UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIENCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS GENÉTICOS VEGETAIS

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As espécies reativas de oxigênio podem controlar o desenvolvimento e maturação dos embriões somáticos de *Cyclamen persicum*?

Florianópolis
2019

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Tese submetida ao Programa de Pós Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do título de Doutora em Ciências.
Orientador: Prof. Dr. Miguel Pedro Guerra
Co-orientador: Dr. Douglas André Steinmacher

Florianópolis
2019

Ficha de identificação da obra elaborada pelo autor,
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Caprestano, Clarissa Alves
As espécies reativas de oxigênio podem controlar o desenvolvimento e maturação dos embriões somáticos de Cyclamen persicum? / Clarissa Alves Caprestano ; orientador, Miguel Pedro Guerra, coorientador, Douglas André Steinmacher, 2019.
102 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Agrárias, Programa de Pós Graduação em Recursos Genéticos Vegetais, Florianópolis, 2019.

Inclui referências.

1. Recursos Genéticos Vegetais. 2. Cyclamen persicum. 3. espécie reativas de oxigênio. 4. oxi-proteômica. 5. glutationa. I. Guerra, Miguel Pedro. II. Steinmacher, Douglas André . III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. IV. Título.

Clarissa Alves Caprestano

As espécies reativas de oxigênio podem controlar o desenvolvimento e maturação dos embriões somáticos de *Cyclamen persicum*?

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Dedico este trabalho aos meus pais

Valmor e Cida

AGRADECIMENTOS

À Universidade Federal de Santa Catarina e ao Programa de Pós-Graduação em Recursos Genéticos Vegetais pela oportunidade de realizar o meu doutorado e pela qualidade da formação acadêmica.

Aos meus pais e irmãs pelo apoio e compreensão durante estes anos de doutorado.

Ao professor Miguel Pedro Guerra pela orientação, confiança desde a graduação.

Ao meu co-orientador Dr. Douglas Steinmacher por todo o suporte durante estes anos e pelo apoio fundamental para que eu conseguisse realizar meu doutorado sanduiche.

À todos os professores do Programa de Pós Graduação em Recursos Genéticos Vegetais por todo conhecimento repassado.

À Berna por todo o apoio logístico ao meu doutorado e pelas boas risadas.

À Sarah Agapito por todo o apoio na parte proteômica e amizade desde os primórdios.

A CAPES e CNPq pelo suporte financeiro.

I thank to Prof. Traud Winkelmann for all support during my period in Germany. Was a pleasure work with you.

To the best technicians in the world: Ewa Schneider, Bäbel Ernst and Friederike Schröder, thank you for all support, media, pipettes and coffee.

To my lab coworks: Melanie, Svenja, Philipp, Mona, Emmanuel, Carina, Stefan, Bunlong, Jane, Mario, Sewwandi, Max, Annmarie, Kristin, Björn, Nhung, Yuru and Hang, was a pleasure work with you.

To Rebecca Cohn for help me with the collecting samples.

To Christin Bünding for all support with the proteomic part and for your friendship.

To Prof. Hans-Peter Braun and his work group for the support with the proteomics experiments, in special to Dr. Holger Eubel, Dr. Christin Lorenz and Dr. Jennifer Senkler.

To Emre Arslantas for your kindness, comprehension and support.

To DAAD and Leibniz Universität Hannover for the financial support during my sandwich period in Germany.

“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.”

Albert Einstein, 1929

RESUMO

Cyclamen persicum Mill. (Primulaceae) é uma planta ornamental de importância mundial cuja propagação se dá por via sexuada. A embriogênese somática, um dos principais sistemas de regeneração de plântulas *in vitro*, permite a redução de custos de propagação associados a um maior rendimento biológico em comparação aos demais sistemas de micropropagação. A propagação de ciclâmen via embriogênese somática foi primeiramente descrita por WICART; MOURAS e LUTZ (1984), sendo estudada por diversos grupos. Entretanto vários gargalos impedem a aplicação da técnica para a multiplicação comercial da espécie. Entre estes se destacam a assincronia do desenvolvimento dos embriões somáticos, resposta embriogenética genótipo- dependente, risco de mutações, perda de capacidade embriogenética, malformações dos embriões somáticos, embriogênese secundária. A modulação da embriogênese somática está ligada ao estresse oxidativo durante a indução e a maturação dos embriões somáticos. Nos últimos anos, a suplementação do meio de cultivo com glutationa, um tiol não proteico e *scavenger* de peróxidos decorrentes do metabolismo celular e do estresse oxidativo, tem sido utilizada para modular o estresse oxidativo durante a embriogênese somática de várias espécies. A presente tese teve como objetivo estudar os fenômenos morfofisiológicos e bioquímicos resultantes dos processos oxidativos durante o desenvolvimento e a maturação de embriões somáticos de *Cyclamen persicum*, visando e superar os gargalos deste sistema embriogenético. A tese foi redigida em quatro capítulos: no primeiro é apresentado o estado da arte. O segundo aborda a influência de concentrações de GSH sobre os estágios iniciais da embriogênese somática em ciclâmen. O terceiro apresenta a quantificação dos conteúdos endógenos de cisteína, glutationa reduzida e oxidada durante a embriogênese somática e suas implicações. O último apresenta um estudo do proteoma e do oxi-proteoma de embriões somáticos de ciclâmen submetidos ou não ao tratamento com GSH.

Palavras-chave: *Cyclamen persicum,* espécie reativas de oxigênio, oxi-proteômica, GSSG, GSH.

RESUMO EXPANDIDO

Introdução

Cyclamen persicum Mill. (Primulaceae) é uma planta ornamental propaganda por sementes, o que requer polinização manual resultando em um alto custo das sementes. A embriogênese somática (SE) pode se tornar uma alternativa para a propagação massal da espécie. Entretanto, o uso da embriogênese somática continua limitada por gargalos como a baixa tolerância a dissecação, um desenvolvimento não sincronizado dos embriões somáticos e a falta de uma etapa de maturação que leva a uma baixa taxa de germinação. Dentre os fatores que podem estar associados com os obstáculos fisiológicos mencionado a cima, as espécies reativas de oxigênio (ROS) são o foco de estudos recentes. ROS são espécies transientes que incluem radicais livres, por exemplo radical superóxido (O_2^-) e outros reativos intermediários como o peroxido de (H_2O_2) e o óxido nítrico (NO). Uma rede complexa de moléculas com baixo peso molecular, juntamente com a glutationa, com uma capacidade antioxidante controla a concentração de ROS e o reparo oxidativo. Tem sido demonstrado que alterações no estado redox da glutationa i.e. a razão glutationa reduzida/glutationa oxidada (GSH/GSSG) pela adição da respectiva forma no meio de cultura resulta em efeitos positivos na qualidade e na quantidade de embriões somáticos formados (BELMONTE et al., 2003, 2005; STASOLLA et al., 2008). No desenvolvimento inicial dos embriões somáticos a suplementação de GSH ao meio de cultura resulta em maior quantidade de proembriões formados, enquanto que a suplementação de GSSG numa fase de maturação resulta numa maior formação de embriões somáticos normais (STASOLLA, 2010).

Objetivos

O presente trabalho visou descrever os fenômenos morfofisiológicos e bioquímicos resultantes dos processos oxidativos durante o desenvolvimento e a maturação de embriões somáticos de *Cyclamen persicum*, com interesse de superar os gargalos deste sistema embriogenético.

Metodologia

Capítulo 2: Foi testado a influência de altas concentrações de GSH (0, 4, 6, 8 e 10 mM GSH) durante as primeiras fases da diferenciação dos embriões somáticos do genótipo 56/2 e duas concentrações (0 e 4mM) para outros dois genótipos (2824 e CNP6). Foram avaliados, o número de embriões somáticos

formados, a porcentagem de conversão viabilidade celular por *fluorescein diacetate* (FDA) como descrito em WINKELMANN et al. (1998) e a localização de superóxidos por azul de tretrazolio descrito PIETROWSKA et al. (2015). **Capítulo 3:** GSH, GSSG e cisteína foram extraídas e quantificadas durante a embriogênese somática de *Cyclamen persicum* seguindo a metodologia descrita por RELLÁN-ALVAREZ *et al.* (2006) com modificações. Para testar a influência das duas formas de glutationa durante a ES foi performado um experimento fatorial de 2x2x2 sendo o primeiro fator a suplementação ou não de 2mM de GSH, o segundo fator foi o tempo de cultivo (7 ou 14 dias) e o terceiro fator a suplementação ou não de 2mM de GSSG após esse período. Além disso, foram analisados o número de embriões somáticos formados e a taxa de conversão destes em plântulas. **Capítulo 4:** Foram performadas análises proteômicas e do oxi-proteoma dos aglomerados celulares embriogênicos de *Cyclamen persicum* submetidos ou não a suplementação de 2mM GSH. As proteínas totais foram extraídas de acordo com RODE *et al.* (2011). A derivatização proteica com Bodipy-Hz foi realizada seguindo os procedimentos descritos por TAMARIT *et al.* (2012) com modificações. Os géis 2D foram executados seguindo a metodologia de MIHR AND BRAUN (2003). Os *spots* de proteínas foram digeridos com tripsina seguindo KLODMANN *et al.* (2010) e analisados em espectrometro de massa Ultraflex II. Avaliados o número de embriões somáticos formados, a viabilidade celular por *fluorescein diacetate* (FDA) (WINKELMANN et al., 1998) e localização de superóxidos por azul de tretrazolio (PIETROWSKA et al., 2015).

Resultados e Discussão

Capítulo 2: A viabilidade celular das células do genótipo 56/2, que foram suplementadas com altas concentrações de GSH (8 e 10mM) foi muito baixa, resultando numa baixa formação de ES aos 28 dias. Não houve diferenças entre o controle e suplementação de 4mM de GSH, sendo que este último resultou em um maior número de ES formados. Entretanto, uma grande variação no número de embriões no estágio torpedo foi observada tanto entre experimentos quanto entre blocos dentro do mesmo experimento. Com esses resultados somente foram testadas estas últimas concentrações nos genótipos CNP6 e 2824. Essa suplementação resultou num aumento do número de ES no estágio torpedo para o genótipo CNP6 e numa diminuição no genótipo 2824. **Capítulo 3:** Foi observado um pico de GSH e cisteína nas culturas celulares após 24 horas de

cultivo. Este pico pode ser relacionado com o processo de peneiração realizado para desagregar as suspenções celulares de ciclamen. A razão GSH/GSSG+GSH mostrou um pequeno decréscimo após 7 dias de cultivo, tornando o meio reduzido. A manipulação experimental do conteúdo endógeno da razão GSH/GSSG+GSH, pela suplementação de glutationa reduzida (GSH) ou oxidada (GSSG) promoveu uma melhor ES em diferentes espécies como *Picea glauca* (BELMONTE *et al.*, 2005), *Araucaria angustifolia* (VIEIRA *et al.*, 2012) e *Pinus taeda* (PULLMAN *et al.*, 2015). Em *Cyclamen persicum* demonstramos que um ambiente reduzido nas fases iniciais do desenvolvimento dos ES gera um aumento de 15% no número de embriões formados quando o GSH é suplementado por sete dias. Entretanto a suplementação de GSSG nas fases posteriores da ES não melhorou o número de embriões formados. **Capítulo 4:** A viabilidade celular foi reduzida a 47% quando da aplicação de GSH diferindo do tratamento controle que teve 56% de clusters viáveis aos 14 dias de cultivo. Uma maior concentração de superóxidos foi observada nos clusters do tratamento controle (63%) quando comparado ao tratado com GSH (42%). O número de embriões somáticos no estádio torpedo, aos 28 dias de cultivo, foi maior nos previamente tratados com GSH (4,1 embriões/200mg) comparado ao controle (2,3 embriões/200mg). Análises do oxiproteoma durante a ES mostrou uma pequena diferença entre o controle e o GSH tratadas. No terceiro dia após o tratamento três proteínas associadas com o metabolismo de carboidratos foram mais oxidadas no tratamento controle do que nos com GSH. A UDP-sugar pyrophosphorylase (ID 208) são enzimas essenciais na síntese de sacarose e percursora da biogênese da parede celular. Durante a ES há uma grande demanda por energia para suportar o processo de divisão celular e alongamento. (NOAH et al., 2013). A oxidação desta proteína no início do desenvolvimento do embrião somático pode reduzir a quantidade de carboidrato no embrião, resultando numa baixa formação de ES.

Considerações finais

Neste trabalho relevantes informações sobre a embriogênese somática em *Cyclamen persicum* foram obtidas. Estas informações podem servir de base para um aprimoramento do protocolo de embriogênese somática da espécie, visando à propagação comercial massal da espécie. Num primeiro momento foi testada a influência de altas concentrações de GSH durante as primeiras fases da

diferenciação dos embriões somáticos. Em dois genótipos ocorreu um efeito promotor do GSH durante a formação de embriões somáticos, entretanto uma grande variação entre a reação das culturas ao GSH foi visualizada. Uma investigação do conteúdo endógeno de GSH, GSSG e cisteína durante a fase de diferenciação dos embriões somáticos de ciclâmen, mostrou que estes compostos não se mantêm estáveis e homogêneos. Um decréscimo dos níveis de GSH foi observado durante os sete primeiros dias de cultivo. Este decréscimo no estado redox da glutationa pode ser revertido pela suplementação de 2mM GSH durante os sete primeiros dias de cultivo. Entretanto, a posterior transferência para um meio oxidado (GSSG) não mostrou efeitos na conversão dos embriões somáticos. Pela primeira vez, um estudo do oxi-proteoma durante a embriogênese somática de ciclâmen foi realizado. Um estudo do oxi –proteoma e do proteoma total de *clusters* de células embrionárias proteínas importantes envolvidas no transporte polar de auxina se mostraram carboniladas no tratamento controle, bem como em maiores concentrações no proteoma total das células tratadas com GSH. Estes resultados sugerem uma oxidação de proteínas importantes para o desenvolvimento do embrião somático e podendo ser revertidas pela suplementação de GSH ao meio de cultivo.

Palavras chave: *Cyclamen persicum,* espécie reativas de oxigênio, oxi-proteômica, GSSG, GSH.

ABSTRACT

Cyclamen persicum Mill. (Primulaceae) is an ornamental crop with high economic relevance worldwide. Somatic embryogenesis, one of the main *in vitro* plant regeneration systems, allows the reduction of propagation costs associated with a higher biological yield compared to other micropropagation systems. WICART; MOURAS e LUTZ (1984) first described the propagation of cyclamen via somatic embryogenesis. However, several bottlenecks prevent the commercial application of the technic. These include the asynchrony of the development of somatic embryos, genotype-dependent embryogenic response, risk of mutations, loss of embryogenic capacity, somatic embryo malformations, and, secondary embryogenesis. The somatic embryogenesis modulation during the induction and maturation is linked to oxidative stress. In recent years, the culture medium supplementation with glutathione, a non-protein thiol, and scavenger of peroxides, resulted from cellular metabolism and oxidative stress has been used to modulate oxidative stress during somatic embryogenesis of various species.
The present thesis aimed to study the morphophysiological and biochemical phenomena resulted from oxidative processes during the development and maturation of *Cyclamen persicum* somatic embryos aiming to overcome the bottlenecks of this embryogenetic system. Therefore, this thesis was written in four chapters for a better exposition of the data. The first chapter presents state of the art. The second chapter deals with the influence of GSH concentrations on the early stages of somatic embryogenesis. The third chapter presents the quantification of the endogenous contents of cysteine, reduced, and oxidized glutathione during somatic embryogenesis and its implications. The last chapter presents a study of the proteome, and oxy proteome of cyclic somatic embryos submitted or not to GSH treatment.

Keywords: *Cyclamen persicum,* oxygen reactive species, oxi-proteomic, GSSG, GSH.

LISTA DE FIGURAS

Capítulo 1

Capítulo 2

Capítulo 3

Capítulo 4

LISTA DE TABELAS

Capítulo 2

Capítulo 4

LISTA DE ABREVIATURAS E SIGLAS

µM – Micromolar
2,4-D – 2,4 ácido diclorofenóxiacético
2-DE – Eletroforese bidimensional
2iP – N6-(2-Isopentanil)adenina
ANOVA – Análise de variância
APX – Ascorbato peroxidase
BSO – Butionina sulfoximina
DHA – Dehidroascorbato
DHAR – Dehidroascorbato redutase
ES/SE – Embriogênese somática
GR – Glutationa redutase
GSH – Glutationa reduzida
GSSG – Glutationa oxidada
H_2O_2 – Peróxido de hidrogênio
IEF – Focalização isoelétrica
IPG – Tiras de gradientes de pH imobilizado
LEC1 – Gene LEAFY COTYLEDON1
LEC2 – Gene LEAFY COTYLEDON2
M – Molar
MDA – Monohidroascorbato
ml – Mililitro
mM – Milimolar
MS – Meio de Murashige e Skoog (1962)
NADPH – Nicotinamida adenina dinucleotídeo fosfato reduzido
PEM – Massa celular proembriogenica
ROS – Espécies reativas de oxigênio
SDS – Dodecil sulfato de sódio
SOD – Superóxido desmutase
Vh – Volts por hora
WUS – Gene WUSCHEL

Sumário

1. OBJETIVOS

11. OBJETIVO GERAL

O presente trabalho visou descrever os fenômenos morfofisiológicos e bioquímicos resultantes dos processos oxidativos durante o desenvolvimento e a maturação de embriões somáticos de *Cyclamen persicum*, visando e superar os gargalos deste sistema embriogenético.

12. OBJETIVOS ESPECÍFICOS

a. Otimizar o meio de cultivo de maturação de embriões somáticos de *Cyclamen persicum* por meio da adição da glutationa ao meio de cultura;

Hipótese: A glutationa oxidada, tornando o meio de maturação oxidado**,** influência positivamente o desenvolvimento dos embriões somáticos por meio de melhorias na estrutura do meristema apical e no desenvolvimento pós-embrionário.

b. Quantificar a glutationa reduzida e oxidada durante o desenvolvimento e maturação dos embriões;

Hipótese: Vários estudos têm relatado o papel crucial de um elevado estado redox da glutationa nos primeiros estágios de desenvolvimento de embriões somáticos. Além disso, sugere-se que a formação de embriões anormais está associada a baixos níveis de GSSG durante o seu desenvolvimento.

c. Localizar superóxido nas células somáticas de *C. perscicum*;

Hipótese: A suplementação de glutationa ao meio de cultura melhora a formação do meristema apical e assim a localização dos ROS é alterada.

d. Quantificar e caracterizar proteínas e proteínas oxidadas expressas durante o desenvolvimento e maturação dos embriões somáticos;

Hipótese: Durante a recuperação metabólica da germinação de sementes *Arabidopsis* foram observados padrões específicos da carbonilação de proteínas, provavelmente necessários para neutralizar e/ou utilizar a produção de ROS resultado da recuperação metabólica durante a germinação de sementes.

2. CAPÍTULO 1: ESTADO DA ARTE

2.1. EMBRIOGÊNESE SOMÁTICA

A embriogênese somática (ES) é o processo de reprodução assexual no qual células somáticas isoladas, ou pequenos grupos delas, sofrem uma série de alterações morfológicas e bioquímicas que resultam no desenvolvimento de embriões somáticos, sendo um processo biológico análogo a embriogênese zigótica e considerado a máxima expressão da totipotencialidade das células vegetais (AMMIRATO, 1983; ELHITI; STASOLLA; WANG, 2013; GUERRA; TORRES; TEIXEIRA, 1999b; MIGUEL; GUERRA, 1997; QUIROZ-FIGUEROA; ROJAS-HERRERA; GALAZ-AVALOS, 2006). Esta rota morfogenética *in vitro* possui aplicações importantes para a multiplicação massal de genótipos superiores e também na obtenção de sistemas biológicos referenciais para estudos de fisiologia, bioquímica, moleculares e morfogenéticos do desenvolvimento embrionário vegetal (GUERRA; TORRES; TEIXEIRA, 1999b; QUIROZ-FIGUEROA; ROJAS-HERRERA; GALAZ-AVALOS, 2006).

Durante a o desenvolvimento das células somáticas é reprogramado para a ativação da rota embriogênica, a qual consiste em três estágios de desenvolvimento: indução, embriogênese e desenvolvimento (Figura 1)(ELHITI; STASOLLA; WANG, 2013)). A embriogênese somática requer uma reprogramação dos padrões da expressão gênica compreendendo a sinalização em cascata de genes que pode ativar ou desativar a sua expressão (FEHÉR; PASTERNAK; DUDITS, 2003).

A aquisição da competência embriogenética é dependente de vários fatores intrínsecos ao explante, tais como o genótipo de origem do explante e sua condição fisiológica. Outros fatores encontram-se ligados às condições de cultivo, tais como a composição do meio de cultivo, a concentração dos reguladores de crescimento e as transferências sequênciais (FEHÉR, 2005; GUERRA; TORRES; TEIXEIRA, 1999b; STEINMACHER; CLEMENT; GUERRA, 2007; STEINMACHER, 2005). A aquisição da competência embriogenética é considerada o fator mais crítico da ES (FEHÉR; PASTERNAK; DUDITS, 2003; STEINMACHER; CLEMENT; GUERRA, 2007; STEINMACHER, 2005).

Desdiferenciação celular é um processo no qual células especializadas (maduras) são convertidas em um estado transiente (*stem cell-like*). Portanto, a

desdiferenciação celular é um processo de regressão celular, resultando em uma célula com competêntencia para se desenvolver para uma determinada direção, de acordo com os estímulos impostos (ELHITI; STASOLLA; WANG, 2013).

