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ONTOGÊNESE E PERFIL BIOQUÍMICO DE SEMENTES DE *Araucaria angustifolia* (BERTOL.) KUNTZE

Dissertação submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Ciências.

Orientadora: Prof.ª Dr.ª Neusa Steiner

Florianópolis

2019

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Goeten, Daniela
ONTOGÊNESE E PERFIL BIOQUÍMICO DE SEMENTES DE Araucaria angustifolia (BERTOL.) KUNTZE / Daniela Goeten ; orientador, Neusa Steiner, 2019.
131 p.

Dissertação (mestrado) - 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. Conífera, . 3. sementes, . 4. pré-fertilização. 5. embriogênese. I. Steiner, Neusa . II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. III. Título.

Ontogênese e perfil bioquímico de sementes de *Araucaria angustifolia* (Bertol.) Kuntze

por

Daniela Goeten

Dissertação julgada e aprovada em 22/02/2019, em sua forma final, pelo Orientador e membros da Banca Examinadora, para obtenção do título de Mestre em Ciências. Área de Concentração Recursos Genéticos Vegetais, no Programa de Pós-Graduação em Recursos Genéticos Vegetais, CCA/UFSC.

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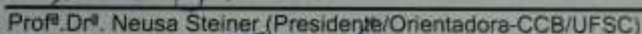

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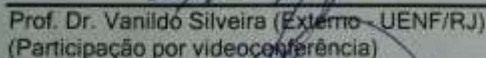

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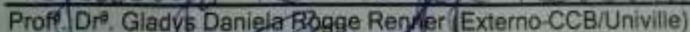

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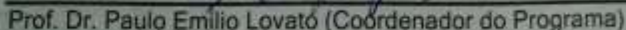

Prof. Dr. Paulo Emílio Lovato (Coordenador do Programa)

Florianópolis, fevereiro de 2019

Aos meus pais, José Hamilton Goeten e Neide de Fátima Bortoli Goeten
os quais são a base de tudo que sou.
Ao meu irmão Alex Sandro Goeten, com quem compartilhei
os melhores momentos da minha infância.
Dedico e ofereço!

AGRADECIMENTOS

A Deus pelo amparo e pelas oportunidades diárias de aprendizado.

A toda minha família, em especial a minha madrinha Marenilse Bortoli, pelo apoio incondicional em cada conquista da minha vida.

À minha orientadora Profª Neusa Steiner pela oportunidade, apoio, confiança depositada em meu trabalho, por toda dedicação e ensinamentos.

À Gladys Daniela Rogger-Renner e Francine Lunardi Farias-Soares que tornaram esse trabalho possível.

Ao LFDGV, em especial ao professor Miguel Guerra, por toda a estrutura cedida.

Aos amigos Luíza G.P., Morgana. L., Ana P.L., Edison C., Anyela R.B., Cristina B., Carolina M., Thiago S.O., Yohan F., Patrick M.D, Oscar L.L, Luan Z., Cláudia D.C., Rosa A.E.S., Vanessa M, por todas as ajudas e momentos de descontração.

A todos os demais amigos e colegas do LFDGV e do LABFISIO que tive o prazer de conhecer.

Ao pessoal do LCME/UFSC, que tornaram possível a realização de todas as análises de microscopia eletrônica presentes nesse trabalho.

A CAPES pelo apoio financeiro através da bolsa de estudos, e ao CNPq pelos recursos financeiros que permitiram a realização desse trabalho e à UFSC por promover um ensino de qualidade.

A todos que de alguma forma colaboraram para a concretização desse trabalho e cujos nomes não foram citados aqui.

“Na simplicidade de nós mesmos encontraremos as respostas para nossas inquietações e superação para nossos medos. No autoconhecimento perceberemos a nossa força latente. Na sinergia com aqueles que comungam desses princípios descobriremos que não estamos sós"

(Mauricio A. Costa)

