UNIVERSIDADE FEDERAL DO PARANÁ

ANA LUIZA DORIGAN DE MATOS FURLANETTO

ESTRESSE OXIDATIVO E TRANSIÇÃO DE PERMEABILIDADE MITOCONDRIAL EM CÉLULAS EMBRIOGÊNICAS SOMÁTICAS DE *Araucaria angustifolia* ESTRESSADAS PELO FRIO.

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Dissertação apresentada como requisito parcial à obtenção do título de Mestre em Ciências-Bioquímica, Setor de Ciências Biológicas - Universidade Federal do Paraná.

Orientação: Prof.ª Dr.ª Silvia Maria Suter Correia Cadena.

Co-orientação: Prof.ª Dr.ª, Glaucia Regina Martinez e Prof.ª Dr.ª Juliana Bello Baron Maurer.

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Ana Luiza Dorigan de Matos Furlanetto

"Estresse oxidativo e transição de permeabilidade mitocondrial em células embriogênicas somáticas de *Araucaria angustifólia* estressadas pelo frio"

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Porque eu sou do tamanho do que vejo
E não, do tamanho da minha altura...

Alberto Caeiro
‘O Guardador de Rebanhos – Poema VII
Heterónimo de Fernando Pessoa

RESUMO

A *Araucaria angustifolia* é classificada como criticamente em perigo de extinção pela União internacional de conservação da natureza (IUCN) lista vermelha de espécies ameaçadas. O desenvolvimento e propagação desta espécie são influenciados por situações de estresse abiótico, como a variação de temperatura. Estudos anteriores evidenciaram a ativação da proteína desacopladora de mitocôndrias de plantas (PUMP) em células embriogênicas de *A. angustifolia* submetidas ao estresse pelo frio, um efeito associado ao estresse oxidativo. No presente estudo, buscou-se avançar no conhecimento da resposta desta conífera ao estresse pelo frio, com enfoque particular no estresse oxidativo. Desta forma, células embriogênicas de *A. angustifolia* foram submetidas ao estresse pelo frio (4 ± 1 ° C por 24h ou 48h) e foram avaliados a viabilidade e morfologia celulares, os níveis de H_2O_2, a lipoperoxidação e a atividade de enzimas antioxidantes. Em mitocôndrias isoladas destas células foram determinadas a atividade das NAD(P)H desidrogenases alternativas e a transição de permeabilidade mitocondrial (TPM). O estresse causado pelo frio não foi capaz afetar a morfologia e a viabilidade das células embriogênicas de *A. angustifolia*, no entanto, promoveu o aumento dos níveis de H_2O_2 em ~ 35% (em 24 e 48h). A lipoperoxidação também foi aumentada em ~ 15% e 30% após 24h e 48h de stress, respectivamente. A atividade da catalase foi reduzida em cerca de 20% após 48h de estresse, enquanto a ascorbato peroxidase (APx) e a dehidroascorbato redutase (DHAR) tiveram suas atividades aumentadas em ~ 100% e ~ 64%, respectivamente. Somente a atividade da monodehidroascorbate redutase (MDHAR) foi aumentada (~172%) no menor tempo de estresse (24h). As atividades das enzimas Glutationa redutase (GR) e superóxido oxidase (SOD) não foram alteradas pelas condições de estresse. Nas mitocôndrias, o estresse induzido pelo frio promoveu uma inibição significativa das NAD(P)H desidrogenases alternativas externas (~40% em 24 horas do stress e ~ 65% em 48 horas do stress), enquanto a transição de permeabilidade mitocondrial (MPT) foi discretamente inibida (em 24h e 48h de stress). Estes resultados mostram que o estresse por baixa temperatura foi capaz de induzir o estresse oxidativo nas células embriogênicas de *A. angustifolia*, o que resultou na ativação das enzimas antioxidantes, em especial as do ciclo glutationa-ascorbato. Esta ativação parece compensar a inibição da catalase e das NAD(P)H desidrogenases externas, uma vez que a viabilidade e morfologia das células não foram alteradas. Este estudo contribui para o esclarecimento da resposta desta gimnosperma a condição de estresse por baixa temperatura e, assim, também para o desenvolvimento de métodos de conservação desta espécie, como a micropropagação *in vitro*.

Palavras-chave

Araucaria angustifolia, Células embriogênicas, Mitocôndrias de plantas, Estresse pelo frio, Estresse oxidativo, Enzimas antioxidantes

ABSTRACT

Araucaria angustifolia is listed as critically endangered by International Union for Conservation of Nature (IUCN) red list of threatened species. The development and propagation of this species is strongly affected by abiotic stress, such as the temperature variation. We previously shown the activation of plant uncoupling mitochondrial protein (PUMP) in embryogenic *A. angustifolia* cells submitted to cold stress, an effect associated to oxidative stress. In this work, we advanced in these studies by submitting these cells to cold stress (4 ± 1°C for 24h or 48h) and evaluating the cellular and mitochondrial response associated to oxidative stress, namely: the H_2O_2 levels, the activity of antioxidant enzymes and lipid peroxidation. In mitochondria from these cells were evaluated the activity of NAD(P)H alternative dehydrogenases and mitochondrial permeability transition (MPT). The cold stress did not affect the morphology and viability of embryogenic *A. angustifolia* cells; however, increased the H_2O_2 levels by ~35% (at 24h and 48h) and lipid peroxidation by ~15% and 30% after 24h and 48h of stress, respectively. The activity of catalase was decreased by ~20% after 48h of cold stress while ascorbate peroxidase (APx) and dehydroascorbate redutase (DHAR) activities were increased by ~100% and ~64%, respectively. For the cells exposition to cold stress by 24h only dehydroascorbate redutase (MDHAR) had the activity increased by ~172%. Glutathione reductase (GR) and superoxide dismutase activities remained unchanged under both stress conditions. In mitochondria, the cold stress promoted a significant inhibition of external alternative NAD(P)H dehydrogenases (~40% at 24h of stress and ~65% at 48h of stress) while the mitochondrial permeability transition (MPT) was slightly inhibited in both, 24h and 48h of stress. The cold stress induces the oxidative stress in embryogenic *A. angustifolia* cells, which result in up-regulation of the enzymatic defense mainly the activation of gluthatione-ascorbate cycle in a compensatory way to the inhibition of catalase and external NAD(P)H dehydrogenases. These results contribute to understanding the pathway to overcoming the cold in this gymnosperm and are important for the development of conservation methods of this species such as in vitro micropropagation.

Keywords

Araucaria angustifolia, Embryogenic cells, Plant mitochondria, Cold stress, Antioxidant enzymes

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REVISÃO DE LITERATURA

ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS E SÍMBOLOS

REVISÃO DE LITERATURA

APx	ascorbato peroxidase
BHAM	ácido benzilhidroxâmico
BHT	butil hidroxitolueno
BSA	albumina de soro bovino
CAT	catalase
DHA	deidroascorbato
DHAR	deidroascorbato redutase
DMSO	dimetilsulfóxido
2,4-D	ácido 2,4-diclorofenóxiacético
EDTA	ácido etileno diamino tetracético
EGTA	ácido etileno glico-bis(β-amino éter) N, N, N', N'-tetracétco
ES	Embriogênese somática
FCCP	carbonil cianida *p*-trifluorometoxifenilhidrazona
GR	glutationa redutase
GSH	glutationa reduzida
GSSG	glutationa oxidada
HEPES	[N-(2 hidroxietil) piperazina N'-(2 ácido etanosulfônico)]
Mal/glut	malato/glutamato
MDA	monodeidroascorbato
MDAR	monodeidroascorbato redutase
MTT	brometo de [3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio]
nda1-2	gene codificador da proteína NDA1-2
NDA1-2	proteína NADH desidrogenase A1-2
ndb1-4	gene codificador da proteína NDB1-4
NDB1-4	proteína NADH desidrogenase B1-4
ndc1	gene codificador da proteína NDC1
NDC1	proteína NADH desidrogenase C1

PBS	solução salina tamponada
PUMP	proteína desacopladora de mitocôndrias de plantas
SDS	dodecil sulfato de sódio
SOD	superóxido dismutase
ERO	espécies reativas de oxigênio
SHAM	ácido salicilhidroxâmico
TBA	ácido tiobarbitúrico
TBARs	substâncias reativas ao ácido tiobarbitúrico
TCA	ácido tricloroacético
UA	unidades arbitrárias (fluorescência)
UCP	proteína desacopladora mitocondrial
UQ	ubiquinona
UQH_2	ubiquinol
Tris	tris (hidrometil) amino metano
TPM	transição de permeabilidade mitocondrial
$\Delta\psi$	potencial elétrico de membrana
NDs	NAD(P)H desidrogenases alternativas do tipo II

SUMÁRIO

1. REVISÃO DE LITERATURA

1.1. *Araucaria angustifolia*

Essa conífera é de ocorrência natural no sul e sudeste do Brasil, ocupando principalmente o estado do Paraná, Santa Catarina, Rio Grande do Sul e, localmente em São Paulo, Minas Gerais e Rio de Janeiro, em áreas de altitudes de 500 a 1800 metros. Também é encontrada em pequenas áreas da Argentina (Misiones) e Paraguay, em altitudes que chegam a 2300 metros (Thomas, P., 2013).

Florestas subtropicais de solo ácido constituem o habitat da *A. angustifolia* no Brasil. Esta espécie é polinizada pelo vento entre os meses de agosto a outubro e os cones das sementes começam a amadurecer dois anos após a polinização. O ciclo completo, que abrange desde carpelo primitivo até as sementes, dura cerca de quatro anos. As árvores jovens só se tornam competentes para a reprodução com no mínimo 12 anos de idade (Thomas, P., 2013).

No Brasil a Floresta de Araucária ou Floresta Ombrófila Mista, como também é chamada, foi muito explorada no século XX e, por isso, atualmente encontra-se em estado crítico de conservação, restando apenas 1-2% da sua área original (Mantovani, A. *et al.*, 2004). Um dos principais componentes dessa floresta é a *Araucária angustifolia* (Bert.) O. Kuntze (Pinheiro do Paraná), cuja exploração ocorreu devido a sua importância econômica, por fornecer madeira e resina de alta qualidade, como também as sementes, muito apreciadas na dieta humana (Mantovani, A. *et al.*, 2004). Somando-se a isso, a fragmentação e principalmente o desmatamento dessa espécie, a fim de fornecer área para o plantio de outras culturas agrícolas, como culturas florestais de *Pinus* e *Eucalyptus* levou, em 2005, ao enquadramento da *A. angustifolia* na categoria CR, (espécies criticamente em perigo de extinção) na lista vermelha de espécies ameaçadas da IUCN (Thomas, P., 2013). Devido a isso, o reflorestamento é essencial para evitar a extinção dessa espécie e, para isto, estudos sobre a biologia dessa planta são fundamentais, bem como o estabelecimento de meios eficientes e rápidos de propagação (Stefenon, V. M. *et al.*, 2009; Klimaszewska, K. *et al.*, 2011). Neste sentido, a embriogênese somática tem se mostrado bastante promissora. Esta se baseia na cultura *in vitro* de células somáticas que dão origem ao embrião, num processo semelhante ao de embriogênese zigótica. Esta técnica é uma importante ferramenta para o estudo da fisiologia desta planta,

possibilitando o desenvolvimento de métodos de propagação mais eficientes (Guerra, M. P. *et al.*, 1999).

1.2. EMBRIOGÊNESE SOMÁTICA

Embriogênese somática (ES) ou também denominada embriogênese assexuada é definida como uma demonstração da totipotência das células vegetais e é um processo de embriogênese análogo ao da embriogênese zigótica, no qual células somáticas são as precursoras do embrião sem que ocorra a fusão de gametas (Guerra, M. P. *et al.*, 1999). Esta ferramenta, além de facilitar os estudos de aspectos fisiológicos e bioquímicos de diferentes vegetais, também possibilita a propagação *ex situ* de diferentes espécies de interesse comercial e ambiental através da micropropagação (Aquea, F. *et al.*, 2008; Klimaszewska, K. *et al.*, 2011; Ma, X. *et al.*, 2012).

A literatura descreve diferentes fatores que podem afetar a indução e manutenção da ES, como a fonte/origem do explante e os componentes do meio de cultura (Astarita, L. V. e Guerra, M. P., 1998; Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005). Para *A. angustifolia* as condições e os fatores que interferem nos primeiros estágios da embriogênese somática já estão bem estabelecidos (Astarita, L. V. e Guerra, M. P., 1998; Astarita, L. e Guerra, M., 2000; Santos, A. L. W. *et al.*, 2002; Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005; Santos, A. L. W. *et al.*, 2008; Dos Santos, A. L. W. *et al.*, 2010). Sabe-se que a taxa de multiplicação e diferenciação celular *in vitro* são influenciadas pela presença do acido abscísico (ABA), agentes osmóticos, carvão ativado, poliaminas e fontes de carbono (Astarita, L. e Guerra, M., 2000; Santos, A. L. W. *et al.*, 2002; Steiner, N. *et al.*, 2005; Silveira, V. *et al.*, 2006; Steiner, N. *et al.*, 2007; Santos, A. L. W. *et al.*, 2008). No entanto, não são conhecidos todos os fatores que afetam a ES, principalmente nos estágio mais avançados de desenvolvimento desse processo (Vieira, L. D. N. *et al.*, 2012; Jo, L. *et al.*, 2014).