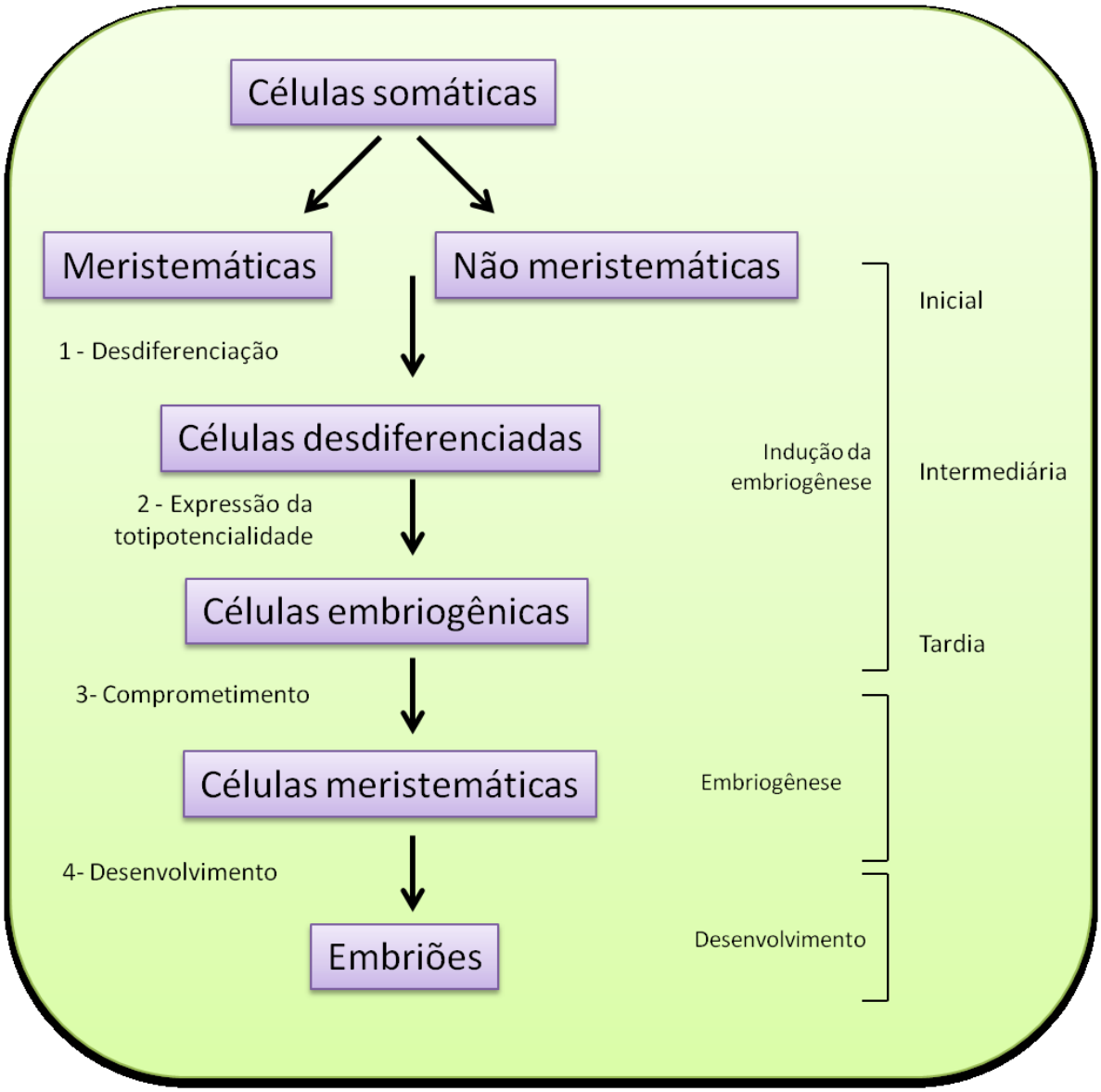

Figura 1. Diagrama esquemático da embriogênese somática *in vitro*. (Adaptado de Elhiti, Stasolla e Wang (2013)). (CAPRESTANO, C.A. 2015)

A rota da ES pode ser iniciada por células diferenciadas, direta ou indiretamente. Sinais endógenos ou exógenos, tais como injúrias, estresses, reguladores de crescimento, entre outros, podem resultar num desbalanceamento do conteúdo endógeno hormonal celular levando a desdiferenciação de células competentes, seguido de embriogênese somática ou pela superproliferação e diferenciação de tecido de calos. Por meio de sinais posteriores a célula desdiferenciada competente pode tornar-se uma célula

embriogênica determinada. Mutações nos genes repressores do desenvolvimento embrionário de células vegetativas (PICKLE chromatin-remodeling ATPase; PKL) e ou a expressão ectópica de fatores de transcrição responsáveis pela identidade do meristema/embrião, tais como WUSCHEL (WUS) ou LEAFY COTYLEDON1 ou 2 (LEC1; LEC2) podem também resultar na diferenciação do embrião somático a partir de células competentes (FEHÉR, 2015)(Figura 2).

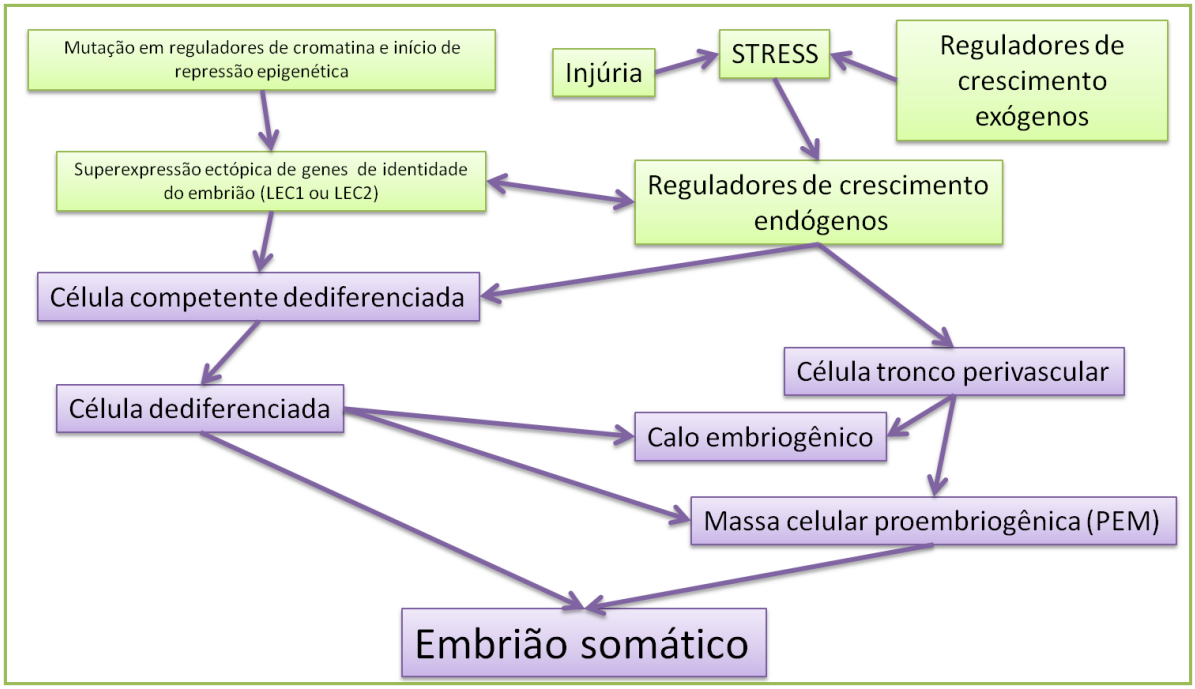

Figura 2. Rota da embriogênese somática (Adaptado de Fehér (2015)). (CAPRESTANO, C.A. 2015)

2.2. *Cyclamen persicum*

Cyclamen persicum Mill. (Primulaceae) (Figura 3) é uma espécie herbácea de importância ornamental, originária do extremo oriente da região mediterrânea (GREY-WILSON, 2002). É uma planta tuberosa que não forma tubérculos secundários, impossibilitando assim a propagação vegetativa. Por outro lado, a reprodução sexuada requer polinização manual, o que, em alguns cultivares, pode resultar em variabilidade da progênie. Como resultado, as suas sementes atingem alto preço, de até 0,20 € por semente (SCHWENKEL, 1999).

Figura 3: *Cyclamen persicum* Mill. (CAPRESTANO, C.A., 2015).

Além disso, parte expressiva dos ciclâmens comercializados são híbridos F1, o que dificulta a produção por depressões endogâmicas em consequência de gerações de autofecundação (WINKELMANN *et al.*, 2011a).

A utilização da ES como estratégia biotecnológica de propagação pode solucionar os problemas anteriormente mencionados. A ES em *Cyclamen persicum* foi descrita primeiramente por Wicart *et al.* (1984) e tem sido alvo de estudos por diversos grupos de pesquisa (OTANI; SHIMADA, 1991; WINKELMANN; HOHE; SCHWENKEL, 1998 JALALI *et al.*, 2010) (Figura 4).

Entretanto, vários gargalos são descritos para a aplicação da embriogênese somática para a produção em larga escala de ciclâmen, incluindo: assincronia do desenvolvimento dos embriões somáticos, resposta embriogenética genótipo- dependente, risco de mutações, perda de capacidade embriogenética, malformações dos embriões somáticos, embriogênese secundária (WINKELMANN *et al.*, 2011a), bem como um defectivo processo de maturação, o que resulta em baixas taxas de conversão dos embriões somáticos (HOENEMANN *et al.*, 2010; SCHMIDT *et al.*, 2006).

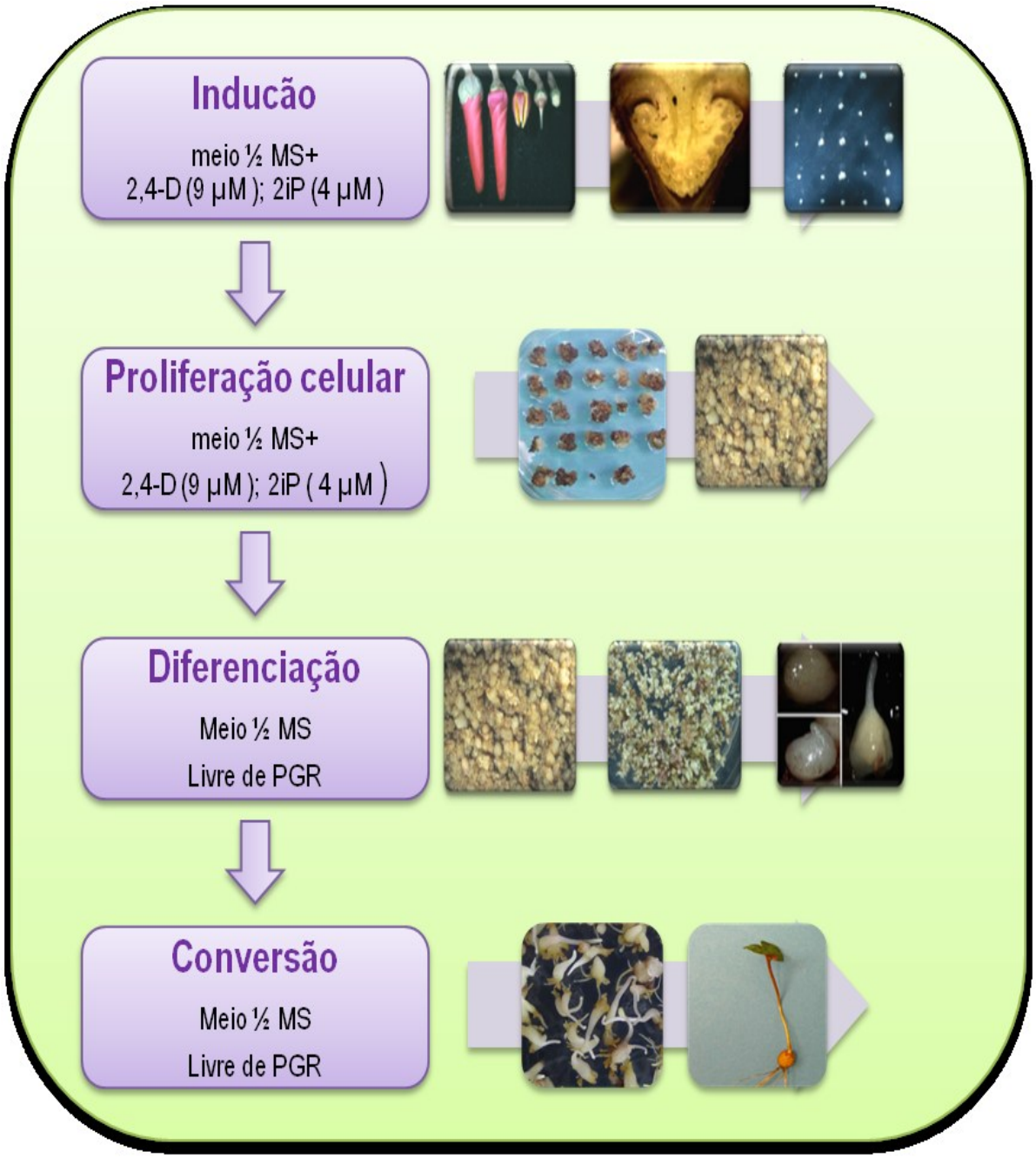

Figura 4: Representação esquemática da embriogênese somática de *Cyclamen persicum* (Adaptado de Winkelmann (2010)). (CAPRESTANO, C.A. 2015)

2.3. ESPÉCIES REATIVAS DE OXIGÊNIO

Espécies reativas de oxigênio (ROS) são espécies transientes que incluem radicais livres como ânion radical superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), radical hidroxil ($^{\bullet}OH$), oxigênio singuleto ($^1O_2$) e óxido nítrico (NO_3) (SAED-MOUCHESHI *et al.*, 2014). A produção de ROS ocorre em diferentes compartimentos da célula vegetal durante os eventos do metabolismo normal e durante a imposição estresses bióticos ou abióticos (Figura 5). A cadeia

transportadora de elétrons do cloroplasto e mitocôndria é responsável pela produção de ROS como superóxido, peróxido de hidrogênio e oxigênio singleto. Já o peróxido de hidrogênio é produzido no peroxissomo, especialmente durante a fotorrespiração e também durante a β-oxidação dos ácidos graxos (RAO; REDDY, 2008).

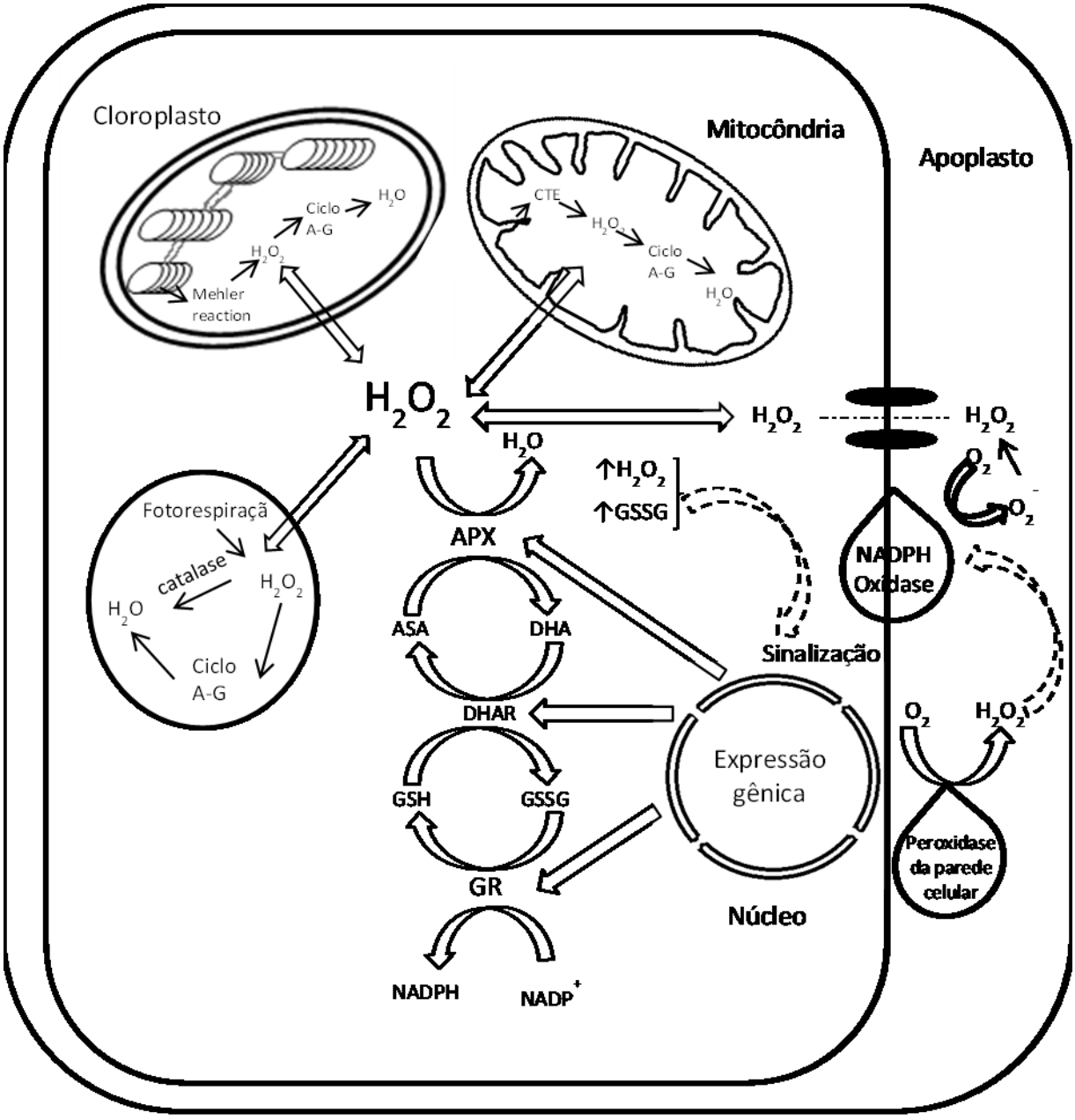

Figura 5: Formação e eliminação de espécies reativas de oxigênio na célula vegetal. (CTE: cadeia de transporte de elétrons; Ciclo A-G: ciclo glutationa-ascorbato) (Adaptado de Rao e Reddy (2008)). (CAPRESTANO, C.A. 2015)

O cloroplasto é uma das grandes fontes de ROS celular por causa das reações altamente energéticas da fotossíntese e sua grande concentração de oxigênio. Alta intensidade luminosa pode levar a um excesso de redução do

fotossistema 1 (PSI), esgotando assim o aceptor de elétrons nicotinamida adenina dinucleotídeo fosfato ($NADP^+$). Sob estas condições o oxigênio compete com o PSI por elétrons, resultando na formação de superóxido pela reação de Mehler. O superóxido então reage com o H_2O_2 produzindo radical hidroxil pela reação de Haber-Weiss. Esta reação é termodinamicamente lenta, entretanto na presença do catalisador ferro a reação de Fenton forma um significante número de radicais hidroxilas. Estes radicais hidroxilas são moléculas altamente reativas que podem causar danos em membranas e outras macromoléculas, tais como pigmentos fotossintéticos, proteínas, DNA e lipídios (FOYER; NOCTOR, 2005, 2013; KARUPPANAPANDIAN *et al.*, 2011; RAO; REDDY, 2008)

A enzima superóxido desmutase (SOD) é responsável pela degradação do superóxido em peróxido de hidrogênio. O excesso de H_2O_2 é eliminado rapidamente pelo ciclo ascorbato-glutationa, caso contrário pode ser convertido em radicais hidroxilas, que são altamente oxidantes. A conversão de H_2O_2 em H_2O se dá pela pela ação da ascorbato peroxidase (APX), utilizando ascorbato como substrato. Este é oxidado à monohidroascorbato (MDA) ou dehidroascorbato (DHA). MDA será reduzido novamente a ascorbato pela ação da dehidroascorbato redutase (DHAR) usando glutationa reduzida (GSH) como substrato, que é oxidada formando glutationa oxidada (GSSG). A reação de redução da GSSG se dá pela ação da glutationa redutase (GR), usando nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH). A baixa afinidade da catalase peroxissomo pelo H_2O_2 não permite a sua completa eliminação, o que é realizada pelo ciclo ascorbatoglutationa (RAO; REDDY, 2008; SLOOTEN; VAN MONTAGU; INZÉ, 1998).

Altas concentrações de ROS são consideradas prejudiciais a homeostase celular, podendo causar danos aos constituintes celulares, por oxidar rapidamente a membrana lipídica, proteínas e organelas celulares, levando à perda de função (PASTERNAK, 2005; WONIASKI-JUNIOR, 2008). O termo estresse oxidativo ainda carece de uma definição precisa, mas tem como características principais: (1) aumento da carga oxidativa (aumento da produção de ROS); (2) potencial de oxidação descontrolado, devido a taxas de produção superior a de metabolismo (3); dano oxidativo aos componentes celulares, supondo que a taxa de oxidação seja superior a reparação ou substituição e resultando: (4) no acúmulo de danos aos componentes celulares, que de alguma forma podem levar à perda da função e morte (FOYER; NOCTOR, 2011).

Entretanto em baixas concentrações as ROS desempenham um papel importante como mensageiros secundários em diversas respostas aos hormônios vegetais, incluindo o fechamento estomatal, o gravitropismo radicular, a germinação, a biossíntese de lignina, a morte celular programada, as reações de hipersensibilidade e os estresses osmóticos (SHARMA *et al.*, 2012).

Uma representação esquemática do efeito da concentração de ROS nas células vegetais é apresentada na Figura 6. ROS são produzidas durante todo o metabolismo vegetal e normalmente são mantidas em níveis basais na célula vegetal. Esse controle é realizado por uma complexa rede de enzimas e moléculas antioxidantes que mantem o equilíbrio redox na célula. O crescimento e desenvolvimento da célula vegetal sob condições ótimas é resultado da ação de um sistema antioxidante capaz de manter a formação de ROS em um nível basal. Quando as plantas são expostas a diferentes níveis de estresse biótico ou abiótico a sobrevivência das células é garantida pela ação de agentes antioxidantes de defesa, quem mantem os níveis de ROS baixos e a manutenção do equilíbrio redox na célula. O estresse oxidativo ocorre quando o equilíbrio redox da célula vegetal é perturbado pelos níveis elevados de ROS ou pelo esgotamento dos sistemas de defesa antioxidante, ou uma combinação de destes, levando ao colapso e morte celular (KARUPPANAPANDIAN *et al.*, 2011).

