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**CRIOPRESERVAÇÃO DE *Araucaria angustifolia* (BERT.) O. KUNTZE:  
ASPECTOS FISIOLÓGICOS E BIOQUÍMICOS**

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## RESUMO

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O longo período para alcançar o estádio reprodutivo, a fenologia irregular e a recalcitrância de sua semente dificultam o estabelecimento de programas de conservação para *Araucaria angustifolia*, arbórea nativa ameaçada de extinção. Técnicas de cultivo *in vitro*, associadas à criopreservação, são ferramentas importantes para conservação *ex situ* de espécies recalcitrantes. O objetivo deste trabalho foi o estudo da criopreservação no sistema *A. angustifolia*. Um protocolo de criopreservação para culturas celulares foi desenvolvido, baseado na otimização das concentrações de DMSO. Apenas o tratamento que utilizou 20% de DMSO apresentou recuperação significativa após cinco semanas após o reaquecimento, sendo o tratamento mais promissor. Observou-se que as células expostas aos crioprotetores apresentaram níveis mais baixos de PAs, ABA e ROS, em comparação às células que não foram expostas aos mesmos. Duas bandas protéicas de baixo peso molecular foram mais intensamente coradas apenas nas células que sofreram exposição aos crioprotetores. O embrião zigótico foi utilizado para o estudo de diferentes aspectos biofísicos do processo de criopreservação como a permeabilidade dos crioprotetores, a capacidade de resposta osmótica e resistência ao congelamento dos tecidos. Embriões foram desidratados por secagem ultrarrápida, meio WPM contendo 2M de sacarose, ou PVS2. As taxas de desidratação foram avaliadas em cinco segmentos do embrião, sendo observada maior resistência nos segmentos intermediários: 2 e 3. Embriões dessecados abaixo de 0,5 g H<sub>2</sub>O.g MS<sup>-1</sup>, por de secagem ultrarrápida (12 h), apresentaram escurecimento e falha para reidratar. Os embriões dessecados a cerca de 0,45 g H<sub>2</sub>O.g MS<sup>-1</sup>, usando 2 M de sacarose (8 h), foram recuperados por reidratação. O conteúdo de água de embriões embebidos em PVS2 (2 h), após pré-tratamento com sacarose 2M, apresentaram conteúdo de água reduzido para 0,35 g H<sub>2</sub>O.g MS<sup>-1</sup>, sem variação entre os segmentos, e também puderam ser recuperados após reidratação. Penetração diferencial de PVS2 foi observada. A análise por microscopia óptica foi realizada em embriões diretamente expostos ao nitrogênio líquido ou submetidos ao tratamento de 2M de sacarose (8 h), seguido de PVS2 (2 h), (seguido ou não de resfriamento) e recuperação em meio contendo 1,2 M sacarose. Observou-se a presença marcante de grãos de amido em todos os tecidos do embrião. Embriões expostos aos diferentes tratamentos apresentaram um maior número de células mortas, regiões de heterocromatina e maior espaçamento intercelular, quando comparados ao controle, não resfriado. O tratamento que envolveu todas as etapas do procedimento de criopreservação foi o que apresentou espaçamentos maiores e mais frequentes. Apesar dos danos acentuados nos embriões resfriados, variação ao longo dos segmentos estudados foi observado. Os resultados deste trabalho permitiram uma melhor compreensão dos diferentes aspectos fisiológicos, bioquímicos e biofísicos durante a criopreservação, em espécies recalcitrantes, auxiliarão o estabelecimento de bancos de germoplasma *ex situ* para *A. angustifolia*.

**Palavras-chave:** *Araucaria angustifolia*. Criopreservação. Semente recalcitrante. Cultura celular. Poliaminas. Ácido abscísico. Espécies reativas de oxigênio. Proteínas.

