was to evaluate the PLBs development pattern in *C. tigrina* for the advancement in knowledge of this morphogenetic route (pathways) and the factors that modulate, and their applications in the conservation and propagation of germplasm. Leaf explants obtained from young plants, *in vitro* micropropagated were inoculated into culture medium MS/2 supplemented with 9μM TDZ for the induction and development of PLBs. The cultures were collected after zero, two, seven, 14, 30, 60 and 100 days of cultivation, for further analysis. Proteomic analysis were performed by combining two-dimensional gels and mass spectrometry. Additionally, we performed standard evaluations of total soluble carbohydrates and starch, sugars qualification and quantification of the IAA (Indole-3-acetic acid) hormone during induction and development of PLBs .Through the results it was possible to check a reprogramming of gene during induction and development of these structures, accompanied by anatomical, biochemical and metabolic changes.The induction of PLBs observed a significant number of proteins expressed to zero days of culture, when compared to other cultivation times. Proteins related to energy and carbohydrate metabolism; defense and response to stress and in protein synthesis showed significant changes in expression during induction of PLBs. During the development of PLBs changes in expression of proteins associated with metabolic processes; energy and carbohydrate metabolism; cell cycle and secondary metabolism and phytohormones, contributed to the high regenerative potential of these structures. After 100 days of cultivation there was greater expression of proteins compared to the other times, which may be related to metabolic activation in relation to the development of PLBs. Regarding the biochemical evaluations, it was observed that the induction and development of PLBs was clearly accompanied by an increase in IAA level and a decrease in sugar content, and this signal appears to be important for cells to differentiate and acquire the necessary competence. The results obtained broaden the

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Keywords: protocorm-like bodies, micropropagation, orchids, hormones, proteomic.

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1. INTRODUÇÃO

O bioma Mata Atlântica é caracterizado pela diversidade de espécies e elevado grau de endemismo, estando entre os 25 *hotspots* em riqueza e número de espécies do planeta, estimando-se a presença de 20.000 espécies de plantas, das quais 40% são endêmicas (Myers et al., 2000). Decorrente do aumento populacional, avanços dos centros urbanos e fronteira agrícola, este bioma é um dos mais ameaçados do planeta resultado de grandes perdas florestais (Silva e Tabarelli, 2000; Metzger, 2009).

Orquídeas compreendem espécies frequentes deste bioma e têm sofrido ameaça continua pela destruição e fragmentação dos habitats naturais em que as mesmas ocorrem, aspecto este agravado pelo fato da maioria delas serem epífitas. Além de contribuir para a manutenção da estabilidade dos ecossistemas florestais em função do seu elevado grau de especialização e de sua adaptação às condições climáticas e oligotróficas extremas (Benzing, 1998), as orquídeas são importantes comercialmente em decorrência da beleza de suas flores. Assim, devido às características ornamentais e paisagísticas apresentam relevante importância econômica, sendo retiradas desordenadamente de seus habitats naturais, levando inúmeras espécies à extinção. *Cattleya tigrina* é um exemplo de espécie de orquídea alvo da coleta predatória no Brasil. O extrativismo ilegal de espécies de orquídeas aliado com a fragmentação da Mata Atlântica faz com que muitas espécies estão desaparecendo em uma taxa alarmante (Hossain, 2008).

Dentro da complexidade das relações da natureza, as orquídeas obedecem a uma rígida organização e elas podem ser consideradas como um símbolo bastante oportuno do cuidado e da conservação da natureza (Meneguce et al., 2004). Assim, são oportunas as estratégias para caracterizar, avaliar e conservar estas espécies, pois na medida em que os recursos genéticos não se conservam adequadamente não se poderá dispor deles ao médio e ao longo prazo (Villamil e Alegría, 1997). Quando se fala de conservação de germoplasma deve-se ressaltar que o objetivo é conservar, com a maior integridade possível a variabilidade genética das populações selecionadas.

Programas de conservação de germoplasma vegetal envolvem diferentes estratégias incluindo estudos genéticos e ecológicos de plantas, manejo de hábitat, e conservação *in situ* e *ex situ*. Uma das estratégias da conservação *ex situ* de orquidáceas seria a utilização de técnicas de propagação sexual (sementes) e assexual (vegetativa).

Na propagação sexual, as sementes podem ser retiradas das matas com baixo impacto ambiental além de obter a variabilidade genética da população original (Hartmann et al., 2002). Porém, este sistema apresenta algumas desvantagens uma vez que a germinação de sementes de orquídeas em seu hábitat natural é baixa, e só ocorre quando associadas a fungos micorrízicos. Tal simbiose torna-se necessária devido ao fato das sementes não possuírem reservas nutritivas suficientes para promover a germinação (Ramos, 1969). Uma alternativa para a propagação de orquídeas com alta taxa germinativa, é o emprego de técnicas de micropropagação através da semeadura, que visa à produção em grande escala de plantas para comercialização, o que contribui para minimizar as coletas predatórias de espécies de orquídeas, garantindo assim a manutenção das populações naturais (Santos et al., 2006).

A propagação vegetativa de orquídeas por meio da divisão de rizomas ou pelo enraizamento de brotos também exige um longo tempo (Mondal et al., 2013). Assim, devido a esta lenta e ineficiente multiplicação por vias naturais de propagação (Junghans e Souza, 2013), a conservação de orquídeas depende de estratégias em longo prazo (Machado Neto e Custódio, 2005).

O cultivo *in vitro* é uma ferramenta amplamente utilizada na conservação de espécies ameaçadas (Sarasan et al., 2006), tanto na propagação clonal por explantes (vegetativa) quanto na germinação de sementes (Fay, 1994; Sarasan et al., 2006). Esta técnica oferece um conjunto de ferramentas que possibilitam a propagação massal de genótipos selecionados, permitindo a captura e fixação de ganhos genéticos (Guerra et al., 1999).

Técnicas de cultura de tecidos permitem a micropropagação clonal massal de genótipos superiores de orquídeas com importância e interesse ornamental. Estas técnicas têm sido largamente utilizadas com sucesso na propagação de diferentes espécies de orquídeas terrícolas e epífitas (Díaz e Álvarez, 2009). Por meio da propagação *in vitro* se obtém mudas uniformes, de alta qualidade e livre de doenças, além de permitir a multiplicação rápida e, em termos ecológicos, pode ser utilizada na preservação e propagação de espécies ameaçadas. Sua importância é evidente pelas oportunidades que se criam para compreender, utilizar e conservar recursos genéticos de plantas, podendo contribuir em todas as etapas do processo de conservação de germoplasma, incluindo coleta, indexação de doenças, quarentena, multiplicação, caracterização e avaliação, armazenamento e distribuição de germoplasma (Withers e Williams, 1998).

A identificação de sistemas regenerativos *in vitro* que podem ser aplicados tanto para a conservação quanto para a propagação em grande escala são estratégias de interesse para inúmeras orquídeas. Alguns padrões de respostas da morfogênese *in vitro*, como os observados em orquídeas apresentam características diferenciadas aos sistemas regenerativos tradicionais, sendo estes definidos como estruturas semelhantes à protocormos - ESPs (protocorm-like bodies – PLBs). Estas estruturas somáticas obtidas *in vitro* são assim referidas devido à semelhança destas com os embriões (protocormos) de origem zigótica (Arditti, 2008).

A competência celular do explante em induzir esta rota regenerativa depende da sua capacidade de resposta aos sinais extracelulares, entre eles a composição basal do meio de cultura, o balanço e os tipos de hormônios e as condições ambientais externas como luz e fotoperíodo (Guerra et al., 1999). Durante a formação destas ESPs ocorrem processos morfogenéticos distintos, ocasionando alterações fisiológicas, bioquímicas e morfoanatômicas. Estudos básicos do metabolismo celular durante a formação das ESPs configuram um modelo interessante para compreensão dos eventos fisiológicos, anatômicos e metabólicos associados à competência celular e histodiferenciação.

Alguns trabalhos foram realizados com o objetivo de compreender os fatores que modulam esse processo em *C. tigrina*. Em trabalho realizado por Liz (2013) elucidando os aspectos envolvidos na morfo-histodiferenciação do desenvolvimento de ESPs, foi possível evidenciar mudanças significativas durante a formação dessas estruturas. A diferenciação dos tecidos ocorreu na primeira semana de cultivo, onde as células epidérmicas começaram a sofrer divisões mitóticas para formação dessas estruturas. A proliferação das ESPs aconteceu gradativamente e em estádios mais avançados foram mais numerosas e mais volumosas. Almeida (2014) analisou a dinâmica das poliaminas, amido, proteínas totais e metilação do DNA durante os 35 dias de cultivo das ESPs e verificou que a ontogênese dessas estruturas está associada às alterações bioquímicas e eventos epigenéticos como a metilação do DNA.

Embora tenham ocorrido avanços na elucidação dos mecanismos associados à modulação desse sistema regenerativo em *C. tigrina*, é necessária uma maior compreensão dos mecanismos bioquímicos e moleculares que regulam esse sistema de regeneração. O estudo da dinâmica dos açúcares e proteínas na indução e regeneração das ESPs é de extrema importância para a compreensão dos eventos que modulam esse processo. O uso de análises morfoanatômicas do material vegetal, utilizando técnicas de microscopia óptica, é de fundamental importância

para a interpretação na identificação e caracterização da formação das ESPs.

A caracterização da dinâmica de proteínas expressas no processo de formação das ESPs permite avaliar quantitativamente e qualitativamente as proteínas que atuam ao longo dos processos de desenvolvimento (Chen e Harmon, 2006). Consequentemente, a identificação de proteínas expressas diferencialmente permite a associação destes polipeptídeos com diferentes eventos fisiológicos que ocorrem nas células, tecidos e órgãos (Blackstock e Weir, 1999; Kormuťák et al., 2006; Wang et al., 2010), permitindo a avaliação das propriedades bioquímicas destas, tais como o perfil das modificações pós-tradução ou a interação com outras biomoléculas (Fitzgerald, 2001).

O presente documento está organizado em três capítulos, precedidos por uma revisão bibliográfica. O primeiro capítulo abrange o estudo proteômico na indução das ESPs. O segundo capítulo se refere à análise proteômica relacionada ao desenvolvimento das ESPs. O terceiro e último capítulo utiliza abordagens hormonais e açúcares para compreender os fatores que modulam a indução e desenvolvimento de ESPs em *C. tigrina.*

2. REVISÃO DE LITERATURA

2.1 Família Orchidaceae

A família Orchidaceae é uma das famílias botânicas mais numerosas e diversificadas, apresentando cerca de 27.000 espécies aceitas, distribuídas em 899 gêneros (The Plant List, 2013). Apresenta distribuição cosmopolita, embora seja mais abundante e diversificada em florestas tropicais da Ásia e das Américas, estando ausente apenas nos polos e nas regiões desérticas (Rodrigues, 2011; Menini Neto et al., 2013) (Figura 1).

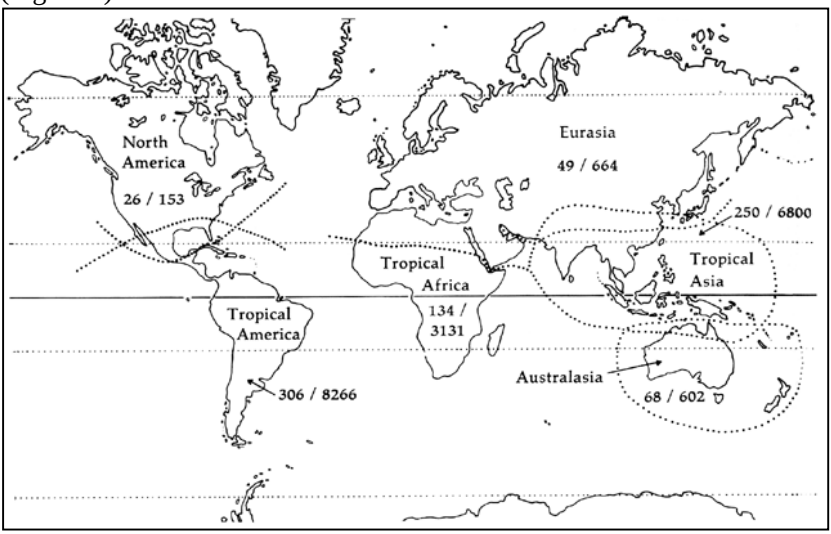

Figura 1: Distribuição da família Orchidaceae no mundo; a esquerda encontra-se o número de gêneros e a direita o número de espécies. (RODRIGUES, 2011) Adaptado de Dressler (1993).

Consiste geralmente de plantas herbáceas epífitas, terrícolas, saxícolas, micoheterotróficas e rupícolas, sendo que a diversidade e a riqueza de espécies desta família são altamente influenciadas pelo relevo e pelas condições climáticas (Waechter, 1996).

No Brasil, segundo a Lista de Espécies da Flora do Brasil (Barros et al., 2016) ocorrem 2.546 espécies distribuídas em 222 gêneros. Apesar de o bioma estar sobre grande ameaça poucas espécies de orquídeas são consideradas em risco de extinção. Na Lista Oficial das Espécies da Flora Brasileira Ameaçadas de Extinção, do Ministério do Meio Ambiente

(Brasil, 2008), das espécies de orquídeas que estão listadas, um número significativo de espécies de orquídeas é considerado com insuficiência de dados. Esses resultados evidenciam a falta de dados do estado de conservação das espécies de orquídeas brasileiras.

Taxonomicamente as orquídeas estão divididas em seis subfamílias: Apostasioideae, Cypripedioideae, Spiranthoideae, Orchidoideae, Epidendroideae e Vandoideae (Dressler, 1990). Dentro da subfamília Epidendroideae, encontra-se dentre outros o gênero *Cattleya*, com enorme valor horticultural com ocorrência natural no Brasil, sendo muito utilizados para a produção de híbridos comerciais (Van Den Berg et al., 2009).

A grande distribuição das orquídeas em todas as formações vegetacionais brasileiras está relacionada com a grande capacidade adaptativa, pois as várias formas estruturais vegetativas presentes na família fazem com que possuam diferentes estratégias relacionadas com a obtenção e reserva de água e nutrientes (Hoehne, 1949). No entanto, muitas destas espécies estão ameaçadas de extinção, tais como a espécie *Cattleya tigrina* (Brasil, 2014). As principais ameaças estão relacionadas à coleta predatória, destruição de habitat e eliminação dos polinizadores. Muitas espécies de distribuição restrita ou microendêmicas são também ameaçadas por eventos estocásticos (Menini Neto et al., 2013).

2.2 Gênero *Cattleya*

Cattleya Lindl. é um gênero de orquídeas exclusivamente neotropical que abrange aproximadamente 120 espécies principalmente epífitas e, mais raramente, rupícolas e terrícolas (CHASE et al., 2003; Van Den Berg, 2009). Este gênero se insere na subfamília Epidendroideae Lindl. tribo Epidendreae Kunth, subtribo Laeliinae Benth. (Chase et al., 2003; Van Den Berg, 2008).

As espécies deste gênero estão distribuídas em duas grandes regiões: nas montanhas do Brasil (a maioria das espécies paralelamente à costa oriental do país) e nos Andes, estendendo-se no sentido Leste através da Venezuela até Trinidade e no sentido Norte através da América Central até o Sul do México (Withner, 1988). A maioria das espécies encontra-se em habitats montanhosos e prefere o frio para se desenvolver, embora algumas tenham se adaptado ao calor e as condições de umidade das planícies (Withner, 1988).

A grande característica desse gênero é a presença de flores de tamanho grande e labelo não fundido a coluna (Withner, 1988). Comercialmente, o cultivo de espécies do gênero *Cattleya* é de grande

importância para o agronegócio florícola mundial devido, principalmente, a ampla capacidade de recombinação genética, beleza, forma, tamanho e durabilidade de suas flores (Zanenga-Godoy e Costa, 2003). A grande frequência com a qual vem ocorrendo essa procura tem levado a uma redução e desaparecimento de várias populações e, consequentemente, várias destas espécies apresentam-se ameaçadas de extinção (Cruz, et al., 2003). O Brasil é o país com maior número de espécies (101), das quais 94 são endêmicas (Barros, et al., 2016). Dentre as orquídeas do gênero *Cattleya* presentes no Brasil, destaca-se a espécie *Cattleya tigrina* (Figura 2). Esta espécie é endêmica do bioma Mata Atlântica e habita trechos de matas ribeirinhas, caracterizados pela alta umidade, sendo encontrada nos estados do Rio Grande do Sul, Santa Catarina, São Paulo, Bahia, Pernambuco e Sergipe (Barros, et al., 2016). Destaca-se pela coloração e número de flores, sendo muito cultivada e comercializada pelos orquidófilos devido seu alto valor comercial (Rocha, 2008). Assim, devido à coleta indiscriminada e a destruição dos seus habitats pela expansão humana, esta espécie está em constante declínio, sendo vulnerável a extinção segundo o Livro Vermelho da Flora do Brasil (Brasil, 2013).

Figura 2. A-B: Aspectos gerais das flores de *Cattleya tigrina*. Barras =1 cm (Buzatto et al., 2010).

2.3 Padrões da morfogênese *in vitro* das estruturas semelhantes à protocormos

As técnicas de cultura *in vitro*, baseadas no princípio da totipotência das células vegetais, ou seja, na potencialidade de células se diferenciarem e regenerarem uma planta completa tem gerado grandes avanços na pesquisa básica, principalmente na fisiologia, bioquímica e genética de plantas, fornecendo ferramentas de ação em áreas práticas como o melhoramento genético vegetal, a farmacologia, a micropropagação e a conservação de germoplasma (Kerbauy, 2008).

Na propagação *in vitro*, tecidos, órgãos ou células podem adquirir novas competências pela ação de determinados sinais químicos (reguladores de crescimento) que ativam seletivamente determinados genes (epigênese). A resposta final é a expressão da morfogênese em dois níveis básicos: organogênese direta ou indireta e embriogênese somática direta ou indireta (Reinert, 1977).

Na organogênese direta se obtém eixos caulinares monopolares originados de gemas pré-existentes. Quando é indireta ocorre a desdiferenciação do explante, resultando na formação de calos, que podem ser definidos como a proliferação de células não diferenciadas, originando meristemóides (Thorpe, 1980).

A embriogênese somática é o processo pelo qual se desenvolvem embriões a partir de uma célula simples ou um grupo de células que não resultaram da fusão de gametas e que se diferenciam os mesmos estádios ontogenéticos observados na embriogênese zigótica (Maheswaran e Wiliams, 1985). Este padrão de expressão da morfogênese é fundamentado através da totipotencialidade celular, postulado pelo fisiologista alemão Haberlandt em 1902, para o qual, uma vez fornecidas às condições e estímulos adequados, cada célula nucleada pode originar uma planta completa (Zimmerman, 1993). 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 da organogênese (Guerra et al., 1999).

Em orquídeas, além da germinação assimbiótica de sementes e da organogênese convencional existe uma rota morfogênica específica para a regeneração *in vitro*. Este tipo de propagação clonal de orquídeas pode ser realizado utilizando explantes somáticos de diferentes origens como, por exemplo, folhas (Chen e Chang, 2001; Gow et al., 2008; Mayer et al., 2010), hastes florais (Chen e Chang, 2000), raízes (Kerbauy & Estelita, 1996) e ápices caulinares (Roy et al., 2007) com a obtenção de estruturas globulares similares aos embriões de origem zigótica. Vários trabalhos

publicados referem-se a esta rota como sendo embriogênese somática, muito provavelmente devido às peculiaridades dos embriões de origem zigótica das orquídeas.