2.4. GLUTATIONA E O SISTEMA REDOX

A glutationa (L-γ-glutamil-L-cisteínilglicina) é um tripeptídio hidrosolúvel formado pelos aminoácidos ácido glutâmico, L-glicina e cisteína. A síntese de glutationa se dá em duas etapas: primeiramente a γ-glutamil cisteína sintetase ou a glutamato cisteína ligase forma, a partir do glutamato e da cisteína, o dipeptídeo γ-GLUCys, e este pela ação da glutationa sintetase combina-se com a glicina formando o GSH (NOCTOR *et al.*, 2011).

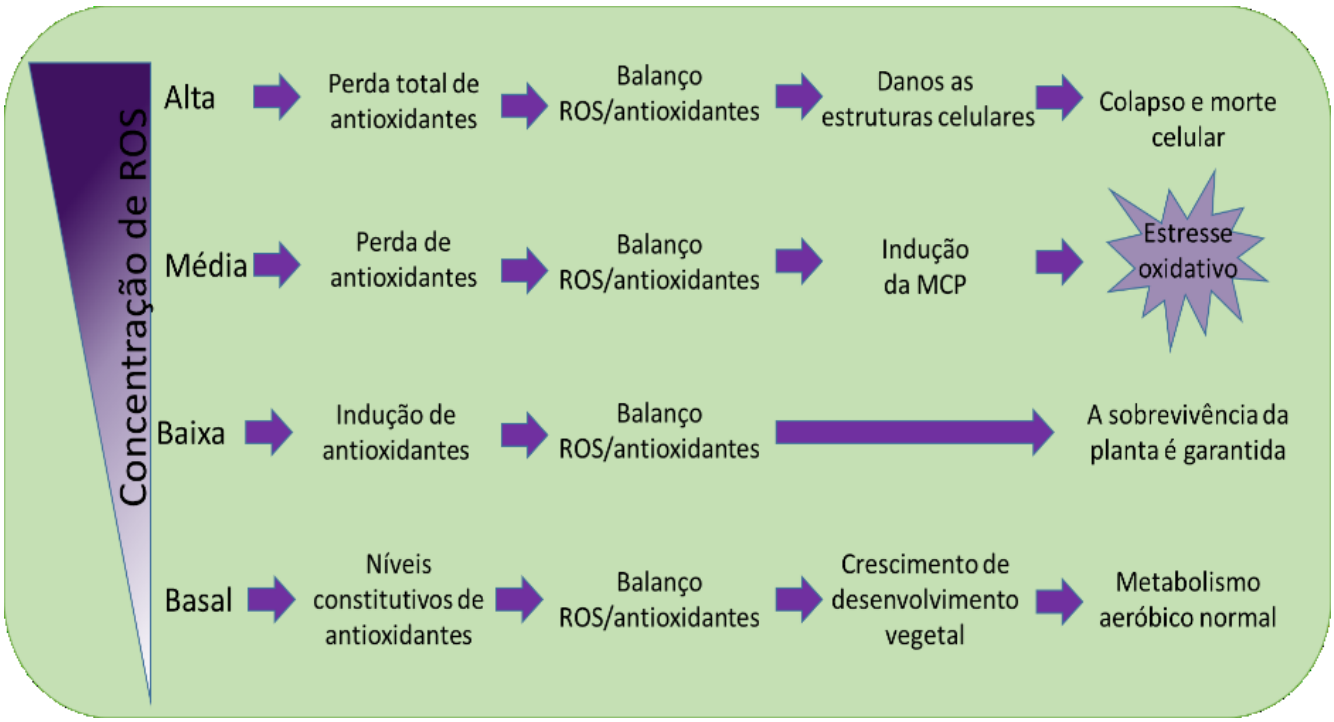

Figura 6: Representação esquemática da concentração das espécies reativas de oxigênio e seu efeito sob a homeostase celular (Adaptado de Karuppanapandian *et al.* (2011)). (CAPRESTANO, C.A. 2015)

A glutationa é o mais abundante tiol não protéico encontrado nos organismos e pode considerada um dos maiores determinantes da redox da homeostase celular (PAVARINO *et al.*, 2013). Como consequência GSH é um eficaz *scavenger* de peróxidos decorrentes do metabolismo celular ou do estresse oxidativo (GARCÍA-GIMÉNEZ *et al.*, 2013).

O ciclo ascorbato-glutationa (Figura 7) é considerado uma das vias mais importantes para a detoxificação do peróxido de hidrogênio produzido nas células vegetais. Sendo particularmente importante no cloroplasto, uma das maiores fontes de peróxido de hidrogênio celular, mas tambem ocorre no citosol, peroxissomos e mitocôndrias (JIMENEZ *et al.*, 1997).

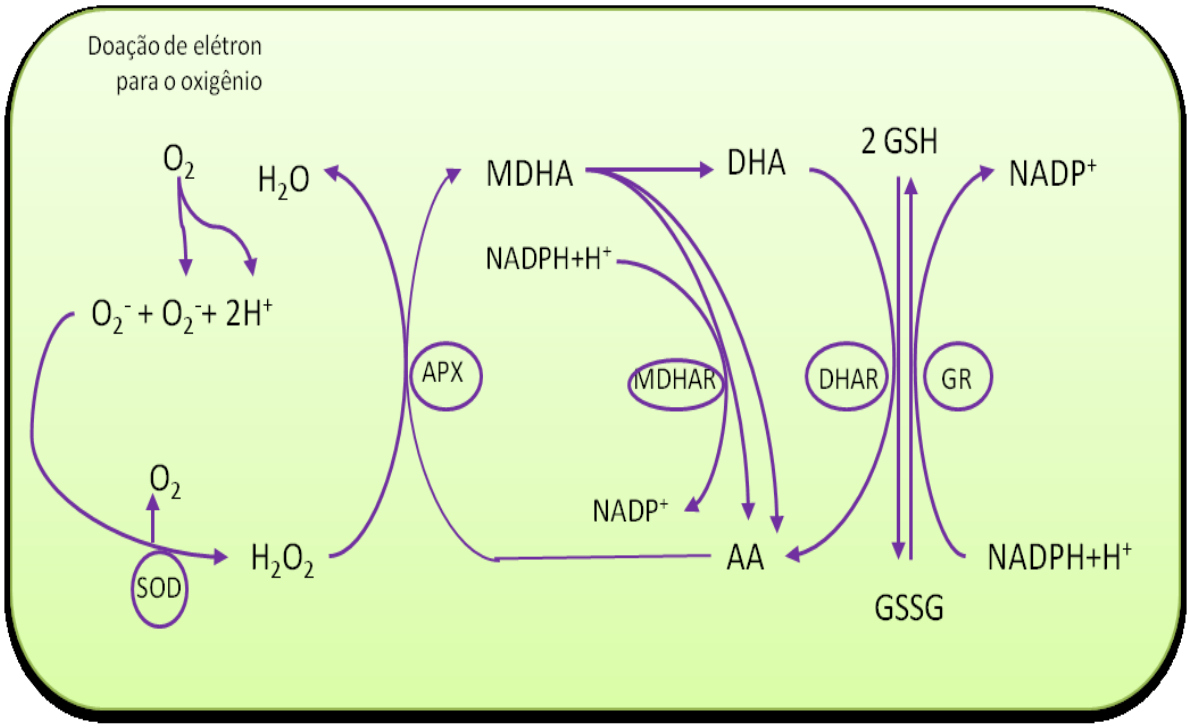

Figura 7: Ciclo ascorbato-glutationa de eliminação enzimática de ROS. Nem todas as reações são representadas estequiometricamente. (Adaptado de Noctor e Foyer (1998). (CAPRESTANO, C.A. 2015)

Neste ciclo, o peróxido de hidrogênio é convertido em água pela ação da ascorbato peroxidase (APX) que oxida o ascorbato em radical monodesidroascorbato (MDHA), o qual é reduzido novamente a ascorbato pela reação mediada pela monohidroascorbato retutase (MDHAR), utilizando NADPH e hidrogênio ou pela ação da dehidroascorbato retutase (DHAR) utilizando 2 GSH como substrato. Com isso o GSH é oxidado em GSSG, sendo essa reação revertida pela ação da glutationa redutase usando NADPH (RAO; REDDY, 2008) (Figura 8).

2.5. ESPÉCIES REATIVAS DE OXIGÊNIO E A EMBRIOGÊNESE SOMÁTICA

Pesquisas recentes têm evidenciado as relações entre ROS e a morfogênese vegetal, tornando evidente que as moléculas de ROS desempenham importantes papéis como moléculas sinalizadoras e reguladoras do desenvolvimento vegetal (CAUSIN *et al.*, 2012; DENNESS *et al.*, 2011; FOREMAN *et al.*, 2003; MORI; SCHROEDER, 2004). O estresse oxidativo serve como um modulador da ES em plantas, induzindo a divisão celular autônoma (PASTERNAK, 2005).

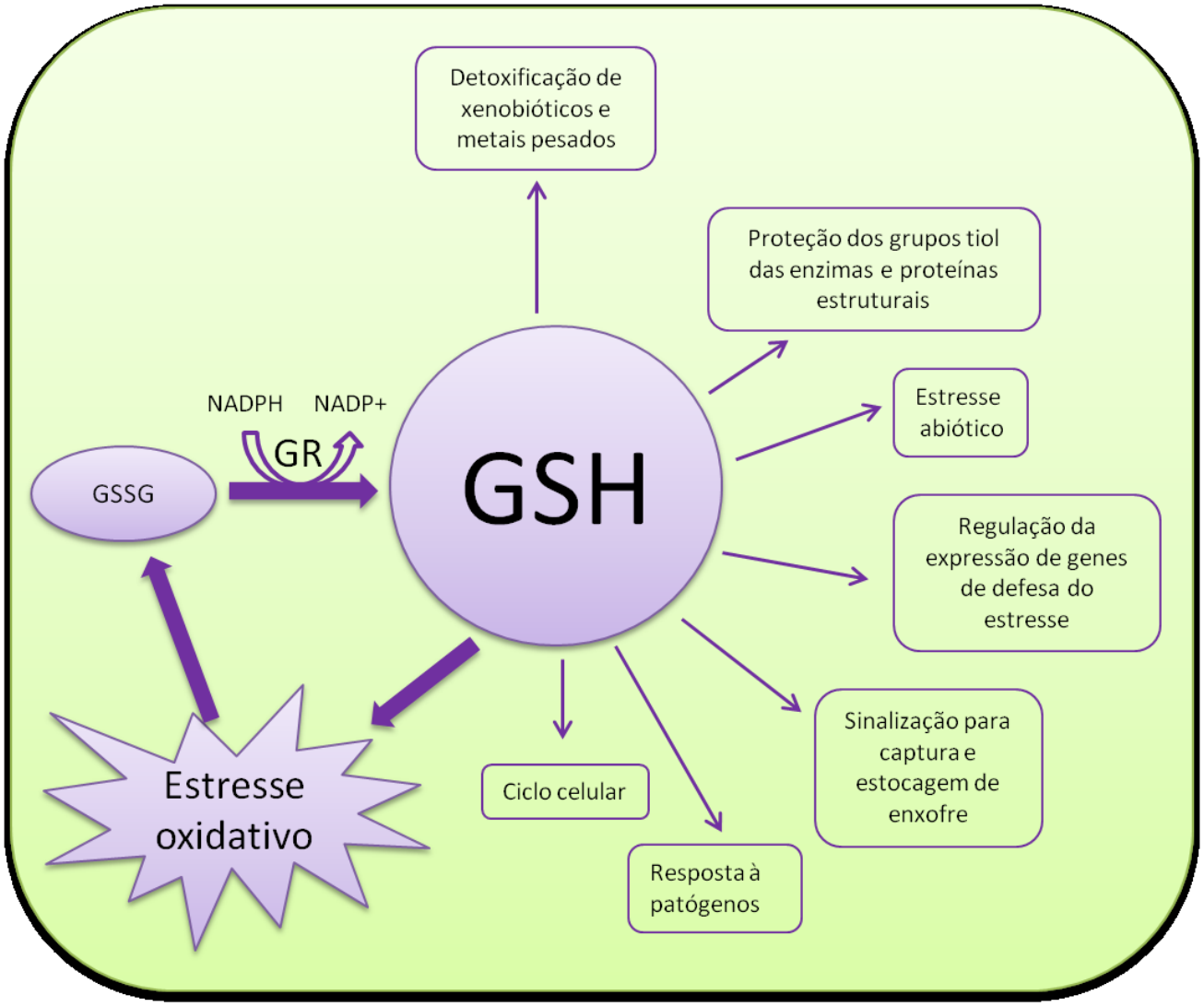

Figura 8: Função da glutationa redutase (GR) e da glutationa (GSH) no metabolismo vegetal. (Adaptado de Rao e Reddy (2008). (CAPRESTANO, C.A. 2015)

Aplicações de butionina sulfoximina (BSO), um inibidor da glutationa redutase, que altera o pool de glutationa celular para a forma oxidada GSSG afetaram positivamente qualidade do embrião de *Picea abies* por meio de melhorias na estrutura do meristema apical e na promoção da maturação do embrião, melhorando assim o desenvolvimento pós-embrionário (BELMONTE; STASOLLA, 2007).

A suplementação de BSO ao meio de cultivo tornando o meio oxidativo parece ser importante para o desenvolvimento das plantas. Em embriões somáticos de *Picea abies* e *Brassica napus*, tratamentos com BSO ou glutationa oxidada aumentaram significativamente o número e qualidade de embriões somáticos (BELMONTE; STASOLLA, 2009; BELMONTE *et al.*, 2003, 2005c, 2005d; STASOLLA *et al.*, 2008).

No entanto os mecanismos controlados pelo maior potencial oxidativo não são conhecidos. JOB e colaboradores (2005), trabalhando com *Arabidopsis thaliana*, concluíram que mudanças observadas nos padrões específicos da carbonilação de proteínas (oxidação) são provavelmente necessárias para neutralizar e/ou utilizar a produção de ROS resultado da recuperação metabólica durante a germinação de sementes.

Em *Picea glauca*, a manipulação da razão GSH/GSSG, pela suplementação destes ao meio de cultivo, afetou a qualidade e quantidade de embriões somáticos formados. No desenvolvimento inicial dos embriões, a suplementação de GSH resultou em uma maior quantidade de pró-embriões, sendo que na fase de maturação a inversão dessa razão pela suplementação de GSSG resultou numa redução na formação de embriões anormais (BELMONTE; YEUNG, 2004).

Os aspectos relacionados à modulação da embriogênese somática por meio das ROS ainda não estão desvendados. Estudos demonstram que a suplementação de BSO ou as formas de glutationa podem melhorar a formação dos meristemas apicais e auxiliar na formação de proteínas de reserva. Entretanto, ainda há dúvidas relativas à essencialidade à localização das ROS nos meristemas. Assim se propõe no presente estudo aprofundar as relações entre as ROS no desenvolvimento e maturação dos embriões somáticos de *Cyclamen persicum,* abordando tanto a localização destas ROS quanto aos seus efeitos na oxidação de proteínas que podem modular essa maturação.

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3. CAPÍTULO 2: EFFECT OF GLUTATHIONE ON THE EARLY DIFFERENTIATION OF *CYCLAMEN PERSICUM* SOMATIC EMBRYOS*.

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*Este manuscrito encontra-se publicado no periódico DGG – Proceedings.

3.1. INTRODUCTION, KNOWLEDGE, OBJECTIVES

Cyclamen persicum (Primulaceae) is an ornamental pot plant and is propagated by seeds. However, seed propagation requires manual pollination and in some cultivar groups results in the variability of the offspring. Somatic embryogenesis (SE) could become an alternative vegetative propagation pathway (SCHWENKEL; WINKELMANN, 1998; WICART; MOURAS; LUTZ, 1984). However, the use of SE for commercial scale propagation is still limited due to physiological disorders, such as low tolerance for desiccation (SEYRING; HOHE, 2005), non-synchronized development of somatic embryos and the lack of a maturation step leading to low germination rates (SCHMIDT *et al.*, 2006).

Among the factors that could be associated with the physiological obstacles above mentioned, reactive oxygen species (ROS) are the focus of recent studies. ROS are transient species that include free radicals, e.g. radical superoxide (O_2^-) and other reactive intermediates, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO). The important role of ROS as signaling and regulatory molecules during plant morphogenesis and development has been shown (SCHMIDT; SCHIPPERS, 2015). A complex network of low molecular weight molecules, among them glutathione, with an anti-oxidative capacity seems to control ROS concentration and oxidative repair. It has been demonstrated that alterations in the glutathione redox state, i.e. the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) by adding the respective form to culture media, resulted in positive effects on the quality and amount of somatic embryos (BELMONTE *et al.*, 2003, 2005; STASOLLA *et al.*, 2008). In the initial development of somatic

embryos, the supplementation of GSH to the culture medium resulted in a higher amount of pro-embryos, while in the later maturation step, the inversion of that ratio by the addition of GSSG led to an increased number of normal embryos (STASOLLA, 2010). Therefore, the aim of this study was to investigate the effect of exogenous GSH on the early embryogenesis in *Cyclamen persicum* in order to increase the number and quality of somatic embryos.

3.2. MATERIAL AND METHODS

Embryogenic cell cultures were initiated from ovules of genotype 56/2 (one single plant of the F1 hybrid cultivar 'Maxora Light Purple' (Varinova BV), as described by Schwenkel and Winkelmann (1998)

The suspension cultures were established following WINKELMANN; HOHE and SCHWENKEL (1998). In experiment 2, cell cultures of two other genotypes (2824 = plant of cv. Miracle White, Syngenta, CNP 6 = plant of cv. Super Series Compact Neon Pink, Morel) were used that had been established in the same way.

The embryogenic suspensions were sieved through a 1000 µm mesh and the density was adjusted to 4 % packed cell volume using liquid plant growth regulator (PGR)-free differentiation medium (SCHWENKEL; WINKELMANN, 1998) with different concentrations of GSH: 0, 4, 6, 8 and 10 mM GSH in the first experiment and 0 and 4 mM GSH in the second experiment. Each treatment consisted of three 100 ml Erlenmeyer flasks containing 20 ml of cell suspension. After 14 days of culture on a rotary shaker at 100 rpm and 24 °C in darkness, the cell cultures were collected on a sieve of 200 µm mesh and 200 mg were plated in 6 cm Petri dishes containing 15 ml of solidified PGR-free differentiation medium and cultured at 24 °C in darkness. The experiment was composed of three blocks and each block contained 3 Petri dishes. The number of somatic embryos in the torpedo stage was evaluated at day 28. The experiments were repeated twice.

After 28 days torpedo stage somatic embryos were collected and transferred to fresh differentiation medium for the conversion into seedlings (germination). After 30 days of culture at 24 °C in darkness, the percentage of converted embryos was evaluated by counting the somatic embryos with cotyledons of at least 1 cm length.

At day 14, i.e. at the end of the treatment in liquid culture, the cell viability was determined by staining with fluorescein diacetate (FDA) as described in WINKELMANN *et al.* (1998). The localization of superoxide (O_2^-) was performed by staining with nitro blue tetrazolium (NBT) following the method described by PIETROWSKA *et al.* (2015). The data were subjected to F-max test to verify the homogeneity of variance. Percentage data were transformed to $\sqrt{(x + 0.5)}$. The data were subjected to ANOVA and mean separation was performed by the Student Newman Keul's (SNK) test, following STEEL and TORRIE (1980).

3.3. RESULTS

After 14 days of GSH treatment in experiment 1, the viability of cell cultures was examined by FDA staining (Figure 1a-c). At the high concentrations of GSH (8 and 10 mM), hardly any viable cells were observed, whereas no differences were recorded between the control (0 mM GSH) and the cultures with 4 mM GSH (a-c). The O_2^- formation was observed in the control and the lower GSH concentrations (0-6 mM) (Figure 1d-e), whereas after treatment with 8 and 10 mM GSH NBT staining localized O_2^- only very rarely (Figure 1f).

After 28 days, high numbers of globular embryos were formed in the control and especially in the 4 mM GSH treatment, but much lower numbers at the higher GSH concentrations (8-10 mM) (Figure 1 g-i). A high variation in the number of torpedo stage somatic embryos was observed, not only between the experiments, but also between the blocks that were derived from different flasks (Table 1). Thus, the slightly increased number of embryos in the 4 mM GSH treatment was not significant when compared to the control ($p>0.05$). Significantly, negative effects of GSH on differentiation were recorded in both replications at 8 mM GSH (Table 1). The conversion rates showed no differences between the concentrations of GSH (0-10 mM) in the first experiment.

Because the higher GSH concentrations had a strong negative effect on cell viability, in experiment 2 only the lowest concentration of 4 mM GSH was tested with two further genotypes (Table 2). While in genotype CNP 6 the number of torpedo stage somatic embryos was significantly increased by the treatment, GSH negatively affected the development of somatic embryos in genotype 2824 which expressed a low embryogenic potential (Table 2).