## ABSTRACT

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The *ex situ* conservation of *Araucaria angustifolia*, Brazilian endangered pine, is limited mainly because the recalcitrance of the seed. *In vitro* techniques associated with cryopreservation are important tools for the conservation of species with recalcitrant seeds. The aim of this work was the study of cryopreservation *A. angustifolia* plant systems. A protocol for cryopreservation of *A. angustifolia* cell cultures was developed based on optimum DMSO concentration. Only the treatment with 20% of DMSO could be recovered with significant amount of cells after five weeks of thawing. Then that was the more promising treatment for cryopreservation of cell cultures of *A. angustifolia* used. It was observed that cells exposed to cryoprotectants showed lower levels of PAs, ABA and ROS compared to cells that were not exposed to them. Two low molecular weight bands could be observed more intensely stained only in the cells that were exposed to the cryoprotectant. The zygotic embryos were used as a model to study different aspects of the biophysical process of cryopreservation such as the permeability of cryoprotectants, osmotic and response characteristics of water freezing in tissue. Embryos were dehydrated flash-drying, WPM media containing 2M sucrose or PVS2. Dehydration rates were evaluated in five segments of the embryo, and a higher resistance to water loss occurred in the intermediate segments: 2 and 3. Embryos desiccated below  $0.5 \text{ g H}_2\text{O.g}^{-1}$  MS (12 h), by flash-drying showed darkening and fail to rehydrate. Embryos desiccated to about  $0.45 \text{ g H}_2\text{O.g MS}^{-1}$  using 2 M sucrose (8 h) were recovered by rehydration. The water content of the embryos embedded in PVS2 (2 hours) after pretreatment with 2M sucrose showed reduction to  $0.35 \text{ g H}_2\text{O.g MS}^{-1}$ , and also could be recovered after rehydration. However, a higher penetration PVS2 the radicle end region (part 1) can be observed. Analysis of the organization and aspects of cellular structures by optical microscopy was performed. Embryos directly exposed to liquid nitrogen or submitted to the treatment of 2M sucrose (8 h), followed PVS2 (2 h) (or not followed by cooling) and recovery in media containing 1.2 M sucrose. We observed a marked presence of starch grains in all tissues of the embryo. The embryos exposed to different treatments showed an increased in number of dead cells, heterochromatic regions in the nucleus and showing greatest intercellular spacing when compared to control not cooled. Among the treatments, the one involving all stages of the cryopreservation showed the intercellular spacings more frequently and visibly. It seems that damages vary along the segments studied. The results of this work led to a better understanding of different physiological, biochemical and biophysical factors during cryopreservation in recalcitrant species and open perspective to the establishment of the *ex situ* conservation of *A. angustifolia*.

**Keywords:** *Araucaria angustifolia*. Cryopreservation. Recalcitrant seed. Cell culture. Polyamines. Abscisic acid. Reactive oxygen species. Protein.

## **INTRODUÇÃO**

Desde a sua descoberta, os recursos florestais envolvendo espécies nativas têm sido amplamente explorados no Brasil de forma indiscriminada, levando a inclusão de várias arbóreas nativas na lista de espécies ameaçadas de extinção (CARVALHO, 1994). A restauração dos ambientes degradados a curto e médio prazo é necessária, não apenas para a recuperação dessas espécies na natureza, mas também para a conservação dos recursos naturais, possibilitando uma exploração sustentável dos mesmos. Nesse sentido, o desenvolvimento de bancos *in situ* e *ex situ* de germoplasma é um pré-requisito para a viabilização dos programas de conservação (FAO, 2009; GROSSNICKLE; SUTTON, 1999).

A formação e manutenção de bancos de germoplasma de variedades locais e parentes silvestres de espécies agrícolas e florestais tem sido uma das temáticas mais relevantes da pesquisa em botânica. Desde o início, diversos programas destinam-se a prevenir a erosão genética e a promover uma melhoria da produtividade agrícola e florestal (IBPGR, 1993). Os estudos nesta área tem foco no desenvolvimento de técnicas para a conservação ao longo prazo da variabilidade genética de espécies vegetais, com a máxima integridade biológica e genética possíveis (BAJAJ, 1995; HÄGGMAN; RUSANEN; JOKIPII, 2008; PANIS; LAMBARDI, 2005; STUSHNOFF; SEUFFERHELD, 1995). A maioria das espécies cultivadas na agricultura é conservada de forma *ex situ*, utilizando estratégias como bancos de sementes, plantio em campo e cultura de tecidos. Na área florestal, a conservação *in situ* das espécies é geralmente integrada à programas de manejo (YEATMAN, 1987), aumentando-se as áreas de reservas florestais gerenciadas, além de áreas de proteção integral (WANG; CHAREST; DOWNIE, 1993).

As sementes são a principal unidade de propagação natural em plantas e, por essa razão, seu armazenamento é a forma mais comum de conservação vegetal *ex situ* (SANTOS, 2001). Dentre os métodos utilizados, a criopreservação é atualmente a única técnica disponível para a manutenção em longo prazo de germoplasma de espécies vegetais que são propagadas vegetativamente ou que apresentam sementes recalcitrantes ou intermediárias (SANTOS, 2000). Entretanto, a capacidade da planta em ser criopreservada depende da sua tolerância à desidratação e à redução da temperatura (STUSHNOFF; SEUFFERHELD, 1995). Durante os procedimentos de criopreservação, as estruturas biológicas são expostas

à condições muito diferentes daquelas encontradas em condições fisiológicas normais. Dessa forma, podem sofrer danos devido à redução da temperatura e/ou congelamento como injúrias nos sistemas lipossomais estruturais ácidos nucleicos (WALTERS et al., 2008). Sob tais condições, diferentes compostos podem ser produzidos pelas células vegetais, sinalizando uma situação de estresse. Dentre estas substâncias, podem-se destacar as poliaminas (PAs), o hormônio ácido abscísico (ABA), espécies reativas de oxigênio (ROS) e proteínas (JARZABEK; PUKACKI; NUC, 2009; RAMON et al., 2002; UCHENDU et al., 2010).