Além dos fatores abióticos citados acima, outros, como variações de temperatura, exposição a luz UV e salinidade, podem induzir a produção de espécies reativas de oxigênio ROS (Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005; Lin, J. *et al.*, 2006a; Shohael, A. M. *et al.*, 2006; Sun, J. *et al.*, 2010). Níveis aumentados de ROS podem causar danos a biomoléculas, como o DNA, proteínas e lipídeos. Ao reagirem com os lipídeos de membrana, ocorre um processo denominado de lipoperoxidação (Figura 1)

que pode desencadear a morte celular programada (MCP) (Gill, Sarvajeet Singh e Tuteja, Narendra, 2010).

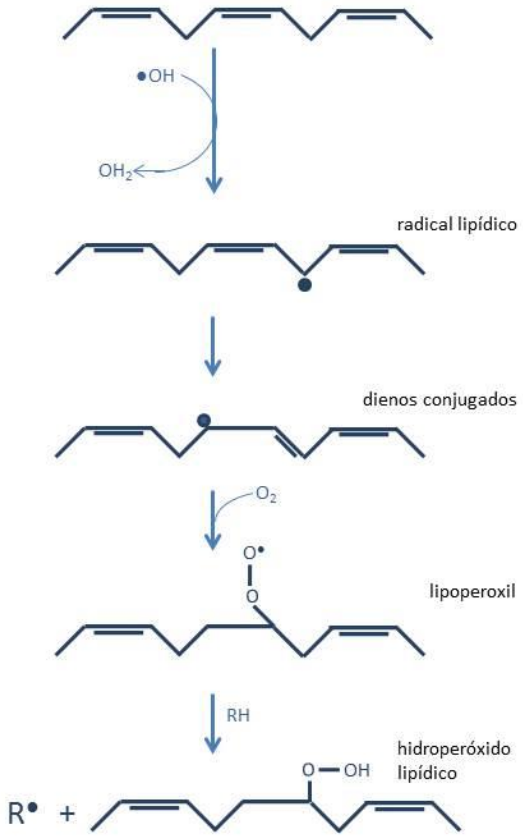


FIGURA 1: LIPOPEROXIDAÇÃO
FONTE: O autor

NOTA: O radical hidroxila (HO•) abstrai um átomo de hidrogênio de lipídeos poliinsaturados formando um radical lipídico (R•). Este por sua vez forma dienos conjugados após rearranjo que podem reagir com o oxigênio formando o radical lipoperoxil. Este radical, pode então abstrair um átomo de hidrogênio de um lipídeo adjacente (RH) ou formar um endoperóxido que tem como um dos produtos de degradação o malondialdeído.

Se por um lado níveis aumentados de ROS estão relacionados à lipoperoxidação, transição de permeabilidade mitocondrial e morte celular, estudos têm mostrado que estas espécies estão envolvidas em importantes vias de sinalização em processos fisiológicos (Ahmad, P. *et al.*, 2008). Neste contexto, Correa-Aragunde, N. *et al.* (2006) demonstraram que o óxido nítrico ($NO^{\bullet}$) é importante na via de indução de genes relacionados ao crescimento radicular. Pequenos aumentos dos níveis de $NO^{\bullet}$ foram relacionados à diferenciação e divisão de células embriogênicas de *Araucaria angustifolia* em cultura (Vieira, L. D. N. *et al.*, 2012). Os autores sugeriram que modificações no

estado redox celular seriam uma estratégia para o desenvolvimento mais eficiente dessas culturas.

1.3. ESPÉCIES REATIVAS DE OXIGÊNIO (ROS) X DEFESAS ANTIOXIDANTES

Nas células vegetais, o aumento dos níveis de ROS durante situações de estresse (biótico ou abiótico) ocorre em mitocôndrias, cloroplastos e peroxissomos. Por outro lado, estas organelas atuam de forma integrada para sobrepor a situação de estresse e manter o balanço redox celular (Ahmad, P. *et al.*, 2008)**.** Em linhas gerais as células vegetais possuem dois mecanismos para manter os níveis de ROS estáveis: (1) Enzimáticos (Figura 2): representados pela superóxido dismutase (SOD), ascorbato peroxidase (APx), glutationa peroxidase (GPX), glutationa redutase (GR) e catalase (EC) e, (2) Não enzimáticos: nos quais se incluem o ascorbato e a glutationa (GSH), bem como o tocoferol, flavonoides, alcaloides e carotenoides (Apel, K. e Hirt, H., 2004; Ahmad, P. *et al.*, 2008).

Em cultura de calos a atividade dos sistemas enzimático e não enzimático já foi descrita para diferentes espécies, sendo que sob condições de estresse abiótico ambos são modulados (Shohael, A. M. *et al.*, 2006; El-Beltagi, H. S. *et al.*, 2011).

Além desses mecanismos, a mitocôndria vegetal possui proteínas exclusivas em sua cadeia transportadora de elétrons capazes de manter e modular significativamente o balanço oxidativo e, por isso, essa organela é considerada o centro de controle deste processo (Pastore *et al*, 2007; Rasmusson, Fernie e Van Dongen, 2009). São estas: as NAD(P)H desidrogenases alternativas, a oxidase alternativa (AOX) e a proteína desacopladora de plantas (PUCP) também denominada de PUMP (Figura 3) (Moller, I. M., 2001; Pastore, D. *et al.*, 2007). Todas estas já foram descritas em *Araucaria angustifolia* (Mariano, A. B. *et al.*, 2008).

1.4. SISTEMAS ENZIMÁTICOS

Condições de estresse oxidativo são caracterizadas pelo o acúmulo de diferentes espécies reativas, como radical superóxido ($O_2^{\bullet-}$), radical hidroxila ($^{\bullet}OH$), oxigênio singlet ($^1O_2$) e peróxido de hidrogênio (H_2O_2). A formação de espécies altamente reativas, como $^{\bullet}OH$, $O_2^{\bullet-}$ e $^1O_2$ podem levar a importantes danos celulares. A superóxido dismutase (SOD) catalisa a dismutação do radical superóxido ($O_2^{\bullet-}$) a H_2O_2 e este, por sua vez, pode

sofrer redução a H_2O e $^1/_2O_2$ pela catalase (Figura 2). A remoção de radicais principalmente $^{\bullet}OH$ evita a iniciação do processo de lipoperoxidação (Halliwell, B. e Gutteridge, J. M. C., 2000).

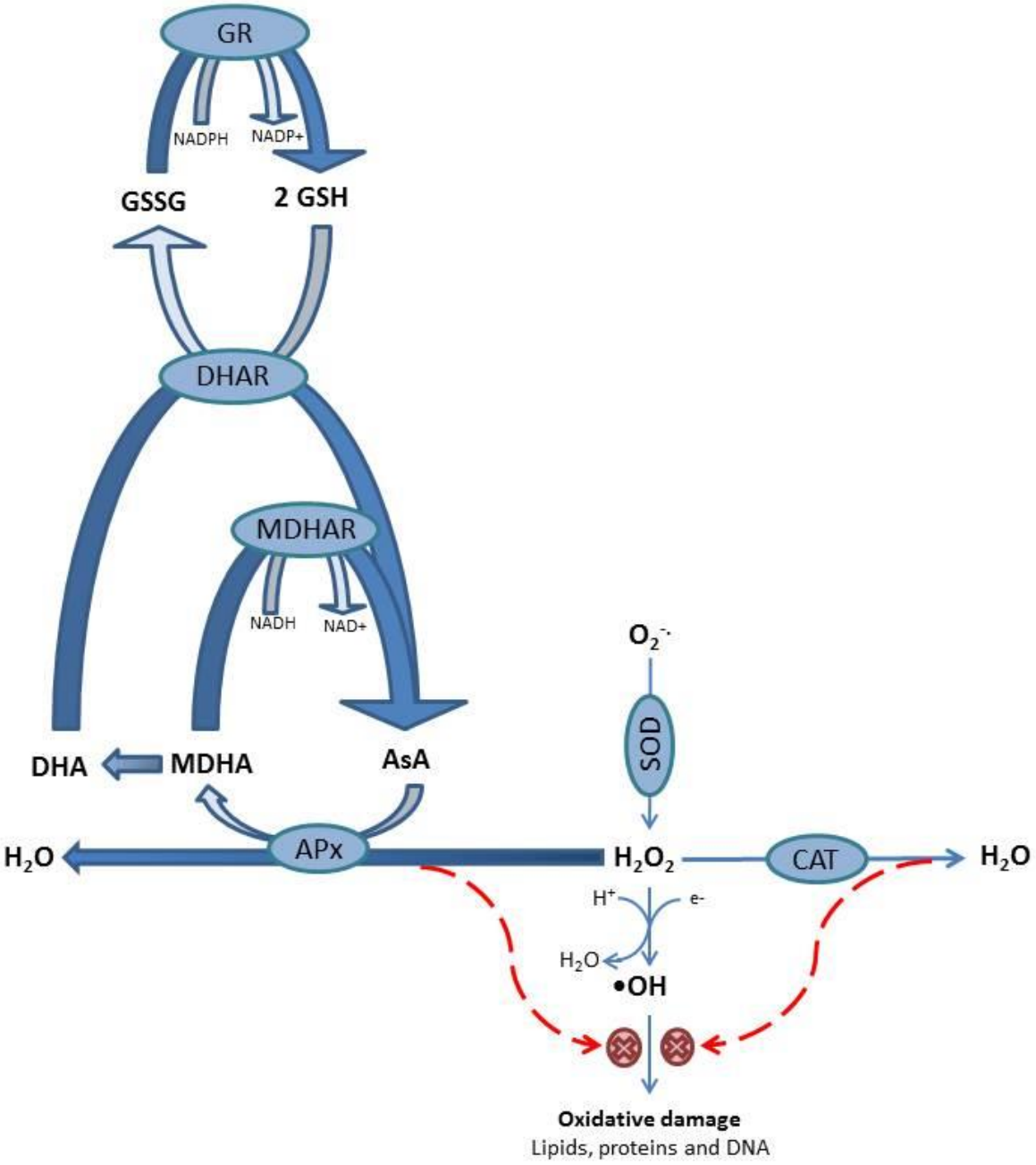


FIGURA 2: PRINCIPAIS ENZIMAS DE DEFESA CONTRA ROS EM PLANTAS.
FONTE: O autor

Interação enzimática entre SOD e CAT e o ciclo Ascorbato-Glutationa (setas espessas). Enzimas antioxidantes: SOD: superóxido dismutase, CAT: catalase, APx: ascorbato peroxidase, MDHAR: monodehidroascorbato redutase, DHAR: dehidroascorbato redutase: GR: glutationa redutase.

Dentro do sistema enzimático de defesa contra ROS, destaca-se o chamado Ciclo do ascorbato-glutationa (Figura 2), que inclui quatro enzimas, ascorbato peroxidase (APx), monodehidroascorbato redutase (MDHAR): dehidroascorbato redutase (DHAR) e glutationa redutase (GR) (Noctor, G. e Foyer, C. H., 1998). A Ascorbato Peroxidase (APx) reduz o H_2O_2 utilizando como doador de elétrons o ascorbato e liberando como produtos a água e o monodehidroascorbato, que é reduzido novamente a ascorbato pela Monodehidroascorbato Redutase (MDHAR) que, por sua vez, utiliza o NADPH como doador de elétrons. O monodehidroascorbato pode também sofrer uma reação de dismutação espontânea a dehidroascorbato que é reduzido pela Dehidroascorbato Redutase (DHAR) a ascorbato utilizando glutationa reduzida (GSH) como doadores de elétrons. Finalmente, a Glutationa Redutase (GR) regenera a glutationa reduzida (GSH) utilizando como doador de elétrons o NADPH (Apel, K. e Hirt, H., 2004).

Vários autores têm descrito a importância desse ciclo no controle do estresse biótico e abiótico (Noctor, G. e Foyer, C. H., 1998; Mittler, R., 2002; Apel, K. e Hirt, H., 2004). Ma, F. e Cheng, L. (2004), por exemplo, demonstraram ativação das enzimas do ciclo Ascorbato-glutationa em maças expostas a radiação solar por um dia. Liu, Y. *et al.* (2011) mostrou que durante o estresse oxidativo induzido por baixas temperaturas (0°C e 4°C) células de *Chorispora bungeana* tiveram a atividade da GR, APx e a concentração intracelular de ascorbato aumentadas, indicando uma maior atividade do ciclo Ascorbato-glutationa em condições de estresse.

Como já descrito, as mitocôndrias vegetais tem papel fundamental no controle dos níveis intracelulares de ROS devido à presença em sua cadeia respiratória de proteínas exclusivas capazes de reduzir os níveis destas espécies, as NAD(P)H desidrogenases alternativas, a oxidase alternativa na (AOX) e proteína desacopladora de plantas (PUMP) também denominada de PUCP (FIGURA 3) (Pastore, D. *et al.*, 2007; Rasmusson, A. G. *et al.*, 2009).

As NAD(P)H desidrogenases alternativas, também denominadas NDs do tipo II são enzimas insensíveis à rotenona, não bombeadoras de prótons que doam seus elétrons diretamente à ubiquinona (UQ) (Moller, I. M., 2001; Logan, D. C., 2007). Rasmusson e colaboradores (2009) descrevem seis diferentes NAD(P)H desidrogenases em plantas, sendo quatro externas e duas internas, possibilitando assim a oxidação de NAD(P)H citosólico e mitocondrial, respectivamente. Em *Arabidopsis thaliana,* Logan, D. C. (2007); Rasmusson, A. G. *et al.* (2008) agruparam as NDs expressas em plantas em três famílias: NDA com 2 membros, NDB com 4 membros e NDC com 1 membro. Sendo as NDA1 E NDA2 NADH internas, NDB1 NADPH externa, NDB2, NDB3, NDB4 NADH externas

NADH e NDC1 NADPH interna. O autor destaca que todos os genes do tipo NDB possuem motivos em sua sequencia de DNA que indicam fortemente a existência de uma região de ligação ao cálcio.