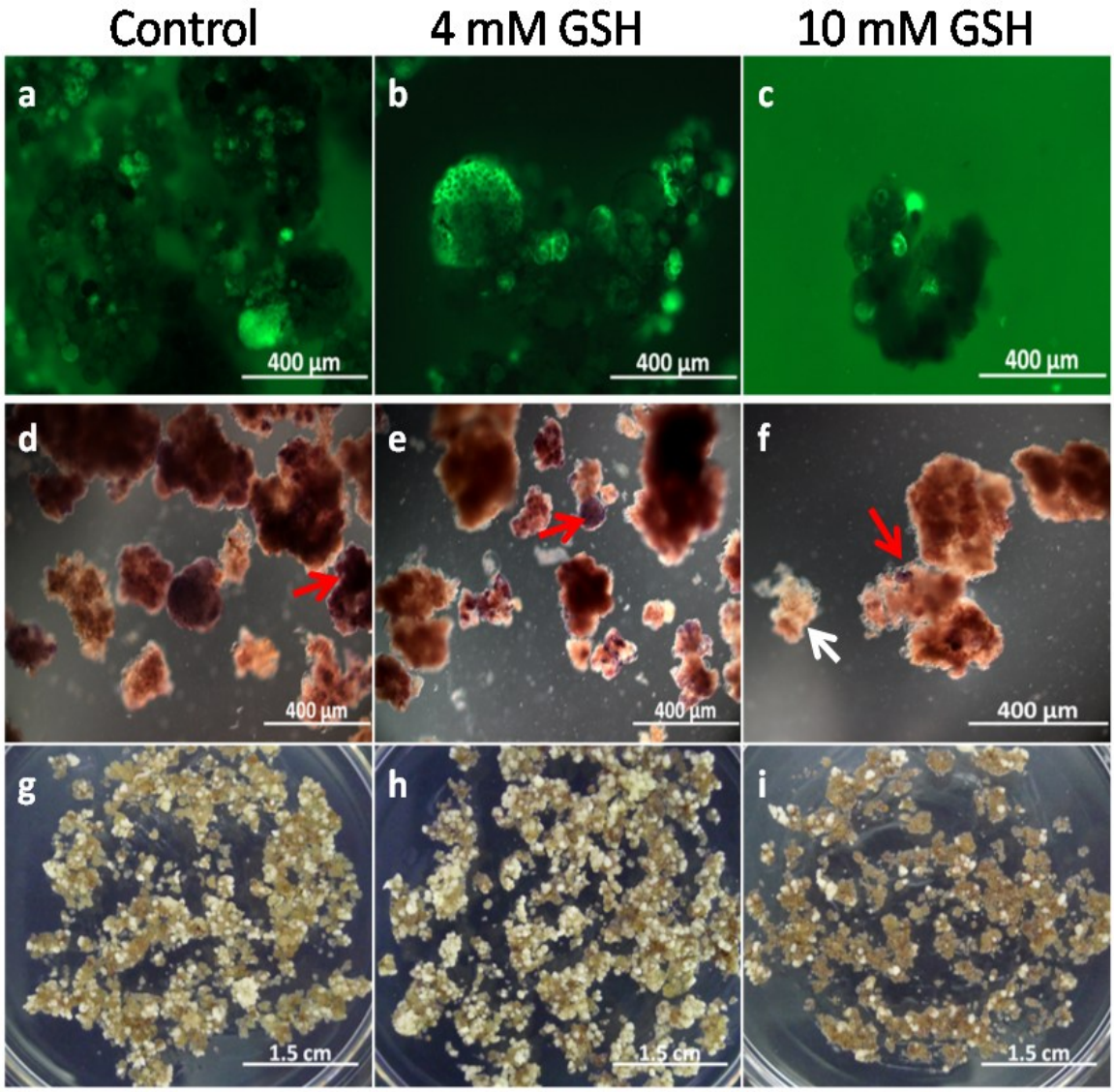

Figure 1 Effects of GSH (0, 4 and 10 mM) during early somatic embryogenesis in *Cyclamen persicum* a – c: Viability of cell cultures after 14 days of treatment. Viable cells express green fluorescence. d – f: Histochemical localization of O_2^- using NBT staining in cell cultures after 14 days of treatment; red arrows shows cell aggregates with NBT staining, white arrows show cell aggrerates negative in the NBT staining. g – i: Morphological aspects of the cultures after 28 days. (CAPRESTANO, C.A, 2015)

Table 1. Effect of GSH in the early differentiation phase of *C. persicum* on the number of torpedo stage somatic embryos after 28 days and their conversion rate after further 30 days of culture in genotype 56/2. Means plus-minus standard deviation followed by different letters indicate significant differences in the SNK test (5%). Rep = Replication.

Number of torpedo stage somatic embryos /200 mg (±SD)

	Control	4 mM	6 mM	8 mM	10 mM	Average
Rep 1	9.8 ± 1.7 ab	12.4 ± 2.1 a	7.8 ± 2.5 bc	3.6 ± 1.3 de	2.6 ± 0.5 de	7.2
Rep 2	7.7 ± 1.6 bc	10.0 ± 1.7 ab	4.3 ± 2.8 cd	2.7 ± 1.0 de	0.0 ± 0.0 e	4.9
Average	8.7 ± 1.9	11.2 ± 2.2	6.1 ± 3.0	3.1 ± 1.1	1.3 ± 1.4	

Conversion rate (%)* (±SD)

	Control	4 mM	6 mM	8 mM	10 mM	Average
Rep 1	61.6 ± 11.2 a	62.4 ± 7.3 a	37.6 ± 15.3 a	57.4 ± 8.5 a	63.9 ± 37.6 a	56.6
Rep 2	58.3 ± 3.8 a	63.9 ± 12.3 a	34.2 ± 29.6 a	58.6 ± 36.7 a	0.0 ± 0.0 b	43.0
Average	59.9 ± 7.7	63.2 ± 9.1	35.9 ± 21.2	58.0 ± 23.8	31.9 ± 42.3	

Means followed by different letters indicate values which differed to the SNK test (5%). * Data transformed in √(x + 0.5).

Table 2. Effect of GSH in the early differentiation phase of *C. persicum* on the number of torpedo stage somatic embryos after 28 days and their conversion rate after further 30 days of culture in genotypes CNP 6 and 2824. Means plus-minus standard deviation followed by different letters indicate significant differences in the SNK test (5%). Rep = Replication. Rep 2 of genotype 2824 was contaminated.

Genotype CNP 6	Number of torpedo stage somatic embryos /200 mg (±SD)		Conversion rate (%) (±SD)	
	Control	4 mM	Control	4 mM
Rep 1	3.2 ± 0.5 b	31.2 ± 12.8 a	42.6 ± 23.1 AB	69.2 ± 6.7 A
Rep 2	10.2 ± 2.3 b	25.0 ± 4.4 a	34.8 ± 0.4 B	52.3 ± 5.0 AB
Average	6.7 ± 4.1	28.1 ± 9.2	38.7 ± 11.2	60.7 ± 8.5

Genotype 2824	Number of torpedo stage somatic embryos /200 mg (±SD)		Conversion rate (%) (±SD)	
	Control	4 mM	Control	4 mM
	3.7 ± 0.6 a	1.2 ± 0.4 b	35.8 ± 13.2 A	27.8 ± 25.5 A

3.4. DISCUSSION

The cellular glutathione pool consists of two interchangeable forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduced glutathione has a high reduction potential, which eliminates the reactive oxygen species, thereby being oxidized (STASOLLA, 2010). When the GSH was supplemented to cyclamen differentiation medium in higher concentrations in the present study, lower contents of superoxide anions were observed in our qualitative localization assay at day 14 (figure 1 d-f). This reactive oxygen species was reduced drastically at 10 mM GSH resulting in the death of the cell culture.

Several authors have reported the crucial role of a high redox state of glutathione in the early stages of the development of somatic embryos (BELMONTE *et al.*, 2003, 2005a; STASOLLA *et al.*, 2008; VIEIRA *et al.*, 2012a): In *Cyclamen persicum* the supplementation of GSH during the early stages of somatic embryogenesis seemed to have an effect on the number of somatic embryos formed, but in a genotype-dependent manner (Table 1,Table 2). However, the high variation between the two replications of the experiments as well as in the reaction of the different genotypes might indicate that the endogenous content of glutathione is also variable. In general, the regulation of generation and detoxification of ROS in plants is considered to be highly complex and interdependent involving many different pathways (SCHMIDT; SCHIPPERS, 2015).

In the present study, the supplementation of GSH during the early stages of SE did not have pronounced influence on the conversion rate. Other studies showed that the maturation and progression of embryos to subsequent germination was improved when the pool is shifted towards glutathione in the oxidized form (STASOLLA, 2010).

3.5. CONCLUSIONS

In two of three cyclamen genotypes under investigation, a promoting effect of 4 mM GSH during the first 14 d of differentiation on the formation of somatic embryos was shown, but the reaction of the embryogenic cell cultures was found to be highly variable. Thus, further investigations are needed to study the effects of GSH in concentrations lower than 4 mM and in different time windows. Measuring endogenous GSH and GSSG concentrations during SE will improve our understanding of their role and offer new opportunities to adjust their ratio in different developmental phases accordingly.

3.6. LITERATURE

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4. CAPÍTULO 3: DYNAMICS OF GLUTATHIONE AND ITS EFFECTS ON *Cyclamen persicum* SOMATIC EMBRYOGENESIS

4.1. RESUMO

Cyclamen persicum (Primulaceae) é uma planta ornamental tuberosa que, por não desenvolver tubérculos secundários, é propagada por sementes gerando maior variabilidade da progênie. A embriogênese somática em ciclâmen é uma técnica promissora para a micropropagação clonal massal. Contudo, diversos fatores fisiológicos limitam seu uso em escala comercial, tais como: desenvolvimento não sincronizado dos embriões somáticos e os fatores limitantes associados à fase de maturação. Estudos têm demonstrado o importante papel de um alto status redox da glutationa nas fases inicias do desenvolvimento embrionário e que a formação de embriões anormais pode estar correlacionada com baixos níveis de GSSG na fase de maturação. No presente trabalho, analisou-se o conteúdo endógeno de GSH, GSSG e cisteína (um dos percursores da GSH) durante a diferenciação dos embriões somáticos de *C. persicum*, com o intuito de compreender a correlação entre a embriogênese somática e o estado redox da glutationa. Os resultados mostraram que o conteúdo endógeno de GSH não foi constante durante a embriogênese somática mostrando um pico deste composto 24 horas após a transferência das culturas embriogênicas para o meio de cultura de desenvolvimento. Após este período a concentração de GSH diminuiu até sétimo dia de cultivo. Este decréscimo do potencial redox de glutationa pode ser revertido pela aplicação exógena de GSH aumentando a formação de embriões somáticos de ciclâmen.

4.2. ABSTRACT

Cyclamen persicum (Primulaceae) is a tuberous ornamental pot plant with no development of secondary tubers, being, therefore propagated by seeds. However, seed propagation requires manual pollination and frequently results in the variability of the progenies. Cyclamen somatic embryogenesis could become an alternative for its mass clonal micropropagation. However, the use of somatic embryogenesis on a commercial scale is limited due to physiological disorders, non-synchronized development of somatic embryos, and the bottleneck associated with the maturation step, including the low tolerance of somatic embryos to desiccation. Several studies have reported the crucial role of a high redox state of glutathione in the early stages of somatic embryo development. Moreover, some authors suggest that the formation of abnormal embryos can be correlated with low levels of GSSG in the late maturation phase. In the present work, we analyzed the contents of endogenous GSH, GSSG, and cysteine (one of the GSH precursors) during the differentiation of somatic embryos of *C. persicum* to better understand the relation between somatic embryogenesis and glutathione redox state. The endogenous GSH redox content was not stable and homogeneous during the somatic embryogenesis, showing a peak 24 hours after the subculture of embryogenic cultures to the differentiation medium. However, these levels decreased after this period until day 7 in culture. This decrease of GSH redox usually could be reversed by exogenous supplementation of GSH, which was found to increase the formation of somatic embryos *in C. persicum* and result in higher uniformity.

4.3. INTRODUCTION

Cyclamen persicum Mill. (Primulaceae) is a perennial tuberous plant with no development of secondary tubers, and thus its vegetative propagation is not possible *in vivo*. On the other hand, sexual propagation requires manual pollination, and this can result in variability among offspring. As a result, the seed has a high price, of up to 0.20€ per seed (SCHWENKEL, 1999).

The use of somatic embryogenesis (SE) for cyclamen propagation may reduce the high costs of seed production and results in a higher uniformity. In *C. persicum* SE was first described by WICART *et al.* (1984) and has been studied for several genotypes (JALALI *et al.*, 2010; OTANI; SHIMADA, 1991; SCHWENKEL; WINKELMANN, 1998). Some bottlenecks were described in cyclamen SE including: lack of synchrony in the development of somatic embryos, genotype-dependent response, risk of mutations and loss of embryogenic potential, malformations of somatic embryos like missing organs, secondary embryogenesis and lack of a maturation step leading to low germination rates (HOENEMANN *et al.*, 2010; SCHMIDT *et al.*, 2006).

Reactive oxygen species (ROS) produced by the plant metabolism usually are associated with stress response, oxidative damage, and cell death. However, the new studies showed that ROS act as secondary messengers in some cellular processes. Because of this dual role of ROS, the cell needs to control high and low levels which are achieved by antioxidative systems comprising of the non-enzymatic as well as enzymatic antioxidants (SHARMA *et al.*, 2012).

Among these scavenging systems, glutathione seems to play an important role in the SE regulation. It has been demonstrated that alterations in the glutathione redox state, i.e., the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) by adding the respective form to culture media, resulted in positive effects on the quality and amount of somatic embryos. In some species as brassica, white spruce, and araucaria the amendment of GSH to the culture medium resulted in a higher amount of pro-embryos a better initial development of somatic embryos (BELMONTE *et al.*, 2003, 2006; VIEIRA *et al.*, 2012b). On the other hand, in the later maturation step, the inversion of that ratio by the addition of GSSG increased the number of normal embryos (BELMONTE *et al.*, 2005b).

In order to better understand the effects of the glutathione redox state on somatic embryogenesis, we evaluated the contents of endogenous GSH, GSSG, and cysteine (one of the GSH precursors) during differentiation of somatic embryos of *Cyclamen persicum*. A better understanding of the physiological processes involved in SE may help to increase the embryos formation rate and the improvement of somatic embryos quality.

4.4. MATERIALS AND METHODS

4.4.1 Plant material

Embryogenic cell cultures were initiated from ovules of genotype 56/2 (one single plant of the F1 hybrid cultivar 'Maxora Light Purple' (Varinova BV)), as described by SCHWENKEL and WINKELMANN (1998). The suspension cultures were established following the procedures described by WINKELMANN *et al.* (1998).

Samples were collected on days 0, 1, 2, 3, 4, 5, 8, 11, 14, 17, 21, and 28 after the transfer of embryogenic cells to the plant growth regulator free (PGR-free) differentiation medium, with four biological replicates in three repetitions each. The embryogenic suspensions were sieved through a 1000 µm mesh and the density was adjusted to 4 % packed cell volume using liquid PGR-free differentiation medium (SCHWENKEL; WINKELMANN, 1998). Each repetition consisted of three 500 ml Erlenmeyer flasks containing 120 ml of cell suspension. After 14 days of culture on a rotary shaker at 100 rpm and 24 °C in darkness, the cell cultures were collected on a sieve of 200 µm mesh and 200 mg were plated in 6 cm Petri dishes containing 15 ml of solidified PGR-free differentiation medium and cultured at 24 °C in darkness.

4.4.2 GSH, GSSG and cysteine extraction and quantification

A 300 mg of fresh mass was collected for each sample for each time point, immersed in liquid nitrogen and kept in a -80°C freezer until extraction proceedings. GSH, GSSG and cysteine were extracted using 5% metaphosphoric acid (MPA) and determined by HPLC coupled to ESI-TOF MS

following the method described by RELLÁN-ALVAREZ *et al.* (2006) with modifications.

4.4.3 Effect of glutathione and the supplementation time in the *Cyclamen persicum* somatic embryogenesis

To test the influence of the two different forms of glutathione during the cyclamen somatic embryogenesis a trifactorial experiment (2x2x2) was designed (Figure 1). The first factor was the supplementation or not of 2 mM of GSH. Each repetition consisted of three 250 ml Erlenmeyer flasks containing 60 ml of cell suspension cultured on a rotary shaker at 100 rpm and 24 °C in darkness. After 7 or 14 days of culture (second factor), the cell cultures were collected on a sieve of 200 µm mesh and 200 mg were plated in five 60 mm diameter Petri dishes containing 15 ml of solidified PGR-free differentiation medium, supplemented or not with 2mM GSSG (factor 3), and cultured at 24 °C in darkness. A randomized-complete block design was used and the experiment was repeated 4 times. The number of torpedo-staged somatic embryos was evaluated at day 28. These somatic embryos were collected and transferred to a fresh differentiation medium for the conversion into seedlings (germination). After 30 days of culture at 24 °C in darkness, the percentage of converted embryos was evaluated by counting the somatic embryos with cotyledons of at least 1 cm length.

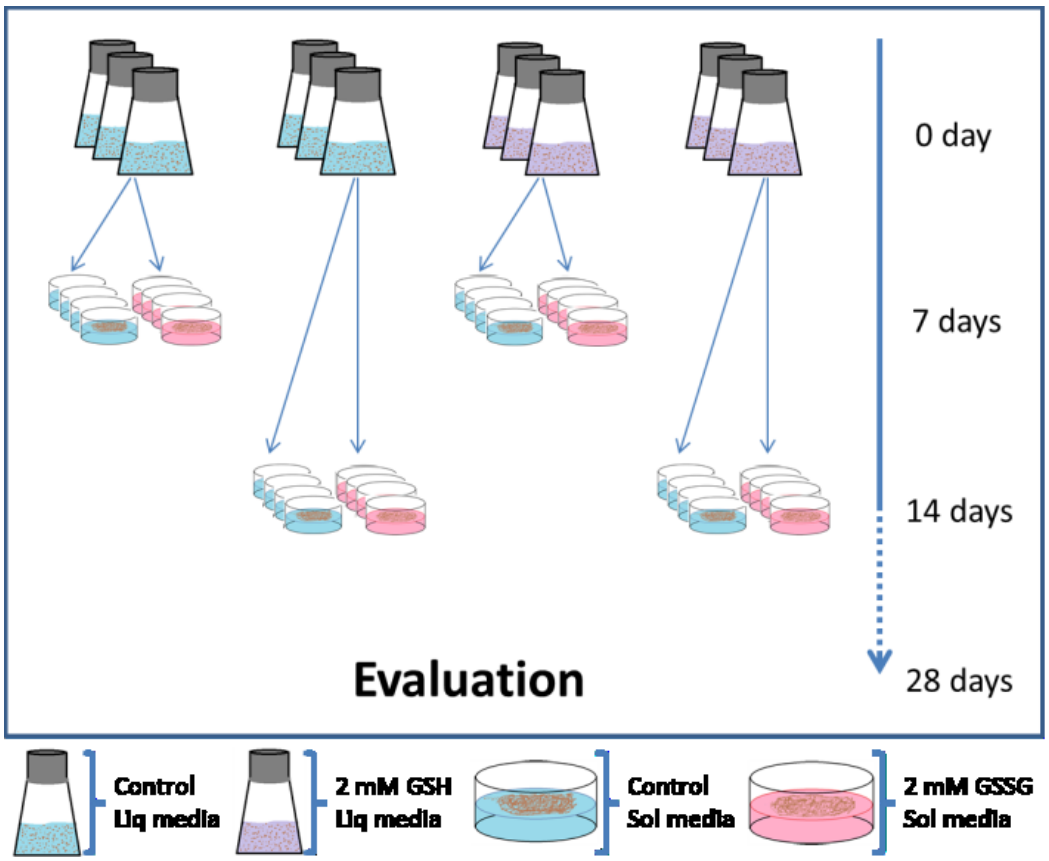

Figure 1) Flow chart of the experimental procedure to test the effect of glutathione and the supplementation time in *Cyclamen persicum* somatic embryogenesis. The experiment was repeated 4 times. Liq: liquid; Sol: solid. (CAPRESTANO, C.A, 2015)

4.4.4 Statistical analyses

The data were subjected to the F-max test to verify the homogeneity of variances. Percentage data were transformed to $\sqrt{(x + 0.5)}$. The data were subjected to ANOVA, and mean separation was performed by the Student Newman Keul's (SNK) test ($p<0.05$) following STEEL and TORRIE (1980).

4.5. RESULTS

To better understand the role of the glutathione redox state during the SE, we quantified the endogenous contents of GSH, GSSG, and cysteine (one of the GSH precursors) during differentiation of somatic embryos of *Cyclamen persicum* (Figure 2). The results showed a peak in cysteine (Figure 2a) and GSH (Figure 2b) concentrations after 24 hours. After that, the content of cysteine remained stable while GSH increased slightly over time. GSSG was detected in lower concentrations than GSH and was observed to increase until day 8 (Figure 2c)

For the glutathione redox ratio, a slight decrease was observed during the firsts 7 days of differentiation (Figure 2d). These results allowed us to design an experiment to choose a better time-lapse for GSH application to improve the SE considering that a high glutathione redox ratio, in the initial phases of SE is required and GSSG during the subsequent phase.

The supplementation of GSH during the initial phase of somatic embryos differentiation increased the number of somatic embryos in 15% (Figure 3a). The change of the differentiation media at day 7 increased the number of embryos formed in 40% as compared to those kept for 14 days in the initial culture medium (Figure 3c). However, the supplementation of GSSG in the second part of the differentiation phase did not increase the number of somatic embryos.

A wide difference in the number of somatic embryos formed was observed among the four experiments. The experiment number two and four had the same mean formation of embryos (3.9 torpedo embryos/200 mg), differing from the third (7.1 torpedo embryos/200mg) and the fourth experiment (9.7 torpedo embryos/200 mg).

The morphological aspect of the cultures after 28 days in the differentiation medium can be seen in figure 4. When all factors are computed simultaneously, it was observed that the supplementation of 2 mM GSH for 7 days and the subsequent transfer to gelled culture medium increased the number of somatic embryos in comparison to the other treatments (Figure 3b and Figure 4).