RESUMO

O pinheiro-brasileiro (*Araucaria angustifolia* (Bertol.) Kuntze) é uma conífera nativa do Brasil dominante na Floresta Ombrófila Mista, ecossistema do bioma Mata Atlântica. Como resultado da intensa exploração e a fragmentação desse ecossistema, seus remanescentes florestais são estimados em 12,6% da sua área original. Em consequência essa Atualmente, a espécie foi catalogada na Lista Oficial de Espécie Brasileiras Ameaçadas de Extinção e encontra-se na *Red List* da IUCN em "perigo crítico" com sua população em declínio. Neste contexto, *A. angustifolia* vem sendo alvo de estudos que deem suporte sua conservação e seus uso sustentável. A compreensão dos aspectos morfológicos, ultraestruturas e fisiológicos que regulam as fases de desenvolvimento das suas sementes recalcitrantessão cruciais para definir estratégias apropriadas de conservação. O presente trabalho buscou caracterizar o desenvolvimento embrionário das sementes de *A. angustifolia*, abrangendo as etapas que ocorrem entre a pré-fertilização e a embriogênese tardia, incluindo os parâmetros morfológicos, ultraestruturas e bioquímicos. O evento da polinização ocorreu entre os meses de agosto a outubro do primeiro ano do ciclo reprodutivo, e no mês seguinte, a célula mãe do megásporo foi observada. No megagametófito, entre dezembro e julho a fase de núcleo livre ocorreu ao longo desse período poucas mudanças no desenvolvimento foram observadas. Cerca de um ano após a polinização a arquegônia amadureceu e a fertilização aconteceu. Nos meses de novembro a julho do segundo ano do ciclo reprodutivo, o embrião se desenvolveu passando pelos estágios pró-embriogenico, embriogênese inicial, embriogênese tardia. O perfil de carboidratos e o acúmulo de dehidrinas em fases específicas do desenvolvimento embrionário também foram estudados. O conteúdo de frutose e glicose foi elevado nos estágios iniciais de desenvolvimento, enquanto a sacarose estava presente em níveis baixos. Na medida em que embrião se diferenciou, durante a embriogênese tardia, a relação sacarose:hexose aumentou e a maltose que estava ausente nos estágios iniciais, passou a ser detectada. A análise de *western blot* não detectou a presença de dehidrinas durante a embriogênese inicial. As dehidrinas foram identificadas durante a embriogenêse tardia em extratos termosensíves dos eixos e cotilédones e termoestáveis somente nos eixos dos embriões zigóticos de *A. angustifolia*. Os resultados ampliam o conhecimento sobre o desenvolvimento reprodutivo de *A. angustifolia*, e sobre aspectos fisiológicos que regulam a embriogênese zigótica,

fornecendo subsidio para o aprofundamento em estudos evolutivos e biotecnológicos visando a sua conservação e seu uso sustentável.

Palavras-chaves: Conífera, sementes, pré-fertilização, embriogênese, dehidrinas, carboidratos.

RESUMO EXPANDIDO

Introdução

A família Araucariaceae compreende trinta espécies distribuídas em três gêneros Agathis, Araucaria e Wollemia. O gênero Araucaria possui dezoito espécies distribuídas na América do Sul, Nova Caledônia, Nova Guiné, Austrália e Nova Zelândia. O pinheiro brasileiro *(Araucaria angustifolia* (Bertol.) Kuntze), concentra-se no Sul e Sudeste do Brasil, com pequenas manchas no noroeste da Argentina e no Paraguai. É uma espécie de conífera nativa do Brasil dominante na Floresta Ombrófila Mista, ecossistema do bioma Mata Atlântica. Como resultado da intensa exploração e a fragmentação desse ecossistema, seus remanescentes florestais são estimados em 12,6% da sua área original. Em consequência a espécie foi catalogada na Lista Oficial de Espécie Brasileiras Ameaçadas de Extinção e encontra-se na *Red List* da IUCN em "perigo crítico" com sua população em declínio. Neste contexto, *A. angustifolia* vem sendo alvo de estudos que dão suporte sua conservação e seus uso sustentável. O pinheiro brasileiro é considerado uma espécie dióica e as plantas masculinas ou femininas adultas produzem microestrobilo e megaestrobilo, respectivamente, que são estruturas reprodutivas. Em particular, pouco se sabe sobre a biologia celular do ciclo reprodutivo da *A. angustifolia*. A literatura disponível sobre biologia sexual reprodutiva do gênero Araucaria é antiga, e alguns aspectos nunca foram descritos ou ilustrados. A maior parte dos estudos nesse contexto, investigou aspectos fisiológicos do desenvolvimento do embrião zigótico de *A. angustifolia*. No entanto, poucos estudos relataram as alterações celulares que ocorrem durante a fertilização até a formação do embrião zigótico de *A. angustifolia* no estágio avançado de desenvolvimento. A compreensão dos aspectos morfológicos, aliados aos perfis bioquímicos, como carboidratos e proteínas *LEA* - do tipo dehidrinas, durante o desenvolvimento embrionário, elucida a função e a importância desses compostos em especial na tolerância à dessecação das sementes. Esses estudos são relevantes para ampliar o conhecimento dos aspectos fisiológicos que regulam a embriogênese zigótica, e o desenvolvimento das sementes, as quais são classificadas como recalcitrantes. Sendo assim, o compilado dos resultados deste estudo, fornece elementos que regulam as fases de desenvolvimento das sementes do pinheiro brasileiro em estágios específicos, o que é crucial para definir estratégias apropriadas de conservação.