O desenvolvimento de um protocolo de criopreservação requer o conhecimento dos mecanismos de respostas das células quando submetidas às condições de desidratação e resfriamento (JARZABEK; PUKACKI; NUC, 2009; STUSHNOFF; SEUFFERHELD, 1995). Nesse sentido, o entendimento dos eventos bioquímicos e fisiológicos, ao longo do processo de criopreservação permite a identificação de padrões e assim, o desenvolvimento de protocolos menos empíricos e mais eficientes.

## **CONSIDERAÇÕES FINAIS**

A utilização da criopreservação, embora reconhecida como estratégia fundamental para a obtenção e estruturação de bancos de germoplasma *ex situ*, não tem sido amplamente explorada no Brasil e, considerando as espécies arbóreas nativas recalcitrantes, como é o caso de *A. angustifolia*, a pesquisa desenvolvida no presente trabalho é inédito no país. Sendo uma conífera de importância tanto econômica quanto ecológica e, devido à necessidade de aumento de conhecimento a respeito do tema, ainda bastante empírico para o alcance de protocolo de sucesso, optou-se pela investigação de diferentes aspectos fisiológicos, químicos e físicos, a fim de obter conhecimentos básicos para fundamentar e conduzir estudos pontuais a cerca do tema como identificação e avaliação de marcadores biológicos e tolerância à dessecação durante o processo, além de sugestões sobre estratégias de metodologia de criopreservação para a espécie estudada.

No presente trabalho foi possível o estabelecimento de um protocolo de criopreservação para células embriogênicas de *Araucaria angustifolia*, como base para o desenvolvimento de bancos de germoplasma em longo prazo. Ressalta-se que as células, por mostrarem-se capazes de adquirir a capacidade de tolerância à dessecação e resfriamento, mesmo pertencendo a uma espécie com sementes recalcitrantes, abrem a perspectiva para estudos comparativos fisiológicos entre os diferentes sistemas de uma mesma espécie sobre o tema. Além disso, por trazer informações inéditas sobre os padrões de variação dos reguladores vegetais durante a criopreservação em espécies recalcitrantes, pode-se utilizar a espécie em estudo como modelo para estudos da criopreservação em plantas sensíveis à desidratação.

A dificuldade em se obter um protocolo de criopreservação dos embriões zigóticos de *A. angustifolia* levou ao questionamento de fatores físico-químicos que impediriam tal sucesso. Provavelmente, o grande tamanho do embrião e a presença massiva de grãos de amido que retém umidade, são fortes candidatos aos fatores que impediram sua criopreservação, já que para o alcance de redução de água necessária ao resfriamento não danoso ao tecido exigem tratamentos com diferentes condições estressantes por tempo prolongado, afetando a recuperação do material. Nesse sentido, um melhor entendimento físico e fisiológico do procedimento de criopreservação em sistemas sensíveis à dessecação foi obtido. Além disso, devido ao

elevado tamanho do embrião zigótico de *A. angustifolia*, estudos a cerca de como os crioprotetores mais comumente utilizados em plantas atuariam nas diferentes células e tecidos, sobretudo em sistemas sensíveis à dessecação, foram realizados de forma inédita. O uso do sistema como modelo de estudos para a criopreservação também permitiu a visualização dos danos decorrentes de cada etapa e como o conteúdo celular poderia influenciar na penetração e atuação dos crioprotetores, assunto que ainda é alvo de discussão neste tópico.

A fim de se investigar de forma mais detalhada os danos físicos causados aos tecidos e de acordo com estudos já realizados para outros sistemas, estudos da ultraestrutura do material permitindo a visualização das membranas celulares, bem como a situação das organelas, vesículas e vacúolos, ao longo do procedimento de criopreservação, é sugerida. Além disso, o perfil das variações dos compostos endógenos produzidos pelos embriões zigóticos ao longo do procedimento poderia ser avaliado, e comparado aos perfis obtidos para as culturas celulares, permitindo a comparação dos fatores fisiológicos dos dois sistemas diferentes em uma mesma espécie vegetal.

Diante do exposto, recomenda-se para a construção de um banco de germoplasma *ex situ* para o sistema *A. angustifolia* ou outros sistemas com sementes sensíveis à perda de água, a conservação de tecidos e células, com utilização de técnicas *in vitro*, como alternativa à conservação de suas sementes, é recomendada. Como sugestão à novas tentativas de criopreservação dos eixos embrionários, estudos utilizando-se o material em processo germinativo avançado e, portanto, com menor conteúdo de grãos de amido (que impedem a retirada de água com eficiência) e maior concentração de açúcares de cadeia mais curta (crioprotetores naturais) são recomendados. A utilização de outros materiais como os ápices caulinares seguido de microestaquia, que apresenta a possibilidade de se criopreservar clones de interesse, também deve ser considerada.

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