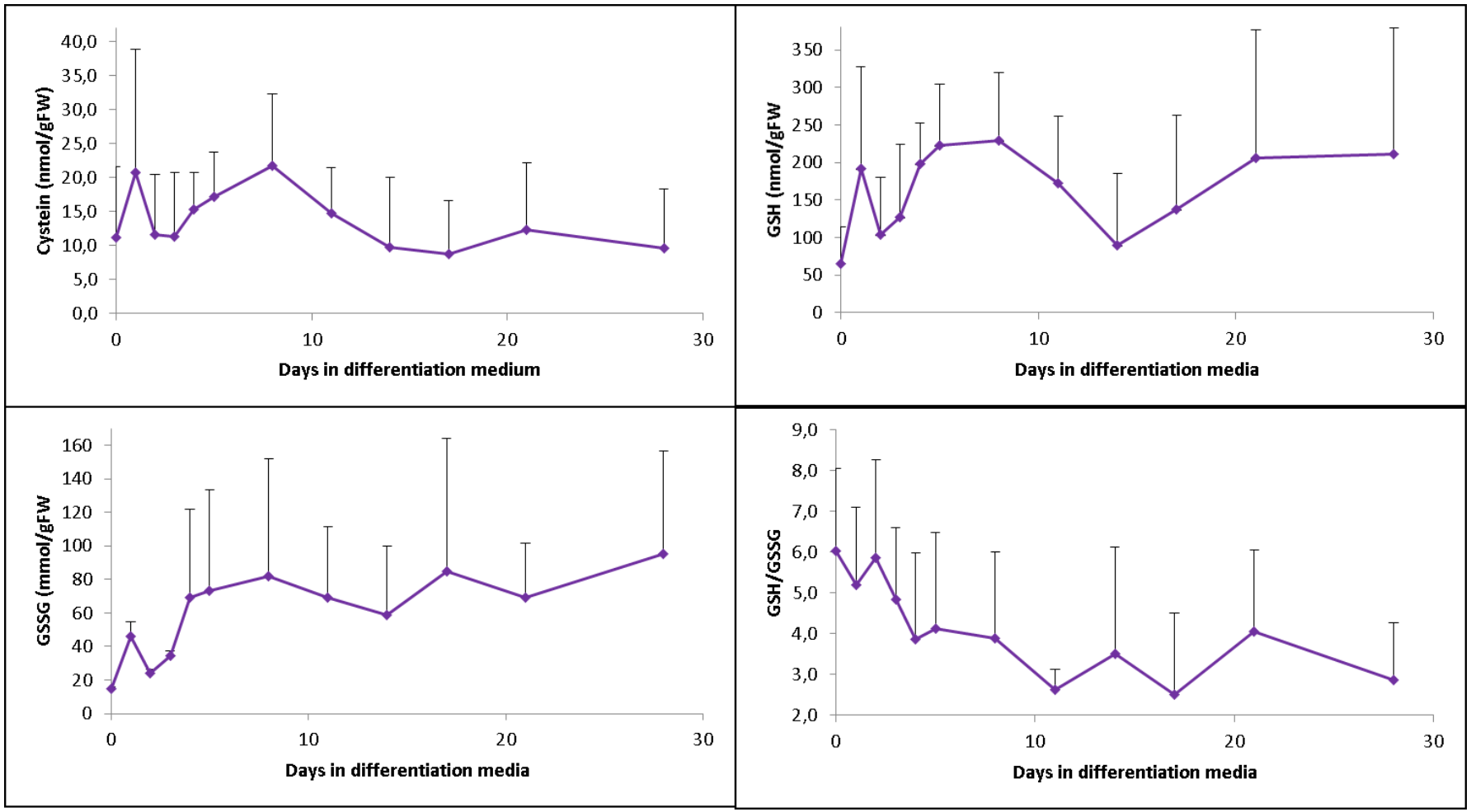

Figure 2: Dynamics of cysteine (A); GSSG (B); GSH (C) and the glutathione redox ratio i.e., GSH/(GSH+GSSG) during *Cyclamen persicum* somatic embryo differentiation phase. n=4. (CAPRESTANO, C.A, 2015)

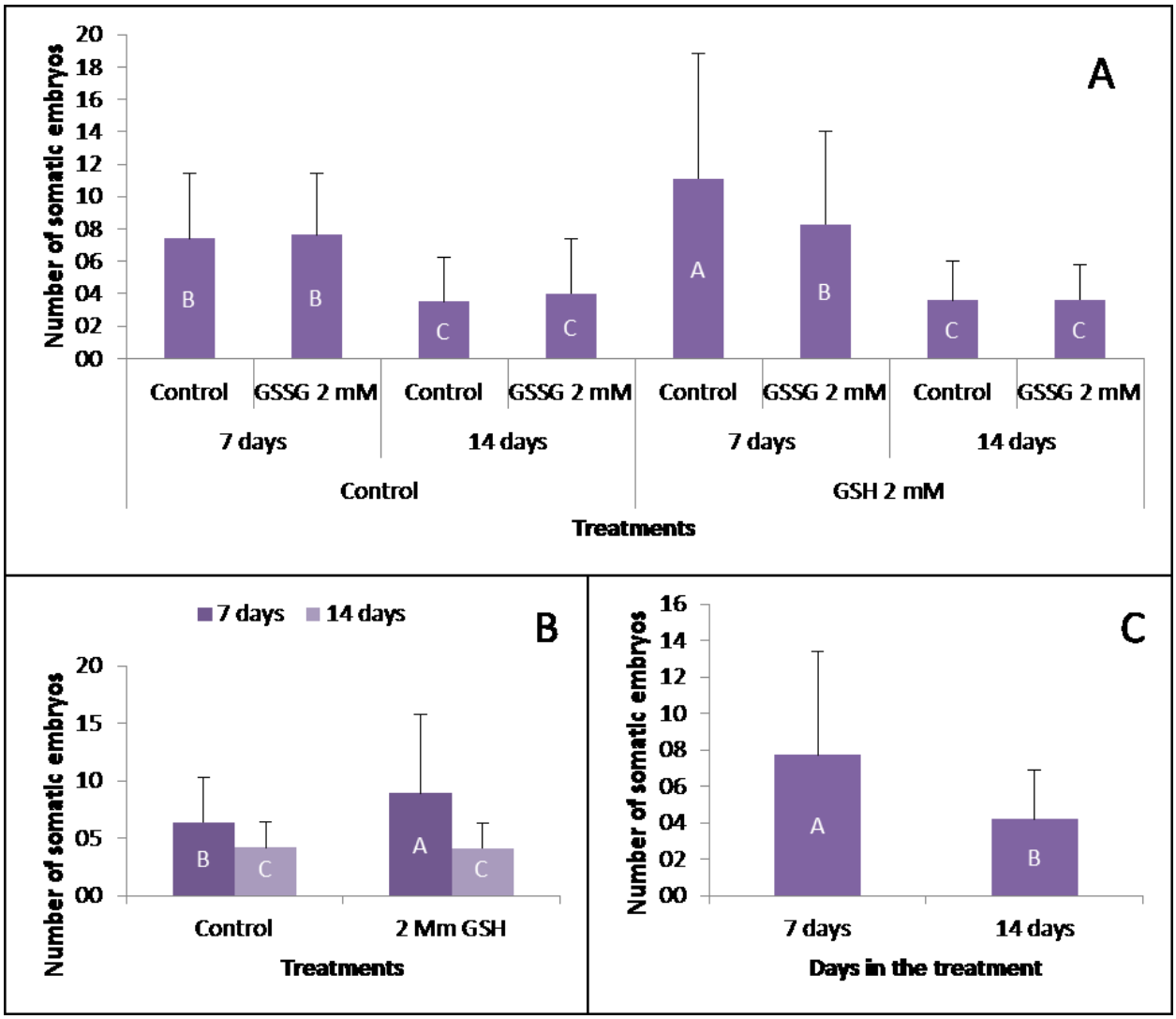

Figure 3) Effect of GSH and GSSG in the differentiation phase of *Cyclamen persicum* on the number of torpedo stage somatic embryos after 28 days. Different letters indicate significant differences in the SNK test (5%).(CAPRESTANO, C.A, 2015)

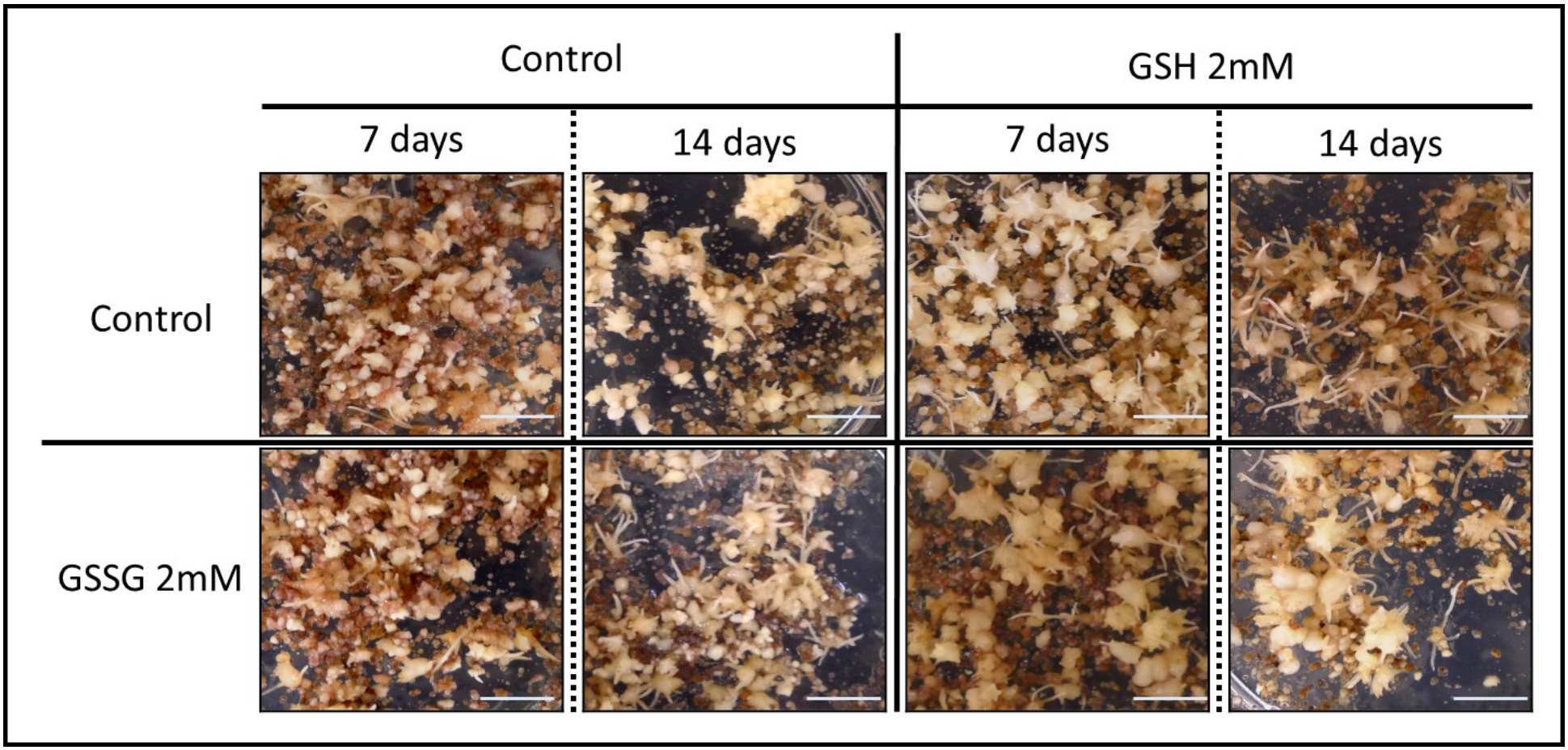

Figure 4) Morphologic aspect of the cultures after 28 days: Effect of GSH and GSSG in the differentiation phase of *Cyclamen persicum*. Bars: 1 cm. (CAPRESTANO, C.A, 2015)

4.6. DISCUSSION

The experimental manipulations of the endogenous ratio GSH/GSSG+GSH, by supplementing reduced (GSH) or oxidized (GSSG) glutathione improved SE in different species. In *Picea glauca,* the initial supplementation of the differentiation media with GSH (7 days) and followed by replacement with GSSG during the remaining time (33 days) increased in 3 times the number of fully developed embryos (BELMONTE *et al.*, 2005). The supplementation of GSH during the early differentiation increased the number of early embryos formed in *Araucaria angustifolia* (VIEIRA *et al.*, 2012) and *Pinus taeda* (PULLMAN *et al.*, 2015).

The cycle ascorbate-glutathione is one of the most efficient scavengers of peroxides produced by cellular metabolism or oxidative stress in plant cells (GARCÍA-GIMÉNEZ *et al.*, 2013). Ascorbate and glutathione are associated with cellular redox signaling networks affecting growth, development, and defense systems (FOYER; NOCTOR, 2005) and are located in chloroplasts, cytosol, peroxisomes, and mitochondria (JIMENEZ *et al.*, 1997).

During the cell proliferation, the GSH seems to be recruited to the nucleus, playing a role in the cell-cycle regulation providing a reduced environment to the beginning of the cell cycle (VIVANCOS *et al.*, 2010). The decrease of the oxidative stress by the antioxidant action of GSH provides a stress-free environment to the cell division protecting gene replication (SANCHEZ-FERNANDEZ *et al.*, 1997; VIVANCOS *et al.*, 2010). In tobacco cell suspension, it was shown that the transition from G_1 to S phase requires an adequate level of glutathione (VERNOUX *et al.*, 2000).

The imposition of a reduced environment with the supplementation of GSH enhanced the somatic embryo capacity, while an oxidized environment with the GSSG supplementation improved the embryo quality in *Picea glauca* (BELMONTE *et al.*, 2005c). The depletion of GSH during the meristem cell developing in *Arabidopsis thaliana* roots raised the number of meristematic cells undergoing mitosis; however, the exogenous application of GSH improved the number of meristematic cells (SANCHEZ-FERNANDEZ *et al.*, 1997).

In the present work, it was observed a peak of cysteine and GSH 24 hours after the subculture to the new culture medium (Figure 2a-b). This increase in the

GSH and cysteine concentration can be related to the sieving process. Cyclamen cell suspensions form cell aggregates which should be disrupted because cell aggregates bigger than 1,000 µm resulted in decreased formation of somatic embryos (HOHE; WINKELMANN; SCHWENKEL, 2001). Previous studies of gene expression of glutathione S-transferases (GST) in cyclamen showed an up-regulation after 4 hours of the transfer to the differentiation medium (HOENEMANN; AMBOLD; HOHE, 2012). These authors concluded that the drought stress imposed on the suspension cells upon transfer to a new culture media might be responsible for this increase in the redox response. In our study, the ratio GSH/GSSG+GSH showed a slight decreased in the first seven days in culture. After 7 days in culture, the ratio increased, turning the environment reduced.

Somatic embryos of *Picea glauca* show a GSH/GSSG+GSH ratio around 0.81 after 7 days in the differentiation media, however when GSH is applied this ratio increase to 0.89. This change increases the conversion frequency of embryos, with an improvement of the embryo morphology as well as alterations in the gene expression during the differentiation period (STASOLLA *et al.*, 2004).

Our experiments demonstrate that the reduced environment in the early stages of somatic embryo development increased the number of somatic embryos formed. Also, in our previous studies, the change in the redox status in the early stages of SE increased the number of somatic embryos. In that case, the supplementation of 4 mM GSH during the first 14 days resulted in an increased formation of somatic embryos in two genotypes. This increase was a genotype-dependent and a high variation between the two replicates but did not improve the SE conversion rate (CAPRESTANO; GUERRA; WINKELMANN, 2015).

Additionally, previous experiments in our research group showed that 2 mM GSH was superior to 4 mM GSH (data not shown) in the embryo somatic embryo. With this background, we designed an experiment to identify the optimal time to apply GSH in *Cyclamen persicum* SE.

The results showed that the transfer from the liquid to gelled culture media at day 7 increased the number of SE when compared with 14 days in liquid culture. When the media supplementation versus time was compared, regarding the SE formation, the best results were obtained when GSH was applied for 7

days. However, for the cultures maintained in the liquid culture for 14 days, the GSH treatment did not improve the number of SE formed. Thus, these results confirm the role of GSH in the earlier phases of SE in this species.

The standard deviation observed between the four replicates of this experiment can probably be explained by variation in the endogenous content of GSH during cyclamen SE. The endogenous content of GSH was quantified four times (twice in June and twice in October 2014) with the same cell lines and also showed a pronounced variation in the GSH content over time.

By switching the glutathione pool to the oxidized state BELMONTE *et al.* (2005c) were able to improve the embryo quality of white spruce. These embryos presented more cotyledons and better post-embryogenic development. A similar response to an oxidized environment was achieved in *Brassica oleracea*, were an oxidized culture medium, improved the pattern of storage product accumulation in the embryos and the architecture of the shoot apical meristems (BELMONTE *et al.*, 2006). This increase in the embryo performance, by an oxidized environment, can be associated with the capacity of GSSG to block the cell expansion, the cell cycle progression and the inhibition of the programmed cell death. Therefore by reducing the cell proliferation, the embryos can properly grow and develop (BOZHKOV; FILONOVA; SUAREZ, 2005; DE GARA *et al.*, 2003; REICHHELD *et al.*, 1999).

However, a GSSG supplementation in the late phases of SE in cyclamen did not improve the number of somatic embryos formed. In addition, the average somatic embryo conversion was not improved by the supplementation of 2 mM GSSG. Lower concentrations of GSSG were previously tested with no increase of embryo conversion ratio in *Cyclamen persicum*.

4.7. CONCLUSION

The endogenous GSH redox content was not stable and homogenous during the SE of cyclamen, showing a peak 24 hours after the transfer of somatic embryos to development media. However, the GSH levels decrease after this period until day 7. This decrease of GSH redox state can be reversed by exogenous supplementation of GSH, resulting in an increasing the SE formation in *Cyclamen persicum*. Notwithstanding, the GSSG supplemented in the latest

phases of SE does not improve the somatic embryo quality. Measurements of GSH, GSSG, and cysteine during SE of another *Cyclamen persicum* genotypes are needed, as well a correlation between these endogenous contents and an embryo formation rate.

4.8. LITERATURE

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5. CAPÍTULO 4: THE ROLE OF GLUTATHIONE IN *Cyclamen persicum* SOMATIC EMBRYOGENESIS: PROTEOME AND OXI-PROTEOME

5.1 RESUMO

Suplementações de GSH, um agente redutor que diminui a presença de ROS, aos meios de cultura, apresentam efeitos benéficos na embriogênese somática de *Cyclamen persicum*, aumentando a produção e a qualidade de embriões somáticos no estágio de torpedo. O oxi-proteoma é uma nova ferramenta de estudo das modificações pós-traducionais causadas pelas espécies reativas de oxigênio. No presente trabalho estudaram-se as mudanças no proteoma e no oxi-proteoma de embriões somáticos de ciclâmen cultivados ou não na presença de GSH em três tempos. A suplementação de GSH ao meio de cultivo reduziu a presença de proteínas oxidadas, as proteínas alvo dos ROS estão relacionadas com a produção ou transporte de auxina. Além disso a suplementação de GSH ao meio de cultivo incrementou proteínas relacionadas com o transporte polar de auxina, as quais também foram positivamente reguladas em resposta à suplementação de GSH, aumentando a produção dos embriões somáticos. Analisados em conjunto estes resultados sugerem que mudanças específicas nos padrões de oxidação das proteínas causada por ROS são deletérias à embriogênese somática em *Cyclamen persicum.*

5.2 ABSTRACT

Cyclamen persicum somatic embryos formation is positively influenced by culture media supplementation with reduced glutathione (GSH), a redactor agent which is a reactive oxygen species scavenger. The oxi-proteome it's a new tool to study the post-transduction changes in the proteome generate by ROS. In the present work, we analyzed the changes in the proteome and oxi-proteome of cyclamen somatic embryos treated or not with GSH in three-time points. GSH supplementation to the culture medium reduced the oxidized proteins related to auxin synthesis and transport. Furthermore, auxin-related proteins were upregulated in the GSH treatment. Our results indicate that the specific changes in the oxi-proteome of *Cyclamen persicum* somatic embryos caused by ROS are deleterious to embryo formation.

5.3 INTRODUCTION

In vitro propagation includes many techniques based on the plant cell totipotency, which enables the mass propagation of selected genotypes, allowing the capture of genetic gains. The application of these techniques is dependent on the induction and control of morphogenesis *in vitro* and plant regeneration is obtained through two routes: organogenesis and somatic embryogenesis (SE).

Somatic embryogenesis is a morphogenetic process in which isolated or small groups of somatic cells undergo a series of morphological and biochemical changes resulting in the formation of somatic embryos. Thus, SE undergoes a pathway analogous to zygotic embryogenesis and is considered as the expression of plant cell totipotency (GUERRA; TORRES; TEIXEIRA, 1999a)

Cyclamen persicum SE was first described by WICART; MOURAS and LUTZ (1984), and target of many groups in the last years (HOENEMANN; HOHE, 2001; JALALI *et al.*, 2010; OTANI; SHIMADA, 1991; WINKELMANN; SPECHT; SEREK, 2006). However, the use of SE in this species on a commercial scale is still limited due to physiological disorders during the development of somatic embryos. Low tolerance for desiccation, non-synchronized development of somatic embryos and the lack of a maturation step are some of the bottlenecks to be overcome (HOENEMANN *et al.*, 2010; SCHMIDT *et al.*, 2006; SEYRING; HOHE, 2005; WINKELMANN *et al.*, 2011b).

Among the factors that could be associated with the physiological obstacles mentioned above, reactive oxygen species (ROS) are the focus of recent studies. ROS are transient species that include free radicals, e.g. radical superoxide (O_2^-) and other reactive intermediates, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) (SAED-MOUCHESHI *et al.*, 2014). The relation between ROS and plant morphogenesis becomes clearer due to the results of recent research which confirm the important role of ROS as signaling and regulatory molecules during plant development (CAUSIN *et al.*, 2012; DENNESS *et al.*, 2011; FOREMAN *et al.*, 2003; MORI; SCHROEDER, 2004). . The oxidative stress works as a possible modulator of somatic embryogenesis in plants, inducing autonomous cell division.