O embrião das orquídeas apresenta um padrão de desenvolvimento muito diferente dos apresentados em outras espécies vegetais. Apesar de George e Debergh (2008) afirmarem ser possível, na fase de protocormo, a diferenciação do meristema apical e radicular, em polos opostos, a literatura no geral afirma que o padrão de desenvolvimento dos embriões de orquídeas é diferente de outras Angiospermas. Estes embriões constituem-se de pequenos corpos elipsoidais formados por relativamente poucas células que acumulam reservas (Arditti, 1992), predominantemente lipídicas. As sementes de orquídeas apresentam, em geral, um padrão bastante uniforme de germinação e desenvolvimento, iniciando-se por intumescimento da semente que acarreta o rompimento do tegumento seminal e a liberação do embrião. Este se desenvolve numa estrutura tuberiforme, geralmente clorofilada, o chamado protocormo (Arditti, 1992).

O termo protocormo (do grego prôtos = primeiro e kormós = tronco de árvore, caule) foi inicialmente proposto por Melchior Treub, em 1888 (Font Quer, 1979) ou 1890 (Arditti e Ernst, 1993), para descrever o estágio inicial do desenvolvimento de licopodíneas, que forma uma estrutura tuberiforme a partir da germinação de esporos. O protocormo é uma estrutura que possui formato geralmente globular, podendo variar entre os táxons, composto principalmente por células parenquimáticas (Batygina et al., 2009). Na parte superior do protocormo são encontradas células menores que darão origem ao meristema apical e aos primórdios foliares. Porém na parte basal não é encontrada uma zona meristemática que dará origem ao meristema radicular como na maioria dos outros embriões, já que nas orquídeas as raízes são de origem adventícia (Batygina et al., 2009).

Em tecidos ou calos de orquídeas cultivadas *in vitro*, são formadas estruturas similares aos protocormos, sendo por esta razão denominada por Georges Morel em 1960 (Arditti, 2008) como estruturas semelhantes à protocormos (protocorm-like bodies). Porém o termo *protocorm-like bodies* é também utilizado para qualquer estrutura originada de orquídeas a partir de explantes meristemáticos (Kraus et al., 2006), como por exemplo mericlones (Kuehnle, 2007), embriões somáticos (Chen e Chang, 2004), brotos (Chen, et al., 2004), segmentos nodais axilares (Shiau et al., 2005; Dohling et al., 2012) e a própria estrutura semelhante à protocormo (Young et al., 2000; Paek et al., 2011). Pouco se tem estudado acerca dessas estruturas, em relação à anatomia, fisiologia e

bioquímica, sendo necessários estes estudos para conhecer a sua estrutura e as rotas da morfogênese *in vitro*.

2.3.1 Auxinas como sinalizador da morfogênese *in vitro*

Os mecanismos que operam durante a morfogênese capacitam o vegetal a adquirir um corpo específico e tecidos com padrões diversos de diferenciação (Goldberg et al., 1994). Os hormônios vegetais desempenham um papel crucial no controle do crescimento e desenvolvimento vegetal. São moléculas regulatórias de ocorrência natural que agem como sinais químicos para regular o processo da morfogênese. Eles são considerados como fatores-chave envolvidos no desencadeamento da morfogênese vegetal, incluindo embriogênese somática e organogênese (Huang et al., 2012).

Entre os reguladores de crescimento destacam-se as auxinas que exercem forte influência nos processos de expansão, divisão celular na definição de órgãos e promoção da diferenciação do sistema vascular (Lui et al., 1993). O ácido indolil-acético (AIA), a principal auxina em plantas tem um papel chave nos processos de desenvolvimento vegetal, como na formação de raízes, dominância apical, tropismo e senescência, atuando também, como sinal para a divisão, alongamento e diferenciação celular (Ljung et al., 2002; Sairanen et al., 2012). Este hormônio ocorre nos tecidos vegetais na forma livre (ativa), ou conjugada, sendo que a manutenção das auxinas no estado conjugado está protegida contra os processos de oxidação, podendo ser enzimaticamente liberada quando necessário (Gaspar et al., 1996).

O mecanismo pelo qual as auxinas agem nos processos fisiológicos e regulatórios está relacionado com a presença de receptores protéicos localizados na membrana da célula, mensageiros no citoplasma e na membrana do núcleo celular. Quando este último recebe o sinal, é desencadeada a ativação de enzimas para a transição de genes envolvidos na regulação da divisão celular (Dodeman et al., 1997).

Nos últimos anos, estudos têm relatado a influência dos níveis endógenos de AIA no controle da embriogênese somática de *Saccharum species* (Guiderdoni et al., 1995), *Prunus sp* (Michalczuk e Druart 1999), *Daucus carota* (Jiménez e Bangerth, 2001), *Araucaria angustifolia* (Steiner et al., 2007), *Acca sellowiana* (Pescador et al., 2012), *Prunus persica* (Pérez-Jiménez et al., 2013) e *Cucurbita pepo* (Leljak-Levanic et al., 2015) e da organogênese (Jiménez, et al., 2001), *Catasetum fimbriatum* (Suzuki et al., 2010) e *Hordeum vulgare* (Hisano et al., 2015). Em contraste, apenas escassos relatos mencionam as mudanças

hormonais endógenas relacionadas com outras vias da morfogênese *in vitro*, como na formação de ESPs.

2.3.2 Carboidratos e proteínas como moduladores da morfogênese

Além dos hormônios vegetais, os carboidratos e as proteínas também afetam o crescimento e desenvolvimento, sendo moduladores da morfogênese. Os carboidratos são fontes de energia para as células e de carbono para processos biossintéticos (Zhang et al., 2012; Kubeš et al., 2014). Eles também atuam como agentes osmóticos contribuindo para a manutenção da integridade da membrana plasmática e são importantes moléculas sinalizadoras que modulam uma variedade de processos no desenvolvimento das plantas (Pareddy e Greyson, 1989; Kubeš et al., 2014; Lastdrager e Smeekens, 2014). As redes moleculares de condução, divisão e expansão celular em grande parte, dependem da disponibilidade de carboidratos para fornecer energia e biomassa (Lastdrager e Smeekens, 2014). Alguns dos efeitos sobre o crescimento e desenvolvimento das plantas sugerem interação da sinalização dos açúcares com a regulação hormonal (Rolland et al., 2006). Estudos recentes têm evidenciado que as auxinas e os açúcares (glicose e sacarose) agem em conjunto, sendo que o metabolismo da auxina é regulado pela disponibilidade de açúcares livres (Sairanen et al., 2013; Ljung et al., 2015). A regulação da biossíntese e degradação da auxina principalmente do IAA, por açúcares requer alterações na expressão de múltiplos genes e metabólitos associados a várias vias da biossíntese de IAA (Sairanen et al., 2013; Ljung et al. 2015).

Dentre os açúcares, a glicose, frutose e sacarose são consideradas essenciais para o processo de desenvolvimento nas plantas. A glicose e a frutose são as primeiras moléculas para atuar na sinalização celular, enquanto que o papel de sacarose pode ser, inicialmente, para fornecer hexoses (Aragão et al., 2015). A sacarose é considerada, como a principal forma de açúcar usada como fonte de esqueleto carbônico e energia pelas células durante o crescimento e desenvolvimento (Tremblay e Lalonde, 1984; Tremblay et al., 1984; Barghchi, 1988; Lastdrager et al., 2014). Sacarose desempenha um papel central no desenvolvimento da planta como uma possível molécula de sinalização regulando um grande número de genes (Coruzzi e Bush, 2001). A glicose também tem seu grande papel no metabolismo, pois pode servir como uma ponte entre a sinalização de carboidratos e fitohormônios (Leon e Sheen, 2003; Roitsch et al., 2003; Hartig e Beck, 2006). Além disso, a glicose tem sido destacada como uma molécula de sinalização em vários processos associados com o

crescimento e desenvolvimento, tais como germinação, alongamento do hipocótilo, expansão dos cotilédones e desenvolvimento foliar (Rolland et al., 2006). De acordo com Weber et al. (1997) as hexoses e a sacarose estão geralmente associadas às fases distintas do desenvolvimento da semente, sendo sugerido que a sacarose regularia a diferenciação celular e o armazenamento de substâncias de reserva, enquanto as hexoses controlariam o crescimento e o metabolismo celular.

Assim como os açúcares, as proteínas também têm um papel chave nos processos da morfogênese, sendo que estão envolvidas na regulação da expansão celular e no estabelecimento das características biofísicas requeridas para a morfogênese (Jiménez, 2001). Sendo assim, essas substâncias podem ser consideradas como marcadores bioquímicos durante a indução e desenvolvimento de ESPs. 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 (Catusse et al., 2008).

A proteômica é o estudo de um Proteoma, ou conjunto de proteínas expressas pelo genoma de um organismo (Wasinger et al., 1995), que permite avaliações quantitativas e qualitativas de proteínas que atuam no metabolismo celular (Chen e Harmon, 2006). Consequentemente, a identificação de proteínas expressas durante o desenvolvimento vegetal permite a caracterização de diferentes eventos fisiológicos que ocorrem nas células, tecido e órgãos das plantas (Blackstock e Weir, 1999; Kormutak et al., 2006; Wang et al., 2010), permitindo a avaliação das propriedades bioquímicas destas, tais como o perfil das modificações pós-tradução ou a interação com outras biomoléculas (Fitzgerald, 2001).

Nos últimos anos, vários trabalhos têm enfocado a caracterização da dinâmica de proteínas no processo de desenvolvimento vegetal de várias espécies incluindo *Vanilla planifolia* (Palama et al., 2010; Tan et al., 2013), *Manihot esculenta* (Li et al., 2010), *Cyclamen persicon* (Bian et al., 2010), *Oncidium shacelatum* (Valadares et al., 2014) entre outras espécies. Esta análise tem sido amplamente empregada no estudo de vários processos do desenvolvimento vegetal, incluindo aspectos bioquímicos e moleculares envolvidos na embriogênese assim como o completo desenvolvimento da semente até a germinação (Nogueira, 2007). A dinâmica das proteínas é influenciada por diversos fatores, seja ele interno ou externo que determinam modificações estruturais e as conformações das proteínas. Assim, o estudo e a caracterização de mapas proteômicos apresentam-se como importantes ferramentas complementares aos estudos anatômicos, fisiológicos e de genômica.

3. OBJETIVO GERAL

Estudar aspectos fisiológicos e bioquímicos associados à indução e desenvolvimento de estruturas semelhantes à protocormos em *Cattleya tigrina* buscando uma melhor compreensão dos fatores que modulam esse sistema de propagação *in vitro*.

3.1 Objetivos Específicos

a) Identificar e caracterizar os diferentes estágios de desenvolvimento das estruturas semelhantes à protocormos em *C. tigrina* mediante a análise histoquímica.

b) Caracterizar as proteínas diferencialmente expressas durante a indução e desenvolvimento das estruturas semelhantes à protocormos em *C. tigrina*.

c) Comparar os teores de açúcares totais e amido durante a durante a indução e desenvolvimento das estruturas semelhantes à protocormos em *C. tigrina*.

d) Avaliar a dinâmica do hormônio Ácido indolil-3-acético (AIA) durante a indução e desenvolvimento das estruturas semelhantes à protocormos em *C. tigrina*.

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4. CAPÍTULO 1. PROTEOMICS IN THE INDUCTION OF *IN VITRO* PROTOCORM-LIKE BODIES OF *Cattleya tigrina* A. Richard.

4.1 ABSTRACT

Cattleya tigrina is an orchid with ornamental characteristics, vulnerable to extinction. *In vitro* propagation is effective for its mass propagation and conservation. Leaves cultured *in vitro*, in the presence of hormones have competent cells to form *protocorm-like bodies* (PLBs) that suffer biochemical and physiological changes allowing the complete formation of a plant. Proteins differentially expressed during the morphogenesis of PLBs in *C. tigrina* were identified and related to the factors that modulate this propagation system. The plant material derived from the culture medium of induction of PLBs were collected at zero, two, seven and 14 days of cultivation. Using the high resolution of the two-dimensional polyacrylamide gel (2-DE) and mass spectrometry, it was evidenced differences between the expressed proteins at zero, two, seven, and 14 days of culture, where it was possible to detect 410, 136, 142 and 109 spots, respectively. 45 proteins differentially expressed through evaluated cultivation time were identified. Proteins related to energy and carbohydrate metabolism (Malate dehydrogenase cytoplasmic, Ribulose bisphosphate carboxylase large chain, Malate dehydrogenase mitochondrial, Fructose-bisphosphate aldolase cytoplasmic isozyme and Acetyl-CoAcarboxylase1), defense and stress response (Heat shock cognate 70KDaprotein, Heat shock protein 81-1 and Heat shock cognate 70KDaprotein 6 chloroplastic) and protein synthesis (Glutamine synthetase, 30S ribossomal protein S8 and Cysteine synthase), have a key role in the process of induction of PLBs. Changes in proteins related to cellular metabolic processes, the assembly of the photosynthetic mechanism and the response to the medium culture stressful contribute to a high induction potential, which characterizes PLBs. To our knowledge, this is the first proteomic analysis performed during the induction process from PLBs in orchids. It may provide important insights into proteins and cellular events involved in the micropropagation of this group of plants.

Keywords: orchid, proteins, protocorm-like bodies, 2-DE, mass spectrometry.

4.2 INTRODUCTION

Cattleya tigrina A. Richard is an endemic Orchidaceae of the Atlantic Forest biome and inhabits part of the riparian forests, characterized by high humidity (Cruz et al. 2003). It is distinguished by the color and number of flowers, being very cultivated and marketed by orchid growers because of its commercial value (Rocha 2008). Coupled with the indiscriminate collection, the specialized life cycle and the destruction of their habitats by human expansion, this species is in constant decline, being vulnerable to extinction (Brasil 2013).

In vitro propagation techniques are considered to be an effective alternative for its mass propagation and maintenance, allowing the capture and fixation of genetic gains (Guerra et al. 1999). These techniques have been widely used with success in the propagation of different species of land and epiphytic orchids (Díaz and Álvarez 2009) as is the case of *C. tigrina* (Fritsche 2012).

Some response standards for *in vitro* morphogenesis have distinct characteristics, as seen in orchids, wherein the regenerative system is defined as protocorm-like bodies (PLBs). These globular structures are so named because of the similarity to the embryos (protocorms) of zygotic origin (Arditti 2008). Its induction has been reported from meristematic apices in *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii 1993) and *Cymbidium* (Subramanium and Taha 2003), from floral stalks in *Phalaenopsis* (Ichihashi 1992), from root apices in *Phalaenopsis* (Tanaka et al. 1976), and through leaf segments in *Doritaenopsis* (Park et al. 2002), in *Aerides crispum* (Sheelavanthmath et al. 2005) and in *Cattleya tigrina* (Fritsche 2012).

For species *C. tigrina,* the understanding of *in vitro* morphogenesis was given by the study of Frische (2012), through an efficient regeneration protocol. More recently, Liz (2013) elucidated aspects involved in the morpho-histodifferentiation of PLBs and Almeida (2014) analyzed the dynamics of polyamines and DNA methylation in the induction of the PLBs. However, it was not yet studied the formation of PLBs using proteomic approaches.

Proteomics is a powerful tool that involves the identification and characterization of proteins (Wang et al. 2010). In addition to providing the large-scale systematic identification of cellular proteins, proteomic techniques can be used to elucidate molecular details that occur during complex procedures such as the formation of callus (Yin et al. 2008), somatic embryos (Silva et al. 2014) and protocorm-like bodies, object of study. The proteomic studies seek to analyze the profile of total proteins

of a particular cell, organelles or tissues (Blackstock and Weir 1999; Wang et al. 2010), allowing the evaluation of biochemical properties thereof, such as the profile of post-translational modifications or the interaction with other biomolecules (Valledor and Jorrín 2011). Proteomic techniques have been used to investigate the tissue culture systems of orchid species, including *Vanilla planifolia* (Palama et al. 2010; Tan et al. 2013) and *Oncidium shacelatum* (Valadares et al. 2014). In this context, this study aimed to identify and characterize the proteins differentially expressed during early morphogenesis of PLBs in *C. tigrina* in order to understand the factors that modulate this process.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

Leaf explants (±1cm) were obtained from young plants, micropropagated and maintained *in vitro* Development Physiology Laboratory and Plant Genetics (LFDGV), the Agricultural Science Center (CCA), Universidade Federal de Santa Catarina (UFSC), Florianópolis, Santa Catarina State, Brazil. The leaf explants were detached and inoculated MS medium (Murashige and Skoog 1962), supplemented with 9 μM TDZ (Thidiazuron) for PLBs induction, which was previously established by (Fritsche 2012).

After inoculation of the explants, they were kept in growth room with an average temperature of 25±2 °C, 16-h light and 8-h dark, with a light intensity of 50 μmol m^{-2} s^{-1}. To zero (which corresponded to the beginning of the assembly of the experiment, being the starting explant), two, seven, and 14 days of cultivation, plant materials were collected for histological and proteomic analysis. For histological studies the five explants of each culture time were collected. With regard to proteomic analysis were collected biological six replicates for each cultivation time, each replica was formed by 500mg of fresh mass starting from a pool of plant material, corresponding basically to 30 leaf explants and stored at -80°C for later analysis.

4.3.2 Histological analysis

The material was fixed in 2.5 % paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. The samples were dehydrated in increasing series of ethanol aqueous solutions (Schmidt et al. 2009). 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).

4.3.3 Proteomic analysis

Protein Extraction

The extraction of total proteins from leaf tissue was performed following the method of Carpentier et al. (2005) with modifications. In brief, 0.5g of leaf tissue collected from each of the selected seedlings per treatment 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 100 mM of ammonium acetate in methanol (1:5 v/v), proteins were maintained overnight at -20 °C. The tube was centrifuged for 30 min at 10,000g 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 (7 M urea, 2 M 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 using 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 (7 M Urea, 2 M Thiourea, 3 % CHAPS, 2 % IPG-buffer, 0.002 % Bromophenol blue, and 0,2 % DTT) containing 600 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, 6 M 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). Gels were stained with Coomassie blue (1 % Coomassie Blue G-250, 2 % H_3PO_4, 8 % $(NH_4)_2SO_4$, 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). The spots were identified and selected based on the comparative analysis of the gels. 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 protein spots that were reproducibly found in at least four biological replicates were included in further examinations. The proteins were found to be differentially expressed between the times of cultivation, while presenting a change of ± 2 times the percentage of volume and representing significant difference (Student's t-test $p < 0.05$) were included in the analysis. 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 Mass Spectrometry Analysis

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 maintained 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 % acetonitrile 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 spectrum was submitted to identification using MASCOT on line (Matrix Science, London, UK) and SwissProt databases. The parameters used for the acceptance of identification were: taxonomy: *Viridiplantae*; enzyme: trypsin, missed cleavages: one; fixed modifications: carbamidomethyl cysteine; variable modifications: oxidized methionine; mass values: MH+, peptide mass tolerance 100 ppm and MS/MS tolerance of 0.5 Da. Positive identification was based on the significative Mascot score ($p<0,05$). Functional categorization and cellular component of proteins was performed according to the GO (Gene Ontology) by searching the protein knowledgebase (UniProtKB).