As NAD(P)H desidrogenases externas atuam cooperativamente com a AOX, evitando a formação de ROS, ao prevenir flutuações nos níveis de redução da ubiquinona (UQ) (Rasmusson, A. G. *et al.*, 2009). Ho, L. H. *et al.* (2007) mostraram que os genes codificantes da NDB4 (uma das variantes de NAD(P)H externa) e AtAOX1c (AOX1), respectivamente, são coexpressos durante o desenvolvimento de cultura vegetal transformada de *A. thaliana.* Os autores destacam que a cooperatividade destas duas enzimas estaria contribuindo para uma menor produção de ROS e maior fornecimento de energia requerido para a divisão celular.

A Oxidase Alternativa (AOX), é uma proteína de 34 kDa localizada na superfície interna da membrana mitocondrial interna (Pinheiro, H. A. *et al.*, 2004). Catalisa a oxidação do ubiquinol com a transferência de elétrons diretamente para o oxigênio reduzindo-o a água, sem o bombeamento de prótons. Essa enzima é insensível ao cianeto, antimicina A ou mixotiazol, mas é inibida por agentes complexantes de ferro, os ácidos hidroxâmicos como o ácido salicilhidroxâmico (SHAM) e o ácido benzohidroxâmico (BHAM) e o tiocianato de potássio. Ácidos orgânicos monocarboxilicos como o piruvato, hidroxipiruvato e glicoxilato e dicarboxilicos como α-cetoglutarato, oxaloacetato, L-malato e succinato são estimuladores da AOX (Millar, A. H. *et al.*, 1996).

Foi demonstrado que ROS promovem a ativação da transcrição e/ou tradução da AOX (Minagawa, N. *et al.*, 1992; Mcintosh, L. *et al.*, 1998). O papel da AOX em tecidos termogênicos já está bem estabelecido, porém, em plantas não termogênicas sugere-se que esta proteína tenha um papel protetor contra os estresses biótico e abiótico, por manter baixos os níveis de ROS (Pastore, D. *et al.*, 2007).

Umbach, A. L. *et al.* (2005) ao comparar um tipo selvagem de *A. thaliana* e um mutante cuja expressão da AOX era diminuída por conter expressas uma AOX anti-senso, constataram que o mutante apresentava uma menor variação da expressão nos genes relacionados com componentes da cadeia respiratória ou do controle oxidativo e, uma expressão aumentada de genes de diversas vias relacionadas ao metabolismo de carbono bem como vias pertencentes ao cloroplasto. Essas modificações, segundo os autores, mostram que a atividade da AOX tem reflexos sobre a geração de ROS e sobre o reservatório de UQ.

A proteína desacopladora de plantas (PUMP) é uma proteína integral de membrana (Vercesi, A. E. *et al.*, 2006), primeiramente isolada de tubérculo de batata (Vercesi, A. E.

et al., 1995). A PUMP facilita a reentrada de prótons na matriz mitocondrial levando ao colapso do gradiente eletroquímico de prótons, sendo inibida por ATP, GDP e GTP, e ativada por ácidos graxos de cadeia longa como o ácido láurico, mirístico, linoléico, oleico e palmítico (Pastore, D. *et al.*, 2000).

Estudos mostram que as proteínas AOX e PUMP possuem funções fisiológicas similares, pois, ambas são dissipadoras de energia (Vercesi, A. E. *et al.*, 1995; Calegario, F. F. *et al.*, 2003; Pinheiro, H. A. *et al.*, 2004; Pastore, D. *et al.*, 2007) e atuam de forma a prevenir o aumento dos níveis de ROS (Pinheiro, H. A. *et al.*, 2004). Apesar disso, são reguladas de modo inverso pelos ácidos graxos livres. A PUMP é ativada pelo aumento da concentração de ácidos graxos, enquanto que a AOX é inibida, sugerindo que estas proteínas não podem apresentar atividade máxima simultaneamente (Sluse, F. E. *et al.*, 1998; Calegario, F. F. *et al.*, 2003). Neste contexto, Valente e colaboradores (2012) demonstraram que em cultura embriogênicas de *A. angustifolia* submetidas ao estresse pelo frio (4°C for 24h and 48h) a atividade da PUMP teve um significativo aumento enquanto a AOX foi sutilmente estimulada, os autores sugerem assim que a PUMP seria responsável pelo controle dos níveis de ROS e também pela superação da condição de estresse.

1.5. TRANSIÇÃO DE PERMEABILIDADE MITOCONDRIAL (TPM)

A TPM é consequência do aumento da permeabilidade da membrana mitocondrial interna, devido à formação de um poro inespecífico, denominado poro de transição de permeabilidade (PTPM). Este permite a passagem de moléculas de massa molecular de até 1,5 kDa (Zoratti, M. e Szabo, I., 1995), o que pode levar ao inchamento mitocondrial, rompimento das membranas interna e externa e, assim, a liberação de fatores indutores de MCP para o citossol. Em plantas a TPM ocorre de forma semelhante à descrita para as mitocôndrias animais, sendo que o inchamento da organela, dependente de Ca^{2+} e fosfato inorgânico, é inibido pela a ciclosporina A (CsA), que parece também ligar-se a ciclofilina-D (Arpagaus, S. *et al.*, 2002). A presença do canal aniônico dependente de voltagem (VDAC), um dos componentes importantes para a PTPM em animais, também já está bem documentado em plantas (Homble, F. *et al.*, 2012).

Como descrito anteriormente níveis aumentados de ROS estão associados a TPM (Vacca, R. A. *et al.*, 2006; Reape, T. J. *et al.*, 2008). Em protoplastos de *Nicotiana tabacum,* por exemplo, o estado redox foi determinante para a abertura PTPM (Lin *et al.*,

2006). O mesmo foi observado por Saviani, E. E. *et al.* (2002) que descreveu a participação do PTPM na indução da morte celular pelo óxido nítrico em culturas de *Citrus sinensis*.

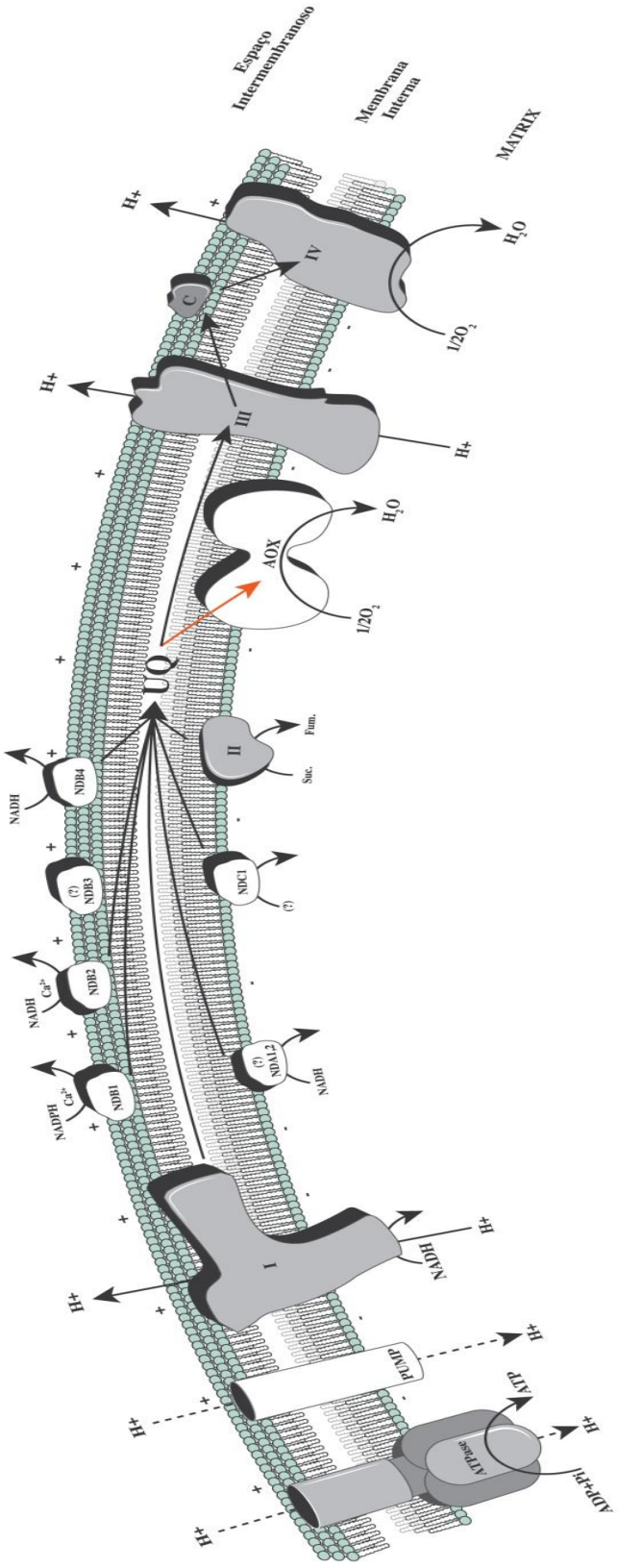


FIGURA 3: TRANSPORTE DE ELÉTRONS EM MITCONDRIAS VEGETAIS.

FONTE:Adaptado de Pastore *et al., 2007; Rasmusson et al.*, 2009

Estão representadas as enzimas da cadeia transportadora de elétrons, a ATP sintase e PUMP. **PUMP:** Proteína desacopladora de planta; **I:** NADH desidrogenase ou complexo I; **ND(A/B/C):** NAD(P)H desidrogenasses alternativas dependentes ou não de cálcio; **UQ:** ubiquinona **II:** succinato desidrogenasse ou complexo II; **AOX:** oxidase alternativa; **III:** Citocromo c oxidoredutase ou complexo III; **C:** citocromo c; **IV:** Citocromo c oxidase ou complexo IV.

2. OBJETIVOS E JUSTIFICATIVA

Nas células vegetais, as ROS podem ser formadas preferencialmente nas mitocôndrias, cloroplastos e peroxissomos. Tais compartimentos estão integrados de forma a manter o balanço oxidativo celular. Quando este balanço é desfeito, o aumento de ROS pode induzir vários danos e até mesmo a morte celular. O aumento de ROS, por sua vez, está associado aos diferentes tipos de estresses aos quais as plantas estão expostas, entre estes, as variações de temperatura. Embora existam na literatura inúmeros estudos sobre este tema, para as gimnospermas os relatos são escassos. A *A angustifolia* é uma gimnosperma que foi de grande interesse econômico nas décadas de 40 a 70 e que, atualmente, encontra-se em risco de extinção. Na tentativa de conservar esta espécie e contribuir para uma exploração sustentável desse recurso, justificam-se todos os esforços no sentido de conhecer os fatores fisiológicos e bioquímicos que possam influenciar seu desenvolvimento e propagação. Desta forma, o objetivo geral deste estudo é investigar a resposta de células embriogênicas de *A. angustifolia* ao estresse promovido por baixas temperaturas, com enfoque particular nos eventos relacionados ao estresse oxidativo. Para tanto, são objetivos específicos deste estudo.