A complex network of low molecular weight molecules, e.g. glutathione, with an anti-oxidative capacity, seems to control ROS concentration and oxidative repair (NOCTOR *et al.*, 2011). The manipulation of cell glutathione redox state,

by adding reduced (GSH) or oxidized glutathione (GSSG) has positive effects on the amount and quality of somatic embryos (BELMONTE *et al.*, 2003, 2005; STASOLLA *et al.*, 2008).

Our previous work with the culture medium manipulating the glutathione redox during the early SE in *Cyclamen persicum* did improve the number of somatic embryos formed (CAPRESTANO; GUERRA; WINKELMANN, 2015). This result shows that the elevated ROS concentration in the early SE in cyclamen may be deleterious to the embryo formation.

However, the mechanism controlled by the oxidative potential as not yet well described. JOB *et al.* (2005) working with *Arabidopsis thaliana* observed that changes on specific patterns of proteins carbonylation (oxidation) are probably necessary to neutralize and/or utilize the production of ROS resulted from metabolic recovery during seed germination. The protein oxidation alters the protein structure leading to a partial or total loss of their function, occurring on lysine, arginine, proline or threonine residues of protein. Removal of contaminating trace metals, O2, and/or inclusion of GSH in the culture media protected the proteins against these processes (GO; JONES, 2013). The oxi-proteome is characterized for the proteome undergo reversible redox reactions and those modified irreversibly by reactive species during oxidative stress (GO; JONES, 2013).

The identification of oxi-proteome components modified can bring some insights into the mechanisms by which these damaged proteins accumulate and potentially affect the SE in *Cyclamen persicum*.

5.4 MATERIAL AND METHODS

5.4.1 Plant material

Following SCHWENKEL and WINKELMANN (1998) embryogenic cell cultures were initiated from ovules of genotype 56/2 (one single plant of the F1 hybrid cultivar ‘Maxora Light Purple’ (Varinova BV). The suspension cultures were established as described by WINKELMANN *et al.* (1998).

The embryogenic suspensions were sieved through a 1,000 µm mesh and the density was adjusted to 4% packed cell volume using liquid plant growth

regulator (PGR)-free differentiation medium (SCHWENKEL; WINKELMANN, 1998) in two different concentrations of GSH: 0 and 2mM. Each treatment consisted of three 500 ml Erlenmeyer flasks containing 120 ml of cell suspension. After 7 days of culture, on a rotary shaker at 100 rpm and 24 °C in darkness, the cell cultures were collected on a sieve of 200 µm mesh, and 200 mg were plated in 6 cm Petri dishes containing 15 ml of solidified PGR-free differentiation medium and cultured at 24 °C in darkness. Samples (100 mg) were collected on day 3, 7 and 14, in biological triplicate. After 28 days in culture, the number of torpedo embryos was collected.

5.4.2 Histological analysis

After 1 hour, 3, 7 and 14 days the cell viability was determined by staining with fluorescein diacetate (FDA) following the procedures of WINKELMANN *et al.* (1998). The localization of superoxide (O_2^-) was performed by staining with nitro blue tetrazolium (NBT) following the method described by PIETROWSKA *et al.* (2015).

Results of cell viability and superoxide are expressed as means± standard errors (SE). Statistical analysis was performed using JMP 5 software (2002; SAS Institute, Cary, NC, USA). Percentage data were transformed to $\sqrt{(x + 0.5)}$. Data were subjected to t-test for comparison of means.

Were collected 25 torpedo-staged somatic embryos at day 28 and fixed for 24 h in a 0.2 M phosphate buffer (pH 7.3) containing 2.5% paraformaldehyde and then dehydrated in a graded ethanol series and embedded in Historesin® (Leica), as described by MITRENGA; WOLFGANG and MAYERSBACH (1974). Sections were sliced in a rotatory microtome HYRAX M55 (Zeiss, Jena, Germany) and fixed into slides by heating. The sections were stained with 0.5% toluidine blue O (TBO) (C.I. 52040) in a 0.2 M phosphate buffer (pH 6.8) for 30 seconds (O'BRIEN; FEDER; MCCULLY, 1964).

5.4.3 Phenolic protein extraction and quantification

Proteins were isolated in three independent extractions from three separate tissue fractions. Each extraction was electrophoretically analyzed by one gel replicate resulting in fifty-four protein extractions and eighteen gels in total. Total

proteins were extracted using phenol combined with ammonium acetate in methanol precipitation according to RODE *et al.* (2011). Therefore, the protein pellet was resuspended in 150 µl of Tris–HCl 20 mM pH 7.5 and 2% SDS. The protein concentrations were determined with Pierce™ BCA Protein Assay Kit, using Bovine Serum Albumin (BSA) as a standard. Three technical repetitions were used for each treatment.

5.4.4 Bodipy-hydrazide derivatization

The Bodipy-Hz protein derivatization was performed following the proceedure described by TAMARIT *et al.* (2012) with modification. Briefly, 100 µL 5 mM of Bodipy-Hz in 0.1 M sodium acetate pH 5, 1 mM EDTA and 1% SDS was added to 200 µl of protein sample containing 500 µg of protein and incubated at 25 °C at 500 rpm for 30 minutes.

To stop and stabilized the hydrazine form was added to the reaction mixture 82.5 µl of 2 M Tris and 22.5 µl of 0.2 M sodium cyanoborohydride and incubated at 25 °C for 30 min. Derivatized proteins were precipitated by adding 1.6 ml of cold acetone and kept for 4 hours at -80°C. Proteins were concentrated by centrifugation at 14,500 rpm for 5 min and the pellets washed by repeating 3 cycles of acetone and then centrifuged at 14,500 rpm 5 min. Pellets were air-dried and resuspended in 350 ml of rehydration buffer (8 M urea, 2 M thiourea, 2 % (v/v) Triton-X, 100 mM DTT, 12 µl/ml DeStreak-reagent (GE Healthcare, Freiburg, Germany), 0.5 % (v/v) IPG-buffer pH3-11 NL (GE Healthcare, Freiburg, Germany).

2D IEF/SDS-PAGE and protein staining

The protein was loaded on Immobiline™ DryStrip gels (18 cm, pH 3-11 NL, GE Healthcare, Freiburg, Germany) and isoelectrically focused for 24 according to MIHR AND BRAUN (2003) using the IPGphor system. For the second dimension separation (SDS PAGE), IPG strips were equilibrated and placed horizontally on a 12 % Tricine SDS PAGE gel. Electrophoresis was carried out for 20 h at 30 mA mm-1 gel layer using the Biorad Protean II XL gel system (Biorad, München, Germany). Bodipy-Hz stained gels were first scanned

Typhoon Trio Scanner (GE Healthcare) and then were stained with colloidal Coomassie Blue 250 G (Merck, Darmstadt, Germany) (NEUHOFF; STAMM; EIBL, 1985; NEUHOFF *et al.*, 1990). For further details see (RODE *et al.*, 2011).

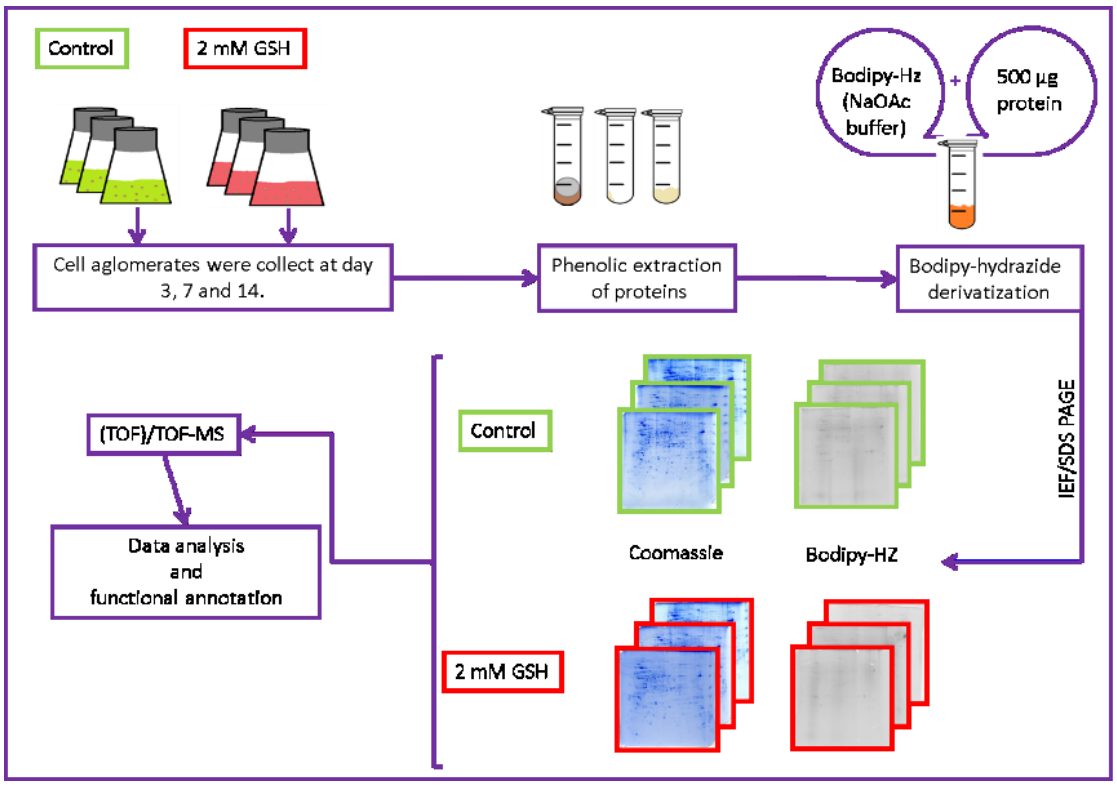

Figure 1. Flow chart of the experimental design for the proteomic and oxiproteome characterization of *Cyclamen persicum* somatic embryos treated or not with oxidized glutahione. (CAPRESTANO, C.A. 2015)

5.4.5 2D gel analyses

Scanned images of Bodipy-HZ and coomassie colloidal stained gels were analyzed using the Delta2D software 4.4 (Decodon, Greifswald, Germany) following BERTH *et al.* (2007). Three replicates of gels per treatment and date (3, 7 and 14 days) were analyzed and spots were automatically detected. Manually corrections of minor corrections of gel disturbances were performed. For determining significant differences in spot patterns six individual comparisons were made between control and treatment in time with Coomassie or Bodipy-HZ stain and six individual comparisons were made between Coomassie and Bodipy-HZ stain in time with the control or treated sample, a Student´s t-test based on the normalized relative spot volume was performed (p-value ≤ 0.05).

Additionally, only spots with a fold change higher than 5 were taken into consideration for protein identification.

5.4.6 In-gel digestion and protein identification by MS/MS

Protein spots were cut from 2D gels and destained and excised protein spots were in-gel-digested with trypsin as described by KLODMANN *et al.* (2010). The supernatant was measured with the (TOF)/TOF-MS analyses were performed with an Ultraflex II mass spectrometer (Bruker, Bremen, Germany).

Proteins were identified using the MASCOT search algorithm against the Arabidopsis protein database (www.Arabidopsis.org; release TAIR 10) or SwissProt (all plants in the database). Identified proteins were functionally classified according to the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html).

5.5 RESULTS

The cell viability was determined after 1 hour, 3, 7 and 14 days of culture, following the methodology described by HOHE; WINKELMANN and SCHWENKEL (1999). The cell viability was not affected after one-hour treatment (Figure 2a). However, the number of viable cells clusters decreased at day 3, in both treatments, but was slightly higher when GSH was applied (Figure 1 b-c). At day 14, 56% of the cells clusters in the control treatment were viable and 47% of the treated cells clusters were viable (Figure 2**Erro! Fonte de referência não encontrada.** d-e).

Lower contents of superoxide anions were observed in our qualitative localization assay after 1 h treatment (Figure 2 f) compared with day 3 when was observed more cells showing superoxide anions (figure 1g and h). As a result of the media change and the remove of GSH at day 14 it was observed an increase of superoxide anions in the cells previously treated with GSH (figure 1 i-j).

The number of torpedo-staged somatic embryos was evaluated at day 28. An increase of the cotyledon stage somatic embryos number was observed when GSH was applied (4.1 embryos/200mg) compared with the control treatment (2.3 embryos/200mg)($P<0.05$).

Somatic embryos derivate of GSH treatment showed a shoot and root meristem (Figure 2 d - f), while in the control treatment some embryos were shoot or root meristem defective (Figure 2 a - c). The procambium was absent in most of the control torpedo-staged embryos (Figure 2 b and c). Embryos, in both treatments, showed an exoderm with intense meristematic activity. The embryos treated with 2mM GSH showed a well-organized cell division resulting in well formed embryos.

Three comparisons were analyzed between control and GSH treatment in the time for both staining (Bodipy-Hz or Coomasie), totalizing six comparisons. We also analyzed the comparison Bodipy-Hz versus Coomassie stain, for both treatments in the time.

A total of 82 spots were selected to be analyzed by MALDI-TOF-MS/MS, and a total of 55 proteins were identified. From this amount, 14 protein showed to be the same isoform probably after a post-translational modification.

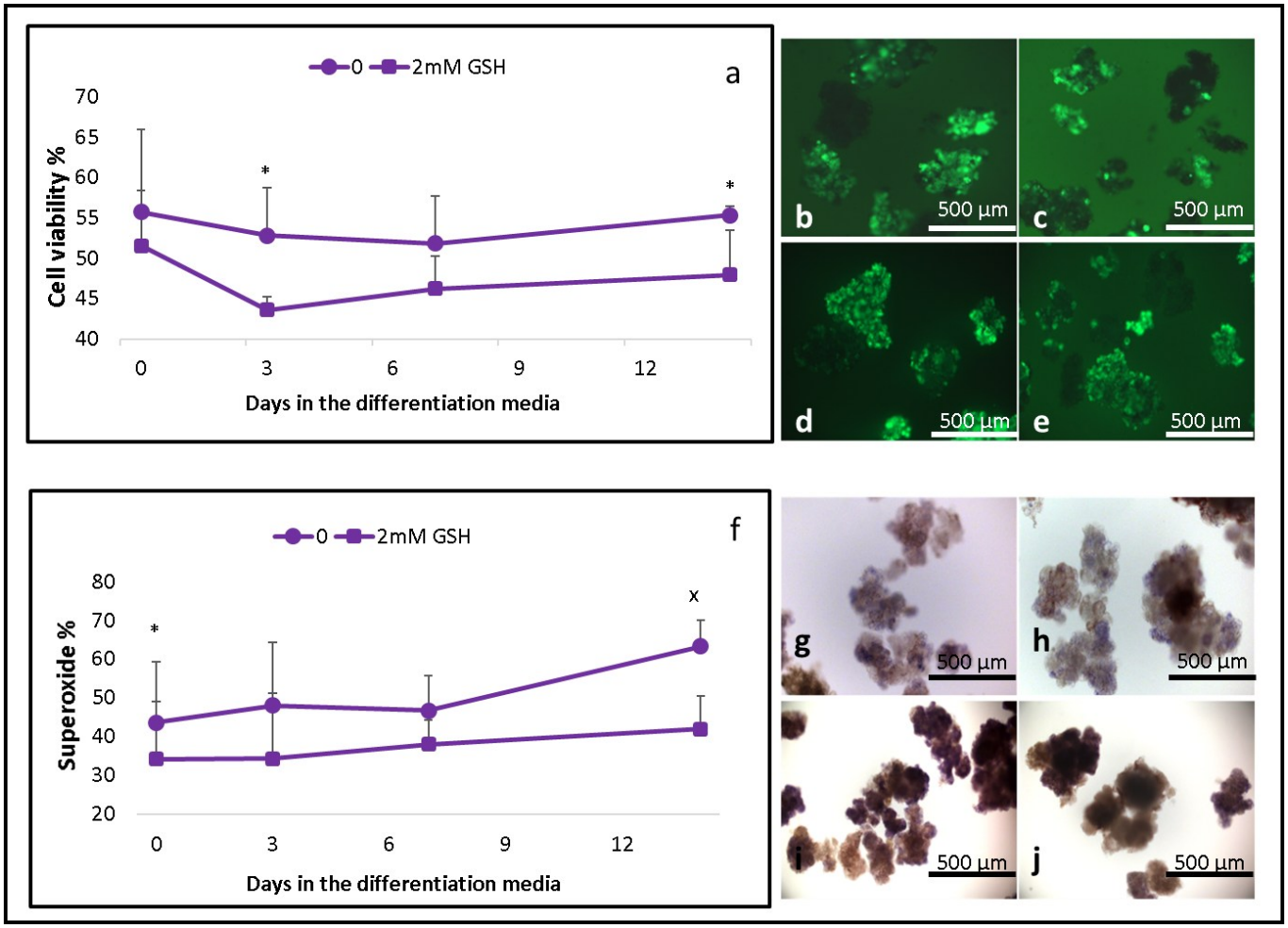

Figure 2. Effects of GSH (0 and 2 mM) during early somatic embryogenesis in *Cyclamen persicum* a) viability of cell clusters; b – c : Viability of cell cultures after 3 days of treatment (b control and c GSH treated), d-e Viability of cell cultures after 7 days of treatment (d control and e GSH treated). Viable cells express green fluorescence.f: g – h: Histochemical localization of O_2^- using NBT staining in cell cultures after 3 days of treatment (g control and h GSH treated); i-j Histochemical localization of O_2^- using NBT staining in cell cultures after 7 days of treatment (i control and j GSH treated); Cell aggregates with NBT staining show a purple stain. (CAPRESTANO, C.A. 2015). *P<0.05, significant difference for control vs 2mM GSH treatment for each equivalent day.

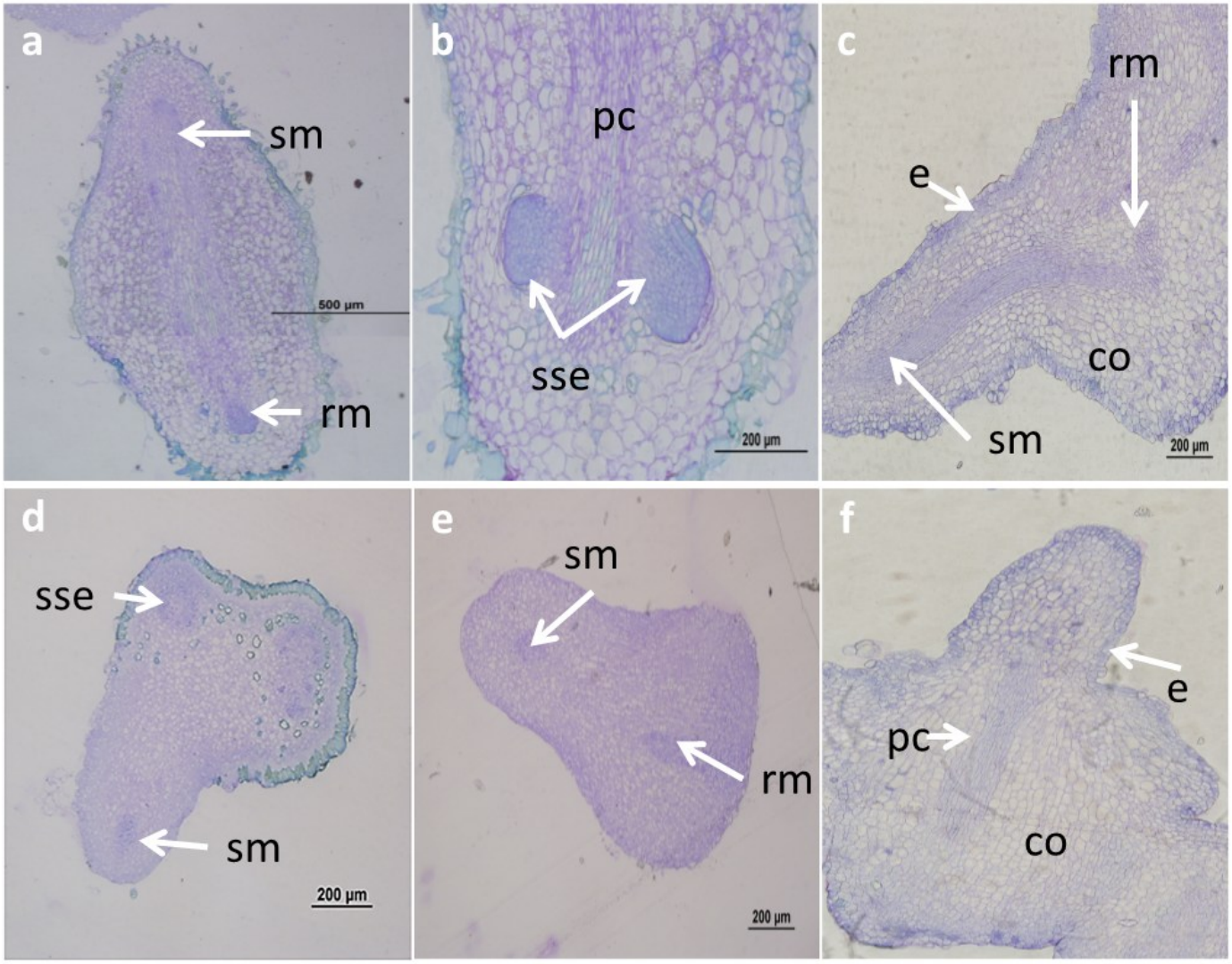

Figure 3. Histological analyses of torpedo-staged somatic embryos of *Cyclamen persicum* cultured in control media (a-c) or presence of 2mM of GSH (d-f) or in *:* a) details of a unipolar embryo without procambium and with secundary somatic embryogenesis; b bipolar embryo without procambium; c bipolar embryo; d,e) details of a bipolar embryo with secunday somatic embryogenesis; f bipolar embryos, or in control media. Rm: root meristem; sm: shoot meristem; pc: procambium; co: cortex; e: epidermis.; sse secunday somatic embryos. (CAPRESTANO, C.A. 2015)

5.5.1. OXIDIZED PROTEOME (BODIPY-HZ STAINING)

For the comparison for the oxidized proteome of the control and GSH treated cells in the 3 time points the identified proteins were matched to the corresponding spots in Figure 4 and the details are shown in the Table 1. At day three there were found 519 protein spots, 65 of them showing a 5 fold difference between the control and GSH treated. From these a total of three oxidized proteins were identified, these proteins showed being higher abundant in the control treatment. These proteins were related to the carbohydrate metabolism (ID 81 and 2836) or to genetic information process (ID 209).