4.4 RESULTS

4.4.1 Early morphogenesis of the protocorm-like bodies

In anatomical analyzes conducted during the induction of *C. tigrina* PLBs, it was observed that at the initial time (control), the basal region of the leaf segments is characterized by the absence of protuberances (Fig. 1a), and cell proliferation (Fig. 1b) is not verified. This lack was also observed at two days of culture, showing no change (Fig. 1c and 1d). But at seven days of culture (Fig. 1e), evidences of PLBs development were shown on the abaxial surface of the basal portion of the leaf. The histodifferentiation of PLBs started from the epidermal cells of the leaf by periclinal division of these cells and subsequent divisions (Fig. 1f).

In this study it was evidenced that although the formations of PLBs have been registered in the basal half of the leaf at seven days, at 14 days of culture, these started to happen mainly in the basal region (Fig. 1g), and at this stage some PLBs have shown to be bulkier.

Cell division and differentiation were observed at seven days of cultivation, but the formation of PLBs was evident only at day 14° of

culture, when the epidermal cells underwent mitotic divisions to the formation of PLBs (Fig.1h).

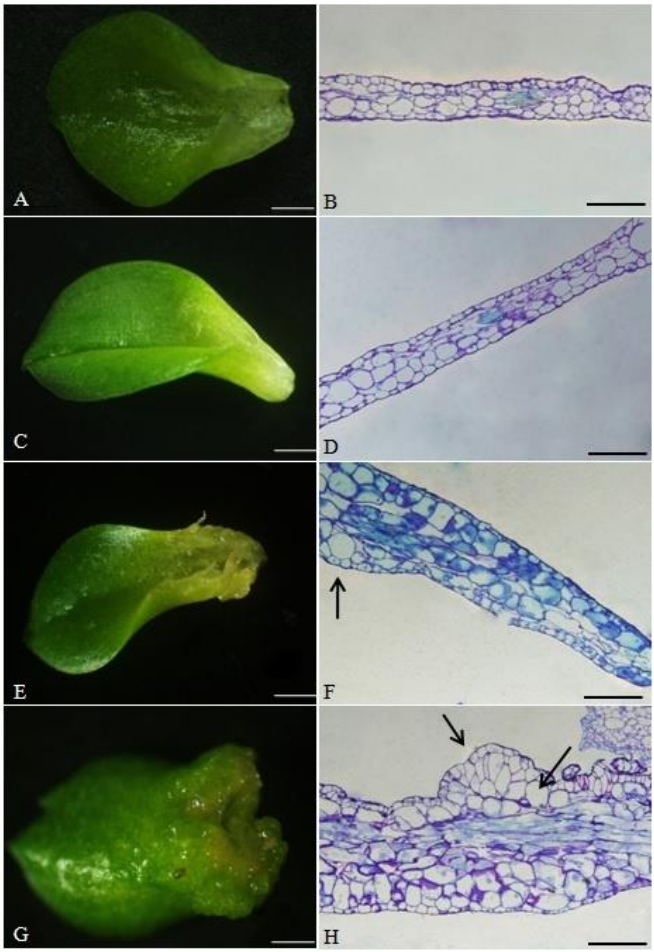


Fig. 1 Histological aspects of protocorm-like bodies (PLBs) from leaf explants of *C. tigrina* A. Rich. **(a)** General view of the adaxial surface of the initial leaf. **(b)** Cross-section of initial explant. **(c)** After two days of culture, overview of the adaxial surface of the leaf. **(d)** Cross-section of leaf induced after two days of culture. **(e)** General view of the adaxial surface of induced leaf after seven days of culture. **(f)** Cross-section of the leaf induced after seven days of culture with evidence of mitotic activity in the epidermis (arrow). **(g)** On the 14th day of culture, the initial

formation of PLBs. **(h)** Cross-section of the leaf induced on the 14th day of culture, with evidence of mitotic activity in the epidermis (arrows), mesophyll cells very vacuolated (arrow). Bars: (a, c, e, g) 1mm; (b, d, f, h) 200μm.

4.4.2 Dynamics of the proteins expressed by two-dimensional electrophoresis

The cell physiological complexity of PLBs at different times of cultivation was clearly evident through proteomic analysis. By analyzing the two-dimensional gels, it were detected 410, 136, 142, 109 spots at zero, two, seven and 14 days, respectively, that were identified in at least four separate gels (Fig.2).

Of the spots detected, 52 were expressed at all times of cultivation, from which 41 showed significant changes ($P<0.05$) in relation to the expression. At zero and two days, it were identified 24 common spots, of which 18 showed statistically significant differences. Seven common spots were expressed at two and seven days of culture, of which three showed significant changes. At seven and 14 days of culture, 24 common spots were detected, of which four showed significant differences. Among the times zero, two and seven days, 23 spots were found, of which 15 showed differences there between. At two, seven and 14 days, of the 16 spots detected, ten were statistically different (Fig. 3a and 3b).

Regarding unique spots, it were detected 311, 14, 20 and 17 spots at zero, two, seven, and 14 days of cultivation, respectively (Fig. 3a). The unique spots common to more than one culture period were only analyzed with MALDI-ToF when presenting volume exceeding 2 %. Still regarding the common spots, it were identified only those with a significant difference of expression (Fig. 3b).

The analysis of the different culture times in the induction of PLBs by 2-DE showed an increase and/or decrease in expression of proteins that may be involved in the induction and formation of these structures (Fig. 2; Table 1). Of the 12 identified proteins, present at all cultivation times, it was found that four proteins (spots 76, 238, 341 and 548) have had their expression decreased simultaneously to the days of cultivation and the formation of PLBs. Three proteins increased the expression in conjunction with the formation of PLBs, corresponding to spots 70, 82 and 125. Four proteins were preferentially expressed at two days of culture (spots 302, 329, 603 and 244). Protein 61 showed differential expression at zero and 14 days of culture (Tab.1). Of the two identified proteins, present at zero and two days, one protein is widely expressed at

day zero (spot 88) and the other strongly expressed at two days of cultivation (spot 536). A protein was identified at seven and 14 days of culture (spot 420) and another protein at zero, two and seven days of culture (spot 106), with significant differences in their expression. A protein was coincident at two, seven and 14 days of cultivation, and its expression was strongly differentiated at two and seven days, presented by the spot 153 (Tab.1).

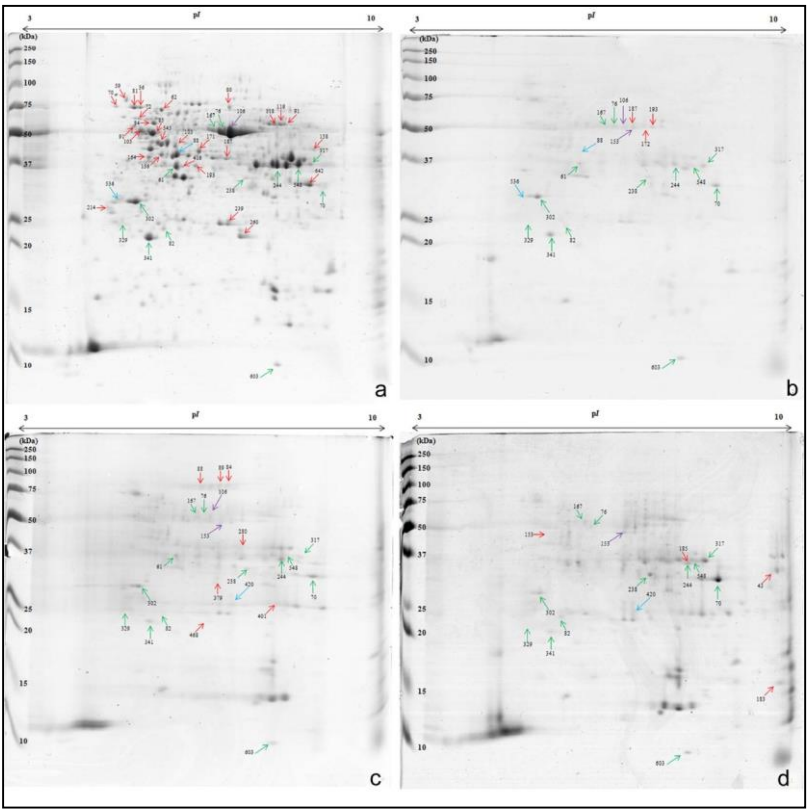


Fig. 2 Two-dimensional gel electrophoresis of different cultivation times of PLB *C. tigrina*. **(a)** Control; **(b)** 2 days; **(c)** 7 days; **(d)** 14 days. Red arrows represent the unique spots in each cultivation time; Green arrows represent sports present in all cultivation times; Blue arrows represent the sports present in two cultivation times and purple arrows represent the spots present in three crop times.

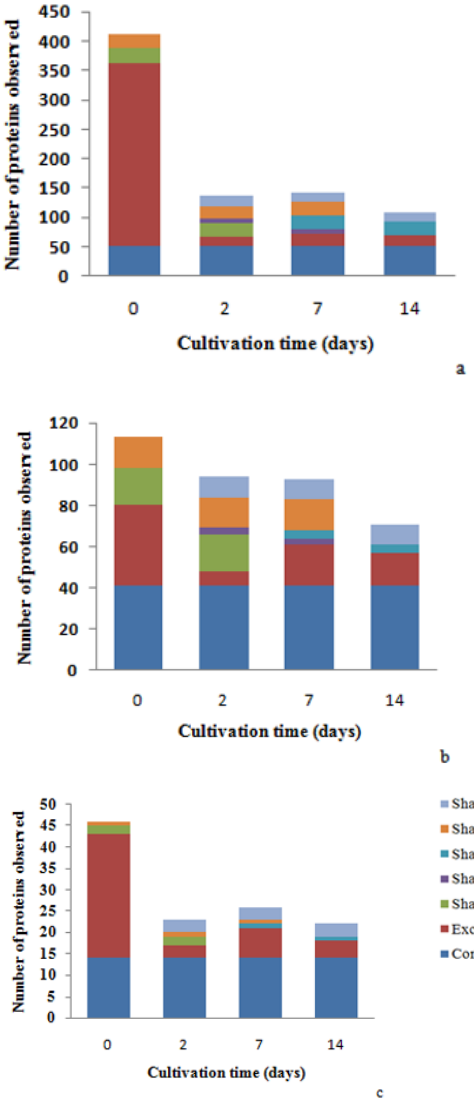


Fig. 3 Number of unique and common proteins in different times of cultivation of PLBs of *C. tigrina*. **(a)** Proteins expressed in different cultivation times; **(b)** Proteins collected for analysis by MALDI-TOF which showed significant differences ($P < 0.05$) compared to the

expression and percentage of volume > 2 %; **(c)** Proteins identified in MALDI-TOF.

4.4.3 Identification of proteins by fingerprint with trypsin/MALDI-TOF

Overall, 141 proteins were selected for identification by MALDI-TOF/MS, of which 64 were identified (Fig. 3c). Of these, 19 proteins were eliminated, which had no homology to any available database. The mass spectra of the peptides were manually inspected.

On the other hand, in each case at least one of the autolytic fragments of trypsin was observed, indicating that the sample was exposed to the protease and that the mass spectrometer operated correctly. The lack of peptides in some samples can be attributed to technical limitations such as small amounts of protein, incomplete digestion of the protein, inadequate extraction of gel peptides or SDS residues which blocked the digestion, as described by Ross et al. (2002) and Lippert et al. (2005).

The experimental masses of tryptic peptides were compared to databases using Mascot program and compared to all higher plants, because the *C. tigrina* genome was not sequenced. The identified proteins are listed in Table 1 and 2, indicating their expression when they were expressed in more than one culture time measured by the normalized spot volume (Software Image Master Platinum/GE Healthcare). The distribution of molecular weight and pI of proteins based on database information can be found in Tables 1 and 2. It were identified more acidic proteins (72.6 %), especially in the pI range of 5-7. Regarding molar mass, it were identified more proteins with molecular weight between 20-100 kDa (Fig. 2; Tab.1 and 2). In most cases, the theoretical molecular weight was similar compared to the experimental values (R^2: 0.70). However, some points migrated differently than expected, probably due to the degradation or modification of the protein.

In relation to the isoelectric point, it can be seen that there was a more dispersed significance of data (R^2:0.19). These observed deviations may have occurred due to unknown post-translational modifications.

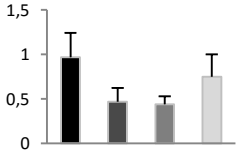
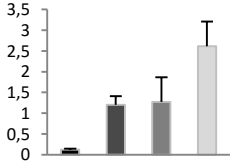
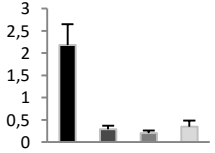
4.4.4 Classification of the identified proteins

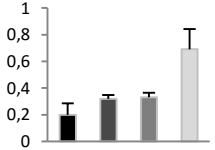
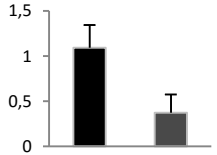
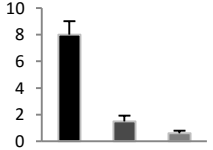
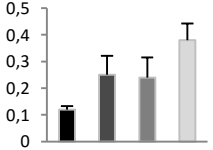
For the interpretation of data related to the proteome or genome expression, the first step is to group the proteins or genes based on their

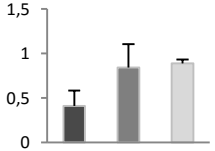
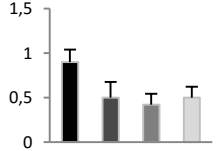
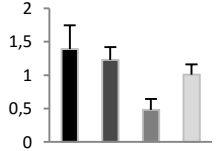
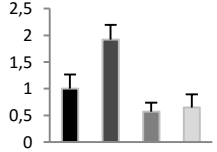
gene ontology (GO). The most complete GO is from *Arabidopsis thaliana* (Rhee et al. 2003).

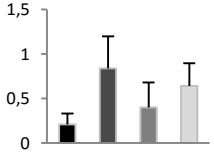
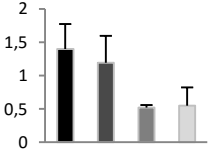
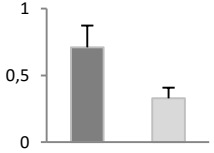
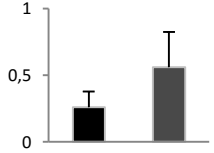
Thus, the proteins identified in each culture time were classified regarding the molecular function, biological process and cellular component (Fig. 4a, 4b and 4c) as defined by terms of the gene ontology in their UniProtKB entries. According to the classification, it was found that most of the proteins were localized in the chloroplasts, followed by the cytoplasm at all cultivation times (Fig. 4a). In relation to the molecular function, at zero, two, seven, and 14 days of cultivation it was found that most of the proteins showed energetic activity with 56; 75; 73 and 71%, respectively, but with decreasing percentages (Fig. 4b). Considering the biological process in which they were involved, it were identified proteins related mainly to photosynthesis, carbohydrate metabolism and defense to stress (Fig. 4c).

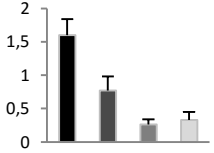
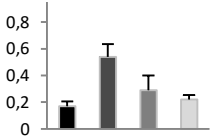
Table 1 Differentially expressed proteins identified by mass spectrometry analysis to the different cultivation times of *C. tigrina* PLBs.

Spot[a]	Access number[b]/ Species	Mascot Scores/ Coverage (%)	Identification	Number Peptides	Mass Th/Ex[c]	pI Th/Ex[d]	% Volume ±DP
61	MDHC_BETVU *Beta vulgaris*	78/ 31	Malate dehydrogenase, cytoplasmic	11	35/35	5.8/5.9	
70	ACC1_ORYSJ *Oryza sativa* subsp. *japonica*	66/8	Acetyl-CoAcarboxylase1	14	25/31	6.0/8.8	
76	RBL_NYPFR *Nypa fruticans*	154/33	Ribulose bisphosphate carboxylase large chain	16	51/51	6.2/6.9	

82	MD37E_ARATH *Arabidopsis thaliana*	56/25	Probable mediator of RNA polymerase II transcription subunit 37e	23	71/23	5.0/5.6	
88	ATPB_BRASC *Brasenia schreberi*	63/35	ATP synthase subunit beta, chloroplastic	11	53/51	5.2/5.2	
106	RBL_DEPGR *Deppea grandiflora*	150/26	Ribulose bisphosphate carboxylase large chain	13	52/53	6.3/6.5	
125	CATA3_MAIZE *Zea mays*	66/15	Catalaseisozyme3	7	57/30	6.4/7.6	

153	MD37D_ARATH *Arabidopsis thaliana*	58/22	Probable mediator of RNA polymerase II transcription subunit 37c	10	71/72	5.0/5.1	
238	MDHM_CITLA *Citrullus lanatus*	96/4	Malate dehydrogenase, mitochondrial	2	36/34	8.8/7.8	
244	ALF_MAIZE *Zea mays*	51/3	Fructose-bisphosphate aldolase, cytoplasmic isozyme	2	39/36	7.5/7.7	
302	PSBO_WHEAT *Triticum aestivum*	182/8	Oxygen-evolving enhancer protein 1, chloroplastic	2	34/29	8.7/4.8	

329	PUR2_ARATH *Arabidopsis thaliana*	63/17	Phosphoribosylamine-glycine ligase, chloroplastic	7	57/27	5.5/4.5	
341	PSBP_FRIAG *Fritillaria agrestis*	93/3	Oxygen-evolving enhancer protein 2, chloroplastic	1	28/22	8.3/5.0	
420	CD48A_ARATH *Arabidopsis thaliana*	64/22	Cell division control protein 48 homolog A	12	90/80	5.3/7.0	
536	RR8_LIRTU *Liriodendron tulipifera*	27/12	30S ribosomal protein S8, chloroplastic	2	15/29	10.1/4.5	

548	CYSK2_ORYSJ *Oryza sativa* subsp. *japonica*	31/7	Cysteine synthase	2	34/14	5.3/7.8	
603	PSAC_AETCO *Aethionema cordifolium*	166/27	Photosystem I iron-sulfur center	2	95/100	6.6/1.7	

[a] the numbering corresponds to the protein spot number in 2-D gels.
[b] Accession number in UniProtKB
[c] Molecular mass, Th theoretical, Ex experimental
[d] pI isoelectric point, Th theoretical, Ex experimental

Table 2 Exclusive proteins identified by mass spectrometry analysis for different cultivation times of *C. tigrina* PLBs.