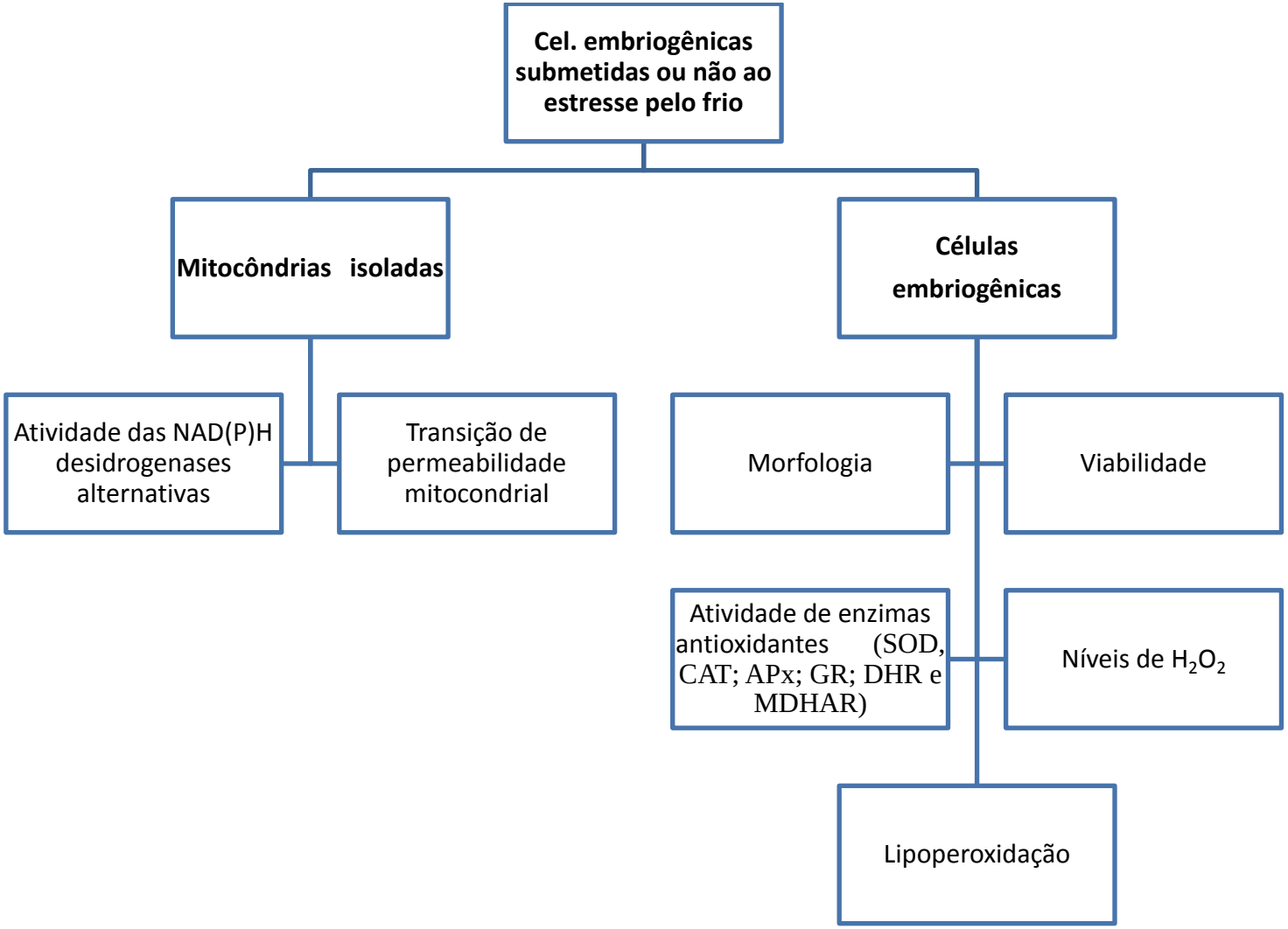
Em células embriogênicas de *A. angustifolia* submetidas ou não ao estresse pelo frio de 4ºC por 24 e 48h, avaliar:

- A morfologia e viabilidade celular;
- Os níveis de H_2O_2;
- A lipoperoxidação;
- A atividade de enzimas antioxidantes (SOD; CAT; APx; GR; DHAR e MDHAR).

Em mitocôndrias isoladas destas células, avaliar:

- A atividade das NADP(H) desidrogenases alternativas;
- A transição de permeabilidade.

3. ESTRATÉGIA EXPERIMENTAL



4. ARTIGO CIENTÍFICO

Cold stress alters the redox balance of *Araucaria angustifolia* embryogenic cells

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4.1. Abstract

Background: *Araucaria angustifolia* is listed as critically endangered by the International Union for Conservation of Nature (IUCN) red list of threatened species. The development and propagation of this species is strongly affected by abiotic stress such as temperature variation. We have previously shown the activation of plant uncoupling mitochondrial protein (PUMP) in *A. angustifolia* embryogenic cultures submitted to cold stress, an effect associated with oxidative stress. In this work, we continued these studies by submitting the cells to cold stress (4 ± 1°C for either 24 h or 48 h) and evaluating the cellular and mitochondrial response associated to oxidative stress.
Results: The cold stress did not affect the morphology and viability of the *A. angustifolia* embryonic cultures cells; however, there were increased H_2O_2 levels and lipid peroxidation after 24 h and 48 h of stress. The catalase activity was decreased after 48 h of cold stress whereas the ascorbate peroxidase (APx) and dehydroascorbate reductase (DHAR) activities were increased. For cells exposed to cold stress for 24 h, only Monodehydroascorbate reductase (MDHAR) activity was increased. Glutathione reductase (GR) and superoxide dismutase (SOD) activities remained unchanged under both stress conditions. In mitochondria, the cold stress promoted a significant inhibition of external alternative NAD(P)H dehydrogenases at 48 h of stress, whereas the mitochondrial permeability transition (MPT) was slightly inhibited after 24 h and 48 h of stress.
Conclusions: Cold stress can induce oxidative stress in *A. angustifolia* embryogenic cultures cells, which results in upregulation of the enzymatic defense pathway—primarily the

activation of the gluthatione-ascorbate cycle as compensation for the inhibition of catalase and external NAD(P)H dehydrogenases. These results contribute to understanding of the pathways involved in overcoming cold stress in this gymnosperm and are important for the development of conservation methods of this species such as *in vitro* micropropagation.

Keywords
Araucaria angustifolia, Embryogenic cultures , Plant mitochondria, Cold stress, Antioxidant enzymes.

4.2. **Background**

The Paraná pine (*Araucaria angustifolia* (Bert.) O. Kuntze) is the primary constituent of the Araucaria forest, which is found in the cold highlands of southern Brazil. The exploration of this gymnosperm in the twentieth century occurred due to its economic importance for providing high quality wood and resin, as well as their edible seeds that are very appreciated in human diet (Mantovani, A. *et al.*, 2004). As a result, *Araucaria angustifolia* is currently classified in the category CR (species critically endangered) by the IUCN red list of threatened species (Thomas, P., 2013).

Reforestation is an essential tool to combat the extinction of this species, and studies on the biology of this plant are vital to establish efficient and rapid strategies of promoting forest growth (Stefenon, V. M. *et al.*, 2009; Klimaszewska, K. *et al.*, 2011). Concerning this, somatic embryogenesis has been demonstrated as a promising approach. This method is based on *in vitro* culture of somatic cells that could form an embryo in a similar process to zygotic embryogenesis. This technique is an important tool for the study of the plant physiology and allows for the development of efficient methods for the micropropagation (Guerra, M. P. *et al.*, 1999). Several authors have been using embryogenic cultures to investigate factors related to stress and cell differentiation, as well as the understanding of the biochemical mechanisms of plant organelles (Silveira, V. *et al.*, 2006; El-Beltagi, H. S. *et al.*, 2011; Valente, C. *et al.*, 2012; Vieira, L. D. N. *et al.*, 2012).

Factors that may affect somatic embryogenesis of *A. angustifolia* are already well established (Astarita, L. V. e Guerra, M. P., 1998; Astarita, L. e Guerra, M., 2000; Santos, A. L. W. *et al.*, 2002; Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005; Santos, A. L. W. *et al.*, 2008; Dos Santos, A. L. W. *et al.*, 2010). It is known that the *in vitro* ratio of proliferation and cell differentiation is influenced by the presence of abscisic acid (ABA), osmotic agents, activated charcoal, carbon sources and polyamines (Astarita, L. e Guerra, M.,

2000; Santos, A. L. W. *et al.*, 2002; Steiner, N. *et al.*, 2005; Silveira, V. *et al.*, 2006; Steiner, N. *et al.*, 2007; Santos, A. L. W. *et al.*, 2008).

Aside from the abiotic factors mentioned above, variations in temperature, salinity and UV light exposure can increase the production of reactive oxygen species (ROS) in plant cells (Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005; Lin, J. *et al.*, 2006a; Shohael, A. M. *et al.*, 2006; Sun, J. *et al.*, 2010). High levels of ROS can damage to biomolecules such as DNA, proteins and lipids. The lipid peroxidation resulting from reaction of ROS with membrane lipids is the primary trigger of programmed cell death (MCP) (Gill, Sarvajeet Singh e Tuteja, Narendra, 2010).

Increased levels of ROS are also associated with the mitochondrial permeability transition (MPT), which is able to promote MCP due to the release of specific proteins from the intermembrane space of mitochondria, including cytochrome c (Vacca, R. A. *et al.*, 2006; Reape, T. J. *et al.*, 2008).

In plant cells, the increase of ROS in response to stress (biotic or abiotic) occurs in a compartmentalized manner, e.g., in mitochondria, chloroplast, and peroxisome. Moreover, these organelles work together to overcome the stressors and maintain the cellular redox balance via enzymatic and non-enzymatic mechanisms (Ahmad, P. *et al.*, 2008). The enzymatic mechanism involves superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione peroxidase (GPX), glutathione reductase (GR), catalase (EC), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), whereas the non-enzymatic mechanism includes ascorbate, glutathione (GSH), tocopherols, flavonoids, alkaloids and carotenoids (Apel, K. e Hirt, H., 2004; Ahmad, P. *et al.*, 2008).

When the production of ROS overcomes the capacity of antioxidants mechanisms, the cell is considered to be under oxidative stress, which is characterized by the accumulation of several reactive species such as superoxide radicals ($O_2^{-}\bullet$), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). These ROS (especially H_2O_2 and $O_2^{-}\bullet$) can be scavenged by enzymatic mechanisms (Fig. 1). Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical ($O_2^{-\cdot}$) to H_2O_2, which can be reduced to ½ O_2 and H_2O by catalase (Fig. 1) (Halliwell, B. e Gutteridge, J. M. C., 2000; Apel, K. e Hirt, H., 2004). Additionally, an important mechanism in plant defense under abiotic and biotic stress has been attributed to the ascorbate-glutathione cycle (Fig. 1) (Noctor, G. e Foyer, C. H., 1998; Mittler, R., 2002; Apel, K. e Hirt, H., 2004), which comprised of four enzymes. In this cycle, the hydrogen peroxide generated during oxidative stress is reduced by APx using ascorbate as electron donor. Then, the oxidized ascorbate [monodehydroascorbate (MDHA)] may be

regenerated by either MDHAR in a single step reaction or by DHAR, which uses the dehydroascorbate (DHA), a dismutation product from MDHA. Finally, the GSH from the reaction catalyzed by DHAR is regenerated by glutathione reductase (GR) (Apel, K. e Hirt, H., 2004). A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in plant cells (Apel, K. e Hirt, H., 2004).

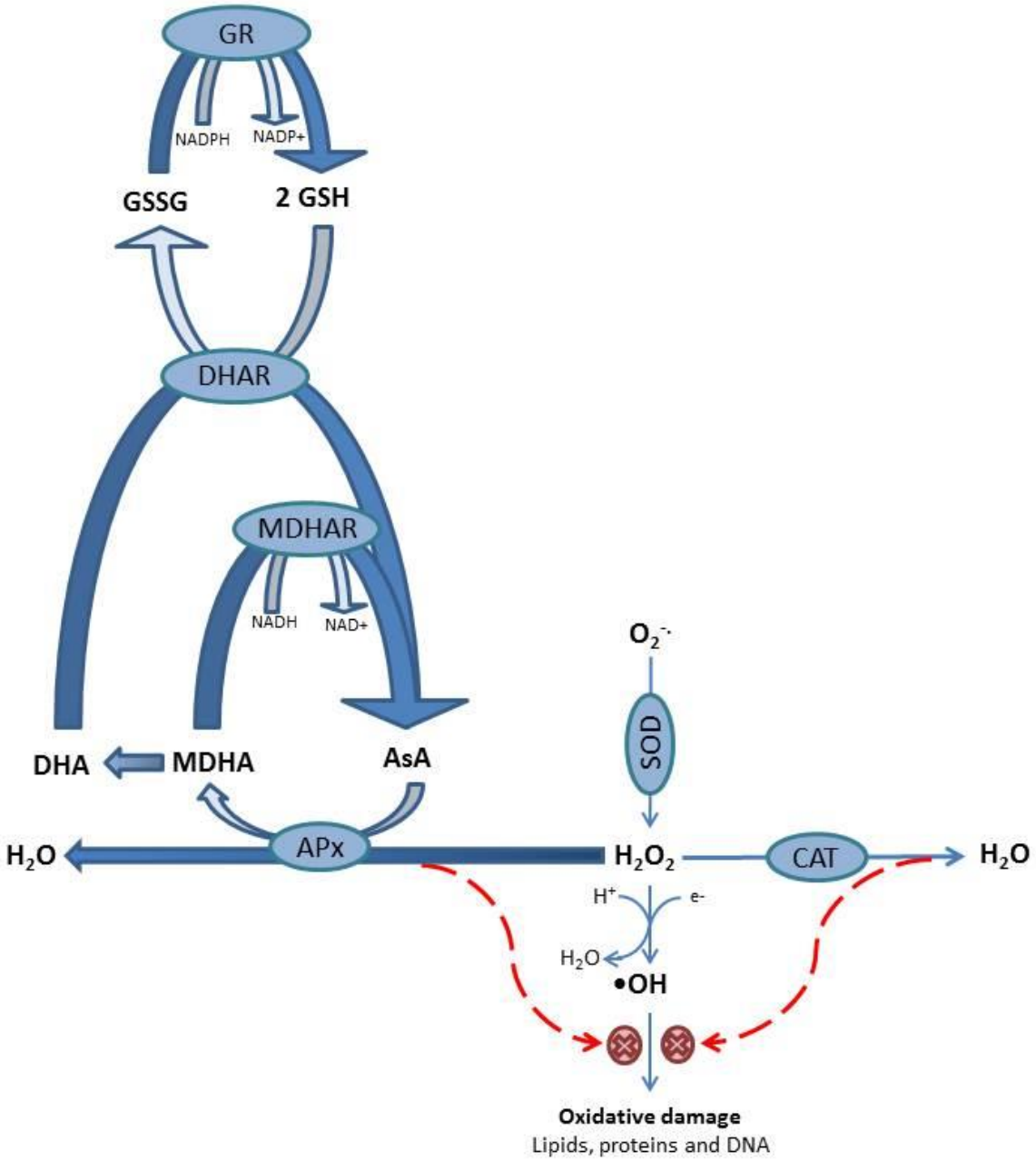

Fig. 1: Enzymatic antioxidant system in plant against the main ROS.
Interaction of enzymes from the ascorbate-glutathione cycle (broad arrows) with SOD and CAT. SOD: superoxide dismutase, CAT: catalase; APx: ascorbate peroxidase; MDHAR: monodehidroascorbato reductase; DHAR: dehydroascorbate reductase, GR: glutathione reductase

In addition to antioxidant enzymes, plant cells possess unique proteins in their mitochondria, specifically NAD(P)H dehydrogenases alternatives (NDs), the alternative oxidase (AOX) and plant uncoupling protein (PUCP) or PUMP, which, aside from participating in oxidative phosphorylation, also contributes to the maintenance of the oxidative balance in cells (Pastore, D. *et al.*, 2000; Rasmusson, A. G. *et al.*, 2009) . We previously described these proteins in *A. angustifolia* embryogenic cultures (Mariano *et al*., 2008; Valente *et al.*, 2012). We also showed that under cold stress (4°C for 24 h and 48 h), the PUMP activity was increased, whereas AOX activity was slightly stimulated in these cells, suggesting that PUMP is involved in the control of ROS levels to overcoming cold stress conditions (Valente, C. *et al.*, 2012).