Objetivo

Estudar e caracterizar aspectos morfo-anatômicos, o perfil de carboidratos e o acúmulo de dehidrinas ao longo do desenvolvimento dos embriões zigóticos de *Araucaria angustifolia*, visando gerar subsídios para a conservação das sementes desta espécie.

Metodologia

Material vegetal: megastrobilo do primeiro e do segundo ano do ciclo reprodutivo, foram coletados mensalmente de agosto de 2011 a julho de 2013. Escalas férteis foram selecionadas, e os óvulos e embriões foram retirados para as diferentes técnicas de microscopia e análises bioquímicas. Análises morfológicas foram realizadas por meio de dupla coloração com azul de Evans e Acetocarmina. Microscópia de luz os megagametófitos foram fixados em paraformaldeído e tampão fosfato, desidratadas em série crescente de soluções de etanol e infiltradas com historesina, as secções foram coradas com PAS, TB-O e CBB. Microscopia confocal os pro-embrioões foram tratados com DAPI e IP. Microscópia eletrônica de transmissão os pró-embriões foram fixados em paraformaldeído, glutaraldeído e tampão cacodilato de sódio, tratados com tetróxido de ósmio e desidratadas em uma série de acetona graduada e então incorporadas à resina de Spurr. Para análise de carboidratos solúveis as amostras foram maceradas em solução de extração e centrifugados e reextraídos, foram identificados e quantificadas usando HPLC. Para o ensaio de Western Blot proteínas foram extraídas e divididas em duas frações termossensíveis e termoestáveis. As proteínas foram resolvidas por SDS-PAGE 17,5% e transferidas para uma membrana de nitrocelulose. As massas moleculares de proteína foram calculadas por meio das ferramentas de análise MW do software Image Lab versão 6.0.1. Análise estatística: análise de variância com dois fatores fixos cruzados, foram realizados, seguido do Student-Newman-Keuls, no software estatístico "R".

Resultados e Discussão

O evento da polinização ocorreu entre os meses de agosto a outubro no primeiro ano do ciclo reprodutivo, e no mês seguinte iniciou a formação do megagametófito, onde foi observado o nucelo com a célula mãe do megásporo rodeada pelas células esporogênicas, e a formação de uma cavidade com núcleos livres. A fase de núcleo livre durou cerca de seis meses, já que no momento da polinização as arquegônias não se encontravam diferenciadas. Cerca de 12 meses após a polinização os

núcleos livres já apresentavam parede celular e estavam organizados formando um tecido homogêneo composto por células protaliais que preenchiam a cavidade. Após 13 meses da polinização foi observado a formação de seis arquegônias. Neste trabalho foi observado que a fertilização ocorreu um ano após a polinização, ou seja, em outubro do segundo ano do ciclo reprodutivo. Após a fecundação, em novembro, aproximadamente quatorze a quinze meses após a polinização foi observado a presença de poliembriões com suas células características, sendo essas, células de capa, embrionárias e do suspensor. Na sequência do desenvolvimento, ocorreu a regressão dos embriões subordinados e apenas o embrião dominante permaneceu na semente matura. O proembrião se desenvolveu, sendo assim, suas células suspensoras sofreram um alongamento, enquanto as células de capa colapsaram, e células embriogênicas deram origem a uma massa embriogênica, assim, originou-se o embrião no estágio inicial de desenvolvimento. A transição do embrião inicial para o embrião tardio foi marcada pela diferenciação celular onde foi observado a protoderme, córtex e procambio. Durante a embriogênese avançada, a diferenciação dos meristemas e apicais caulinar e radicular foi evidente. Entre os processos bioquímicos que ao longo da embriogênese zigótica proteínas e carboidratos estão envolvidos no metabolismo basal e energético, sendo essenciais no desenvolvimento das sementes. A análise de carboidratos realizada durante todos os estágios específicos do desenvolvimento do embrião zigótico e seu respectivo megagametófito, mostrou um comportamento semelhante entre os tecidos. Os estágios proembrionário e embrionário inicial, foram marcados por altos conteúdos de hexoses (frutose e glicose) e baixo conteúdo de sacarose. Corroborando com nossos resultados, estudos mostraram que durante os estágios iniciais de desenvolvimento, ocorre a alta atividade de enzimas invertase, responsável pela hidrólise de sacarose em frutose e glicose, aumentado o conteúdo de hexose nas células. Estágios iniciais de desenvolvimento foram caracterizados pela intensa atividade mitótica, visando o crescimento do embrião, e estudos apontam o papel da glicose como uma molécula de sinalização na divisão celular. Em nosso estudo, a transição do estágio embrionário inicial para o estágio embrionário avançado, maltose passou a ser detectada e a relação hexose: sacarose diminuiu, sendo que, o conteúdo de hexose reduziu e a sacarose se tornou o açúcar mais abundante. Diante desse comportamento, sugerimos que sacarose e a maltose estão relacionadas com os processos de sinalização para a diferenciação celular. A suplementação de maltose em embriões somáticos de coníferas, durante a embriogênese tardia é