At day seven 388 spots were found in the comparison of the control and GSH treatments. No statistically differences in the oxidized proteome were found.

At day 14, 575 spots were found with three spots showing a 5 fold difference between control and GSH treated cells. We were able to identify only two of these code proteins for the same isoform of Importin subunit B1 (ID 508 and 512), more abundant in the GSH treatment, rolled in the genetic information process.

Table 1. Identification of oxidize proteins from spots with changed abundance in comparison between the treatment control and the GSH treated cells clusters of *Cyclamen persicum*. A Student´s t-test was performed (p-value ≤0.05) to determine significant changes in spot volume on the basis of normalized relative spot volume. Only alterations of at least 5-fold in spot volume were considered to represent true alterations in protein level

ID[1]	Reg [2]	Accession name[3]	DB[4]	Protein name	Gene name	Organism	KEGG[5]	Score[6]	PI Calc[7]	MW Calc [8]	Pep [9]
3DB 81	0.020	AT5G65690.1	Tair	Phosphoenolpyruvate carboxykinase	At5g65690	*Arabidopsis thaliana*	Carbohydrate metabolism	132	6.0	72.8	3
3DB 208	0.095	USP_ARATH	SwissProt	UDP-sugar pyrophosphorylase	USP	*Arabidopsis thaliana*	Carbohydrate metabolism	254	6.1	67.8	5
3DB 209	0.177	RH52C_ORYSJ	SwissProt	DEAD-box ATP-dependent RNA helicase 52C	Os11g0599500	*Oryza sativa subsp. japonica*	Genetic Information Processing	514	9.0	65.9	9
14DB 508 AND 512	0.009	IMB1_ARATH	SwissProt	Importin subunit beta-1	KPNB1	*Arabidopsis thaliana*	Genetic Information Processing	287	4.6	96.2	7

1) ID represents the number of a oxidize protein spot in the 2D PAGE gels. Corresponding spots of all gels are labeled with the same ID. 3DB stands for 3 days with Bodipy-Hz stain and 14DB for 14 days with Bodipy-HZ staining; 2) Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (GSH treatment/ control); 3) Accession number of the identified protein in the database where the spot was searched; 4) Database which was used for protein search: Tair (Tair10 release (Arabidopsis)) or SwissProt (Plant category of SwissProt, all plants in the database)); 5) Functional classification according to the KEGG Pathway Database; 6) The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation); 7) Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) a protein database; 8)Calculated molecular mass (MW) in kDa obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a protein database; 9) Number of peptides matched to the protein through the database search.

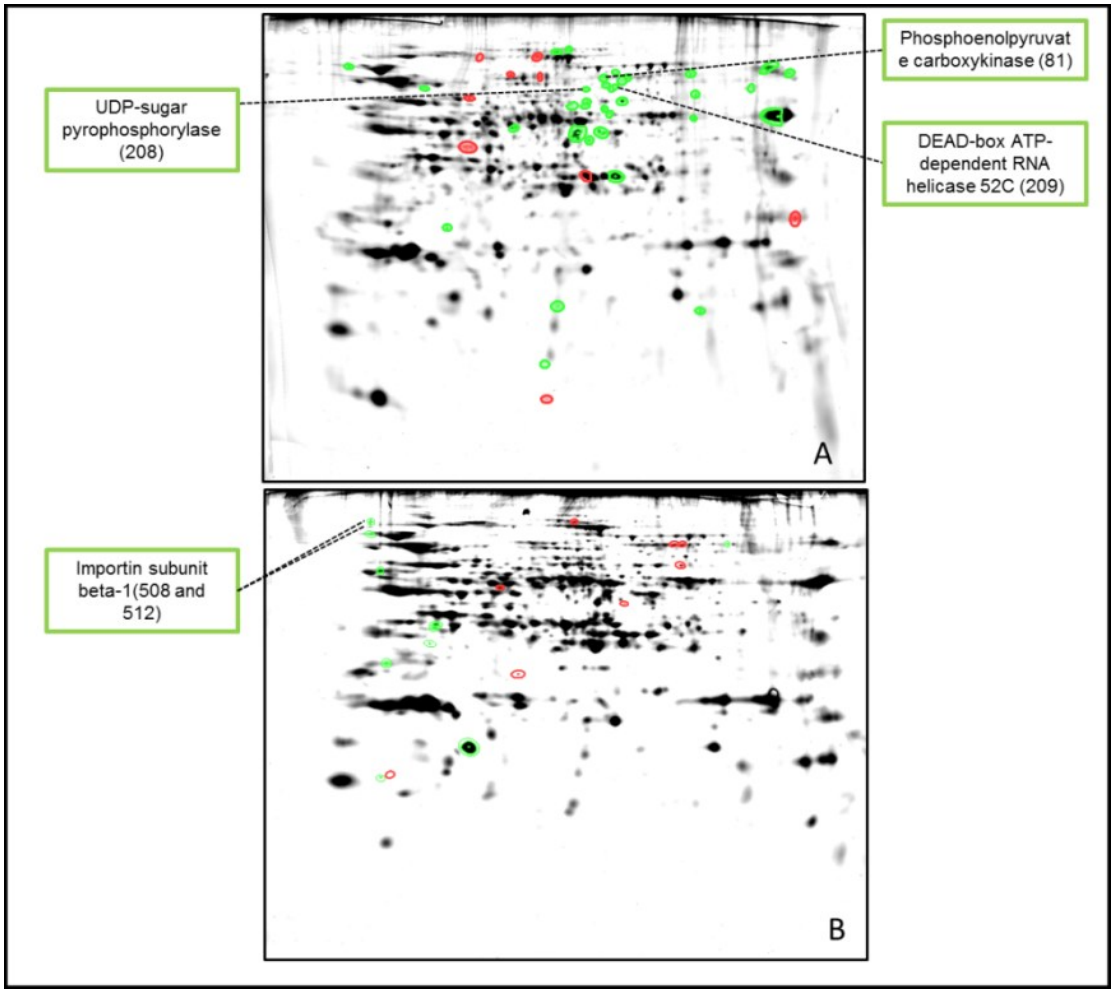

Figure 4. Identification of oxidized proteins from spots with changed abundance in comparison between the treatment control and the GSH treated cells clusters *Cyclamen persicum*: A at 3 days and B at 14 days. Red spots represent proteins higher abundant in gels from control plants, green spots represent proteins higher abundant in gels from GSH treated cell cluster. Numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis. Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused image over all gels.

5.5.2. Total proteome (coomassie blue staining)

For the comparison for the total proteome of the control and GSH treated cells in the 3 time points the identified proteins were matched to the corresponding spots in Figure 5 and the details are shown in Table 2.

At day three, 848 spots were found,17 of them showing a 5 fold difference between control and GSH treated cells. We successfully identified 14 proteins. Four of them showed the same isoform, probably after a post-translational modification (ID 747 and 748; ID 718 and 720). From the 10 protein identified, eight were upregulated (5 fold) in the GSH treated cells. From these, four are related to the genetic information process (ID 145, 604, 681,726 and 718-720), one with carbohydrate metabolism (ID 738) and one related to aminoacids biosynthesis (ID 747-748). It was also possible to identify two enzymes associated with the protein refolding and possibly involved in embryo development (ID 680 and 688). Two proteins were upregulated in the control treatment, being related to genetic information processing (ID 604 and 296).

At day seven, 779 spots were found and nine of them showed a five fold difference between control and GSH treated cells. We successfully identified 5 proteins, one upregulated in the control treatment, associated with genetic information processing (ID 686), and four upregulated in the GSH treated cells: three associated genetic information processing (ID 610, 657 and 681); and one protein from the germin like family was identified (ID 615).

At day 14, 793 spots were found, 14 of them showing a five fold difference between control and GSH treated cells. We successfully identified 8 proteins, two upregulated in the GSH treated cells: ABC transporter F family member 1, implicated in detoxification processes (ID 485) and Short-chain dehydrogenase/reductase SDRA involved in the peroxisomal activation of 2,4-dichlorophenoxybutyric acid (2,4-DB) (ID 199). Six proteins were upregulated in the control cells: four codifying for ribosomal proteins (ID 704, 709, 773 and 238/771); an enzyme associated with the protein refolding and may regulate the embryo development (ID 769), and a molecular chaperone roled in the processing of secreted materials (ID 513).

Table 2. Identification of proteins from spots with changed abundance in the comparison between the treatment control and the GSH treated cells clusters of *Cyclamen persicum*. A Student´s t-test was performed (p-value ≤0.05) to determine significant changes in spot volume on the basis of normalized relative spot volume. Only alterations of at least 5-fold in spot volume were considered to represent true alterations in the protein level.

ID[1]	Reg [2]	Accesion name[3]	DB[4]	Protein name	Gene name	Organism	KEGG[5]	Score[6]	PI Calc[7]	MW Calc[8]	Pep[9]
3DC 145	8.20	HSP81_ORYSI	SwissProt	Heat shock protein 81-1	HSP81-1	*Oryza sativa subsp. indica*	Genetic Information Processing	311	4.8	80.1	8
3DC 296	0.11	SIR1_TOBAC	SwissProt	Sulfite reductase 1 [ferredoxin], chloroplastic	SIR1	*Nicotiana tabacum*	Energy metabolism	245	9.7	77.9	6
3DC 604	0.14	H2B11_ARATH	SwissProt	Histone H2B.11	At5g59910	*Arabidopsis thaliana*	Genetic Information Processing	154	10.5	16.4	5
3DC 681	6.13	RL12_PRUAR	SwissProt	60S ribosomal protein L12	RPL12	*Prunus armeniaca*	Genetic Information Processing	198	9.7	17.9	3
3DC 738	5.06	AT5G49460.1	Tair	ATP-citrate synthase beta chain protein 2	ACLB-2	*Arabidopsis thaliana*	Carbohydrate metabolism	386	8.5	65.8	8
3DC 747 and 748	15.25	METE_CATRO	SwissProt	5-methyltetrahydropteroyl triglutamate-homocysteine methyltransferase	METE	*Catharanthus roseus*	Biosynthesis of amino acids	387	6.1	84.8	8
3DC 680	16.75	AT2G29960.1	Tair	Peptidyl-prolyl cis-trans isomerase CYP19-4	CYP19-4	*Arabidopsis thaliana*	Unclassified	81	9.6	21.5	2
3DC 688	23.40	CYPH_CATRO	SwissProt	Peptidyl-prolyl cis-trans isomerase	PCKR1	*Catharanthus roseus*	Unclassified	131	9.5	18.3	4

3DC 726	39.88	AT1G07660.1	Tair	Histone H4	At1g07660	*Arabidopsis thaliana*	Genetic Information Processing	121	12.0	11.4	3
3DC 718 and 720	30.87	AT3G05560.1	Tair	60S ribosomal protein L22-2	RPL22B	*Arabidopsis thaliana*	Genetic Information Processing	128	10.1	14.0	2
7DC 610	17.42	SAR1A_ARATH	SwissProt	GTP-binding protein SAR1A	SAR1A	*Arabidopsis thaliana*	Genetic Information Processing	365	7.7	22.0	8
7DC 615	11.42	GL11_ORYSJ	SwissProt	Germin-like protein 1-1	GER4	*Oryza sativa subsp. japonica*	Unclassified	103	9.2	22.5	2
7DC 657	6.51	RL31_PANGI	SwissProt	60S ribosomal protein L31	RPL31	*Panax ginseng*	Genetic Information Processing	136	10.6	14.0	4
7DC 681	30.73	H4_ARATH	SwissProt	Histone H4	At1g07660	*Arabidopsis thaliana*	Genetic Information Processing	120	12.0	11.4	3
7DC 686	0.13	AT2G45710.1	Tair	40S ribosomal protein S27-1	RPS27A	*Arabidopsis thaliana*	Genetic Information Processing	106	10.6	9.4	2
14DC 513	0.07	ENPL_CATRO	SwissProt	Endoplasmin homolog	HSP90	*Catharanthus roseus*	Genetic Information Processing	959	93.4	4.7	21

14DC 485	10.93	AT5G60790.1	Tair	ABC transporter F family member 1	ACBCF1	*Arabidopsis thaliana*	Genetic Information Processing	255	66.8	6.0	6
14DC 199	12.25	AT4G05530.1	Tair	Short-chain dehydrogenase/reductase SDRA	SDRA	*Arabidopsis thaliana*	Metabolism of cofactors and vitamins	150	26.7	9.3	3
14DC 704	0.20	RS5_CICAR	SwissProt	40S ribosomal protein S5	RPS5	*Cicer arietinum*	Genetic Information Processing	386	22.0	10.5	8
14DC 709	0.15	RL11_MEDSA	SwissProt	60S ribosomal protein L11	RPL11	*Medicago sativa*	Genetic Information Processing	508	20.7	10.6	13
14DC 769	0.10	CYPH_CATRO	SwissProt	Peptidyl-prolyl cis-trans isomerase	PCKR1	*Catharanthus roseus*	Unclassified	154	18.3	9.5	3
14DC 238 and 771	0.17	AT3G05560.1	Tair	60S ribosomal protein L22-2	RPL22B	*Arabidopsis thaliana*	Genetic Information Processing	139	14.0	10.1	2
14DC 773	0.19	RL301_ARATH	SwissProt	Putative 60S ribosomal protein L30-1	RPL30A	*Arabidopsis thaliana*	Genetic Information Processing	178	12.3	10.4	4

1) ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labeled with the same ID. 3DC stands for 3 days with Coomassie blue stain, 7DB for 7 days with Coomassie blue and 14DB for 14 days with Coomassie blue; 2) Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (GSH treatment/ control); 3) Accession number of the identified protein in the database where the spot was searched; 4) Database which was used for protein search: Tair (Tair10 release (Arabidopsis)) or SwissProt (Plant category of SwissProt, all plants in the database)); 5) Functional classification according to the KEGG Pathway Database; 6) The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation); 7) Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) a protein database; 8)Calculated molecular mass (MW) in kDa obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a protein database; 9) Number of peptides matched to the protein through the database search.

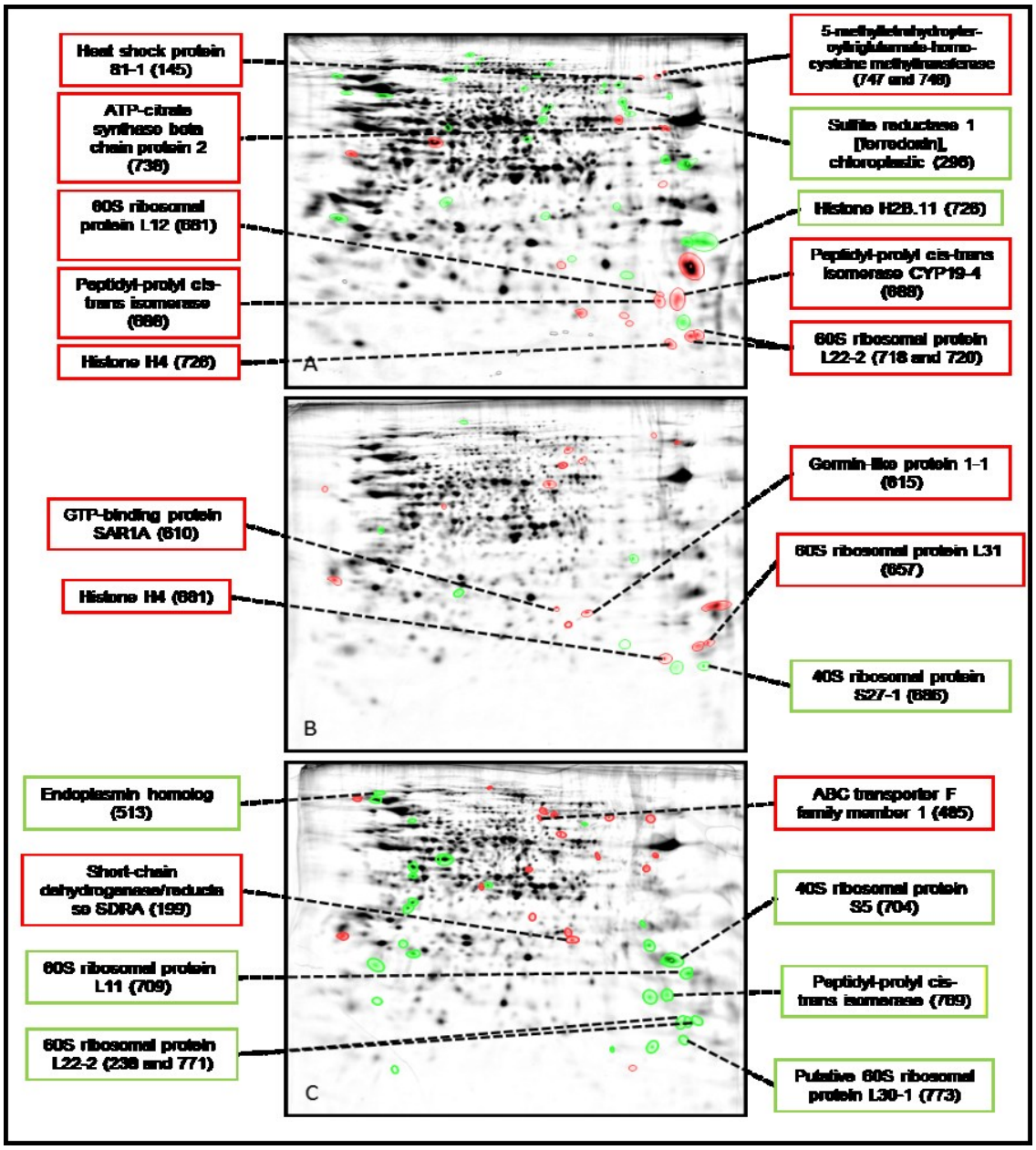

Figure 5. Identification of proteins from spots with changed abundance in the comparison between the treatment control and the GSH treated cells clusters of *Cyclamen persicum* : A at 3 days; B at 7 days and C at 14 days. Red spots represent proteins higher abundant in gels from control plants, green spots represent proteins higher abundant in gels from GSH treated cell clusters. The numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis. Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused image overall gels.

5.5.3. Comparision Bodipy-hz versus Coomassie Blue stain

To compare the oxi-proteome and the total proteome six comparisons were performed: Body-Hz versus Coomassie blue, in the control or GSH treated cells, in three time points (3, 7, 14 days). These comparisons were analyzed and the 1.5 fold differences were calculated. After that, the proteins spots which were 1.5 fold upregulated in the oxiproteome of control cells but not in the treated cells were excised and analyzed.

On day three the comparison of Bodipy-Hz and Coomassie Blue in the control treatment revealed 876 spots, 236 of them differing 1.5 fold. In the GSH treated cells, 900 spots were found with 116 differing in 1.5 fold. From these spots, with differential expression 13 showed to be present only in the control comparisons. From these residual spots, 8 were successfully identified. Two codifying to actin, involved in the cellular process (ID 284/271 and 805/806), and two involved in the genetic information process codifying to heat shock proteins (ID 1 and 2). Proteasome subunits, closely aligned with the cell proliferation process were also oxidized in a higher amount in the control treatment (ID 494 and 531). Proteins involved in carbohydrate metabolism and energy metabolism were also identified (ID 192 and 193 respectively).

On day seven the comparison of Bodipy-Hz and Coomassie Blue in the control treatment revealed 784 spots, 114 of them differing 1.5 fold. In the GSH treated cells 786 spots were found, 102 of them differing 1.5 fold. From these spots with differential expression, 11 were present only in the control comparisons. From these residual spots, 7 were successfully identified. A succinate dehydrogenase involved in complex II of the mitochondrial electron transport chain (ID 197), two ATP synthase (ID 673 and 713/775), a tubulin essential for the structure of the cytoskeleton (ID 712), one involved in the amino acid metabolism (ID 226), a ribosomal protein (ID 680); an enzyme (Peptidyl-prolyl cis-trans isomerase) associated with the protein refolding and may regulate the embryo development (ID 698).

On day 14 the comparison of Bodipy-Hz and Coomassie Blue in the control treatment revealed 818 spots, 126 of them differing 1.5 fold. In the GSH treated cells, 840 spots were found, 85 of them differing 1.5 fold. From these spots with differential expression 10 were only in the control comparisons. From these residual spots 9, were successfully identified. Three connected with aminoacid

metabolism (ID 160, 302 and 316), one actin involved in the regulation of hormone-induced plant cell proliferation (ID 302), a protein involved carbohydrate metabolism (ID 268), an energy metabolism (ID 788), a RuBisCO subunit (ID 776) and peptidyl-prolyl cis-trans isomerase (ID 532).

Table 3. Identification of proteins oxidized from spots with changed abundance in the comparison between the treatment control and the GSH treated cells clusters of *Cyclamen perisicum.* A Student´s t-test was performed (p-value ≤0.05) to determine significant changes in spot volume on the basis of normalized relative spot volume. Only alterations of at least 5-fold in spot volume were considered to represent true alterations in the protein level.