In this study, we evaluated the effects of cold stress on *A. angustiolia* embryonic stem cells with regards to H_2O_2 levels, the activity of antioxidant enzymes and lipoperoxidation. We also focused on the primary organelle that control ROS levels in these cells - the mitochondria - by evaluating type II NDs and the mitochondrial permeability transition.

4.3. **Results and discussion**

4.3.1. Morphology and viability of *A. angustifolia* embryogenic cultures

We previously showed that cold stress induces cell responses related to oxidative stress, specifically the activation of PUMP (Valente, C. *et al.*, 2012). As this study aimed to explore these responses, cells were submitted to the same conditions of stress, 4 °C ± 1 °C for 24 h and 48 h.

The microscopic analyses of *A. angustifolia* cells using Evans blue and acetocarmine double staining showed two primary cell types (Fig. 2) as previously described (Silveira, V. *et al.*, 2006; Valente, C. *et al.*, 2012). The embryonic cells are reactive to acetocarmine, which stains the cells red by reacting with glycoproteins, chromatin and DNA. Embryogenic cells have a large nuclei and a dense cytoplasm. Suspensor cells are elongated, vacuolated and permeable to Evans blue dye (Fig. 2a-2 and b). The acetocarmine is difficult to locate in these large vacuolated cells because of the Evans blue staining (Gupta, P. e Holmstrom, D., 2005). Embryogenic and suspensor cells are organized in aggregates known as the proembryogenic mass (PEM) (Silveira, V. *et al.*, 2006). The cold treatment did not promote any significant alterations on cell morphology (Fig. 2c-e)

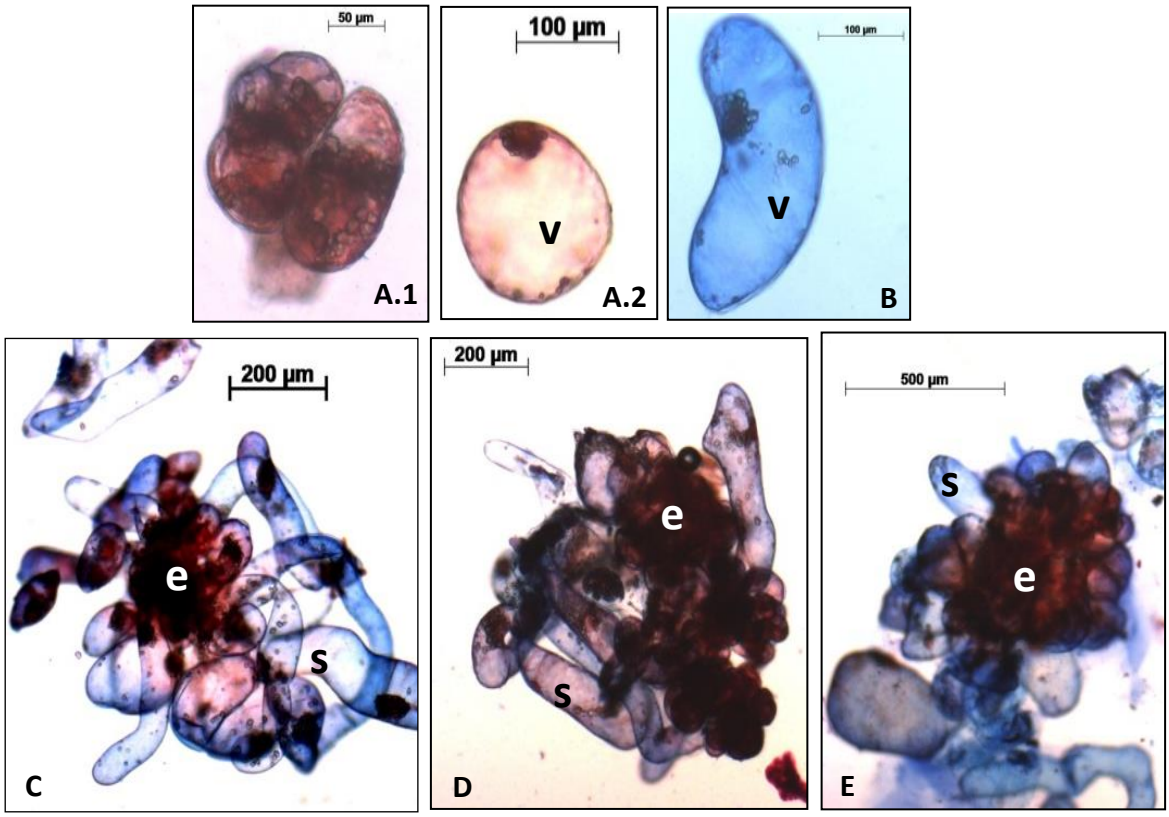

Fig. 2: Effect of cold stress on the morphology of *A. angustifolia* cells in culture.
Microscopic view of Araucaria control cells (15 days of culture) double stained with Evans blue and acetocarmine. Two cells types are identified: a) embryonic cells, which are smaller, grouped in cells aggregates and stained by acetocarmine, and b) suspensor cells, which are elongated, vacuolated and stained by Evans blue.
a.1. Embryonal-like cells following cell division of control; a.2. Vacuole enlargement. b. Cell elongation with cytoplasm reduction. c. PEMs of control cells. d. PEMs of cells subjected to 24 h of cold stress; e. PEMs of cells subjected to 48 h of cold stress. [PEMs = proembryogenic masses; ec = embyonic cells; sc = suspensor cells; v = vacuole]

To assure that the *A. angustifolia* cells were metabolically viable, MTT assays were performed. MTT is reduced to formazan by mitochondrial dehydrogenases only in viable cells with fully active mitochondria (Castro-Concha, L. *et al.*, 2006). Two controls were used in these experiments (Fig. 3): cells maintained in ideal culture conditions (25 ± 1 °C in the dark, 100% viability) and cells disrupted by 3 cycles of boiling-freezing (cell death control, ~30% viability). Cold stress for 24 h and 48 h had no discernable effect on cell viability (Fig. 3).
The absence of effects of cold stress on the morphology and viability of *A. angustifolia* embryogenic cultures indicates that this condition did not promote significant cell damage in accordance with our previous report (Valente, C. *et al.*, 2012). In this study, these assays were repeated to ensure that cells did not change their morphology pattern during their time in culture (Filonova, L. H. *et al.*, 2000; Silveira, V. *et al.*, 2006).

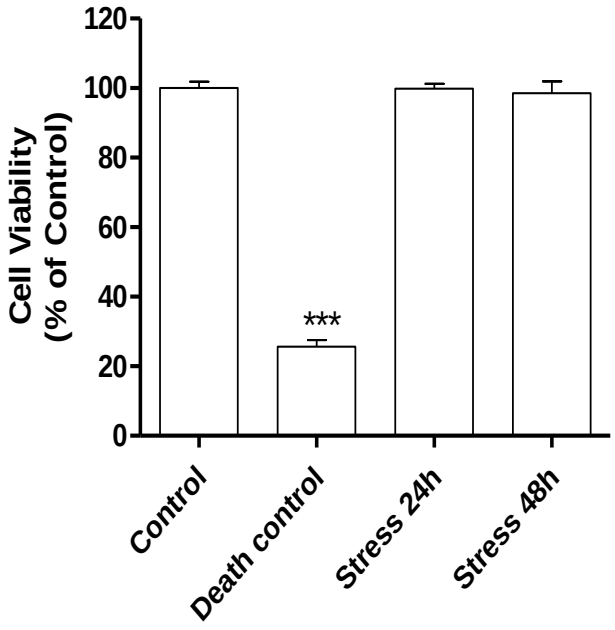

Fig. 3: The effect of cold stress on viability of *A. angustifolia* embryogenic cultures. Cells (15 days of culture - 330 mg fresh weight) were incubated with PBS in the presence of MTT at a final concentration of 1.25 mM for 8 h in the dark at 25°C. The formazan salts were extracted twice with 750 µL of 1% SDS (w/v) in 50% methanol by incubating for 30 min at 60°C. After centrifugation (9000 × g for 5 min), the absorbance of the supernatant was measured at 570 nm. Death control, cells subjected to freezing and boiling cycles; Control, cells incubated at 25 ± 1°C; Stress 24 h and 48 h, cells incubated at 4 ± 1°C. The data are represented as the mean ± S.D. from three independent experiments. The values are expressed as a percentage of the control. A value of 100% corresponds to 0.289 of absorbance. *** $P <$ 0.0001 vs. control.

4.3.2. H_2O_2 levels and lipid peroxidation

It is well known that abiotic factors such as variations in temperature, salinity and UV light exposure can increase the ROS levels in plant cells (Steiner, N. V., F.N.; Maldonado, S., Guerra, M.P. , 2005; Lin, J. *et al.*, 2006a; Shohael, A. M. *et al.*, 2006; Silveira, V. *et al.*, 2006; Sun, J. *et al.*, 2010; Valente, C. *et al.*, 2012; Vieira, L. D. N. *et al.*, 2012), which causes damage to biomolecules such as DNA, proteins and lipids.

Although increased levels of ROS are related to the induction of cell death, studies have shown that these species are involved in important signaling pathways in physiological processes (Mittler, R., 2002; Mittler, R. *et al.*, 2004; Moller, I. M. *et al.*, 2007; Theocharis, A. *et al.*, 2012). In this context, Correa-Aragunde, N. *et al.* (2006) demonstrated that nitric oxide

(NO) is important gene modulator during lateral root formation. Small increases in NO levels were shown to be important for the differentiation and division of *Araucaria angustifolia* embryogenic cultures (Vieira, L. D. N. *et al.*, 2012). The authors suggest that changes in the cellular redox state could be a strategy for more efficient development of these cultures.

The compartmentalized increase of ROS in mitochondria, chloroplasts and peroxisomes in response to biotic and abiotic stress occurs primarily during respiration and photorespiration (Ahmad, P. *et al.*, 2008). Hydrogen peroxide, the relatively stable product of superoxide dismutase reaction, is a potent generator of hydroxyl radicals by the Fenton reaction, which are more reactive and harmful to cells (Halliwell, B. e Gutteridge, J. M. C., 2000). In *A. angustifolia* cultures submitted to cold stress (4 ± 1º C) for 24 h and 48 h, the H_2O_2 levels were increased by 35% compared to the control cells (25 ± 1º C) (Fig. 4). To evaluate the possible damage caused by the increase of H_2O_2 levels, we evaluated the lipid peroxidation by the TBARS method. ROS, particularly the hydroxyl radicals produced from the Fenton reaction, may react with polyunsaturated fatty acids to form malondialdehyde (MDA), which in many situations is the most abundant aldehydic lipid breakdown product and is used to monitor the cellular response to abiotic stress, thus reflecting lipid injury (Halliwell, B. e Gutteridge, J. M. C., 2000; Moller, I. M. *et al.*, 2007). The results of these assays are presented in Fig. 5, which shows an increase of lipid peroxidation by 15% and 30% after 24 h and 48 h of cold stress, respectively. This result is in agreement with the increase of H_2O_2 in the same conditions (Fig. 4). Shohael, A. M. *et al.* (2006) described an increase in lipid peroxidation and H_2O_2 levels in the somatic embryos of *Eleutherococcus senticosus* grown under different light treatments in comparison to embryos cultured in the dark (control). Similar results were reported in 3-week-old *Cicer arietinum L.* (kaka genotype) subjected to cold stress (-10°C for 10 min) (Nazari, M. *et al.*, 2012) and in different genotypes of chickpea (*Cicer Arietinum L.*) under drought stress during different stages of development (Patel, P. K. e Hemantaranjan, A., 2012). Our results are similar to these reports, and their relevance is reinforced considering that little is known about oxidative stress parameters under cold conditions in gymnosperms.