comum e eficiente para conversão de embriões. Devido a hidrólise lenta da maltose muitos autores defendem a teoria do "déficit de carboidratos", reduzindo a disponibilidade de hexose e como consequência as células diminuem a divisão e passam a se diferenciar. O conteúdo elevado de sacarose no final do desenvolvimento pode estar relacionado ao potencial osmótico, ao crescimento celular, proteção contra dessecação além de composto de reserva. A arabinose esteve presente durante todas as fases de desenvolvimento, já que é um componente de parede celular. Além do perfil dos carboidratos, proteínas do tipo dehidrinas foram analisadas por Western Blot, estas pertencem ao segundo grupo das proteínas LEA (*late embryogeneses accumulated*), as quais são acumuladas durante as fases finais da embriogênese em resposta à secagem e a sua expressão cessa rapidamente após embebição. Estas proteínas têm natureza hidrofílica, se ligam a moléculas de água impedindo a sua saída das células, consequentemente mantem a estabilização de membranas e outras proteínas. Devido a esta característica, as dehidrinas eram tidas como proteínas de sementes ortodoxas e ausentes em sementes recalcitrantes. Não foi detectada a presença dessas proteínas durante o estágio inicial de desenvolvimento, o que é justificado, já que dehidrinas são proteínas *LEA*. Como esperado, proteínas dehidrinas passaram a ser detectadas a partir da transição da embrigênese inicial para a avançada onde fração proteica termossensíveis de 21,5, 23, 26 e 28kDa estavam presentes em eixos embrionários e nos cotilédones. Com o desenvolvimento subsequente, banda de 28kDa não foi detectada enquanto a de 23kDa estava presente somente no eixo. Um estudo com sementes maturas de *A. angustifolia* usando a técnica de imunolocalizações *in situ* mostrou dehidrinas nos eixos embrionário e cotilédones, e a nível subcelular estas se encontravam associadas aos corpos proteicos, microcorpos e a cromatina no núcleo. Devido à importância ecológica, econômica, social e ao risco de extinção da *A. angustifolia,* nosso trabalho fornece embasamento para a compreensão dos aspectos morfoanatômicos, bioquímicos e fisiológicos durante o desenvolvimento de sementes da *A. angustifolia.* Caracterizar estágios do desenvolvimento embrionário, em paralelo, elucidar a função dos carboidratos solúveis durante estes estágios e estudar em qual período ocorre maior acúmulo de dehidrinas, auxilia na otimização da época de coleta para armazenamento das sementes, garantindo sua viabilidade. Uma vez, que a espécie tem natureza recalcitrante, não tolera dessecação e perde a viabilidade quando armazenadas por longos períodos. Além disso, estudos durante estágios específicos do desenvolvimento embrionário das sementes de *A.*

angustifolia, auxilia na compressão dos processos metabólicos envolvidos durante a embriogênese somática, gerando informações importantes para otimização do protocolo *in vitro,* o qual é uma alternativa para a conservação dessa espécie.

Considerações Finais

Estudos morfológicos sugeriu que *Araucaria angustifolia* é uma espécie mais primitiva quando comparado a Cupressaceae, Pinaceae e Taxodiaceae. No presente trabalho, foi possível identificar três aspectos, por meio de análises morfo-histológicas, que suportam a primitividade da espécie. (i) uma fase prolongada dos núcleos livres, (ii) a presença de células de capa e (iii) a ausência de proembrionia por clivagem. Além disso, caracterizamos a formação do megagametófito e o tempo de ocorrência da fertilização. Indicamos um tempo de treze meses até a formação da archegonia matura e cerca de quatorze meses entre a polinização e a penetração do tubo polínico no núcleo. Pela primeira vez, foi caracterizado os três estágios de desenvolvimento embrionário zigótico para esta espécie: proembriogênese, embriogênese inicial e avançada. Descrevemos o processo de morte celular em células de capa e suspensoras, bem como a diferenciação dos tecidos em embrião tardio. Nossos dados descrevem pela primeira vez o desenvolvimento completo do embrião zigótico e que levam 23 meses entre a fertilização até o embrião maturo de uma conífera nativa e ameaçada de extinção. Em *A. angustifolia*, os estágios de desenvolvimento embrionário também podem ser caracterizados por alterações no perfil bioquímico. Este é o primeiro trabalho que descreveu em conjunto o metabolismo dos carboidratos e o estado das dehidrinas ao longo do desenvolvimento embrionário. Indicamos uma correlação positiva entre a síntese de carboidratos e de dehidrinas. Os estágios iniciais de desenvolvimento apresentaram altos teores de hexoses e baixos teores de sacarose. No entanto, durante os estágios finais de desenvolvimento, observou-se um aumento na relação sacarose: hexose e detectou-se maltose. As deidrinas não são suficientes para conferir tolerância à dessecação, uma vez que são acumuladas nos estágios tardios da embriogênese e na maturação das sementes. Este estudo fornece uma melhor compreensão dos aspectos bioquímicos e fisiológicos envolvidos durante o desenvolvimento e maturação de embriões zigóticos de *A. angustifolia*. Além disso, indicamos parâmetros metabólicos que podem ser utilizados para melhorar o protocolo de embriogênese somática.