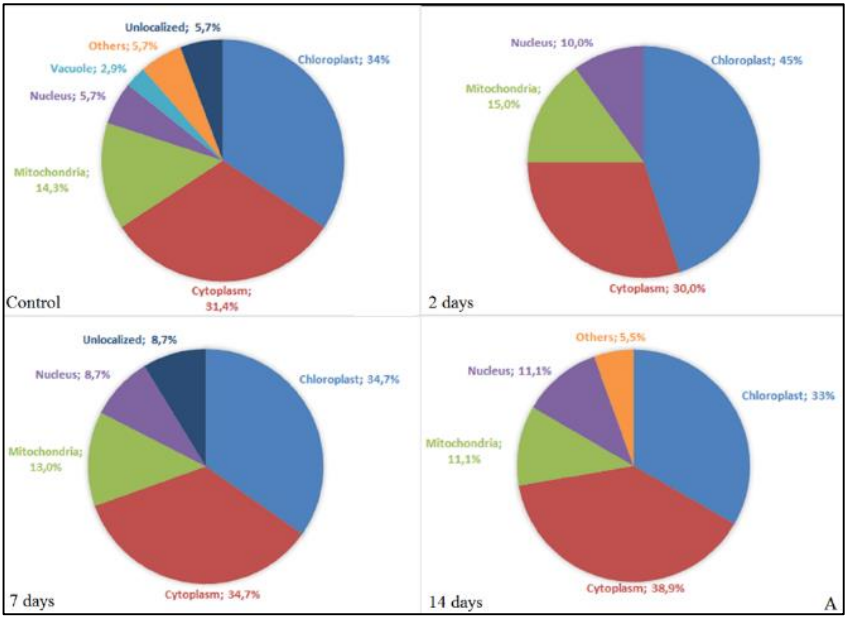
Spot[a]	Access number[b]	Mascot Scores	Coverage (%)	Identification	Taxonomy	Number Peptides	Mass Th/Ex[c]	pI Th/Ex[d]	Relative Volume ±SD
Control									
36	HSP7C_PETHY	96	28	Heat shock cognate 70 kDa protein	*Petunia hybrida*	15	71/ 74	5.1/ 5.0	0,35±0,94
59	HSP81_ORYSI	98	28	Heat shock protein 81-1	*Oryza sativa* subsp. Indica	18	80/ 80	5.0/ 4.8	0,30±0,07
62	VATA_DAUCA	121	33	V-type proton ATPase catalytic subunit A	*Daucus carota*	16	69/ 67	5.2/ 5.3	0,33±0,05
70	HSP7F_ARATH	63	11	Heat shock 70 kDa protein 6, chloroplastic	*Arabidopsis thaliana*	7	76/ 75	5.0/ 4.8	0,37±0,12
81	MD37E_ARATH	90	29	Probable mediator of RNA polymerase II transcription subunit 37e	*Arabidopsis thaliana*	16	72/ 71	4.9/ 5.0	0,74±0,12
84	ATPA_PHAAO	165	5	ATP synthase subunit alpha, chloroplastic	*Phalaenopsis aphrodite*	12	55/ 57	5.3/ 5.1	0,47±0,17

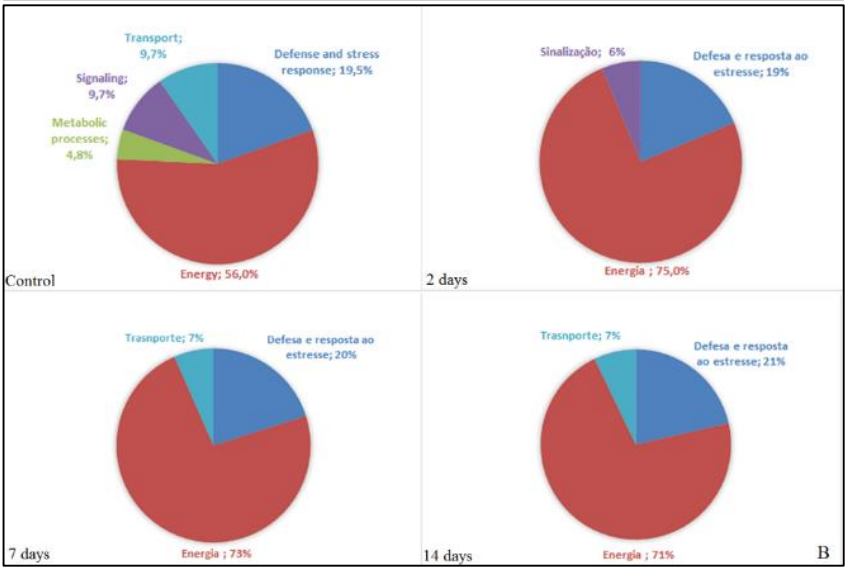
91	VATB2_ARATH	92	34	V-type proton ATPase subunit B2	*Arabidopsis thaliana*	15	54/ 53	5.0/ 4.3	0,29±0,09
103	ATPBM_NICPL	113	42	ATP synthase subunit beta, mitochondrial	*Nicotiana plumbaginifolia*	17	59/ 50	5.9/ 5.0	0,63±0,21
104	ETR1_TOBAC	31	2	Ethylene receptor	*Nicotiana tabacum*	1	82/ 45	7.3/ 5.7	0,29±0,03
119	CATA1_ORYSI	67	21	Catalase isozyme A	*Oryza sativa* subsp. *Indica*	9	57/ 57	6.5/ 7.8	0,37±0,04
156	CAB_HORVU	70	35	Ribulose bisphosphate carboxylase/oxygenase activase B, chloroplastic	*Hordeum vulgare*	13	47/ 40	7.5/ 6.4	0,30±0,10
158	FDH_SOLTU	89	3	Formate dehydrogenase, mitochondrial	*Solanum tuberosum*	1	42/ 38	6.6/ 8.8	0,36±0,09
171	PGKH2_ARATH	63	16	Phosphoglyceratekinase2, chloroplastic	*Arabidopsis thaliana*	6	50/ 41	8.2/ 6.2	0,48±0,14
187	GLN15_ARATH	67	4	Glutamine synthetase cytosolic isozyme 1-5	*Arabidopsis thaliana*	2	39/ 39	6.2/ 6.8	0,32±0,07
214	1433_MESCR	69	35	14-3-3-like protein	*Mesembryanthemum crystallinum*	9	30/ 30	4.7/ 4.7	0,31±0,10
239	TPIS_MAIZE	66	35	Triosephosphate isomerase, cytosolic	Zea mays	7	27/ 28	5.5/ 7.2	0,25±0,07

260	CAH2_FLALI	61	5	Carbonicanhydrase2	*Flaveria linearis*	1	21/ 26	6.2/ 7.3	0,40±0,07
416	MDH_TOBAC	72	33	Malate dehydrogenase	*Nicotiana tabacum*	9	36/ 36	5.9/ 6.3	0,66±0,39
2 days									
172	RBL_BAMML	59	12	Ribulose bisphosphate carboxylase large chain (Fragment)	*Bambusa multiplex*	7	49/ 55	6.6/ 6.9	0,61±0,13
187	TI441_ARATH	14	2	Mitochondrial import inner membrane translocase subunit TIM44-1	*Arabidopsis thaliana*	1	54/ 53	6.6/ 6.4	0,98±0,45
7 days									
88	METE_CATRO	47	2	5-methyltetrahydropteroyltriglutamate —homocysteine methyltransferase	*Catharanthus roseus*	1	85/ 81	6.1/ 6.4	0,51±0,17
89	ATPB_LOTJA	61	30	ATP synthase subunit beta, chloroplastic	*Lotus japonicus*	9	53/ 82	5.3/ 6.7	0,53±0,20
280	FDH_HORVU	41	3	Formate dehydrogenase, mitochondrial	*Hordeum vulgare*	1	41/ 41	6.9/ 7.1	0,44±0,10
379	EX84C_ARATH	22	1	Exocyst complex component EXO84C	*Arabidopsis thaliana*	1	87/ 31	4.1/ 6.7	0,30±0,03

401	PDRP1_ORYSI	27	1	Probable pyruvate, phosphate dikinase regulatory protein, chloroplastic	*Oryza sativa* subsp. *indica*	1	51/ 25	9.5/ 7.6	1,0±0,38
468	DRL10_ARATH	27	1	Probable disease resistance protein RF45	*Arabidopsis thaliana*	1	11/ 24	6.9/ 6.5	0,39±0,08
14 days									
43	DF141_ARATH	8	14	Defensin-like protein 141	*Arabidopsis thaliana*	1	92/ 33	8.0/ 9.7	0,75±0,15
153	ATPBM_MAIZE	103	36	ATP synthase subunit beta, mitochondrial	*Zea mays*	13	59/ 51	6.0/ 5.2	0,58±0,16

[a] the numbering corresponds to the protein spot number in 2-D gels.
[b] Accession number in UniProtKB
[c] Molecular mass, Th theoretical, Ex experimental
[d] pI isoelectric point, Th theoretical, Ex experimental


Unlocalized; 5,7%
Others; 5,7%
Vacuole; 2,9%
Nucleus; 5,7%
Mitochondria; 14,3%
Chloroplast; 34%
Cytoplasm; 31,4%
Control
Nucleus; 10,0%
Mitochondria; 15,0%
Chloroplast; 45%
Cytoplasm; 30,0%
2 days
Unlocalized; 8,7%
Nucleus; 8,7%
Mitochondria; 13,0%
Chloroplast; 34,7%
Cytoplasm; 34,7%
7 days
Others; 5,5%
Nucleus; 11,1%
Mitochondria; 11,1%
Chloroplast; 33%
Cytoplasm; 38,9%
14 days
A


Transport; 9,7%
Signaling; 9,7%
Metabolic processes; 4,8%
Defense and stress response; 19,5%
Energy; 56,0%
Control
Sinalização; 6%
Defesa e resposta ao estresse; 19%
Energia ; 75,0%
2 days
Trasnporte; 7%
Defesa e resposta ao estresse; 20%
Energia ; 73%
7 days
Trasnporte; 7%
Defesa e resposta ao estresse; 21%
Energia ; 71%
14 days
B

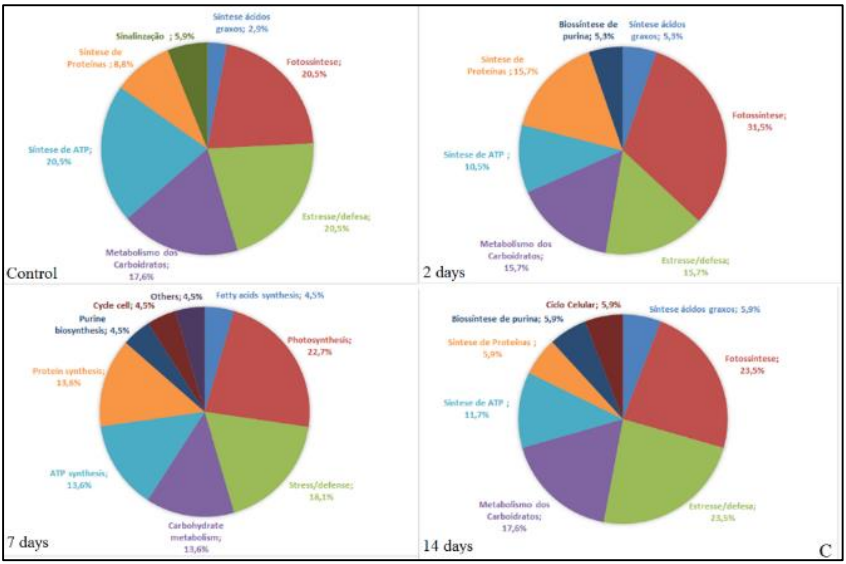


Fig. 4 Ontology classification of differentially expressed proteins in PLBs cultivation times in *C. tigrina*. **(a)** Cellular component; **(b)** Molecular function; **(c)** Biological process.

4.5 DISCUSSION

4.5.1 Early morphogenesis of the protocorm-like bodies

Histological evaluation showed distinct morphological features during the cultivation times. These data are consistent with the results found by Liz (2013), in which leaves of *C. tigrina*, inoculated and cultured *in vitro*, showed evidence of PLBs induction at seven days of culture, when epidermal cells began to undergo mitotic divisions, giving birth to cell differentiation and formation of the PLBs tissues. Proliferation of PLBs happened gradually, and at 14 days of cultivation these structures had become more numerous and more voluminous.

Studies of the induction and formation of PLBs in *C. tigrina* were performed by Fritsch (2012), wherein it was verified that this morphogenetic route can be induced from leaf explants. Furthermore, it was found that the basal region had the highest percentage of induction, compared to the medial region. This result was similar to that found in our work, showing the efficiency of this regeneration protocol. Devi et al. (2013) observed that the basal portion of *Aerides odorata* Lour leaves had

meaningful response in the formation of PLBs when compared to the medial and apical region of the leaf.

According to Firoozabady and Moy (2004), the basal region of the leaf may respond better to the process of embryogenesis and/or organogenesis, as it is closer to the axillary meristem, and may contain both meristematic regions and newly formed tissues, with significant metabolic activity. The meristems and the newly originated tissues in shoots of plants have high auxin synthesis (Taiz and Zeiger 2009), and therefore such endogenous levels of leaf tissues close to the axillary meristems in *C. tigrina* should support the higher morphogenic activity in the differentiation of PLBs.

4.5.2 Comparison of the protein profiles between cultivation times in the initial formation of PLBs

Protein expression patterns associated with the induction of *C. tigrina* PLBs were screened using the 2-DE technology coupled to MALDI-ToF/MS and available databases, where it was possible to generate significant and innovative information regarding proteomic aspects at functional and molecular level. This study constituted the first proteomic analysis in *C. tigrina.*

In this study, many proteins were expressed at day zero (410 spots), decreasing the expression in other cultivation times. Between two and seven days of cultivation, morphological changes begin to occur, being identified unique and differentially expressed proteins. At 14 days, when it was possible to demonstrate the formation of PLBs, 109 spots were detected. Tan et al. (2013), working with nodal explant of *Vanilla planifolia*, found the expression of 265 proteins in nodal explant (initial explant) and 179 proteins at 15 days of culture, when there was initial formation of callus.

The accumulation of proteins during the development of somatic embryos of *Acca sellowiana* was studied by Cangahuala-Inocente et al. (2009), in which it was observed that relatively few proteins were present in mature stages of somatic embryos, especially in the cotyledonary stage, and in the remaining stages there was greater expression of proteins. These results show that proteins are dependent on the developmental stages and the tissue analyzed, and in our study and in the study by Tan et al. (2013), few proteins were detected in the initial formation of PLBs and callus, respectively, compared to the initial explant.

This study indicated that the initial phase of induction and formation of PLBs caused significant changes in relation to the protein

profile. Of the 45 identified proteins, 18 proteins were expressly exclusive to zero time (initial explant), two proteins were expressed at two days, six proteins expressed at seven days, and at 14 days, two proteins were expressed. In addition to these proteins, many were differentially expressed in the different culture times. These observations suggest that some genes would be involved in the regulation of the initial formation of *C. tigrina* PLBs. The main identified proteins are discussed below based on their functional classification.

4.5.3 Energy metabolism and carbohydrates

The proteins involved in metabolic and energetic processes represent the largest group of differentially expressed proteins in this study, demonstrating the important role in the formation of PLBs. Plant cells adapt to environmental conditions, altering gene expression and rearranging the metabolic pathways and physiological processes (Rode et al. 2012).

In this study, among the identified proteins related to metabolic and energetic processes, the proteins Malate dehydrogenase cytoplasmic (spot 61), Ribulose bisphosphate carboxylase large chain (spot 76), Malate dehydrogenase, mitochondrial (spot 238) and Fructose-bisphosphate aldolase, cytoplasmic isozyme (spot 244) were abundantly expressed at day zero of culture, reducing their expression at two and seven days, increasing again at 14 days. High levels of expression of proteins at day zero may have been triggered by the exogenous supply of sucrose during *in vitro* cultivation (Winkelmann et al. 2006). The increased expression of these proteins at 14 days of culture most likely occurred due to the energy demand caused by rapid growth and cell division in the formation of PLBs. The Ribulose bisphosphate carboxylase large chain (spot 76) and Fructose-bisphosphate aldolase (spot 244) are glycolytic enzymes involved in the organogenesis *in vitro* and in the somatic embryogenesis processes (Fortes et al. 2008). Lyngved et al. (2008), working with *Cyclamen persicum*, found a significant increase of glycolytic enzymes and proteins involved in energy metabolism in the development of embryos. Palama et al. (2010) found that an increase in glycolytic enzymes would probably be related to cell division and differentiation in *Vanilla Planifolia* callus. Not with standing, Tan et al. (2013) found that proteins related to energetic and metabolic processes were expressed only at the nodal tissue of *V. planifolia*, and, as this tissue is chlorophylled, it is expected that a significant number of abundant proteins present therein is involved in

energy metabolism. According to the authors, further experiments are needed to determine whether some of these proteins are truly involved in the initial formation of callus.

The enzyme Malate dehydrogenase-MDH (spot 61 and 238) was also present in the PLBs induction process. This enzyme catalyzes the final reaction of the tricarboxylic acid cycle, regenerating the oxaloacetate (Bouthour et al. 2012). In work with grape berry ripening, Sharathchandra et al. (2011) reported that the MDH expression level was low at the early stage of maturation, increasing after ripening. The accumulation of MDH transcripts has been reported to be involved in cell division and differentiation and in salt stress tolerance (Yao et al. 2011).

The acetyl-CoAcarboxylase1 (ACCase 1) is a multifunctional enzyme that catalyzes the first step of the synthesis of fatty acids, the carboxylation of acetyl-CoA, to form malonyl-CoA (O'Hara et al. 2002). This enzyme was expressed at all cultivation times, and its expression increased gradually with the advancing formation of PLBs, demonstrating essential role in this process (spot 70). Most fatty acids are used for the synthesis of membranes and storage lipids, and fatty acid synthesis is required for the initial phase of cell growth and development (Sasaki and Nagano 2014).

The growth of embryogenic cultures is usually accompanied by changes in the synthesis and mobilization of proteins, carbohydrates and lipids (Cangahuala-Inocente et al. 2009), in which they act as specific signals in the signal translation chain or in the supply of substrates and energy required to cell division (Nomura and Komamine 1995).

In the development of secondary embryos of cassava (*Manihot esculenta*), the abundance of proteins related to energy metabolism was attributed to the high metabolic activity required to maintain intense cell division in developing embryos (Baba et al. 2008). In this study, it was observed the significant presence of proteins related to the metabolic and energetic processes, suggesting the direct involvement of those with the induction and formation of PLBs.

4.5.4 Defense and stress response

The stress-response proteins have been identified as the second largest group of differentially expressed proteins in this study. These proteins have been reported in dividing cells and tissues (Correia et al. 2012), having among other functions the protection. Similar results were found in callus of *Oryza sativa* (Yin et al. 2008) and *V. planifolia* (Tan et al. 2013), where the great majority of the proteins identified in the early

stages of callus differentiation were classified as stress/defense proteins. According to Sharifi et al. (2012), it is possible that the prevalence of the stress-response proteins is assigned regarding the conditions under which the cells are subjected during *in vitro* cultivation. Thus, in the present study, the explants used for the induction of the PLBs were isolated from the mother plant causing injury/wound in the explant base. Of the stress-related proteins identified, three heat shock proteins (HSP), heat shock cognate70-KDa protein (spot 36), heat shock protein 81-1 (spot 59) and heat shock cognate 70-KDa protein 6, chloroplastic (spot 70), were exclusively expressed in the initial explant (day zero). Whereas they were absent in other cultivation times (two, seven, and 14 days), these HSPs may have been expressed in response to the condition of the culture medium used during the initial stages of culture. According to Tan et al. (2013), the presence of heat shock protein exclusively in the nodal explant relates to plant growth regulators used during the initial stages of culture.