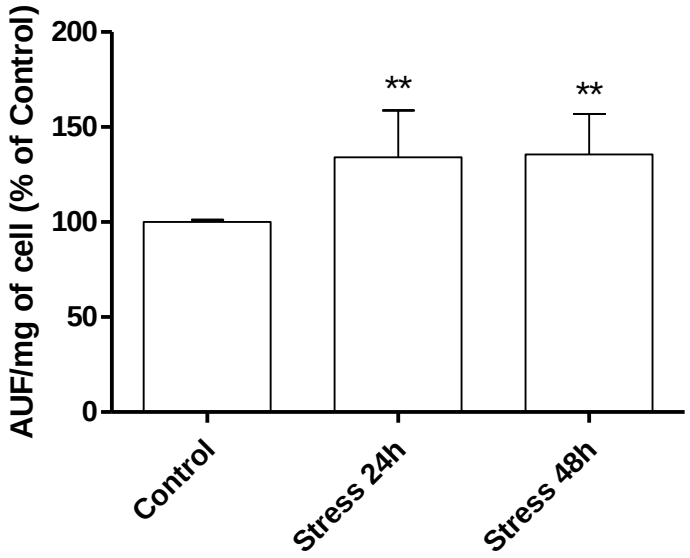

Fig. 4: Effect of cold stress on H_2O_2 levels in *A. angustifolia* embryogenic cultures.
Cells (15 days of culture - 100 mg) were homogenized in 1 ml of sodium phosphate buffer, pH 6.5, and the extract was centrifuged. The H_2O_2 concentration was determined fluorimetrically at excitation and emission wavelengths of 571 and 585 nm, respectively. The data are represented as the mean ± S.D. from three independent experiments and were calculated using a calibration curve for peroxide. The values are expressed as the percentage of the control. A value of 100% corresponds to 0,3284 µM H_2O_2/mg per cell. * $P < 0.05$ vs. control.

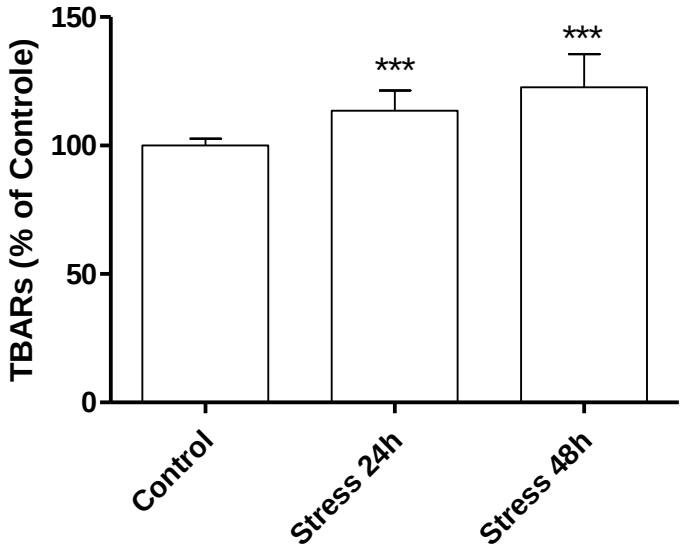

Fig. 5: Lipid peroxidation in *A. angustifolia* embryogenic cultures.
Cells (15 days of culture – 200 mg) were homogenized in 4 ml of 0.1% TCA. The extract was centrifuged, mixed with 20% TCA and 0.5% TBA and incubated at 95°C for 30 min. The concentration of TBARS was measured at 532 nm using $\mathcal{E} = 1.54 \times 10^5\ M^{-1}\ cm^{-1}$. The data are represented as the mean ± S.D. from three independent experiments. The values are expressed as a percentage of the control. A value of 100% corresponds to 1.95 $nmol \cdot ml^{-1} \cdot g^{-1}$ of TBARS. * $P < 0.05$ vs. control.

4.3.3. Effects of cold stress on antioxidant enzymes activities

Although cold stress increased H_2O_2 levels and lipid peroxidation (Fig. 4 and 5), this condition was not sufficient to either reduce cell viability or promote morphological changes in *A. angustifolia* cells (Fig. 3 and 2). These results suggest that the mechanisms of ROS detoxification may be active in these cells to prevent major damage. In general, plant cells employ two mechanisms to maintain nontoxic levels of ROS: (1) enzymatic, which includes SOD, APx, GPX, GR, CAT, MDHAR and DHAR, and (2) non-enzymatic, which include ascorbate, glutathione (GSH), tocopherols, flavonoids, alkaloids and carotenoids (Apel, K. e Hirt, H., 2004; Ahmad, P. *et al.*, 2008). In this study, we sought to evaluate the enzymatic mechanism, which may function synergistically (Mittler, Ron *et al.*, 2004; Guo, F.-X. *et al.*, 2006; Liu, Y. *et al.*, 2011).

Figure 6 shows that cold stress did not change the SOD activity but promoted a decrease of catalase activity by ~22% after 48 h of exposure to low temperatures. This inhibition could explain the increase in H_2O_2 levels and lipid peroxidation observed at 48 h (Fig. 4 and Fig. 5); however, the APx and DHAR activities were increased by ~100% and ~64%, respectively. For the cells exposed to cold stress for 24 h, only MDHAR showed any increase in activity (~172%), and the GR activity remained unchanged under both stress conditions (data not shown). The interpretation of these results is complicated due to the synergy between the antioxidant defenses and the redox state of the cells as a consequence of stress conditions (Guo, F.-X. *et al.*, 2006; Shohael, A. M. *et al.*, 2006; Xu, C. *et al.*, 2008). It is suggested that the increase of ROS levels, particularly H_2O_2, are significantly higher after 48 h compared to 24 h; however, this increase is countered by the higher activity of antioxidant enzymes (APx and DHR) in 48 h of cold stress. Additionally, plant cells have other enzymes, e.g., glutathione peroxidases, that could function with the enzymes from glutathione-ascorbate cycle in the removal of H_2O_2 (Moller, I. M. *et al.* (2007).

Moreover, the lipid peroxidation was more pronounced after 48 h of cold stress (Fig. 5), which does not exclude the increase of other ROS such as $O_2^{\cdot-}$, •OH and $^1O_2$ in this condition (Gill, S. S. e Tuteja, N., 2010).

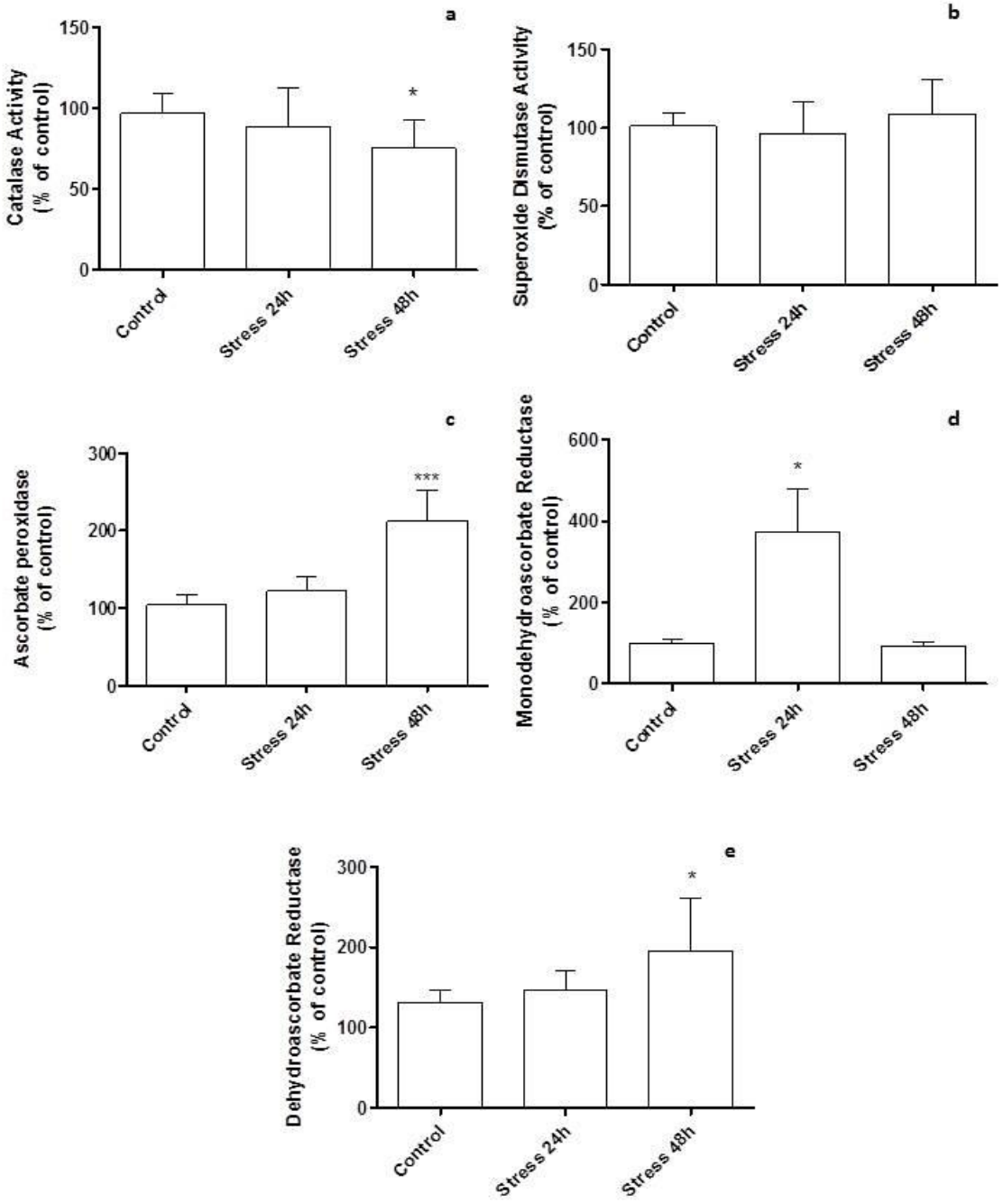

Fig. 6: Effect of cold on antioxidant enzymes from *A. angustifolia* embryogenic cells. The enzymatic assays were performed as described in the Methods section. (a) Catalase: the data are represented as the mean ± S.D. from four independent experiments and expressed as a percentage of the control. A value of 100% corresponds to 2.23 ± 0.55 U of catalase. (b) SOD: the data are represented as the mean ± S.D. from five independent experiments and expressed as a percentage of the control. A value of 100% corresponds to 49.44 ± 67.13 U of SOD. One unit of SOD was calculated as the amount of enzyme required to inhibit 50% of epinephrine oxidation under the experimental conditions. (c) APx: the data are represented as the mean ± S.D. from four independent experiments and expressed as a percentage of the control. A value of 100% corresponds to 0.308 µmol of ascorbate oxidized·min^{-1}·mg^{-1} of protein. (d) MDHAR: the data are represented as the mean ± S.D. from four independent experiments and expressed as a percentage of the control. A value of 100% corresponds to 29.91 nmol of NADH oxidized·min^{-1}·mg^{-1} of protein. (e) DHAR: the data are represented as the mean ± S.D. from four independent experiments and expressed as a percentage of the control. A value of 100% corresponds to 37.34 nmol of ascorbate·min^{-1}·mg^{-1} of protein. * $P <$ 0.05 vs. control.

The synergism between antioxidant defenses was also shown by Guo, F.-X. *et al.* (2006) in suspension cultures of *C. bungeana* cells incubated for 15 days at -8°C. In comparison to the control cells (grown at 25°C), stressed cells (grown at -8°C) did not show a decrease of viability despite of the increase of MDA levels, which peaked on the first day and gradually decreased. The authors also observed increased peroxidase (POD) activity during the first days of stress that was compensatory to the reduction of CAT activity during the same period. APx, DHAR and GR, which are key enzymes in the glutathione-ascorbate cycle (Fig. 1), were strongly activated during the 15 days of stress. Thus, the authors suggested that this synergism between the antioxidant enzymes in a low state of autoxidation contribute to the protection of the cell membrane. Similar to our results, (Liu, Y. *et al.*, 2011) showed an increase of H_2O_2 levels and lipid peroxidation in *Chorispora bungeana* cells subjected to cold stress at either 0 °C or 4 °C. In these cells, the CAT activity was decreased compared to the control cells incubated at 25 °C. Additionally, the decrease in CAT activity was accompanied by an increase of ascorbate peroxidase (APx) and glutathione reductase (GR) activities as well as the production of ascorbate (AsA), which indicates that glutathione cycle was stimulated to compensate for the decrease of CAT activity. Our results show that the increase in MDHAR and DHAR activities after 24 h and 48 h of stress also may indicate the upregulation of the ascorbate-glutathione cycle to overcome the stress conditions. Additionally, the different profiles between the time of stress induction suggests an acclimation to this condition, which may involve non-enzymatic antioxidants such as phenolics and flavonoids (Shohael, A. M. *et al.*, 2006) and the accumulation of cryoprotectants as carbohydrates and amino acids (glycine and proline) (Theocharis, A. *et al.*, 2012). This modulation in response to abiotic stress was observed by Patade, V. *et al.* (2012) in sugar cane cultures exposed to salt stress for 15 days. The authors showed an increase of TBARS, proline and glycine levels. Also, Shohael, A. M. *et al.* (2006) demonstrated that *Leutherococcus senticosus* somatic embryos cultured under fluorescent light had increased levels of total phenolic and flavonoid constituents along with increased H_2O_2 levels and lipoperoxidation.

4.3.4. Effect of cold stress on mitochondrial permeability transition

In addition to antioxidant enzymes, plant cells possess unique proteins in their mitochondria, namely NAD(P)H dehydrogenases alternatives (NDs), the alternative oxidase (AOX) and plant uncoupling protein (PUCP) also called PUMP. Aside from participating in oxidative phosphorylation, these proteins also contribute to maintaining oxidative balance in

the cells (Pastore, D. *et al.*, 2007; Rasmusson, A. G. *et al.*, 2009). We previously described these proteins in *A. angustifolia* embryogenic cultures (Mariano, A. B. *et al.*, 2008; Valente, C. *et al.*, 2012). We also showed that under cold stress (4°C for 24 h and 48 h), the PUMP activity increased whereas the AOX activity was slightly stimulated in these cells, suggesting that PUMP is involved in the control of ROS levels to overcome the stress condition (Valente, C. *et al.*, 2012).