Palavras-chave: Conífera, sementes, pré-fertilização, embriogênese, dehidrinas, carboidratos.

ABSTRACT

The Brazilian pine *(Araucaria angustifolia* (Bertol.) Kuntze) is a native conifer of dominant Brazil in the Ombrophilous Mixed Forest, an ecosystem of the Atlantic Forest biome. As a result of the intense exploitation and fragmentation of this ecosystem, its forest remnants are estimated at 12.6% of its original area. As a result, this species is currently listed in the Official List of Brazilian Species Endangered Species and is on the IUCN Red List in "critical danger" with its declining population. In this context, *A. angustifolia* has been the object of studies that support its conservation and its sustainable use. Understanding the morphological, ultrastructural, and physiological aspects that regulate the developmental stages of their recalcitrant seeds are crucial to defining appropriate conservation strategies. The present work aimed to characterize the embryonic development of *A. angustifolia* seeds, covering the stages that occur between pre-fertilization and late embryogenesis, including morphological, ultrastructural and biochemical parameters. The pollination event occurred between August and October of the first year of the reproductive cycle, and the following month, the mother cell of the megaspore was observed. In the megagametófito, between December and July the free nucleus phase occurred during this period few changes in the development were observed. About a year after pollination archegonia has matured and fertilization has taken place. In the months of November to July of the second year of the reproductive cycle, the embryo developed through the pro-embryogenic stages, initial embryogenesis, late embryogenesis. The carbohydrate profile and accumulation of dehydrins at specific stages of embryonic development were also studied. The fructose and glucose content was high in the early stages of development, while sucrose was present at low levels. As embryo differentiated, during late embryogenesis, the sucrose: hexose ratio increased and maltose that was absent in the early stages, was detected. Western blot analysis did not detect the presence of dehydrins during the initial embryogenesis. The dehydrins were identified during late embryogenization in thermosensitive extracts of the axes and cotyledons and thermostable only in the axes of the zygotic embryos of *A. angustifolia*. The results amplify the knowledge on the reproductive development of *A. angustifolia* and on the physiological aspects that regulate zygotic embryogenesis, providing subsidy for the deepening in evolutionary and biotechnological studies aiming its conservation and its sustainable use.

Key words: Conifer, seeds, pre-fertilization, embryogenesis, dehydrins, carbohydrates.

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Capítulo I

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1. ANTECEDENTES E JUSTIFICATIVA

Araucaria angustifolia (Bertol.) Kuntze, popularmente conhecida como Pinheiro do Paraná, Araucária ou Pinheiro-brasileiro, é uma conífera dióica e perenifólia (Zanette et al. 2017). Ocorre exclusivamente na América do Sul, na região da Floresta Ombrófila Mista, formação florestal que recebe o nome de “Floresta das Araucárias” - devido à abundância e ao porte da espécie, a qual imprime a fitofisionomia da floresta (Mattos 1994, 2011). Essa espécie é endêmica das regiões Sul e Sudeste do Brasil, e em pequenas manchas no Noroeste da Argentina e no Paraguai, encontra condições ideais para o desenvolvimento em altitudes entre 300 e 1800 m (Farjon; Filer 2013).

Na região Sul do Brasil a *A. angustifolia* é a gimnosperma de maior importância ecológica, econômica e social (Guerra et al. 2008; Zanette et al. 2017). Ela propicia um ambiente ideal para o desenvolvimento de espécies vegetais em seu caule, galhos e no dossel inferior, o qual é sombreado por sua copa e suas sementes servem de alimento para a fauna selvagem. Em relação aos aspectos econômicos, possui madeira de alta qualidade, sendo empregada para construção civil, móveis e para a produção de celulose (Mattos 1994; Guerra et al. 2002). Além do mais, a coleta e a venda de sementes se constituem em relevante atividade econômica para um considerável número de famílias que vivem nas regiões de ocorrência (Carvalho, 1994; Silva, 2009).