Other heat shock proteins expressed in this work are the proteins Probable mediator of RNA polymerase II transcription subunit 37e (spot 82, present at all cultivation times) and Probable mediator of RNA polymerase II transcription subunit 37c (spot 153, present at times two, seven and 14). These proteins are molecular chaperones that assist in the folding of proteins that are unfolded or deformed under stress conditions. In cooperation with other chaperones (Hsp70s), these proteins stabilize proteins against preexisting aggregation, mediating the folding of polypeptides recently translated in the cytosol, as well as within organelles (Osborn and Greer 2015).

HSPs represent a class of proteins that are expressed at high levels because of the many stresses, including high temperature (Liu et al. 2012; Yin et al. 2012). The vast majority of HSPs belongs to a class of molecular chaperones, being expressed under various types of stress, including maintenance of cells during the initial culture or during plant development *in vitro* (Imin et al. 2010). They are also known for their roles in maintaining the correct folding of synthesized proteins, preventing protein aggregation (Tonietto et al. 2012). The heat shock proteins have been reported to be highly expressed during somatic embryogenesis of *Vitis vinifera* (MARSONI et al., 2008), *Cyphomandra betacea* (Correia et al. 2012) and *Elaeis guineensis* (Silva et al. 2014). The results of this study suggest that increased levels of chaperone proteins can play a key role in the initiation of PLBs possibly for easing tensions associated with complete reprogramming during the initiation of these structures.

At two days of culture, it was observed the presence of an exclusively expressed protein, the protein Mitochondrial import inner

membrane translocase subunit TIM44-1 (spot187). This protein is a key component of the TIM complex (Translocase Complexes of the inner membrane), a complex necessary for the translocation of proteins from the inner membrane of the mitochondria matrix. This complex is composed of subunits, wherein the TIM44 subunit recruits the mitochondrial heat shock proteins (heat shock 70 kDa) into the matrix using ATP as an energy source (Ishihara and Mihara 1998; Murcha et al. 2003). Hence, it is supposed that this protein may be interconnected with the heat shock proteins, serving as an adapter to occur the transport and signaling of these proteins.

Another stress-related protein identified in our study was the Catalase isozyme 3 (spot 125), found at all times of cultivation, the increased expression being proportional to the induction of PLBs. At 14 days of culture, when the cells were in full cell division, with high proliferation of PLBs, the expression of Catalase was highly evident, playing an essential role in the protection against reactive oxygen species (ROS). According to Foyer and Noctor (2011), when the photosynthetic and respiratory rates rise, or when the plant undergoes stress, high levels of reactive oxygen species (ROS) are generated, whose homeostasis is controlled by a network of enzymatic and non-enzymatic components, including catalase, superoxide dismutase (SOD) and glutathione-ascorbate cycle enzymes. Catalase, together with the ascorbate peroxidase, have a fundamental role in the removal of ROS produced during photosynthesis or during some stress that the plant suffers (Gill and Tuteja 2010), which may explain their different expression in the induction of PLBs in *C. tigrina*. The ROS are highly reactive and toxic molecules, being produced continuously as by-products of various metabolic pathways that are located in different cellular compartments of plants (Gill and Tuteja 2010; Zhang et al. 2013). High levels of ROS can damage proteins, lipids, carbohydrates and DNA, which can ultimately lead to cell death (Mirzaei et al. 2012). The accumulation of proteins related to oxidative stress has been considered as an essential component of the stress induced by somatic embryogenesis (Sharifi et al. 2012). Recent proteomic studies indicated that the proteins induced after oxidative stress are linked with increased rate of cell division (Holmes et al. 2006; Tan et al. 2013). These results emphasize the great functional role of some proteins related to ROS in the regulation of cell division.

4.5.5 Protein synthesis

Physiological and metabolic changes during cell reprogramming require the production, assembly, and stabilization of newly synthesized proteins (Correia et al. 2012). Such changes are necessary to establish a new cellular phenotype (Sharifi et al. 2012). In work with saffron, Sharifi et al. (2012) reported that about 20 % of the proteins found in embryogenic and non-embryogenic callus were involved in the protein synthesis and processing, reporting that these are necessary for the development of embryos. So it is not surprising that in this study proteins are involved in the metabolism of amino acids and proteins.

The glutamine synthetase - spot 187 (present at day zero) is known to play a key role in the nitrogen metabolism, increasing the synthesis of amino acids necessary for protein synthesis (Miflin and Habash 2002). The 30S ribosomal protein S8- spot 536 (present at zero and two days of cultivation) is one of the major rRNA-binding proteins, coordinating the processing of the 30S subunit, being involved in protein biosynthesis (Davies et al. 1996). The enzyme Cysteine synthase (spot 548) was also expressed in this study, at all times of cultivation, and this enzyme is related to amino acid synthesis (Wirtz and Hell 2006).

Correia et al. (2012) found that one of the major functional groups of proteins found in embryogenic callus had relation with biosynthesis, suggesting that the protein metabolism is a key factor in the induction of somatic embryogenesis. Palama et al. (2010) reported that the proteins glutamine synthetase and proteasome complex have been abundantly expressed in *Vanilla* callus during the early stages of differentiation. Tan et al. (2013) also showed the presence of an abundant protein in *Vanilla* callus (elongation factor 1-delta1), involved in the metabolism of amino acids and proteins.

There are also the identified proteins that do not reside in these three functional groups, for example, the spot 420, which was identified as Cell division control protein 48 homolog A (CDC48). This protein is essential for cytokinesis, cell expansion and differentiation in plants (Park et al. 2008). The presence of this protein was checked at seven and 14 days of culture, wherein it was verified increased cell division, thereby demonstrating a key role in the process of PLBs induction. Sharifi et al. (2012), working with embryogenic and non-embryogenic callus in saffron also reported the presence of this protein, demonstrating an important role in the proliferation and development of somatic embryos. Hirt et al. (1991) reported that the cell cycle genes play a key role in

somatic embryogenesis. CDC48 is a conserved homohexameric chaperone-like AAA-ATPase required for a variety of cellular processes.

4.6 CONCLUSION

This is the first proteomic analysis performed during the early development of PLBs in *C. tigrina*. The determination and comparison of the protein profile by means of proteomic techniques throughout the different culture times of PLBs showed differences in the expression pattern of proteins. It was possible to determine proteins whose expression and abundance were significantly altered during the induction process of the PLBs. Furthermore, the analyses allowed to identify unique proteins and those that were expressed simultaneously at the different times of cultivations.

The initial explant showed a different protein pattern compared to other times of cultivation, resulting in changes in this protein pattern during the induction of PLBs. This suggests the occurrence of changes in cellular metabolism and the subsequent initiation of a new development program, which depend on the synthesis of new proteins appropriate to ensure that their functions are carried out.

This study represents a basis for further research that will provide a complete understanding of the molecular mechanisms underlying the initiation and development of PLBs in *C. tigrina*.

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5. CAPÍTULO 2. DIFFERENTIALLY EXPRESSED PROTEINS IN THE DEVELOPMENT OF PROTOCORM-LIKE BODIES OF *Cattleya tigrina* A. Richard

5.1 ABSTRACT

During the development of *protocorm-like bodies* (PLBs) of *Cattleya tigrina*, differential protein profiles were obtained and analyzed. Leaves cropped in culture medium Murashige and Skoog (MS) supplemented with 9 μM TDZ for PLBs development were collected after 30, 60 and 100 days in culture. Samples were macerated in liquid nitrogen, followed by total protein extraction using phenol as extraction buffer. Later, two-dimensional electrophoresis and protein identification by mass spectrometry in the MALDI-TOF-MS were conducted. Images analyzed using ImageMaster 2D Platinum v. 7.0 software resulted in detection of 122, 132 and 447 protein spots 30, 60 and 100 days respectively. 36 proteins were identified, and their differential expression during PLBs development. Proteins related to energy and carbohydrate metabolism, cell proliferation, protein processing and secondary metabolism and phytohormones have a key role in the process of development of PLBs. Enolase was present only for 100 days of cultivation appears to be a strong candidate as a molecular marker in this development phase. Our results suggest profound metabolic changes in the development of PLBs in *C. tigrina*.

Keywords: 2-DE, Orchid, protocorm like-bodies.

5.2 INTRODUCTION

Cattleya tigrina is an epiphyte or rupicolous species endemic to the Atlantic Forest biome that occurs in the Northeast, Southeast and South of Brazil (Barros 2016). Drastic reduction of its habitat, indiscriminate collection and population decline culminated in the inclusion of this species to the Red Book of Brazilian Flora, as "vulnerable to extinction" (Brasil 2013). Therefore, *in vitro* propagation techniques are seen as an effective alternative to its propagation and conservation. In addition, *in vitro* propagation techniques have been widely used with success in the propagation of different terrestrial and epiphytic orchids (Díaz and Álvarez 2009), such as the case of *C. tigrina* (Fritske 2012). During *in vitro* culture establishment, a morphogenic response pattern defined as

protocorm-like bodies (PLBs) has been described for various orchid species (Park et al. 2003; Yang et al. 2010; Fritske 2012; Gantait et al. 2012).

These globular structures were named after their similarity to embryos of zygotic origin (protocorms) (Arditti 2008). Its induction has been reported from meristematic apices in *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii 1993) and *Cymbidium* (Subramanium and Taha 2003), from floral stalks in *Phalaenopsis* (Ichihashi 1992), from root apices in *Phalaenopsis* (Tanaka et al. 1976), and through leaf segments in *Doritaenopsis* (Park et al., 2002), *Aerides crispum* (Sheelavanthmath et al. 2005) and *Cattleya tigrina* (Fritske 2012). For *C. tigrina*, some studies aimed to optimize the culture medium composition, in order to induce and develop PLBs (Fritske 2012), and to characterize the induction and development process, in terms of morpho-anatomical and biochemical changes (Liz 2013; Almeida 2014). Moreover, efforts were made recently to characterize differentially expressed proteins during PLBs induction in *C. tigrina* (De Conti et al. 2014) using proteomic approaches. However, proteins associated with the development of these structures have not been studied. Proteomics is an overall analysis strategy that provides information about a plurality of processes in complex events, such as PLBs induction (De Conti et al. 2014). Proteins directly influence cellular biochemistry, providing a more precise cell change analysis during growth and development (Chen and Harmon 2006). Therefore, the study of proteomics on tissue culture may help detect proteins that are regulated by phytohormones (Takác et al. 2011), which allows PLBs formation in our case.

In orchids, proteomic studies have been mainly conducted to investigate protein profile changes during germination (Valadares et al. 2014) and callus formation (Palama et al. 2010; Tan et al. 2013), showing relevant information on genes and expressed proteins. However, there are few studies related to the morphogenic response pattern defined as protocorm-like bodies (De Conti et al. 2014). Thus, understanding molecular mechanisms at protein level in explants with efficient morphogenic potential for PLBs formation is of great interest to improve micropropagation protocols. In this context, this study aimed to identify and characterize differentially expressed proteins during PLBs development in *C. tigrina*, in order to understand the factors that modulate this development pattern.

5.3 MATERIALS AND METHODS

5.3.1 Plant material

Leaf explants (±1cm) were obtained from young plants, micropropagated and maintained *in vitro* in the Development Physiology Laboratory and Plant Genetics (LFDGV), the Agricultural Science Center (CCA), Universidade Federal de Santa Catarina (UFSC), Florianópolis, Santa Catarina State, Brazil. The leaf explants were detached and inoculated MS medium (Murashige and Skoog 1962), supplemented with 9 μM TDZ (Thidiazuron) for PLBs induction, which was previously established by (Fritsche 2012).

After inoculation of the explants, they were kept in growth room with an average temperature of 25±2 °C, 16-h light and 8-h dark, with a light intensity of 50 μmol m^{-2} s^{-1}. To thirty, sixty and hundred days of cultivation, plant materials were collected for histological and proteomic analysis. For histological studies the five explants of each culture time were collected. With regard to proteomic analysis were collected biological six replicates for each cultivation time, each replica was formed by 500 mg of fresh pasta starting from a pool of plant material, corresponding basically to 30 leaf explants and stored at -80 °C for later analysis.

5.3.2 Histological analysis

The material was fixed in 2.5 % paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. The samples were dehydrated in increasing series of ethanol aqueous solutions (Schmidt et al. 2009). 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).

5.3.3 Proteomic analysis

Protein Extraction

The extraction of total proteins from leaf tissue was performed following the method of Carpentier et al. (2005) with modifications. In brief, 0.5g of leaf tissue collected from each of the selected seedlings per treatment 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 100 mM of ammonium acetate in methanol (1:5 v/v), proteins were maintained overnight at -20 °C. The tube was centrifuged for 30 min at 10,000g 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 (7 M urea, 2 M thiourea, 3 % CHAPS, 2 % IPG-buffer, 1,5 % DTT), by mild vortexing and stored at -20 °C. Protein quantification was determined by means os copper-based method using 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 (7 M Urea, 2 M Thiourea, 3 % CHAPS, 2 % IPG-buffer, 0.002 % Bromophenol blue, and 0,2 % DTT) containing 600 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, 6 M 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). Gels were stained with Coomassie blue (1 %

Coomassie Blue G-250, 2 % H_3PO_4, 8 % $(NH_4)_2SO_4$, 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). The spots were identified and selected based on the comparative analysis of the gels. 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 protein spots that were reproducibly found in at least four biological replicates were included in further examinations. The proteins were found to be differentially expressed between the times of cultivation, while presenting a change of ± 2 times the percentage of volume and representing significant difference (Student's t-test $p < 0.05$) were included in the analysis. 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 Mass Spectrometry Analysis

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 % acetonitrile 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 spectrum was submitted to identification using MASCOT on line (Matrix Science, London, UK) and SwissProt databases. The parameters used for the acceptance of identification were: taxonomy: *Viridiplantae*; enzyme: trypsin, missed cleavages: 1; fixed modifications: carbamidomethyl cysteine; variable modifications: oxidized methionine; mass values: MH+, peptide mass tolerance 100 ppm and MS/MS tolerance of 0.5 Da. Positive identification was based on the significative Mascot score ($p<0,05$). Functional categorization and cellular component of proteins was performed according to the GO (Gene Ontology) by searching the protein knowledgebase (UniProtKB).

5.4 RESULTS

5.4.1 Histological analysis

Leaf explants cultured on induction medium allowed PLBs proliferation. After 30 and 60 days of cultivation (Fig. 1A to 1F), it was possible to verify the formation of large PLB amounts arising from the formation of new PLBs over the preexisting ones (Fig. 1A and 1D). After 60 days of cultivation, it was also possible to observe the beginning of leaf primordial formation (Fig.1E). After 100 days of cultivation, besides the formation of new PLBs over the preexisting ones, well-developed leaf primordial were found (Fig. 1G to 1I).

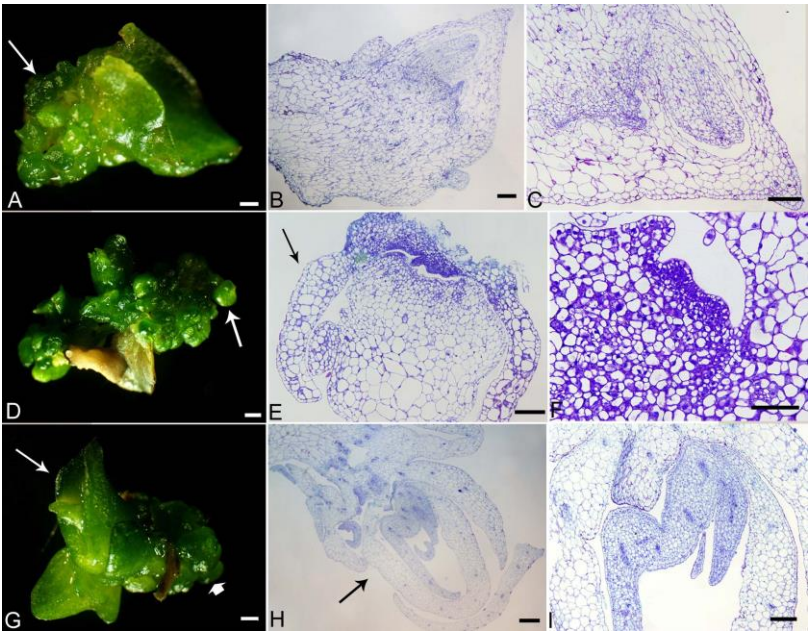


Fig. 1 Protocorm-like bodies (PLBs) formed at 30, 60 and 100 days after inoculation of leaf explants of *C. tigrina*. **a** General view of leaf inoculated at 30 days of culture, with lots of PLBs (arrow) concentrated in the leaf base. **b-c** Longitudinal section subjected to TB-O. **d** General view of leaf inoculated at 60 days of culture, yellowish already showing deterioration, but loads of PLBs (arrow) present in the leaf base. **e** Longitudinal section showing the beginning of the formation of leaf (arrow). **f** Longitudinal section subjected to TB-O. **g** General view of leaf inoculated at 100 days of culture with lots of PLBs (broad arrow) and leaf developed (arrow).**h** PLBs longitudinal sections showing developed leaves (arrow).**i** Longitudinal section subjected to TB-O. Bars: (A, B, D, G): 2mm; (C, E, F, I): 200µm; (H): 500µm.

5.4.2 Comparison of protein profiles in the development of PLBs

Protein profiles analysis during PLBs development showed qualitative and quantitative changes, in which it was possible to observe the cellular complexity of these structures at molecular level. Using 2D electrophoresis, it was possible to detect 122, 132 and 447 spots between 30, 60 and 100 days of cultivation, respectively, which were identified at

least in four separate gels (Fig.2). Comparative analysis during PLB development demonstrated that a large number of proteins were exclusively observed at each cultivation period, in which 39, 28 and 383 spots were detected after 30, 60 and 100 days of cultivation, respectively. 43 spots were common to all cultivation periods; 40 spots were common to both 30 and 60 days of cultivation, and 21 spots were common to 60 and 100 days of cultivation. Spots that were exclusive and common to more than one cultivation period were only analyzed through MALDI-TOF when their volume exceed 2%. In addition, common spots were only identified when they showed significant expression difference ($P < 0.05$).

5.4.3 Protein identification

Based on score significant values ($p < 0.05$) calculated by MASCOT software, 42 proteins were identified. Of these, six proteins were eliminated, as they were not homologous to any data base available. Peptide mass spectra were manually inspected.

Tryptic peptide experimental masses were compared to databases using the MASCOT program. In addition, they were compared to all higher plants, as the *C. tigrina* genome was not sequenced. Identified proteins were listed in Tables 1 and 2, indicating their expression when expressed in more than one cultivation period, measured by normalized spot volume (Image Master Platinum Software/GE Healthcare). Protein molar mass and pI distribution, based on database information, are found in Tables 1 and 2. Acidic proteins showed the highest number (61.1%), especially in the pI range between 5 and 7. Regarding molar mass, proteins with molecular weight between 20 and 100 kDa had the highest number (Fig. 2, Tab.1 and 2). In most cases, theoretical molecular weight was similar compared to experimental values (R^2: 0.62). However, some points migrated differently than expected, probably due to protein degradation or modification.