Here, we evaluated the effects of cold stress on NAD(P)H dehydrogenases alternatives (type II), which are rotenone-insensitive and catalyze the transfer of electrons directly to ubiquinone (UQ) without proton pumping (Moller, I. M., 2001; Logan, D. C., 2007). Logan, D. C. (2007); Rasmusson, A. G. *et al.* (2008) proposed that plants contain 3 families of type II NDs that are located internally and externally in the inner mitochondrial membrane: NDA, with two members; NDB, with four members and NDC, with one member. All NDB genes encode external NDs; NDB1 and NDB2 are regulated by Ca^{+} and oxidize NADPH and NADH, respectively, unlike NDB3, NDB4, which are not regulated by Ca^{+} and oxidize NADH. However, the NDC and NDA families are internal NDs. NDAs oxidize only NADH, and NDCs oxidize both NADH and NADPH and are the only internal NDs regulated by Ca^{+}.

Cold stress promoted a decrease in the activity of external alternative NAD(P)H dehydrogenases but did not affect internal alternative NADH dehydrogenases (Fig. 7). In line with our results, Svensson, Å. S. *et al.* (2002) demonstrated that the transcription of nda1 and ndb1, which corresponds to internal NADH and external NADPH, respectively, are negatively modulated by cold stress treatment (5°C during 5 days) in potato leaves, with this effect being more prevalent with nda1. Compared to the control leaves grown at 25°C, the authors showed a decrease in the activity of internal NADH and external NAD(P)H under cold treatment. The decrease in the external alternative NAD(P)H dehydrogenase activities observed in our study (Fig. 7) may be related to the increased levels of H_2O_2 under the same conditions (Fig. 3). In this sense, Sweetlove, L. J. *et al.* (2002), using mitochondria from *Arabidopsis* cell cultures submitted to treatment with H_2O_2 for 16 h, showed a decrease in the respiratory ratio when NADH, malate or pyruvate were used as the substrate; however, when cytochrome c was used as the electron donor, the ratio of the respiratory chain was not affected compared to the control cells (absence of H_2O_2). The authors suggested that H_2O_2 stress promoted the inhibition of external and internal NADH dehydrogenases.

Although our results suggest that cold stress promotes a decrease in the activity of external NAD(P)H dehydrogenases alternatives, the activation of PUMP in these cells

(Valente, C. *et al.*, 2012) may be sufficient to maintain the sublethal levels of ROS and overcome this stress condition.

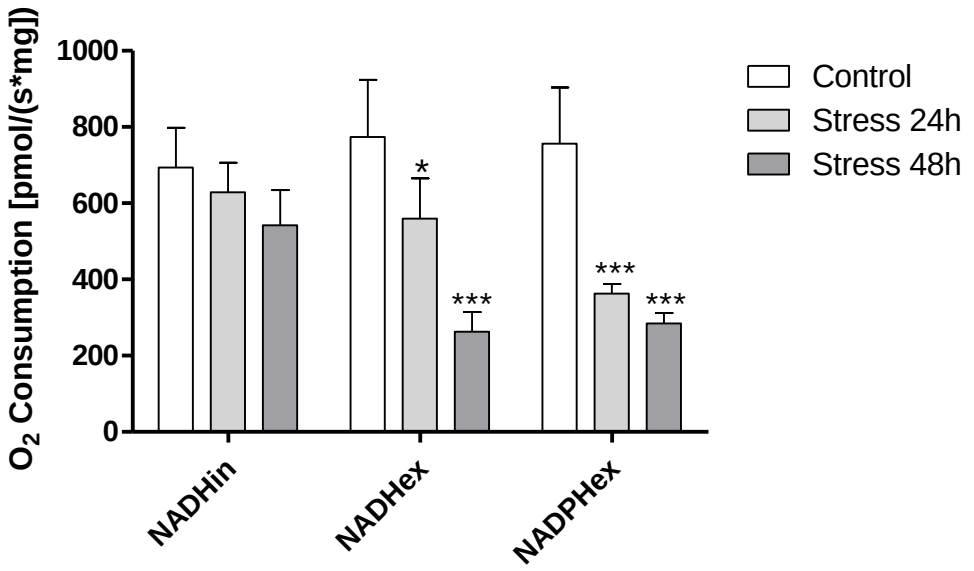

Fig.7: Effect of cold stress on alternative NAD(P)H dehydrogenases.
The activities of the type II NAD(P)H dehydrogenases were determined by monitoring the oxygen consumption as described in the Methods section. The target substrates used were 10 mM sodium glutamate plus 10 mM sodium malate in the internal NADH dehydrogenase assays and 2 mM of either NADH or NADPH for the external NADH and NADPH dehydrogenase assays. The data are represented as the mean ± S.D. from six independent experiments. * $P < 0.05$ vs. control.

4.3.5. Effect of cold stress on mitochondrial permeability transition

The primary stores of calcium in plant cells are the vacuole, endoplasmic reticulum and apoplast, with the vacuole the central storage compartment where calcium can reach a concentration of 80 mM (Stael, S. *et al.*, 2012). The modulation of the intracellular calcium concentration is important for activation of signal transduction events, which may culminate in different responses during oxidative stress, including programed cell death (PCD) (Mittler, R., 2002; Reape, T. J. e Mccabe, P. F., 2010). The increase in the cytosolic calcium concentration and ROS are associated to abiotic stress conditions (Reape, T. J. e Mccabe, P. F., 2010; Theocharis, A. *et al.*, 2012) and are able to induce PCD via the mitochondrial permeability transition (MPT). As demonstrated in *Nicotiana tabacum* protoplasts, the redox state is crucial for opening the PTPM (Lin, J. *et al.*, 2006b; Vacca, R. A. *et al.*, 2006).

Upon MPT, the permeability of inner mitochondrial membrane is increased due to the formation of a non-specific pore called the permeability transition pore (PTP). PTP formation/opening allows the passage of molecules 1.5 kDa in size (Zoratti, M. e Szabo, I., 1995), thus promoting organelle swelling and the rupture of the inner and outer membranes, which results in the release of MCP-inducing factors into the cytosol (Arpagaus, S. *et al.*, 2002; Reape, T. J. e Mccabe, P. F., 2010). MPT in plants occurs in a similar manner as described in animal mitochondria, as organelle swelling is Ca^{2+} dependent and inhibited by cyclosporin A (CsA) and dithioerythritol (DTT) (Arpagaus, S. *et al.* (2002); (Saviani, E. E. *et al.*, 2002; Tiwari, B. S. *et al.*, 2002; Virolainen, E. *et al.*, 2002).

In this study, we showed mitochondrial swelling induced by the formation/opening of PTP in *A. angustifolia* cells (Fig. 8). MPT occurred in the presence of high concentrations of Ca^{2+} (25 mM-50 mM) and was dependent on phosphate. We previously reported that the mitochondrial calcium uptake in these cells occurs by a phosphate-dependent uniporter (Mariano et al., 2008). The PTP formation/opening was characterized by the addition of EGTA (which impedes mitochondrial swelling by chelating Ca^{2+}), dithioerythritol (DTT) (which reduces disulfide bridges) and cyclosporine (CsA) (which inhibits cyclophilin D). The inhibition of PTP-induced swelling by CsA and DTT is a feature typical of plant mitochondria (Arpagaus, S. *et al.* (2002).

Our results show that the cold stress (24 h and 48 h) promoted a slight inhibition of PTP-induced swelling, suggesting that this abiotic stress would not able to induce PCD through this pathway in these cells. This result is in accordance with the viability and morphology data of the cells, which were not affected by cold stress (Fig. 2 and Fig. 1).

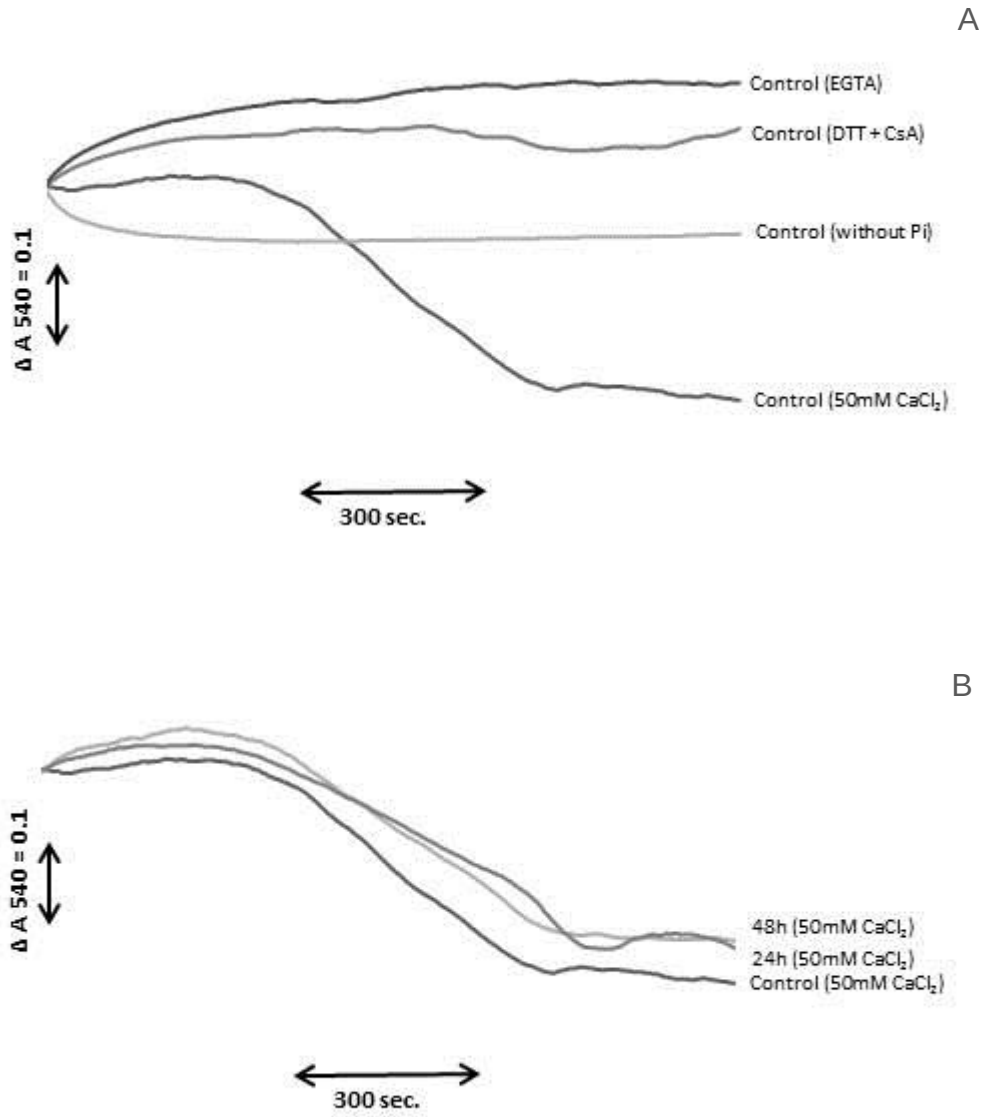

Fig. 8: Effect of cold stress on mitochondrial permeability transition.
(A) Characterization of PTP in mitochondria from control cells (25 ± 1 ºC). (B) Permeability transition in mitochondria from stressed and control cells. The reaction medium was comprised of 0.250 mg/ml mitochondrial protein, 200 mM sucrose, 10 mM MOPS, 5 mM succinic acid, 10 µm EGTA, 2 µM rotenone, 1 µg/ml oligomycin, 1 mM H_3PO_4 and 1,6 µM CsA, 1 mM DTT and 50 mM of $CaCl_2$ where indicated. The mitochondrial swelling was monitored as described in the Methods section. The traces are representative of four 4 independent experiments.

4.4. **Conclusions**

Cold stress was able to induce oxidative stress in *A. angustifolia* embryonic cultures, which resulted in upregulation of the enzymatic defense mechanism, specifically the glutathione-ascorbate cycle, to compensate for the inhibition of catalase and external NAD(P)H dehydrogenases. The Ca^{2+} - and phosphate-dependent MPT demonstrated in these cells for the first time was not induced by cold stress. These results contribute to the understanding of the pathways involved in overcoming stress in this gymnosperm and are important for the development of conservation methods of this species such as *in vitro* micropropagation.

5. Methods

5.1. **Chemicals**

Thiamine, glycine, casein hydrolyzate, 2,4-dichlorophenoxyacetic acid, benzylaminopurine, kinetin, myo-inositol, l-glutamine, phytagel, cysteine, NADH, NADPH, ADP, GSSG, GSG, Ascorbate oxidase, DHA, EGTA, BSA, HEPES, glutamate, pyridoxine, nicotinic acid, Pi, $MgCl_2$, linoleic acid, oligomycin, Rotenone, FCCP and MTT were purchased from Sigma Chemical Co. (St. Louis, USA). The *Kit Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit* was purchased from invitrogen®. All other reagents were commercial products of the highest available purity.