Devido às suas características de alto interesse, a *A. angustifolia* foi alvo de intensa exploração ao longo do século passado (Guerra et al., 2002). Em decorrência da vasta exploração e fragmentação, seus remanescentes florestais estão estimados em 12,6% da sua extensão original, e a maioria dos fragmentos possuem menos de 50 hectares (Ribeiro et al., 2009). A drástica redução da paisagem, culminou na inclusão da espécie na categoria “perigo crítico” da *Red List* de espécies ameaçadas de extinção, pertencente a International Union for Conservation of Nature and Natural Resources (IUCN, 2018). A espécie também se encontra catalogada na Lista Oficial das Espécies da Flora Brasileira Ameaçadas de Extinção, elaborada pelo Ministério do Meio Ambiente (MMA) (Instrução Normativa nº 06, de 23 de setembro de 2008). No entanto, mesmo após a inclusão de *A. angustifolia* na lista oficial de espécies brasileiras ameaçadas de extinção, remanescentes florestais continuaram a ser explorados (Stefenon et al., 2009).

Diante deste cenário, *A. angustifolia* é uma das espécies nativas do Brasil com grande potencial para estudos que auxiliem sua conservação genética por meio do estabelecimento de coleções de germoplasma *in situ* e *ex situ* (Guerra et al., 2008). O manejo dos remanescentes florestais e o uso da biotecnologia são ferramentas chaves para uso e conservação dessa espécie, no entanto, estudos básicos são necessários para garantir a eficácia dessas técnicas. Sementes da *A. angustifolia* são classificadas como recalcitrantes, perdendo sua viabilidade quando desidratadas e não tolerando baixas temperaturas, dificultando o armazenamento por longos períodos (Farrant; Pammenter; Berjak, 1989; Panza et al., 2002; Amarante et al., 2007; Farias-Soares et al., 2013). Esses aspectos reforçam a importância de estudos que visem auxiliar no estabelecimento de protocolos e técnicas eficiente para o seu uso e conservação.

Em particular, pouco se sabe o desenvolvimento do embrião zigótico de *A. angustifolia*. A literatura disponível sobre o ciclo reprodutivo do gênero Araucária é antiga (Burlingame, 1914, 1915; Johansen, 1950; Haines; Prakash, 1980; Haines, 1983). Em estudos mais recentes sobre a sua biologia reprodutiva evidenciamos a existência de lacunas consideráveis, como o momento exato de fertilização, a ocorrência de estágio de núcleo livres, a poliembrionia polizigótica, regressão dos embriões subordinados, e caracterização do estágio embrionários (Mantovani; Morellato; Reis, 2004; Anselmini; Zanette; Bona, 2006; Kuhn; Mariath, 2014; Sant'Anna et al., 2013; Rogge-Renner et al., 2017; Zanette et al., 2017). Sendo que, a maior parte dos trabalhos investigaram aspectos bioquímicos e fisiológicos do desenvolvimento do embrião zigótico de *A. angustifolia,* (Panza et al., 2002; Dos Santos et al., 2006, 2016; Agapito-Tenfen et al., 2011; Balbuena et al., 2011a; Farias-Soares et al., 2013; Shibata et al., 2016; Shibata; Maria; Coelho, 2016). Negligenciando aspectos morfo-anatômicos e caracterizando bioquimicamente estágios específicos de desenvolvimento embrionários que não correspondem aos mesmos.

Visando elucidar o mapa do destino do desenvolvimento biológico das sementes de *A. angustifolia*, o presente estudo descreveu as características morfológicas, histológicas e ultraestruturais que ocorrem desde a pré-fertilização até a embriogênese tardia. Alguns processos são mostrados aqui pela primeira vez e fornecem subsídios para o aprofundamento em estudos evolutivos. Considerando que os carboidratos e as proteínas *LEA* estão intimamente associados à tolerância e sensibilidade à dessecação de sementes, este estudo amplia o

conhecimento sobre o comportamento e acúmulo de carboidratos e dehidrinas durante estágios específicos do desenvolvimento embrionário. Estes resultados são relevantes para ampliar o conhecimento dos aspectos fisiológicos que regulam a embriogênese zigótica, e o desenvolvimento das sementes. Além disso, este estudo fornece elementos para o desenvolvimento de protocolos regenerativos *in vitro* baseados na embriogênese somática, visando o uso sustentável e a conservação dessa espécie.

2. REVISÃO BIBLIOGRÁFICA

2.1 *Araucaria angustifólia*

O termo *Araucaria* deriva de Arauco, região do Chile de onde o gênero procede, e *angustifolia* vem do latim *angustus*, estreito e pontudo e *folium*, folha. *A. angustifolia* é uma conífera dióica, raramente monóica após trauma ou doença, com estruturas unissexuais (Reitz; Klein, 1966).