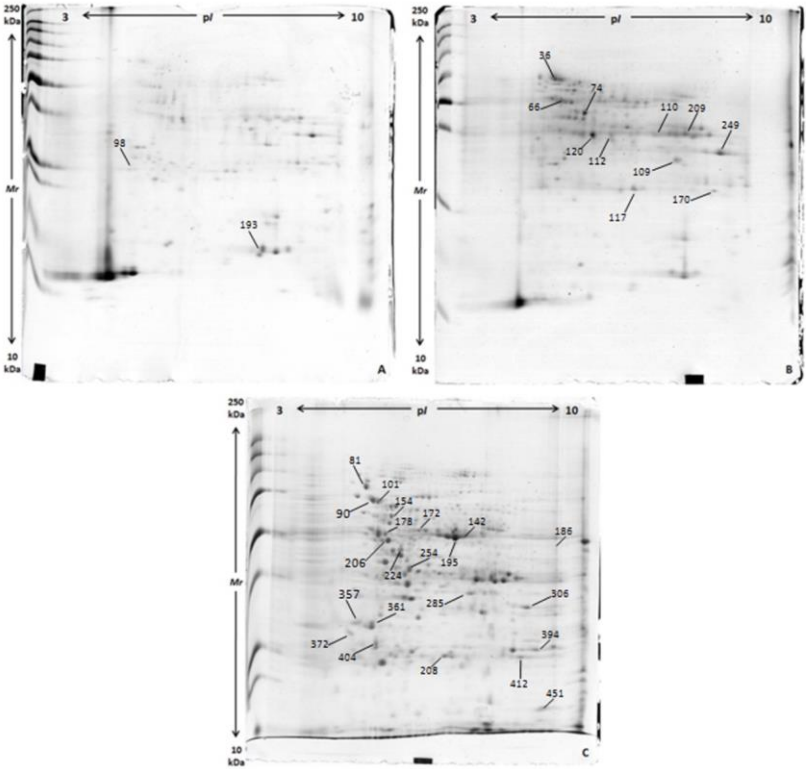
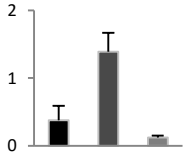
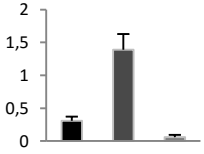
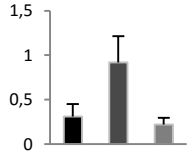
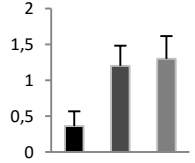
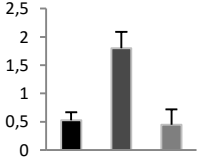
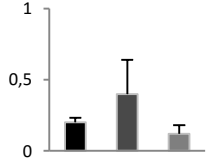
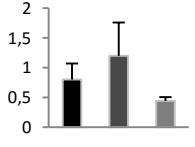
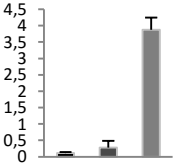
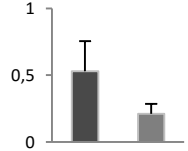
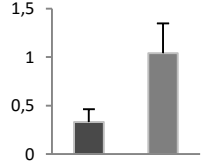


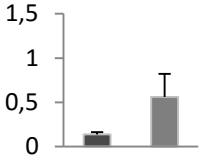
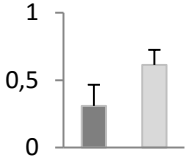
Fig. 2 Two-dimensional gel electrophoresis of different cultivation times of PLB *C. tigrina*. **(a)** 30 days; **(b)** 60 days; **(c)** 100 days. The numbered spots refer to the proteins identified by MALDI-TOF-MS.

Table 1: Differentially expressed proteins identified by mass spectrometry analysis to the different cultivation times of *C. tigrina* PLBs.

Spot[a]	Access number[b]/ Species	Mascot Scores/ Coverage (%)	Identification	Number Peptides	Mass Th/Ex[c]	pI Th/Ex[d]	% volume ± DP ■30 days ■60 days ■100 days
66	ATPBO_ARATH *Arabidopsis thaliana*	96/ 29	ATP synthase subunit beta-3, mitochondrial	15	60/48	6.1/5.0	
110	YCF1_CUCSA *Cucumis sativus*	57/ 11	Putative membrane protein ycf1	15	22/37	9.8/71	
112	SDLCA_SOYBN *Glycine max*	60/ 22	Dynamin-related protein 12A	9	69/66	8.1/8.0	

209	G3PC1_ARATH *Arabidopsis thaliana*	44/ 4	Glyceraldehyde-3-phosphate dehydrogenase GAPC1, cytosolic	1	37/37	6.6/7.5	
120	PME36_ARATH *Arabidopsis thaliana*	65/ 33	Probable pectinesterase/pectin esterase inhibitor 36	11	58/58	9.4/8.9	
170	ODP23_ARATH *Arabidopsis thaliana*	58/ 20	Dihydrolipoyllysine-residue acetyltransferase component 3 of pyruvate dehydrogenase complex, mitochondrial	8	59/24	7.9/8.4	
193	IFT74_CHLRE *Chlamydomonas reinhardtii*	69/ 23	Intraflagellar transport protein 74	13	71/19	8.9/7.5	

195	RBL_ABIMR *Abies mariesii*	129/ 35	Ribulose bisphosphate carboxylase large chain (Fragment)	14	49/48	6.5/7.5	
74	ACT1_SORBI *Sorghum bicolor*	68/ 28	Actin-1	8	42/42	5.4/5.2	
109	3HIDH_ARATH *Arabidopsis thaliana*	68/ 8	Probable 3-hydroxyisobutyrate dehydrogenase, mitochondrial	6	37/33	7.0/7.7	

117	MATK_CANSA *Cannabis sativa*	58/ 16	Maturase K	7	61/23	9.3/6.8	
224	SR541_HORVU *Hordeum vulgare*	66/ 32	Signal recognition particle 54 kDa protein 1	13	54/44	9.3/5.9	

[a] The numbering corresponds to the protein spot number in 2-D gels.
[b] Accession number in UniProtKB
[c] Molecular mass, Th theoretical, Ex experimental
[d] pI isoelectric point, Th theoretical, Ex experimental

Table 2: Exclusive proteins identified by mass spectrometry analysis for different cultivation times of *C. tigrina* PLBs.

Spot[a]	Access number[b]	Mascot Scores	Coverage (%)	Identification	Taxonomy	Number Peptides	Mass Th/Ex[c]	pI Th/Exp[d].	Relative Volume ± SD
30 days									
98	CSPLE_RICCO	96	58	CASP-like protein 4D1	*Ricinus communis*	4	18/ 28	7.7/ 5.0	0,40±0,07
60 days									
36	MD37E_ARATH	64	25	Probable mediator of RNA polymerase II transcription subunit 37e	*Arabidopsis thaliana*	13	72/ 72	5.0/ 5.0	0,31±0,12
249	CLPC1_ARATH	59	13	Chaperone protein ClpC1, chloroplastic	*Arabidopsis thaliana*	14	10/ 34	6.4/ 8.5	0,79±0,13
100 days									
81	HSP81_ORYSI	89	18	Heat shock protein 81-1	*Oryza sativa subsp. indica*	13	80/81	5.0/ 5.0	1,19±0,26
90	MD37E_ARATH	84	27	Probable mediator of RNA polymerase II transcription subunit 37e	*Arabidopsis thaliana*	13	71/70	5.0/ 5.1	1,20±0,11
101	HSP70_MAIZE	82	18	Heat shock 70 kDa protein	*Zea mays*	9	70/69	5.2/ 5.2	0,60±0,07

115	RBL_BURIN	99	19	Ribulose bisphosphate carboxylase large chain (Fragment)	*Bursera inaguensis*	11	51/52	6.0/ 6.4	0,45±0,10
142	RBL_CATSP	88	23	Ribulose bisphosphate carboxylase large chain (Fragment)	*Catesbaea spinosa*	14	52/52	6.3/ 7.3	0,50±0,21
154	ATPA_CERDE	80	23	ATP synthase subunit alpha, chloroplastic	*Ceratophyllum demersum*	10	55/56	5.4/ 5.5	0,48±0,09
172	C76C1_ARATH	60	23	Cytochrome P450 76C1	*Arabidopsis thaliana*	11	57/52	7.2/ 6.2	0,51±0,10
178	ATPB_ACRAL	144	38	ATP synthase subunit beta, chloroplastic	*Acrocomia aculeata*	17	53/54	5.2/ 5.1	0,44±0,10
186	LEA2_CICAR	66	37	Late embryogenesis abundant protein 2	*Cicer arietinum*	7	16/49	9.0/ 9.4	0,54±0,11
206	ENO_SOLLC	64	15	Enolase	*Solanum lycopersicum*	6	48/48	5.7/ 5.5	1,31±0,08
208	CHLN_PINCO	58	17	Light-independent protochlorophyllide reductase subunit N	*Pinus contorta*	5	52/56	6.1/ 6.3	0,58±0,08
254	RCA_ORYSJ	80	19	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Oryza sativa subsp. japonica*	10	51/39	5.4/ 60	0,83±0,21
285	DAO_ORYSI	62	18	2-oxoglutarate-dependent dioxygenase DAO	*Oryza sativa subsp. indica*	5	32/34	5.5/ 7.4	0,57±0,11
306	TPT_SOLTU	31	2	Triose phosphate/phosphate translocator, chloroplastic	Solanum tuberosum	2	45/32	9.7/ 87	0,74±0,11

357	1433_HELAN	68	22	14-3-3-like protein	*Helianthus annuus*	8	29/29	4.6/ 4.8	0,60±0,21
361	SPLE_RICCO	64	38	CASP-like protein 4D1	*Ricinus communis*	4	18/29	7.8/ 5.2	0,98±0,15
372	1433_MESCR	90	27	14-3-3-like protein	*Mesembryanthemum crystallinum*	9	30/27	4.8 /4.6	0,43±0,08
394	AUL1_ARATH	60	8	Auxilin-like protein 1	*Arabidopsis thaliana*	10	16/25	4.9/ 8.8	0,48±0,13
404	ACCO_ACTDE	61	25	1-aminocyclopropane-1-carboxylate oxidase	*Actinidia deliciosa*	8	36/26	5.3/ 5.2	0,47±0,17
412	RBS3B_SOLLC	58	41	Ribulose bisphosphate carboxylase small chain 3B, chloroplastic	*Solanum lycopersicum*	5	20/24	6.7/ 8.4	0,51±0,02
451	P2C03_ORYSJ	58	20	Probable protein phosphatase 2C 3	*Oryza sativa subsp. japonica*	5	52/54	8.5/ 9.1	0,62±0,10

[a] The numbering corresponds to the protein spot number in 2-D gels.
[b] Accession number in UniProtKB
[c] Molecular mass, Th theoretical, Ex experimental
[d] pI isoelectric point, Th theoretical, Ex experimental

5.4.4 Functional classification of proteins

Based on the biological process, most of proteins identified on both cultivation periods were involved in cellular processes (Fig. 3A). Regarding molecular function, most proteins were related to the energy process, followed by metabolic processes (Fig. 3B). For the cellular component category, most proteins were localized in the chloroplast, followed by cytoplasm, in all cultivation periods (Fig. 3C).

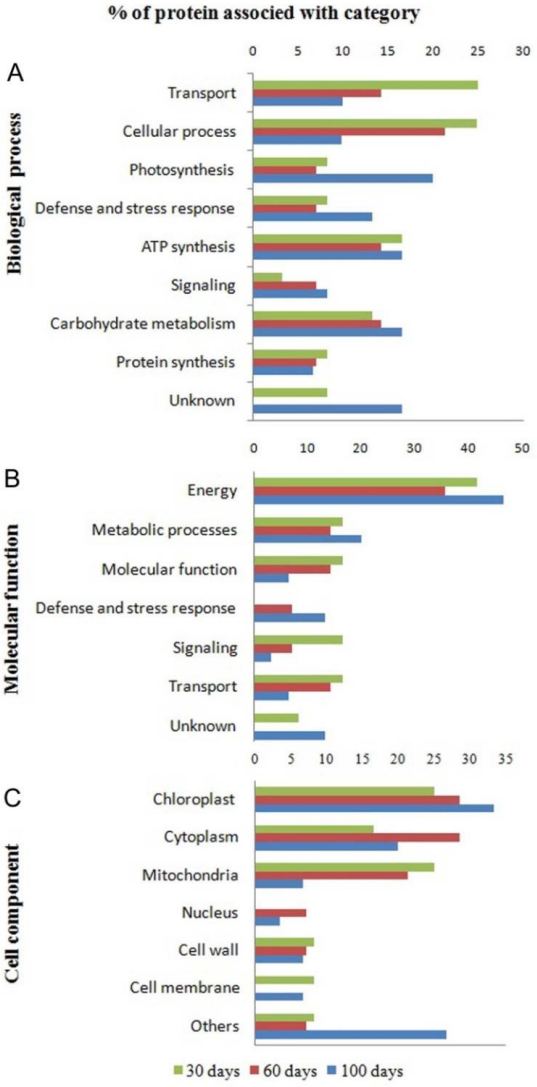


Fig. 3 Ontology classification of differentially expressed proteins in PLBs cultivation times in *C. tigrina*. **(a)** Biological process; **(b)** Molecular function; **(c)** Cellular component.

5.5 DISCUSSION

5.5.1 Histological analysis

Histochemical assessment showed distinct morphological characteristics during PLBs development in the respective cultivation periods. During PLBs development, new PLBs are formed over preexisting ones, representing a cyclic regeneration proposed by Haccius (1978), which always starts with formation over the previous PLBs layer. These data corroborate findings by Liz (2013), in which *C. tigrina* leaves inoculated and cultured *in vitro* showed PLBs cyclic regeneration after 30 and 60 days of cultivation days. Batygina et al. (2003) also observed the formation of numerous secondary protocorms from initial protocorm epidermal cells during *in vitro* cultivation. Leaf primordia initial formation after 60 days and primordia development after 100 days denote morpho differentiation evidence.

According to Fritsche (2012), *C. tigrina* showed that plant regeneration signals occurred from the second or third subculture, in a culture medium free of growth regulators. The author also found that this process occurred asynchronously, preventing seedling obtainment for constant and large-scale acclimation.

5.5.2 Comparison of protein profiles in the development of PLBs.

PLB development was accompanied by protein pattern changes during the synthesis of new proteins. In addition, PLBs development accompanied the reduction or complete disappearance of other proteins. In the present study, a higher number of proteins were expressed after 100 days of cultivation when compared to the other periods (30 and 60 days of cultivation). One possible explanation for higher protein levels during the cultivation time aforementioned may be related to metabolic activation in relation to PLBs development. Tan et al. (2013) found that several proteins were expressed the most in the final stage (callus with 45 days of cultivation) when compared to calluses in the early stage (15 days), while studying *Vanilla planifolia* nodal explant. Cellular adaptation related to environmental conditions is usually accompanied by changes in gene expression, signal transduction pathway organization and physiological process (Fehér et al. 2003). Therefore, physiologic and metabolic changes during cellular differentiation and reprogramming require the assembly and stabilization of newly synthesized proteins, as well as peptide modification and removal (Chugh et al. 2002; Bian et al.

2010). In this study, 36 proteins were identified, whose expression was altered during PLBs development. Some of these proteins may play important roles in the development of these structures. In addition, expression changes may trigger direct consequences on plant tissue cellular reprogramming during *in vitro* conditions. Given the above, the most important proteins, according to their functional classification, will be discussed.

5.5.3 Carbohydrate and energy metabolism associated proteins

PLBs growth and development is defined by metabolic changes. In order to keep cell division, a large amount of energy is required. In the present study, many proteins related to metabolic and energy processes were identified, and many of them were exclusive to 100 days of cultivation (spots 115, 142, 154, 178, 206, 254, 306 and 412). The *enolase* protein (spot 206), which is a glycolytic enzyme that catalyzes the 2-phospho-D-glycerate conversion for phosphoenolpyruvate (PEP) (Wold and Ballou, 1957; Van Der Straeten et al., 1991), was only exclusively after 100 days of cultivation. This enzyme has been correlated with cellular ATP use in higher plants (Wang et al. 2009). Andriotis et al. (2010) found maximum enolase transcript mounts in *Arabidopsis* torpedo embryos. Likewise, Lippert et al. (2005) and Tonietto et al. (2012) only found elevated expression of this protein in the embryonic torpedo stage of *Picea glauca* and *Coffea arabica*, respectively. Therefore, the authors suggested that enolase would be an interesting candidate for molecular marker in the embryo maturation stage.

Other identified proteins involved in energy synthesis were *glyceraldehyde-3-phosphate dehydrogenase GAPC1, cytosolic* (spot 209) and *Ribulose bisphosphate carboxylase large chain-Fragment* (spot 195), which were present during all cultivation periods and were abundantly expressed after 100 days. These glycolytic enzymes are often involved in *in vitro* somatic organogenesis and embryogenesis (Nogueira et al. 2007; Fortes et al. 2008). Therefore, glycolysis increase after 60 and 100 days of cultivation is probably involved in cellular division and differentiation. According to Palama et al. (2010), who studied *Vanilla planifolia*, glycolytic enzyme increase would probably be related with cellular division and differentiation. Proteins related to energy and metabolic processes are the largest group among proteins identified in *C. tigrina* PLBs induction (De Conti et al. 2014), having a key role in the formation of these structures. In the development of cassava (*Manihot esculenta*)

secondary embryos, the abundance of proteins related to energy metabolism was attributed to high metabolic activity, which is required to keep high cell division in developing embryos (Baba et al. 2008).

5.5.4 Cell proliferation

The cell cycle dependent on the synthesis of new proteins, which cause morphological and biochemical changes associated with mitotic activity (Dewitte et al. 2003). According to Silva et al. (2014), cell division increase requires proteins involved in cell wall loosening, degradation or biosynthesis. In this study, among identified proteins, *Actin-1* (spot 74), which was present after 30 and 60 days of cultivation, and *Probable pectinesterase/pectinesterase inhibitor 36* (spot 120), which was present during all cultivation periods, were involved in wall cell formation. Actin is related to the development of the cytoskeleton, in which, according to Ketelaar et al. (2004), organization, cellular division and growth require the normal functioning of that protein. The *Probable pectinesterase/pectinesterase inhibitor 36* protein acts by modifying the cell wall through cell wall pectin demethylesterification. This protein is involved in the first step of the sub pathway that synthesizes 2-dehydro-3-deoxy-D-gluconate to pectin. Marsoni et al. (2008), who compared *Vitis vinifera* embryogenic and non-embryogenic calli, found that all proteins involved in cell division (including actin) had high somatic embryogenesis levels, according to active embryogenic cell division. Cell division reactivation in somatic plant cells is required for dedifferentiation (Nagata et al. 1994) and embryogenic capacity establishment (Dudits et al. 1991).

5.5.5 Protein processing

Cellular reprogramming requires post-translational modifications and protein translocation and folding. Heat shock proteins (HSPs) were found after 60 and 100 days of cultivation, including members of HSP70 (spot 36, 90 and 101) and HSP90 (spot 81) families. HSPs are a large family of molecular chaperone proteins whose biological role is to keep cellular homeostasis, both under optimal growth and stress conditions (Rodziewicz et al. 2014). Moreover, they are responsible for the correct protein folding, translocation and degradation, preventing aggregation (Efeoğlu 2009). These proteins are divided into five main families (HSP60, HSP70, HSP90, HSP100 and small HSP-sHSPs) and are located in various cellular components. In this study, the following two types

were identified: HSP70 and HSP90. Proteins belonging to the Hsp90 family play an essential role in protein folding and signal transduction regulation (Buchner, 1999; Pratt and Toft 2003). Proteins belonging to the HSP70 family prevent the aggregation of newly synthesized proteins, besides assisting in the proper folding. These proteins are also involved in the transport and proteolytic degradation of unstable proteins (Su and Li 2008). Both families (Hsp70 and Hsp90) cooperate, and their central role is the protection of organisms against environmental and genetic stress, besides protein folding (Gonçalves 2012).