5.2. **Culture conditions**

A. angustifolia embryogenic cells were grown on BM culture (Gupta, P. K. e Pullman, G. S., 1991; Santos, A. L. W. *et al.*, 2002) supplemented with 2 mg/L glycine, 0.5 mg/L pyridoxine HCl, 0.5 mg/L nicotinic acid, 1 mg/L thiamine HCl, 500 mg/L casein hydrolysate, 100 mg/L myo-inositol, 1g/L l-glutamine, 30 g/L sucrose, 2 g/L phytagel, 2 µM 2,4 dichlorophenoxyacetic acid, 0.5 µM benzylaminopurine and 0.5 µM kinetin. The pH of the culture medium was adjusted to 5.8 with KOH prior to autoclaving at 121 ºC (1 atm) for 30 min. Vitamin, casein hydrolyzate and l-glutamine solutions were filter-sterilized and added to the medium after autoclaving. The cells were maintained in this semi-solid medium at 25 ± 1 ºC in the dark and subcultured every 20 days. For all assay the cells were collected between 13-15 days of culture.

5.3. **Stress Conditions**

The cold stress was induced by the cells exposition to 4 ± 1°C for 24h and 48h in the dark. The control cells were cultured in an incubator at 25 ± 1°C in the dark.

5.4. **Morphology of *A. angustifolia* cells**

The evaluation of the cell culture was performed macroscopically and by light microscopy using a double stain with acetocarmine and Evans's blue (Gupta, P. e Holmstrom, D., 2005; Valente, C. *et al.*, 2012). The cells were incubated with two drops both acetocarmin 2% (w/v) and Evans Blue 0.5% (w/v) for 3 min. After that, the excess dye was removed and the cellular mass was washed with PBS and analyzed using an Axiovert 40 CFL microscope (Carl Zeiss).

5.5. **Cell viability**

The cell viability was evaluated by MTT method (1-[4,5-Dimethlthiazol-2-yl]-3,5-diphenylformazan). This assay is based on the activity of the mitochondrial dehydrogenases from viable cells, which are able to reduce the tetrazolium to insoluble formazan. For the assay, the cells (330 mg fresh weight) were incubated during 8h in the dark at 25 ± 1°C in PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.2) and MTT 1.25mM. Then, the formazan salts were solubilized in 1.5 mL of 1% SDS (w/v) in 50% methanol (v/v) by incubation for 30 min at 60 ºC with shaking. This solution was centrifuged (9000 x g for 5 min) and the absorbance of the supernatant was measured at 570 nm (Castro-Concha, L. *et al.*, 2006).

5.6. **Hydrogen peroxide measurement**

Quantitative measurement of H_2O_2 was performed using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes®) following the manufacturer's instructions. Briefly, samples were prepared from 100 mg of cells that were grounded in liquid nitrogen and immediately mixed with 1 mL of phosphate buffer 20 mM (pH 6.5). This solution was centrifuged (10000 × g for 10 minutes) at 4 °C (Wu, A. *et al.*, 2012), and 50 µL of the supernatant was incubated with 50 µl of the *Amplex® Red* reaction buffer containing 100 µM *Amplex® Red* and 0.2 U/mL of peroxidase. The fluorescence was monitored for 30 minutes at 25 °C using 571 nm and 585 nm as the excitation and emission wavelengths, respectively.

5.7. **Determination of lipid peroxidation**

Lipid peroxidation in *A. angustifolia* cells was measured using malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, with thiobarbituric acid (TBA) (Hodges, D. M. *et al.*, 1999). Cells (1 g) were ground in liquid nitrogen and mixed immediately with 1 mL of 0.5% TCA. The mixture was then centrifuged at 19000 × g for 20 min at 4 ºC, and 0.5 mL of the supernatant was mixed with 2.5 mL of 20% acid trichloroacetic (TCA) and 0.5% thiobarbituric acid (TBA). The solutions were heated at 95 °C for 30 min, cooled in ice bath and centrifuged at 10000 × g for 10 min. The absorbance of the supernatant was measured at 532 nm and 600 nm (A_{600} represents the nonspecific absorbance that was subtracted from the A_{532} values corresponding to the absorbance of the TBA/MDA adduct). Lipid peroxidation was determined from these values using $\varepsilon_{532} = 155\ mM^{-1}\ cm^{-1}$ for the TBA/MDA adduct (Halliwell, B. e Gutteridge, J. M. C., 2000).

5.8. **Antioxidants enzymes assays**

The embryogenic cultures embryonic stem cells from *A. angustifolia* were stored in cryovials and maintained at -80 °C until analysis (Lynch, P. T. *et al.*, 2011). For the assays, cells (0.5 g fresh weight) were grounded with liquid nitrogen and mixed with 1 ml of specific extraction buffer for each enzyme. This solution was filtered through cheesecloth and then centrifuged at 22000 × g for 20 minutes at 4°C. When necessary, the supernatant was re-centrifuged to obtain a clear solution (Shohael, A. M. *et al.*, 2006). This solution was used as the enzyme source. The protein concentration in the supernatant was estimated by the Bradford method (Bradford, M. M., 1976).

The extraction buffer used to measure the activities of ascorbate peroxidase (APx), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) consisted of 100 mM sodium phosphate buffer (pH 7.0), 5 mM ascorbate, 10% glycerol and 1 mM EDTA (Shohael, A. M. *et al.*, 2006). The assays were performed in a UV-microplate well at 25°C in a final volume of 0.2 mL. The fluorescence was determined in Tecan Infinite® 200 Fluorometer.

APx (EC 1.11.1.11) activity was determined in a reaction medium containing 50 mM sodium phosphate buffer (pH 7.0), 0.25 mM L-ascorbic acid (AsA) and 5 mM H_2O_2. The decrease of absorbance at 290 nm was monitored for 5 minutes, and the activity was calculated using the extinction coefficient of ascorbate ($\varepsilon = 2.8\ mM^{-1}cm^{-1}$) and expressed as a

percentage of the control (100%). DHAR (EC 1.8.5.1) activity was measured by the absorbance increase at 290 nm, which corresponds to the reduction of dehydroascorbate, in a reaction medium containing 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH and 0.2 mM DHA. The reaction was monitored for 5 min, and the DHAR activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as a percentage of the control (100%). MDHAR (EC 1.6.5.4) activity was determined in a reaction medium containing 50 mM HEPES buffer (pH 7.6), 2.5 mM AsA, 0.25 mM NADH and 0.4 U/well ascorbate oxidase. The reaction was monitored at 340 nm for 5 min by the decrease of absorbance corresponding to NADH oxidation and calculated using the extinction coefficient of NADH (6.22 $mM^{-1}cm^{-1}$) (Murshed, R. *et al.*, 2008). The results are expressed as a percentage of the control (100%)

Prior to measurement of the glutathione reductase (GR) and catalase (CAT) activities, the enzymes were extracted into a buffer containing 100 mM sodium phosphate buffer (pH 7.0) and 1 mM EDTA (Shohael, A. M. *et al.*, 2006). The GR (EC 1.6.4.2) activity was assayed in a UV-microplate well at 25°C in a final volume of 0.2 mL in a reaction medium containing 50 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 0.25 mM NADPH and 0.5 mM GSSG. The absorbance at 340 nm was monitored for 5 min, and the activity calculated using the extinction coefficient of NADPH (6.22 $mM^{-1}cm^{-1}$(Murshed, R. *et al.*, 2008) and expressed as a percentage of the control (100%). CAT (EC 1.11.1.6) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm using a reaction buffer containing 100 mM sodium phosphate buffer (pH 7), 100 mM EDTA and 10 mM H_2O_2, in a final volume of 3 mL at 20 °C. The extinction coefficient of H_2O_2 (0,394 $mmol^{-1}\cdot L\cdot cm^{-1}$) was used to calculate the CAT activity (Aebi, H., 1984). The results are expressed as a percentage of the control (100%)

Cells were homogenized with extraction medium containing 100 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVPP) and 0.5% (v/v) Triton X-100 to determine superoxide dismutase (SOD, EC 1.15.1.1) activity. The enzyme activity was measured at 30 °C in 3 mL of reaction medium comprised of 50 mM sodium carbonate/bicarbonate buffer (pH 9.8), 0.1 mM EDTA and 0.6 mM epinephrine. The formation of adrenochrome was monitored at 475 nm for 3 min. The SOD activity is expressed in units, in which one unit of SOD corresponds to 50% inhibition of epinephrine oxidation (Verma, S. e Dubey, R. S., 2003).

5.9. **Isolation of *A. angustifolia* embryogenic cells mitochondria**

Mitochondria were isolated from embryogenic cells (13-15 days of culture) by differential centrifugation (Mariano, A. B. *et al.* (2008). Briefly, to 35 g of fresh cells was added 200 mL of isolation medium containing 0.25 M sucrose, 3 mM cysteine, 2 mM EGTA, 0.2% BSA and 10 mM HEPES (pH 7.6). The cells were homogenized using van Potter–Elvehjem homogenizer and disrupted in a Turratec homogenizer by 4s- burts. The homogenate was filtered through a nylon cloth, and the pH was adjusted to7.2. The filtrate was centrifuged for 10 min at 1000 x g. The supernatant was centrifuged for 10 min at 15,000 × g, and each pellet was resuspended in wash medium (0.25 M sucrose, 0.25 mM EGTA, 0.2% BSA and 10 mM Hepes at pH 7.2), transferred to a single tube and centrifuged for 10 min at 1000 × g. The supernatant was centrifuged for 10 min at 15,000 × g, and the pellet was resuspended in wash medium. The resulting mitochondrial suspension (~10 mg protein/mL) was stored. The mitochondrial suspension was purified by ultracentrifugation at 45,000 x g for 45 min with Percoll (Sigma®) gradient 13.5%, 21% e 45% (v/v) (Jackson, C. *et al.*, 1979; Millar, A. H. *et al.*, 2001). After centrifugation the fraction correspondent to mitochondria fraction (between 21% and 45%) was collected centrifuged at 15,000 x g for 15 min for Percoll removing . The pellet containing the purified mitochondrial was resuspended in a small volume of wash medium and stored (10 - 15 mg protein/ml).

5.10. **Protein determination**

Protein concentrations were determined by the method described by (Bradford, M. M., 1976).

5.11. **Oxygen uptake**

The mitochondrial respiration was measured by high-resolution respirometry in Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria) using two chambers at 28°C under gentle agitation following the experimental conditions described by Valente, C. *et al.* (2012). The reaction medium containing 0.25 M sucrose, 10mM Hepes, 2mM KCl and 2 mM Pi supplemented with 5mM $MgCl_2$, 0.1 mM EGTA, 10 mM sodium glutamate/sodium malate, 0.25 mg/ml of mitochondrial protein and 0.2 mM ADP. The respiratory coefficient control (CCR) was used as a determinant of the quality of the preparation (Estabrook, R. W., 1967). Only mitochondrial preparations with respiratory control above 2.5 were used (data not shown).

5.12. **Determination of type II NAD(P)H dehydrogenases activity**

The activities of type II NAD(P)H dehydrogenases were determined by monitoring the oxygen consumption as described in item 1.11 except with the addition of 0.1 mM Ca^{2+}, 1 µM FCCP and 10 µM rotenone to the reaction medium. As oxidizable substrates were used 10 mM sodium glutamate plus 10 mM sodium malate for the internal NADH dehydrogenases assays and 2 mM NADH or NADPH for the external NADH and NADPH dehydrogenases (Estabrook, R. W., 1967; Svensson, Å. S. *et al.*, 2002).

5.13. **Determination of mitochondrial permeability transition**

The mitochondrial permeability transition (MPT) was monitored by analyzing the decrease in absorbance at 540 nm due to mitochondrial swelling as a consequence of the formation/opening of the permeability transition pore (PTP) induced by calcium (Arpagaus, S. *et al.* (2002). The reaction was monitored for 20 min at 25 ºC in a reaction medium containing 200 mM sucrose, 10 mM MOPS, 5 mM succinic acid and 0.25 mg/mL of mitochondrial protein and supplemented with 10 µm EGTA (pH 7.4 adjusted with Tris-base), 2 µM rotenone, 1 µg/mL oligomycin, 1 mM H_3PO_4, 1.6 µM CsA, 1 mM DTT, and 50 mM of $CaCl_2$ when indicated.

5.14. **Statistical analyses**

The data were statistically analyzed by variance analysis (ANOVA) and by Tukey's test for average comparison. Mean values ± S.D. were used; values were considered significant when $P < 0.05$.

6. Acknowledgements

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7. CONCLUSÃO

Os resultados mostram que o estresse causado pelo frio não afetou a viabilidade e morfologia das células embriogênicas de *A. angustifolia*, no entanto, foi capaz de induzir o estresse oxidativo caracterizado pelo aumento dos níveis de H_2O_2 e lipoperoxidação. A resposta destas células frente ao estresse oxidativo foi a ativação do sistema enzimático antioxidante, em especial as enzimas do ciclo glutationa-ascorbato. Esta ativação parece compensar a inibição da catalase e das NAD(P)H desidrogenases externas, uma vez que a viabilidade e morfologia das células não foram alteradas. Apesar do estresse pelo frio não ser capaz de modular significativamente a transição de permeabilidade mitocondrial, esse processo foi demonstrado pela primeira vez em células de *A. angustifolia*, sendo dependente de Ca^{2+} e fosfato. Este estudo contribui para o esclarecimento da resposta desta gimnosperma a condição de estresse por baixa temperatura e, assim, também para o desenvolvimento de métodos de conservação desta espécie, como a micropropagação *in vitro*.

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