As árvores são de grande porte, apresentando 30 - 52 m altura com galhos dispostos na parte superior dos trocos. Os fustes são eretos e cilíndricos, raramente ramificados, a casca é grossa e resinosa. O sistema radicular é muito desenvolvido, para sustentação da copa e as folhas são simples, côncavas e pequenas de 5 – 6 cm de comprimento e 0,5 mm de espessura. Os ramos secundários, chamados grimpas, são persistentes e revestidos pelas folhas (Mattos, 1994, 2011; Reitz; Klein, 1966). As araucárias demoram de 12 a 15 anos para alcançar a idade reprodutiva (Carvalho, 1994; Zanette et al., 2017).

A. angustifolia ocorre naturalmente no Brasil, na província de Missiones na Argentina e no Departamento de Alto Paraná no Paraguai (Guerra et al., 2008; Farjon; Filer, 2013). No Brasil, a espécie era encontrada principalmente nos Estados do Paraná (40% da superfície), Santa Catarina (31%) e Rio Grande do Sul (25%). Manchas esparsas eram observadas no Sul de São Paulo (3%), internando-se até o sul de Minas Gerais e Rio de Janeiro (1%) (Carvalho, 1994; Guerra et al., 2002, 2008). Nestes locais, a araucária é encontrada em regiões de altitude elevada (500 - 1800 m) no bioma Mata Atlântica; em distintas formações edáficas e em clima subtropical, com temperaturas variando de 10º C a 21º C (Reitz; Klein, 1966; Farjon; Filer, 2013).

A Araucária é uma planta pioneira e heliófita, criando um ambiente ideal para o desenvolvimento de outras espécies tolerantes à sombra. As

sementes servem de alimento para a fauna selvagem, sendo ricas em energia e disponíveis durante os meses mais frios, enquanto as outras espécies da floresta não estão produzindo frutos (Guerra et al., 2000, 2002, 2008). Por esse motivo, a conservação da araucária implica também na manutenção das cadeias tróficas. Do ponto de vista social e econômico, as sementes servem de alimento com grande valor nutricional, sendo que a coleta e a venda das sementes estão intimamente ligadas à sobrevivência de famílias de baixa renda, que vivem nas regiões de ocorrência, em épocas de outono e inverno (Carvalho, 1994; Silva; Reis, 2009). *A. angustifolia* possui madeira de alta qualidade, motivo pelo qual, no início do século XX, foi altamente explorada sendo empregada especialmente para construção civil, de móveis, e para a produção de celulose. (Carvalho, 1994; Mattos, 1994, 2011; Guerra et al., 2002, 2008). Além da madeira, usa-se também, a resina exsudada da casca (Reitz; Klein, 1966).

Devido à exploração intensa da madeira e o desmatamento visando à agricultura, restaram atualmente alguns remanescentes isolados, os quais representam aproximadamente 12,6% da área total de sua ocorrência natural (Ribeiro et al, 2009). A fragmentação gerou endogamia, redução da diversidade genética e, por conseguinte, também a perda da capacidade adaptativa às mudanças ambientais (Guerra et al., 2008). Como consequência essa espécie foi incluída na lista vermelha da IUCN (*International Union for Conservation of Nature and Natural Resources*) de espécies ameaçadas e classificada como em perigo crítico de extinção (IUCN, 2017), bem como catalogada na Lista Oficial das Espécies da Flora Brasileira Ameaçadas de Extinção, elaborada pelo Ministério do Meio Ambiente (MMA) (Instrução Normativa nº 06, de 23 de setembro de 2008). No entanto, mesmo após seu nome incluso na lista oficial de espécies brasileiras ameaçadas de extinção, remanescentes florestais, contendo essa espécie, continuam a ser explorados (Stefnon et al., 2009).

Diante deste cenário, *A. angustifolia* é uma espécie nativa do Brasil com alto potencial para estudos, os quais visem a sua conservação. Sementes desta espécie são classificadas como recalcitrantes, perdendo sua viabilidade quando desidratadas e não tolerando baixas temperaturas, impossibilitando o seu armazenamento por longos períodos de tempo (Farrant; Pammenter; Berjak, 1989; Panza et al., 2002; Farias-Soares et al., 2013; Shibata et al., 2016; Shibata; Maria; Coelho, 2016).

2.2 MORFOLOGIA E CICLO REPRODUTIVO

Estróbilos masculinos apresentam de 10 cm a 15 cm de comprimento são constituídos por escamas coriáceas com 10 a 25 anteras alongadas, presas na fase ventral de cada escama. As escamas encontram-se arranjadas em forma de espiral e se abrem deixando o pólen livre para ser transportado pelo vento até o estróbilo feminino. A estrutura reprodutiva feminina localiza-se no ápice do ramo e é constituída por inúmeras brácteas coriáceas com o óvulo, inseridas sobre um eixo central (Reitz; Klein, 1966; Guerra et al., 2008, 2012).