ID[1]	Reg [2]	Accesion name[3]	DB[4]	Protein name	Gene name	Organism	KEGG[5]	Score[6]	PI Calc[7]	MW Calc[8]	Pep[9]
3d BC 1	1.84	HS902_ARATH	SwissProt	Heat shock protein 90-2	HSP90-2	*Arabidopsis thaliana*	Genetic Information Processing	570	4.8	80.0	13
3d BC 2	1.86	HSP80_SOLLC	SwissProt	Heat shock cognate protein 80	HSC80	*Solanum lycopersicum*	Genetic Information Processing	1218	4.8	80.1	27
3d BC 192	1.61	DLDH2_ARATH	SwissProt	Dihydrolipoyl dehydrogenase 2, mitochondrial	LPD2	*Arabidopsis thaliana*	Carbohydrate metabolism	306	6.6	54.0	7
3d BC 193	1.73	ATPAM_HELAN	SwissProt	ATP synthase subunit alpha, mitochondrial	ATPA	*Helianthus annuus*	Energy metabolism	427	6.0	55.5	8
3d BC 284 and 271	1.62	ACT_GOSHI	SwissProt	Actin	N/A	*Gossypium hirsutum*	Cellular processes	653	5.2	41.6	12
3d BC 494	3.60	PSA4_PETHY	SwissProt	Proteasome subunit alpha type-4	PAC1	*Petunia hybrida*	Genetic Information Processing	217	5.5	27.2	4
3d BC 531	1.60	PSB6_ARATH	SwissProt	Proteasome subunit beta type-6	PBA1	*Arabidopsis thaliana*	Genetic Information Processing	173	5.2	25.1	3

3d BC 806 and 805)	1.62	AT5G09810.1	Tair	Actin-7	ACT7	*Arabidopsis thaliana*	Cellular processes	828	5.2	41.7	14
7d BC 197	1.59	SDHA_ORYSJ	SwissProt	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDH1	*Oryza sativa subsp. japonica*	Carbohydrate metabolism	467	6.7	68.8	13
7d BC 226	1.80	ILVD_ARATH	SwissProt	Dihydroxy-acid dehydratase, chloroplastic	DHAD	*Arabidopsis thaliana*	Amino acid metabolism	213	5.8	64.9	3
7d BC 673	2.92	ATP5H_ARATH	SwissProt	ATP synthase subunit d, mitochondrial	At3g52300	*Arabidopsis thaliana*	Energy metabolism	89	4.9	19.6	3
7d BC 680	1.59	AT5G35530.1	Tair	40S ribosomal protein S3-4	RPS3C	*Arabidopsis thaliana*	Genetic Information Processing	382	10.0	27.4	9
7d BC 698	3.02	CYPH_CATRO	SwissProt	Peptidyl-prolyl cis-trans isomerase	PCKR1	*Catharanthus roseus*	Unclassified	225	9.5	18.3	5
7d BC 712	2.06	TBB5_WHEAT	SwissProt	Tubulin beta-5 chain	TUBB5	*Triticum aestivum*	Cellular processes	605	4.6	50.3	14
7d BC 713 and 775	1.78	ATPBM_NICPL	SwissProt	ATP synthase subunit beta, mitochondrial	ATPB	*Nicotiana plumbaginifolia*	Energy metabolism	1043	5.9	59.8	18
14d BC 160	1.94	AT5G62530.1	Tair	Delta-1-pyrroline-5-carboxylate dehydrogenase 12A1, mitochondrial	ALDH12A1	*Arabidopsis thaliana*	Amino acid metabolism	321	6.3	61.7	6

14d BC 254	2.13	IF4A9_TOBAC	SwissProt	Eukaryotic initiation factor 4A-9	N/A	*Nicotiana tabacum*	Genetic Information Processing	335	5.4	46.8	8
14d BC 268	2.18	AT5G13420.1	Tair	Aldolase-type TIM barrel family protein	T22N19_70	*Arabidopsis thaliana*	Carbohydrate metabolism	311	6.0	47.7	7
14d BC 302	1.78	AT5G09810.1	Tair	Actin-7	ACT7	*Arabidopsis thaliana*	Cellular processes	717	5.2	41.7	13
14d BC 302	1.78	GLN14_ARATH	SwissProt	Glutamine synthetase cytosolic isozyme	GLN1-4	*Arabidopsis thaliana*	Amino acid metabolism	218	5.0	39.0	6
14d BC 316	1.58	GLNA1_MEDSA	SwissProt	Glutamine synthetase cytosolic isozyme	N/A	*Medicago sativa*	Amino acid metabolism	210	5.4	39.1	6
14d BC 532	1.66	CYPH_CATRO	SwissProt	Peptidyl-prolyl cis-trans isomerase	PCKR1	*Catharanthus roseus*	Unclassified	136	9.5	18.3	3
14d BC 776	1.97	RUBB_PEA	SwissProt	RuBisCO large subunit-binding protein subunit beta, chloroplastic	N/A	*Pisum sativum*	Genetic Information Processing	937	5.8	62.9	20
14d BC 788	1.79	VATB2_ARATH	SwissProt	V-type proton ATPase subunit B2	VHA-B2	*Arabidopsis thaliana*	Energy metabolism	572	4.9	54.3	12

1) ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labeled with the same ID. 3d BC stands for 3 days, 7d BC for 7 days and 14d BC for 14 days blue; 2) Regulation of a spot according to the comparison between groups. Regulation is given as the ratio between spot abundance (Bodipi-Hz/Coomassie blue); 3) Accession number of the identified protein in the database where the spot was searched; 4) Database which was used for protein search: Tair (Tair10 release (Arabidopsis)) or SwissProt (Plant category of SwissProt, all plants in the database)); 5) Functional classification according to the KEGG Pathway Database; 6) The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation); 7) Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) a protein database; 8)Calculated molecular mass (MW) in kDa obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a protein database; 9) Number of peptides matched to the protein through the database search.

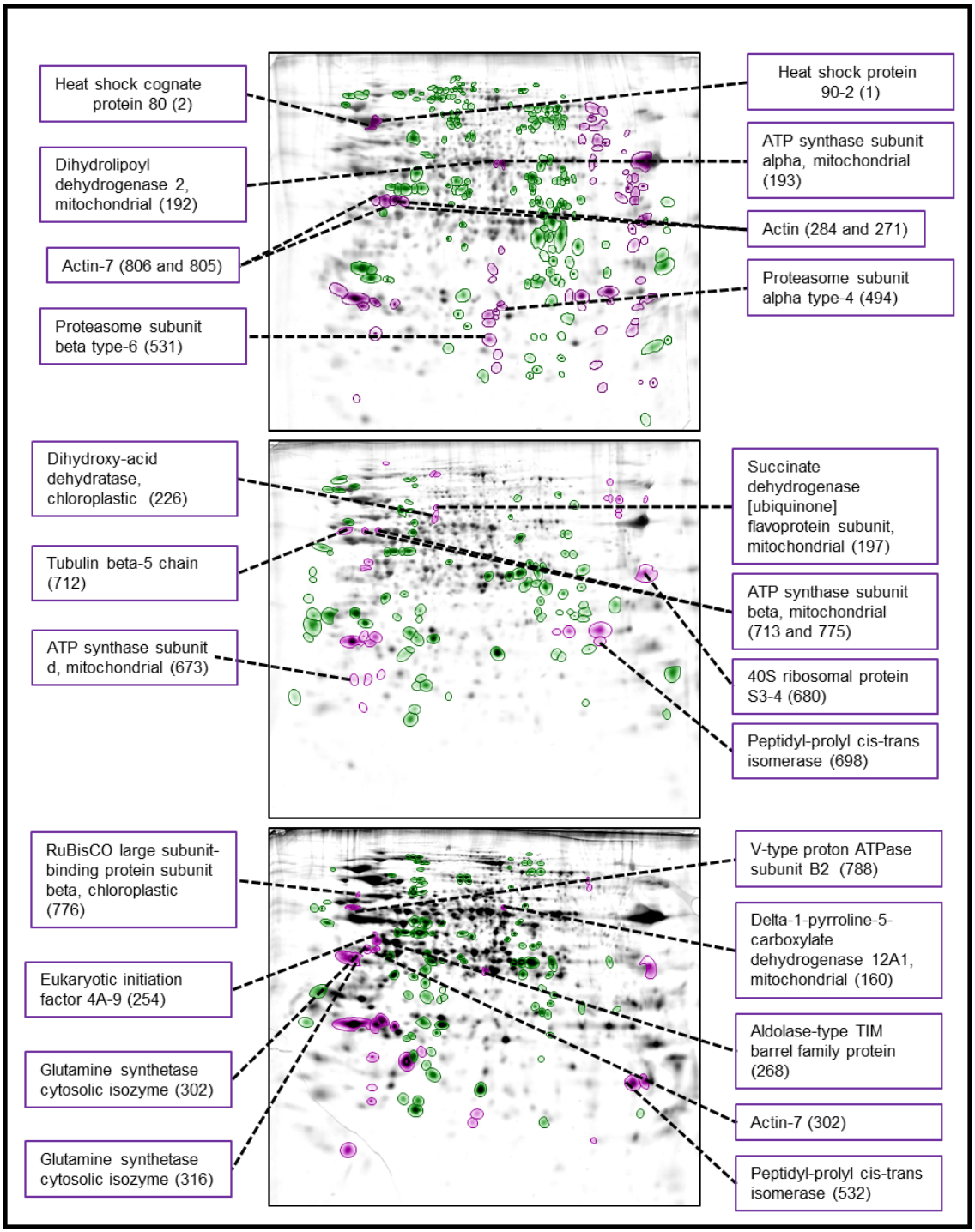

Figure 6. Identification of proteins from spots with changed abundance in the comparison between Bodipy-Hz and Coomassie blue the treatment control and the GSH treated cells clusters: A at 3 days; B at 7 days and C at 14 days. Purple spots represent proteins higher abundant in gels from control plants, green spots represent proteins higher abundant in gels from GSH treated cell cluster of *Cyclamen persicum*. The numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis. Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused image over all gels. (CAPRESTANO, C.A. 2015)

5.6 DISCUSSION

Two interchangeable forms are present in the cellular glutathione pool: reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduced glutathione has a high reduction potential, which eliminates the reactive oxygen species, thereby being oxidized (STASOLLA, 2010). Our previous studies the levels of GSH and GSSG were measured during *Cyclamen persicum* the SE showed to be unstable and heterogeneous (CAPRESTANO et al, 2016). The decrease of GSH redox in the early stages of SE can be reversed by exogenous supplementation of GSH, increasing the formation of somatic embryos.

In the present study, the supplementation of 2 mM GSH improved the number of somatic embryos in 1.5 times. GHS seems to be recruited to the nucleus during the cell proliferation providing a reduced environment to the cell cycle (VIVANCOS *et al.*, 2010). However, in our study, GSH showed to reduce the initial number of viable cells (Figure 2a). This can be a result of the optimization of the embryogenic cell proliferation by eliminating nonembryogenic cells. The reduced environment formed by supplementation of GSH can be observed by the percentage of cells cluster which shows a purple coloration in response to NBT stains. Studies showed that the transitions from G_1 to S phase requires an adequate level of glutathione (VERNOUX *et al.*, 2000).

Cyclamen persicum is a dicotyledonous plant were one of the two cotyledons is suppressed very early; the shoot and root meristem being connected by procambial strands (WICART; MOURAS; LUTZ, 1984). In the present work, somatic embryos formed in the GSH treatment showed an enhanced embryo formation with the shoot and root meristem connected by a procambium tissue. Somatic embryos formed in the control treatment present an altered morphology, containing only shoot or root meristem, without procambium, however embryos with a complete morphology. In both treatments the epidermis of somatic embryos showed a rough and irregular epidermis structure regular and irregular epidermis, typical for somatic embryos of Cyclamen (HOENEMANN *et al.*, 2010). In both treatments, secondary SE was observed, a common feature in cyclamen (HOENEMANN *et al.*, 2010; WINKELMANN; SPECHT; SEREK, 2006).

5.6.1. The oxi-proteome

Carbonyl groups, derivate from ROS formation, attack the amino-acid side chains of proline, lysine, threonine, and arginine (MAISONNEUVE *et al.*, 2009). The protein oxidation is a deleterious posttranslational modification that can alter protein structure leading to a partial or total loss of their function.

Analyses of the oxi-proteome in cyclamen SE showed a slight difference between the control and GSH treatment. On day three using a protein carbonyl detection method based on fluorescent Bodipy-Hz only three proteins were identified to be five times more oxidized in the control treatment, as compared to the GSH treatment. These proteins are related to carbohydrate metabolism (ID 81, 208) or genetic information process (ID 209).

The phosphoenolpyruvate carboxykinase (ID 81) occurs in developing seeds involved in the metabolism and transport of assimilates (WALKER *et al.*, 1999). In date palm, phosphoenolpyruvate carboxykinase was associated with the inorganic carbon fixation in proliferating embryos (MASMOUDI *et al.*, 1999). UDP-sugar pyrophosphorylase (ID 208) are enzymes essential for the sucrose synthesis as well to another nucleotide sugar, being a precursor of the cell wall biogenesis (GIBEAUT, 2000). This protein was already identified in the cyclamen somatic embryos, associated with the sucrose supplementation in the culture media (WINKELMANN *et al.*, 2006).

During SE a heavy demand for energy is observed to support the process of cell division and elongation (NOAH *et al.*, 2013). The higher expression of carbohydrate metabolism was described for several species, including *Cyclamen persicum* (RODE *et al.*, 2011; WINKELMANN *et al.*, 2006). The oxidation of this protein at the beginning of embryo development can reduce the inflow of carbohydrates to the embryo, resulting in a reduced formation of the somatic embryo as observed in this work.

Another protein identified was DEAD-box ATP-dependent RNA helicase 52C (ID 209), a protein involved in regulatory events in particular during organ maturation, differentiation and cellular growth (TUTEJA; TUTEJA, 2004).

On day 14 using one proteins two isoform of the same protein was identify to be five times more oxidized in the control treatment, compared with the GSH

treatment: Importin subunit beta-1 (ID 508/509) a protein which facilitates the nuclear protein import, binding to nuclear, or directly where the protein acts as an autonomous nuclear transport receptor.

5.6.2. The total proteome

In the total proteome, at day 3, two proteins were upregulated in the control group. A sulfite reductase 1 (ID 296), required in assimilatory sulfate reduction pathway in the primary and secondary metabolism, involved in the development and growth (KHAN *et al.*, 2010). However, the supplementation of GSH in the culture media may decrease the gap of sulfite reductase formation (KHAN *et al.*, 2010). And a histone H2B1.1 (ID 604), another histone H4 was up-regulated in the GSH treatment (ID 724). Histones are a core component of nucleosome playing a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability (KAPROS *et al.*, 1992).

The peptidyl-prolyl cis-trans isomerase (ID 680 and 688) was upregulated in the GSH treatment, has a protein refolding activity and is localized in the endoplasmatic reticulum (ANDERS *et al.*, 2008). This protein interacts with GNOM a member of a large family of ARF guanine nucleotide exchange factors that are required for proper polar localization of the auxin efflux carrier PIN1 (CHAUDHURY *et al.*, 2001).

Heat shock protein 81-1 (ID 145) is a protein from HSP-90 family an essential molecular chaperone in eukaryotic cells and plays key roles in the folding and activation of proteins involved in signal transduction and control of the cell cycle (SANGSTER; QUEITSCH, 2005). The concurrency of this chaperone family in the cytoplasm and in another subcellular compartments suggests a role in plant cell signal transduction (KRISHNA; GLOOR, 2001)

At seven day a Germin-like protein 1-1 (ID 615) was up regulated in the GSH treatment. Germin-like proteins constitute a large and highly diverse family of ubiquitous plant proteins, being glycoproteins somehow associated with the extracellular matrix (BERNIER; BERNA, 2001). Germin-like proteins bound ionically to the walls of preglobular somatic embryos of *Pinus caribea* but not in the nonembriogenic callus, planning a role in the initiation and termination of the embryo cell wall expansion (DOMON *et al.*, 1995; PATNAIK; KHURANA, 2001).

The histone H4 (ID 681) showed up regulated also at day seven in the GSH treated cells.

At day 14, 6 proteins were upregulated in the control treated cells; five connected with the genetic information process (ID 513, 704, 709, 773 and 238/771). A peptidyl-prolyl cis-trans isomerase, mentioned above, is for the first time upregulated in the control cells.

In the GSH treated cells an ABC transporter F family member 1 (ID 485) was 10 fold upregulated. ABC transport family is one of the largest and most ubiquitous transporter families, associated with auxin transport and embryo polarity (SHARMA; BRYAN; MILLAM, 2007; ZAZÍMALOVÁ *et al.*, 2010). Short-chain dehydrogenase/reductase (ID 199), upregulated in the GSH treatment, is a protein family of NAD(P)(H)-dependent oxidoreductases (KAVANAGH *et al.*, 2008) which participates preferentially in auxin synthesis.

The upregulation in the GSH treatment of proteins involved in the auxin synthesis and transport at 3 and 14 days could explain better anatomy found in the embryos treated with GSH. Auxin regulates the cell expansion, growth, and division, contributing to the cell differentiation and dedifferentiation. The polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry during plant embryogenesis (LIU, 1993). The inhibition of auxin transport can result in abnormal somatic embryos (LARSSON *et al.*, 2008). In tobacco it was demonstrated that the auxin flows upward and accumulates in the early proembryo, therefore it flows in different directions and accumulates in the hypophysis and cotyledon tips of the differentiated embryo (CHEN *et al.*, 2010).

5.6.3. Bodipy-Hz versus Coomassie Blue

On day three, 8 oxidized proteins were up regulated only in the control treatment were identified. Actin (ID 284/271 and 805/806) is an important protein for the mentation of cell cycle, provide mechanical support and movement, functions in intracellular transportation and definition of cell polarity and orientation of cell division (HENG; KOH, 2010; SCHWARZEROVÁ *et al.*, 2010).

A heat shock protein (HSP90) (ID 1) an essential molecular chaperone in eukaryotic cells was oxidize on day 3. These proteins have also been identified

in *Pinus massonina* (ZHEN *et al.*, 2012), *Araucaria angustifolia* (BALBUENA *et al.*, 2011) and *Carica papaya* (VALE *et al.*, 2014) somatic embryogenesis. This protein family is associated with maturation, stabilization and protein activation, and important signal molecules for protein kinases, hormone receptors and transcriptions factors (KADOTA; SHIRASU, 2012). Another gene HSP gene was identified as a heat shock cognate protein HSC 80, a gene expressed in young and dividing cells and low expressed in maturate cells of carrots (KONING; ROSE; COMAI, 1992).

The identification of oxidized proteins connects with genetic information and the cellular process on day 3 could explain the high number of the formed embryo.

On day seven, in the comparison of Bodipy-Hz and Coomassie Blue in the control treatment was successful identified 7 proteins. From these proteins, two were associated with energy metabolism: ATP synthase (ID 673 and 713/775). A succinate dehydrogenase involved in complex II of the mitochondrial electron transport chain (ID 197)

Tubulin an essential for the structure of the cytoskeleton (ID 712), is involved in cell division and elongation with a role during the mitosis(PAN *et al.*, 2009). During *Citrus sinensis* somatic embryogenesis an upregulation of tubulin was observed until the globular state (PAN *et al.*, 2009).

On day 14 the comparison of Bodipy-Hz and Coomassie Blue 10 showed to be present only in the control comparisons. From these residual spots, 9 were successfully identified.

The enzyme glutamine synthetase catalysis the amidation of glutamate to generate glutamine, one key compound in the biosynthesis of nitrogen (RODRÍGUEZ *et al.*, 2006). This enzyme was found mainly in the procambial cells of SE and ZE of Pine, is a molecular marker of the early stage of vascular differentiation in pine. In facto somatic embryos formed in the control treatment, appears more incomplete, with some embryos with the absence of procambium cells (RODRÍGUEZ *et al.*, 2006). Thus, this protein is being synthesized but with the absence of procambium cells the loss of function induced by carbonylation.

5.7 CONCLUSION

This is the first report of redox-proteome during somatic embryogenesis. Important proteins involved in the auxin synthesis and transportation were identified to be oxidized in the control treatment or upregulated in GSH treated cells these results suggest an important role of GSH in the protein oxidation protection.

5.8 LITERATURE

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6. CONSIDERAÇÕES FINAIS

Neste trabalho relevantes informações sobre a embriogênese somática em *Cyclamen persicum* foram obtidas. Estas informações podem servir de base para um aprimoramento do protocolo de embriogênese somática da espécie, visando à propagação comercial massal da espécie.

Num primeiro momento foi testado a influência de altas concentrações de GSH durante as primeiras fases da diferenciação dos embriões somáticos. Em dois genótipos um efeito promotor do GSH na formação de embriões somáticos, entretanto uma grande variação entre a reação das culturas ao GSH foi visualizada.

Uma investigação do conteúdo endógeno de GSH, GSSG e cisteina durante a fase de diferenciação dos embriões somáticos de cyclamen, mostrou que estes compostos não se mantem estáveis e homogêneos. Um decréscimo dos níveis de GSH foi observado durante os sete primeiros dias de cultivo. Este decréscimo no estado redox da glutationa pode ser revertido pela suplementação de 2mM GSH durante os sete primeiros dias de cultivo. Entretanto a posterior transferência para um meio oxidado (GSSG) não mostrou efeitos na conversão dos embriões somáticos. Novos trabalhos correlacionando o estado redox e a formação de embriões somáticos em outros genótipos de ciclâmen podem auxiliar na elucidação desta correlação. Testes com maiores concentrações de GSSG em diferentes etapas do desenvolvimento do embrião somático de ciclâmen são necessárias para avaliar a importância da alteração do estado redox na diferenciação dos embriões somáticos.

Pela primeira vez um estudo do oxi-proteoma durante a embriogênese somática de ciclâmen foi realizado. Um estudo do oxi –proteoma e do proteoma total de clusters de células embrionárias de ciclâmen suplementadas ou não com 2mM GSH em três tempos (3,7 e 14 dias) foi performado. Proteínas importantes envolvidas no transporte polar de auxina se mostraram carboniladas no tratamento controle, bem como em maiores concentrações no proteoma total das células tratadas com GSH. Estes resultados sugerem uma oxidação de proteínas importantes para o desenvolvimento do embrião somático e podendo ser revertidas pela suplementação de GSH ao meio de cultivo.