HSP prevalence after 60 and 100 days of cultivation in this study suggests that these proteins would be responsible for every newly synthesized protein or unstable protein folding. In addition, HSPs are responsible for the refolding of damaged proteins, which is caused by *in vitro* cultivation stress in *C. tigrina* PLBs development. Moreover, the presence of such proteins may be attributed to increased cell division, as there is high PLBs formation and primordial leaf development in this period, showing morpho differentiation advance. According to Correia et al. (2012), thermal shock proteins have been reported in cells and tissues under division, with the function of protector.

HSP70 family members were also found in *Picea glauca* embryogenic tissue (Lippert et al., 2005), *Vitis vinifera* callus (Marsoni et al., 2008) and *Cyclamen persicum* somatic embryos (Winkelmann et al., 2006), showing the important role of these proteins in these processes. According to Bond and Schlesinger (1987), HSPs may play a role in cell proliferation, differentiation and embryogenesis.

5.5.6 Secondary metabolism and phytohormones

The *1-aminocyclopropane-1-carboxylate oxidase* (spot 404) protein, which was involved in ethylene (a key phytohormone involved in plant regulation and development) biosynthesis and perception (Schaller 2012), was also detected in this study. The 1-aminocyclopropane-1-carboxylate oxidase (ACC) synthase enzyme, which catalyzes the first step in ethylene biosynthesis (Kende and Zeevaart 1997), was present after 100 days of cultivation.

Ethylene affects both plant growth and development (Abeles et al., 2002). In terms of growth, ethylene is usually associated with cell size regulation, often limiting cell elongation, although it may also regulate cell division. Regarding development, ethylene is most commonly seen as an "aging" hormone, as it accelerates processes such as ripening, senescence and abscission (Schaller 2012).

This phytohormone was also positively correlated with *Pinus sylvestris* somatic embryo development (Lu et al. 2011), in which it was verified that this regulator was heavily involved in the maturation of somatic embryos from this species. Many physiological and molecular studies have shown that ethylene synthesis and perception are necessary for in vivo plant growth (Smalle and Van Der Straeten 1997; Kepczyn'ski and Kepczyn'ska 2005). Kepczyn'ska et al. (2011) found that ethylene product ion inhibition in *Medicago sativa*, which was due to the presence of two ethylene biosynthesis inhibitors during the proliferation stage, was correlated with somatic embryo formation inhibition. This result suggests that ethylene is necessary for cellular proliferation.

Another protein related to secondary metabolism identified in this study was *Cytochrome P450 76C1* (spot 172), which was responsible for diterpenoid biosynthesis. This protein was present after 100 days of cultivation. Recent studies have reported that the Cytochrome 450 (CYP450) family is actively involved in the biosynthesis of plant growth regulators, such as gibberellic acid, abscisic acid and brassinosteroids (Krochko et al. 1998, Fujioka and Yokota 2003). This indicates the great importance of the CYP450 enzyme complex in the evolutionary process of vascular plant tolerance against biotic and abiotic factors (González-Mendoza 2007).

5.5.7 Proteins related to other functions

Protein phosphorylation is an essential post-translational modification in plant life, affecting thousands of proteins. The process is reversible and may result in conformational changes that cause protein activity changes. All eukaryotic cells have protein modules that act as sensors for the phosphorylation state of specific phospho-sites, such as the 14-3-3 protein (De Boer et al. 2013). The result of the binding of 14-3-3 protein to its targets depends on the target itself, which may alter enzyme activity, prevent or induce protein degradation or change protein subcellular localization (Igarashi et al. 2001; Sirichandra et al. 2010; Boer et al. 2013). This protein plays plant development roles in primary metabolism, in pathways related to stress and in hormonal control (Cotelle et al. 2000; Sirichandra et al. 2010; Boer et al. 2013; Yoon et al. 2013). Thus, the different biological functions associated with this protein family are clearly observed. The 14-3-3 protein was identified in this study after 100 days of cultivation (spot 357 and 372). However, information about the specific function of this protein and how it overlaps functions in a single organism is still scarce in the literature (Aitken et al.

2011). Thus, the actual function of 14-3-3-like protein in PLB development was not clearly evident in this study.

Another protein identified in this study was *Maturase k* (spot 171), observed after 60 and 100 days of cultivation. The transcription machinery plays an important role in abiotic stress adaptation (Pang et al. 2010). This protein is associated with development, transcription and post-transcriptional modification and gene expression regulation by forming miRNA/siRNAas a response to plant stress (Sunkar et al. 2007). *Maturase K* protein expression changes are very complex in plants grown under abiotic stress, depending on plant species and stress type (Pandey et al. 2005). According to the author, *Maturase K* was induced when the plant underwent high pressure related to ROS. In contrast, *Maturase K* protein was regulated to be low in maize treated with salt (Zorb et al. 2010). In this study, increased expression of this protein during PLBs development may be associated with increased cell division and differentiation.

5.6 CONCLUSION

The results of this study describe protein differential expression during PLBs development. Thus, the physiological and biochemical events responsible for this process were defined. Changes related to protein profile observed in this study indicate that some proteins could be candidates as markers in the development process of this morphogenetic pathway.

Protein differential expression during *C. tigrina* PLBs development is mainly related to energy metabolism and carbohydrate intensification, in addition to cell division resumption. Thus, morphogenic response is acquired, and proteins proved to be essential in this process.

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6. CAPÍTULO 3. DETERMINATION OF ENDOGENOUS IAA AND CARBOHYDRATES DURING THE INDUCTION AND DEVELOPMENT OF PROTOCORM-LIKE BODIES OF *Cattleya tigrina* A. Richard.

6.1 ABSTRACT

Tissue culture techniques have been employed for orchids mass propagation by means of the morphogenetic route of protocorm like-bodies (PLBs). The study aimed to analyze and compare Indole-3-acetic acid (IAA) and sugar endogenous levels in protocorm-like bodies (PLBs) induction and development in *Cattleya tigrina*, in order to better understand this process and to optimize micropropagation procedure protocols. Leaves grown on culture medium (Murashige and Skoog) MS, suplemented with 9 μM TDZ for PLBs induction and development were collected after 0, 2, 7, 14, 30, 60 and 100 days of cultivation, for further analysis. Increase of IAA and reduction of sugar levels are strongly related to morphogenetic response, that is, PLBs formation over the preexisting ones and leaf primordia formation. Sucrose, fructose and glucose presence in this study is related to cell signaling. Thus, hormonal signals and carbohydrates alter metabolism, triggering PLBs initiation and development in *C. tigrina.*

Keywords: orchid, protocorm-like bodies, IAA, sugars.

6.2 INTRODUCTION

Orchidaceae is the second largest angiosperm family (Dressler 2005; Govaerts et al. 2014), which is associated with various life forms (Waechter 1996). Due to ornamental and landscape features, orchids are inordinately taken from their natural habitats, leading many species to extinction and significantly threatening biodiversity maintenance (Swarts and Dixon 2009). Plant tissue culture technique development has been widely used with success in the propagation of different land and epiphyte orchid species (Diaz and Alvarez 2009), allowing rapid multiplication. In ecological terms, plant micropropagation can be used in the preservation and propagation of endangered species. Furthermore, this approach can be used important as a model to study metabolism, differentiation, and cell morphogenesis, providing information on physiological and biochemical events leading to *in vitro* morphogenesis modulation.

During *in vitro* establishment, a morphogenic response pattern defined as protocorm-like bodies (PLBs) has been proposed for several orchid species, such as the species under study. PLBs are globular structures named after their similarity with zygotic origin embryos (protocorms) (Arditti, 2008). The induction of these structures has been reported by apical meristems in *Phalaenopsis*, *Doritaenopsis* (Tokuhara and Mii 1993) and *Cymbidium* (Subramanium and Taha 2003), by flower stalks in *Phalaenopsis* (Ichihashi 1992), by root apices in *Phalaenopsis* (Tanaka et al. 1976), and by foliar segments in *Doritaenopsis* (Park et al. 2002), *Aerides crispum* (Sheelavanthmath et al. 2005) and *Cattleya tigrina* (Fritske 2012). The study of this morphogenetic route has been conducted with *C. tigrina* through morphoanatomical (Liz 2013) and biochemical analysis (Almeida 2014; De Conti et al. 2016), in order to relate the signaling events that trigger and regulate PLBs formation. However, endogenous IAA and sugar levels in PLB induction and development were not studied.

Both endogenous and exogenous plant hormones are closely related to *in vitro* cell differentiation (Senger et al. 2001; Jiménez 2005; Barreto et al. 2010; Feng et al. 2010; Rakshit et al. 2010; Silva 2010; Sun and Hong 2010). They are considered key factors in triggering plant morphogenesis, including somatic embryogenesis and organogenesis (Huang et al. 2012). In the last decade, several studies investigated the association between endogenous hormone levels and plant morphogenesis, with special focus in somatic embryogenesis routes in *Saccharum* species (Guiderdoni et al. 1995), *Prunus sp* (Michalczuk and Druart 1999), *Daucus carota* (Jiménez and Bangerth 2001), *Araucaria angustifolia* (Steiner et al. 2007), *Acca sellowiana* (Pescador et al. 2012), *Prunus persica* (Pérez-Jiménez et al. 2013) and *Cucurbita pepo* (Leljak-Levanic et al. 2015). In contrast, only few reports mention endogenous hormonal changes associated with other *in vitro* morphogenesis routes. Among growth regulators, auxin is highlighted, which has a strong influence on a wide variety of development responses (Lui et al. 1993; Sairanen et al. 2012). Indole-3-acetic acid (IAA), the primary auxin in plants, is associated with histodifferentiation embryogenic pattern regulation, and is an integral regulator in cell expansion, division and differentiation (Sairanen et al. 2012).

In addition to plant hormones, carbohydrates also affect growth and development. Carbohydrates are energy sources for cells and carbon sources for biosynthetic processes (Zhang et al. 2012; Kubeš et al. 2014). They also act as osmotic agents, helping maintaining plasma membrane integrity, besides being important signaling molecules that modulate

several processes in plant development (Pareddy and Greyson, 1989; Tremblay and Tremblay 1991; Kubešet al. 2014; Lastdrager and Smeekens 2014). Cell conduction, division and expansion molecular net works largely depend on carbohydrate availability to provide energy and biomass (Lastdrager et al. 2013). There are several mechanisms that coordinate hormonal processes, so that they are energetically compatible with the plant carbon state. These mechanisms may act modulating hormone synthesis, transport and signaling, so that hormonal responses that promote growth are inhibited under limited carbon conditions (Eveland and Jackson 2012; Ljung et al. 2015).

Thus, the aim of this study was to analyze and compare IAA and sugar endogenous levels in *C. tigrina* PLBs induction and development, in order to better understanding this process and to optimize micropropagation procedure protocols.

6.3 MATERIALS AND METHODS

6.3.1 Plant material

Leaf explants (±1cm) were obtained from young plants, micropropagated and maintained *in vitro* Development Physiology Laboratory and Plant Genetics (LFDGV), the Agricultural Science Center (CCA), Universidade Federal de Santa Catarina (UFSC), Florianópolis, Santa Catarina State, Brazil. The leaf explants were detached and inoculated MS medium (Murashige and Skoog 1962), supplemented with 9 μM TDZ (Thidiazuron) for PLBs induction, which was previously established by (Fritsche 2012).

After inoculation of the explants, they were kept in growth room with an average temperature of 25±2°C, 16-h light and 8-h dark, with a light intensity of 50 μmol m^{-2} s^{-1}. To zero (which corresponded to the beginning of the assembly of the experiment, being the starting explant), 2º, 7º, 14º, 30º, 60º and 100º days of cultivation, plant materials were collected for histological, hormonal and sugars analysis. For histological studies the five explants of each culture time were collected. For hormone analysis, about 50 mg lyophilized samples were processed in triplicate for each cultivation time. With regard to sugar analysis were collected biological three replicates for each cultivation time, each replica was formed by 500mg of fresh pasta starting from a pool of plant material, corresponding basically to 30 leaf explants and stored at -80°C for later analysis.

6.3.2 Histological analysis

The material was fixed in 2.5 % paraformaldehyde in 0.2 M (pH 7.2) phosphate buffer overnight. Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions (Schmidt et al. 2009). 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 double-stained with Periodic Acid-Schiff (PAS) to identify neutral polysaccharides (Gahan 1984) and Coomassie Brilliant Blue (CBB) 0.4% in Clarke's solution to identify proteins (Gahan 1984). Sections were analyzed with a camera (Olympus® DP71) attached to a microscope (Olympus® BX-40).

6.3.3 Determination IAA

IAA content was determined according to Ludwig-Muller et al. (2008) with modifications. The lyophilized samples were extracted with a mixture of 1mL isopropanol and acetic acid (95:5v/v). Exactly 0,5 µg [$^{13}C_6$]-IAA (Cambridge Isotopes, Inc.) was added to each sample as internal standards. The extracts were then incubated with continuous shaking for 40 min at 4°C. The samples were centrifuged for 10 min at 25.000g at 4°C, the supernatant was removed and evaporated to the aqueous phase under SpeedVac until only 50µL. The aqueous phase was then extracted with ethyl acetate and water, the organic fraction was removed and the sample was taken up in methanol. The sample was evaporated to the aqueous phase using SpeedVac as described above. Purified samples were evaporated, resuspended in 50µL pyridine followed by a 60 min derivatization at 92°C using 50µL N-tert-Butyldimethylsilyl-N methyltrifluoroacetamide. The analysis was performed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. The samples were injected in the splitless mode; linear velocity 33,9cm/seg. Column DB-5 MS (30m, ID 0.25 mm, 0.25 µm thick internal film). Column flow: 0,83 mL/min. The temperature program for metabolite analysis was: 2 min at 100°C, followed a ramp of 10°C min^{-1} to 140°C, 25°C min^{-1} to 160°C, 35°C min^{-1} to 250°C, 20°C min^{-1} to 270°C and 30°C min^{-1} to 300°C. The injector temperature was 250°C, and the following MS operating parameters were used: ion source temperature 230°C; and interface temperature, 260°C. Solvent cut 5min

– mode SIM (0,30 sec). Ions with a mass ratio/charge (m/z) of 244, 202 and 130 (corresponding to endogenous IAA), 250, 208 and 136 (corresponding to [$^{13}C_6$]-IAA) were monitored. Endogenous IAA concentrations were calculated based on extracted chromatograms at m/z 244 and 250. The data were submitted to ANOVA and presented as means of three biological replicates.

6.3.4 Total soluble carbohydrates content

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.

6.3.5 Thin-layer Chromatography (TLC)

Qualitative analysis of sugars was carried out on the extracts described previously by TLC on aluminum plates coated with silica G60 (Alugram®). Mobile phase consists of 2-Propanol/Ethyl acetate/Nitroethane/Acetyl hydroxide /water (45:25:10:1:19). Detection of carbohydrates was performed by spraying solution of orcinol, sulfuric acid and ethanol followed by heating the plate. They were used as markers fructose, glucose, xylose, maltose, and sucrose.

6.3.6 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 3,000rpm 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.

6.4 RESULTS

6.4.1 Induction and development of PLBs

Inoculated leaf explants cultured on induction medium allowed PLBs proliferation. Through anatomical analysis, it was observed that after zero and two days of cultivation, the basal region of the leaf segment was characterized by protrusion absence (Fig. 1A-1F). After PAS and CBB double staining, starch granules associated with the protein material (Fig.1C and 1F), in both cultivation times, was observed. PLBs histodifferentiation began on the 7th day of cultivation (1G-1I). However, on the 14th day of cultivation, PLBs formation was observed, in which elongated cells in the epidermis were observed (1J-1L). Starch grains and protein material were present on the 7th and 14th days of cultivation, with gradual increase through the analyzed times (1I and 1L). Significant predominance of starch and protein material reserves was observed on the 30th day of cultivation, when, by histochemical analysis, the formation of large PLB amounts was verified, which resulted from new PLB formation over the preexisting ones (Fig. 1M-1O). Leaf primordia formation began on the 60th day of cultivation, and the complete primordia formation was concluded on the 100th day of cultivation (1Q-1Q). In the 60th and 100th days of cultivation, there was a gradual decrease in relation to neutral polysaccharides and increased protein material, mainly on the 100th day of cultivation (1R and 1U).

6.4.2 Endogenous hormone

The endogenous levels of IAA is clearly altered during the induction of PLBs and development (Fig. 2). In zero, 7th and 100th days of cultivation, there was progressive and substantial IAA accumulation in relation to the other cultivation times. However, in the other cultivation times, a sharp IAA decrease began, ranging within relatively low levels, except in the 7th and 60th days of cultivation, where there was IAA concentration increase.

6.4.3 Sugar contents

Total soluble carbohydrate content varied during PLBs induction and development (Fig. 3). Larger soluble sugar amounts were observed on the 2nd, 7th, 14th, 30th and 60th days of cultivation after induction, without statistically differing from each other. The only difference occurred on the 100th day of cultivation, when there was a significant concentration decrease (8.21 mg g^{-1} FM). Regarding the initial explant (day zero), there was a slight decrease in total sugar amount, with significant differences in relation to the 2nd and 100th days of cultivation.

As for starch contents, it was observed that the highest starch concentration occurred on the 30th day (7.1 mg g^{-1} FM), statistically differing from the other cultivation times, with the exception of the 14th day after starting induction. On the zero, 7th, 14th and 60th days of cultivation, there were no statistical differences. In the 2nd and 100 days of cultivation, starch levels were lower, with concentrations of 3.11 and 2.63 mg g^{-1} FM, respectively (Fig. 3).

Composition analysis of soluble sugars in the different cultivation times, during PLB induction and development, showed sugar alcohols presence, especially glucose, fructose and sucrose (Fig.4), in all culture times.

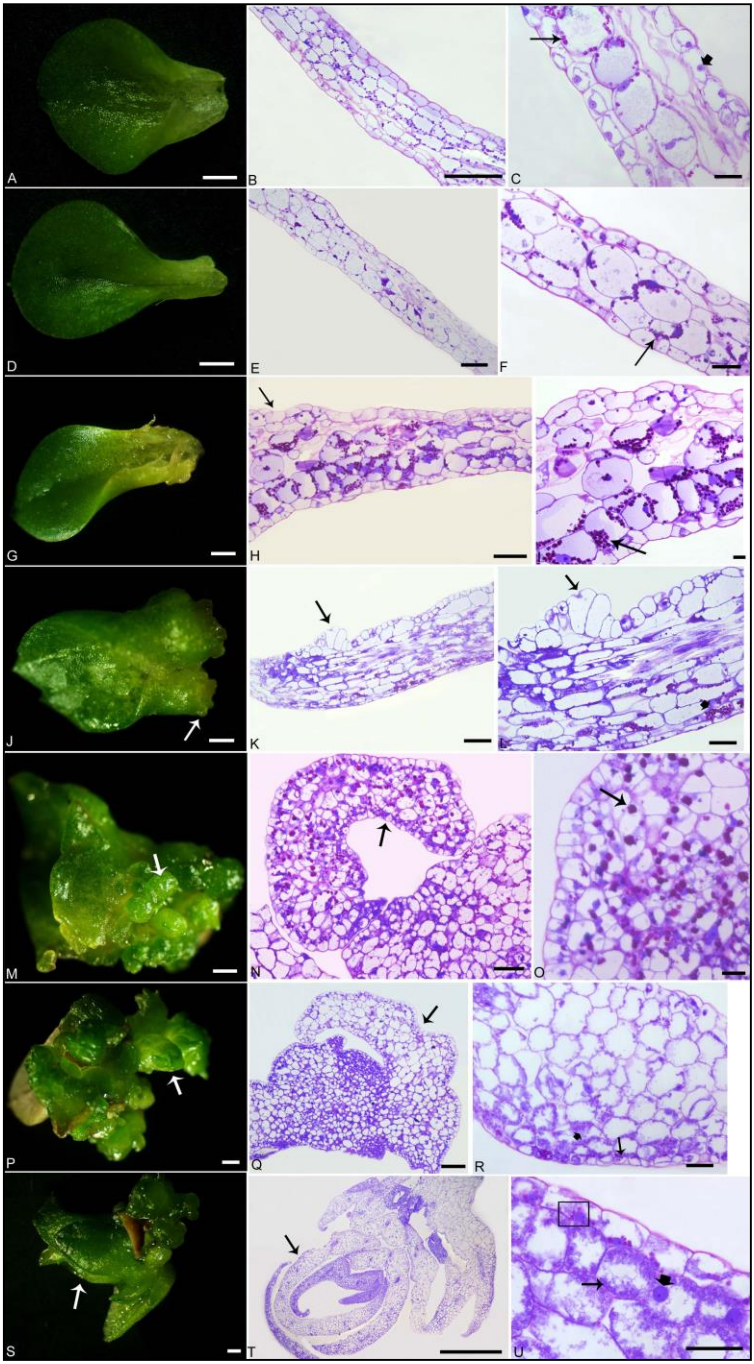
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Fig. 1 Histological (double-stained: PAS/CBB) aspects of protocorm-like bodies (PLBs) from leaf explants of *C. tigrina* A. Rich. **(a)** General view of the adaxial surface of the initial leaf. **(b)** Cross-section of initial explant. **(c)** Longitudinal section. Blue-stained prominent nuclei (broad arrow) and red-stained starch granules (arrow). **(d)** After two days of culture, overview of the adaxial surface of the leaf. **(e)** Cross-section of leaf induced after two days of culture. **(f)** Red-stained starch granules (arrow). **(g)** General view of the adaxial surface of induced leaf after seven days of culture. **(h)** Cross-section of the leaf induced after seven days of culture (arrow). **(i)** Longitudinal section. Red-stained starch granules (arrow). **(j)** On the 14 days of culture, the early formation of PLBs (arrow). **(k)** Cross-section of the leaf induced on the 14th day of culture, with evidence of mitotic activity in the epidermis (arrow). **(l)** Longitudinal section. Elongated cells in the epidermis (arrow) and red-stained starch granules (broad arrow). **(m)** After 30 days of culture, with lots of PLBs (arrow) concentrated in the leaf base. **(n)** Longitudinal section of a PLB **(o)** Red-stained starch granules (arrow). **(p)** General view of leaf inoculated at 60 days of culture, yellowish already showing deterioration, but loads of PLBs (arrow) present in the leaf base. **(q)** Longitudinal section showing the beginning of the formation of leaf (arrow). **(r)** Blue-stained prominent proteins (broad arrow) and red-stained starch granules (arrow). **(s)** General view of leaf inoculated at 100 days of culture with leaf developed (arrow). **(t)** PLBs longitudinal sections showing developed leaves (arrow). **(u)** Blue-stained prominent nuclei (broad arrow), proteins decorated with blue (arrow) and red-stained starch granules (square).

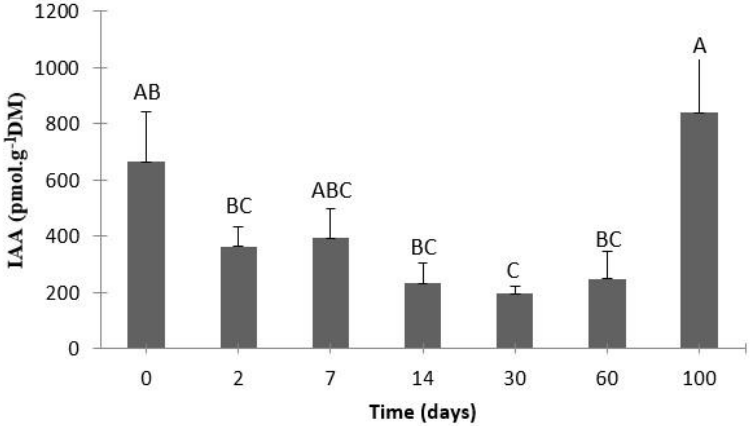

Fig. 2 Changes in endogenous indole-3-acetic acid (IAA) content during the induction and development of PLBs in *C. tigrina*. Vertical bars represent the standard deviation for each measure. Means followed by different letters are significantly different according to the Tukey test at 5%.

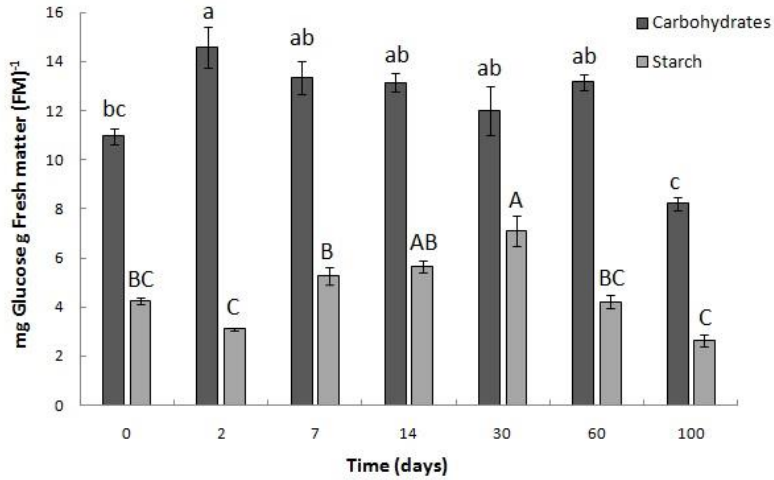

Fig. 3 Changes in the total soluble carbohydrates (mg g^{-1} FW) during the induction and development of PLBs in *C. tigrina* after explant inoculation. Vertical bars represent the standard deviation for each measure. Means followed by different letters are significantly different according to the Tukey test ate 5%.

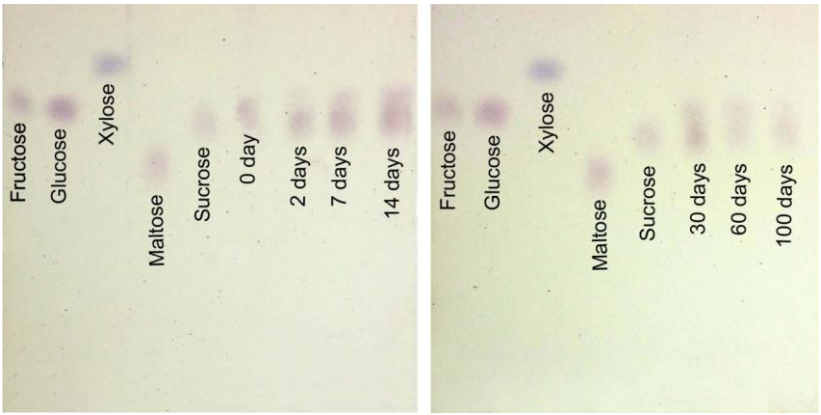

Fig. 4 Qualification of sugars by Thin-layer Chromatography during the induction and development of PLBs *C. tigrina.* Fructose, glucose, xylose, maltose and sucrose were used as markers.

6.5 DISCUSSION

Regeneration from organogenesis or somatic embryogenesis is well known, and it is known that regeneration is strongly correlated with the concentration of endogenous hormones or hormones externally applied to the culture medium (Bhaskaran and Smith, 1990). IAA, the main natural representative of the auxin group, is responsible for regulating plant growth and development, besides being related to a wide variety of plant responses (Sairanen et al. 2012). IAA levels on the 2nd, 7th, 14th and 30th days of cultivation are strongly related to cell division and differentiation. During these cultivation times, *C. tigrina* leaves showed evidence of PLBs development and formation, whose epidermal cells began to undergo mitotic division, initiating cell differentiation and PLBs component tissues formation. Significant IAA level increase on the 100th day of cultivation is strongly related to morphogenetic response. This peak occurred in the period coinciding with the moment in which cells are becoming irreversibly affected by a specific morphogenic response (leaf primordia formation, in this case). Furthermore, on the 100th day of cultivation, there was PLBs formation over the preexisting ones, which is cyclic regeneration. According to Sairanen et al. (2012), auxins perform functions in cell elongation, cell division beginning, organ definition and vascular system differentiation processes. High auxin concentrations indicate their importance for zygotic embryo development, especially with respect to cell elongation and division, IAA

characteristics (Taiz and Zeiger 2009). Substantial evidence has shown that IAA homeostasis regulation is a central feature of many environmental and development responses (Normanly 2010).

Regarding soluble sugar amounts, sharp increase on the 2nd day of cultivation may be due to sucrose presence on the culture medium. Similar data were found in *Medicago sativa* somatic embryo development (Horbowicz et al. 1995) and in *Cedrela fissilis* organogenesis (Aragão et al. 2015), in which high sugar content was associated with high carbon concentrations on the culture medium. According to Gemas and Bessa (2006), in *Anacardium occidentale* nodal explants, carbohydrate absorption from the culture medium in the beginning of cultivation is important to start sprout induction and promote their growth. Similar results were observed in sprouts obtained through *Digitalis lanata* callus (Fátima et al. 2009). Total sugars decrease on the 100th day of cultivation is related to increased energy demand for the metabolic processes occurring there. Cangahuala-Inocente et al. (2013), while working with *Acca sellowiana* somatic embryogenesis, observed that total soluble sugar contents differed in various stages of embryo development, with similar values in cordiform and torpedo stages, followed by decrease in the pre-cotyledon stage. Soluble sugars influenced plant growth in two ways, as follows: serving as carbon sources, from which energy is derived for glycolysis and respiration, and acting as signaling molecules through receptor kinases action (Rolland et al. 2006; Wind et al. 2010). Both ways lead to cell induction and differentiation (Lülsdorf et al. 1992) in the present study, during PLB formation.

The same pattern is observed for starch content, and its increase on the 14th and 30th days of cultivation may serve as carbon temporary storage reserves, which can be used later for biosynthesis of other storage materials. He et al. (2011), while working with somatic embryogenesis (SE) in soybean, also found starch content increase in SE development, followed by sharp decrease. According to the authors, starch may serve as a carbon temporary storage reserve, which can be later used for the biosynthesis of other seed storage materials, such as oil (Norton and Harris 1975) or oligosaccharide (Yazdi-Samadi et al. 1977). On the 60th day of cultivation, there was a significant starch content decrease, and this apparent catabolism may be associated with cell proliferation and differentiation occurrence, observed in this cultivation period. Starch contents remained constant during the first 30 days in *Acca sellowiana* somatic embryo development, although these values significantly decreased compared to the initial inoculum (Cangahuala-Inocente et al. 2013). According to Silva et al. (1998), starch content decrease may be

related to its degradation to produce glucose as an energy source for various metabolic reactions that occur during cell vital metabolism growth and maintenance. In addition, it is also a glucose source for cellulose biosynthesis.

In this study, glucose, fructose and sucrose were observed in all cultivation times. According to Aragão et al. (2015), glucose and fructose are the first molecules to act in cell signaling, while the role of sucrose may initially be to provide hexoses. Thus, sucrose subsequently acts as the signaling molecule in *A. occidentale* sprout development (Gemas and Bessa 2006). Sucrose is considered the main sugar form used by cells as carbon skeleton and energy source during growth and development (Tremblay and Lalonde 1984; Tremblay et al. 1984; Barghchi 1988; George 1993; Lastdrager et al. 2014). Sucrose plays a central role in plant development as a possible signaling molecule, regulates a number of genes (Coruzzi and Bush 2001). Glucose also has a big role in metabolism, as it may serve as a bridge between carbohydrate and phytohormone signaling (Leon and Sheen 2003; Roitsch et al. 2003; Hartig and Beck 2006). In addition, glucose has been highlighted as a signaling molecule in various processes associated with growth and development, such as germination, hypocotyl elongation, cotyledon expansion and leaf development (Rolland et al. 2006). According to Weber et al. (1997), hexoses and sucrose are generally associated with different seed development stages. The authors suggested that sucrose regulates cell differentiation and reserve substances storage, while hexoses control cell growth and metabolism.

Recent studies showed that auxins and sugars (glucose and sucrose) act together, and auxin metabolism is regulated by free sugars availability (Sairanen et al. 2013; Ljung et al. 2015). Biosynthesis regulation and auxin degradation (mainly IAA) by sugars requires changes in the expression of multiple genes and metabolites associated with various IAA biosynthesis routes (Sairanen et al. 2013; Ljung et al. 2015). In order to study soluble sugar effects in auxin homeostasis, wild *Arabidopsis* seedlings were incubated with various glucose concentrations after 10 days of germination. Then, it was possible to verify the induction of the expression of several genes that encode enzymes in auxin biosynthesis from tryptophan, including YUCCA8 and YUCCA9 (Sairanen et al. 2013). Previous reports found that a YUCCA corn putative gene was strongly induced by glucose (Le and Chourey 2010).

Therefore, it was observed that plant growth and development plasticity is exemplified by the complex sugar and hormone signaling interaction (León and Sheen 2003).

6.6 CONCLUSION

The results of this study show the dynamics of physiological and biochemical changes that occur during PLBs induction and development in *C. tigrina*. Endogenous IAA and sugar biochemical profiles indicate that these biomolecules play an important role in cell events. IAA content increase and sugar content decrease are strongly related to morphogenetic response, that is, PLBs formation over the preexisting ones and leaf primordial formation. This study is the first to report the relation between endogenous levels of IAA and carbohydrates during PLB induction and development.

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7. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A realização deste trabalho possibilitou a obtenção de resultados importantes e inéditos relacionados aos aspectos fisiológicos e bioquímicos durante a indução e desenvolvimento de estruturas semelhantes à protocormos em *Cattleya tigrina*. Os resultados obtidos possibilitaram a compreensão de vários pontos chaves, em especial aqueles relacionados com a função das proteínas, açúcares e hormônios no processo da morfogênese que originam as estruturas semelhantes à protocormos (ESPs).

Os resultados apresentados no capítulo 1 indicaram proteínas cuja expressão e abundância foram significativamente alteradas ao longo da indução das ESPs. O maior grupo de proteínas identificadas estava relacionado ao metabolismo energético e carboidratos sendo que estas proteínas estão relacionadas à divisão e diferenciação celular. Também foram identificadas proteínas de resposta ao estresse sendo que estas proteínas estão envolvidas com a estabilização e conformação de outras proteínas, o que pode auxiliar na resposta dessas estruturas ao estresse do ambiente *in vitro*. Também foram encontradas proteínas relacionadas à síntese de outras proteínas, sendo que a sua biossíntese é um fator chave na indução das ESPs. A presença das proteínas *glutamine synthetase* e *cysteine synthase* são de extrema importância, pois estas proteínas estão relacionadas à síntese de aminoácidos necessários para a síntese de outras proteínas. Futuros estudos focados na quantificação de aminoácidos podem complementar estes resultados. A presença da proteína *Cell division control protein 48 homolog A* (CDC48) também se mostrou de extrema importância para a indução das ESPs visto que esta proteína é essencial para a citocinese, expansão celular e diferenciação em plantas.

No capítulo 2, estudando o desenvolvimento das ESPs através de análise proteômica, pode-se verificar a expressão de proteínas relacionadas principalmente ao metabolismo energético e carboidrato e processos metabólicos, demostrando serem essenciais na aquisição desta resposta morfogênica. Além disso, a presença de proteínas relacionadas à energia é de suma importância para o desenvolvimento das ESPs. Nesta etapa, são observadas células em constante divisão celular, tanto na formação de ESPs sobre as preexistentes quanto na formação de primórdios foliares. A enzima *enolase* presente de forma exclusiva aos 100 dias de cultivo parece ser um interessante candidato como marcador molecular nesta fase de desenvolvimento. Além disso, foram observadas proteínas do ciclo celular, que podem ser essenciais durante o desenvolvimento das ESPs. Sabe-se, portanto, que o ciclo celular é

dependente da síntese de novas proteínas para promover alterações morfológicas e bioquímicas associadas à atividade mitótica. Também foram identificadas diversas proteínas de choque térmico. Essas proteínas são responsáveis pela correta dobragem, translocação e degradação de proteínas, prevenindo também a sua agregação auxiliando na resposta morfogenética *in vitro*. Por fim, a identificação de diversas proteínas envolvidas direta ou indiretamente com o metabolismo secundário gerou fortes indícios de que o desenvolvimento das ESPs pode estar sintetizando estes compostos.

O estudo proteômico realizado neste trabalho levanta algumas limitações quanto à baixa disponibilidade de sequências da família Orchidaceae nas bases de dados, que intervém na identificação das proteínas. Isto, considerando que algumas proteínas poderiam ser únicas das orquidáceas ou apresentar modificações específicas. Assim, torna-se de suma importância o estudo gênico nas espécies de orquídeas, onde os resultados obtidos possam contribuir com esse tipo de informação.

No terceiro e último capítulo, o estudo da ação do hormônio AIA e dos açúcares na indução e desenvolvimento das ESPs foram visivelmente relatadas. O desenvolvimento de ESPs sobre as preexistentes e a formação de primórdios foliares foi claramente acompanhado por um acréscimo das concentrações de AIA, e um decréscimo nas concentrações de açúcares. Esta oscilação, parece ter sido importante para que as células se diferenciassem e adquirissem a necessária competência. A presença dos açúcares glicose, frutose e sacarose, parecem exercer importante papel de sinalização celular, além de fornecer energia para os processos metabólicos que ali ocorrem. A presença de açúcares durante a indução e desenvolvimento das ESPs, pode ser fundamental para avaliar a dinâmica nos diferentes tempos de cultivo e o que essa dinâmica influencia no desenvolvimento das ESPs. Além disso, a quantificação de demais hormônios como o ABA e citocininas dariam um embasamento maior da regulação da indução e desenvolvimento das ESPs.

O controle da indução e desenvolvimento de ESPs em *C. tigrina* é bastante complexo, pois uma série de fatores diferentes age ao mesmo tempo e no mesmo tecido. Alguns componentes importantes foram abordados no presente trabalho, contudo, a compreensão das possíveis inter-relações depende ainda, de estudos mais aprofundados. Entretanto, as informações contidas neste trabalho, por dar ênfase às alterações bioquímicas observadas na indução e desenvolvimento das ESPs, contribuem para um melhor entendimento dessas inter-relações.