O ciclo reprodutivo da araucária dura cerca de 20 a 24 meses (Mantovani et al., 2004; Rogge-Renner et al., 2017) e foi apresentado por Guerra et al. (2008) e reproduzido na Figura 1. A liberação dos grãos de pólen dos microestróbilos pelos microsporângios ocorre entre agosto e setembro, do primeiro ano do ciclo reprodutivo De acordo com Shimoya (1962), o início da polinização Minas Gerais, ocorre no final de setembro, enquanto Mantovani et al. (2004) observaram no Estado de São Paulo a liberação de pólen em agosto e setembro. No sul do Brasil, este fenômeno ocorre entre os meses de agosto a outubro (Anselmini; Zanette, 2008). Grãos de pólen são transportados pelo vento e depositados nas escamas ovulíferas dos megaesporângios, onde inicia a germinação *in situ*. A germinação ocorre na parte externa, sem penetrar na nucela, formando uma delicada teia entre outubro e dezembro (Shimoya, 1962). Os tubos polínicos penetram a ponta nucelar e, ali permanecem, durante toda a fase de núcleo livre (Owens et al., 1995).

O momento certo da fecundação não é relatado por Guerra et al., (2008). No entanto, Rogge-Renner et al., (2017) reportou a ocorrência da fertilização um ano depois da polinização, entre setembro e outubro, do segundo ano do ciclo reprodutivo. A presença de uma fase de núcleos livres após a fertilização foi observada. Após a formação dos núcleos livres ocorreu a elongação celular e formação de poliembriões, que apresentam células da capa, embrionárias e do suspensor (Guerra et al. 2008; 2012). Apenas um embrião dominante permanece na semente e se desenvolve até a forma madura, enquanto os embriões subordinados regridem (Burligame, 1915; Johasen, 1950; Guerra et al. 2008; 2012). Quanto à dispersão de sementes, os principais atores são aves e roedores (Carvalho, 2003).

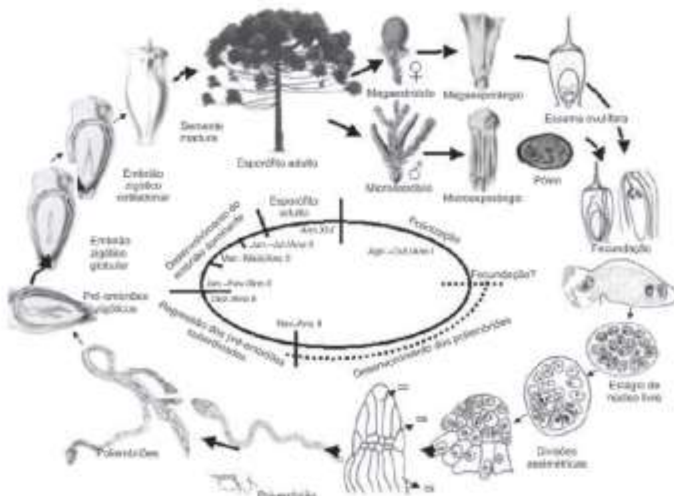

Figura 1: Ciclo de vida da *Araucaria angustifolia* (Guerra et al., 2008, 2012)

2.1.1. Megasporogênese

Megasporogênese é o processo pelo qual a célula mãe do megásporo (megasporócito) dentro do óvulo, sofre meiose dando origem a quatro megásporos. Apenas o megásporo mais próximo da extremidade chalazal sobrevive, o qual sofre mitose e torna-se multicelular, originando o megagametófito (Burlingame, 1915; Johansen, 1950; Shimoya, 1962; Owens et al. 1995; Mattos, 2011; Williams, 2009).

A fase inicial do megagametófito, dentro do nucelo é marcada pelo surgimento dos núcleos livres, seguidos por uma organização celular com arranjo elipsoide e posterior formação da parede celular para cada núcleo livre (Shimoya, 1962; Owens et al. 1995). Simultaneamente ao início da formação do megagametófito, ocorre a penetração dos tubos polínicos, que crescem no nucelo e causam destruição celular, estimulando a produção de alguma substância, provavelmente hormonal, determinante da divisão celular, que resultará na formação de células protaliais ou protalo a partir dos núcleos livres (Owens et al. 1995; Shimoya, 1962) e mais tarde neste tecido protalial ocorre a formação de arquegônias, geralmente numerosas (Shimoya, 1962), cada qual com sua célula ovo. A arquegônia é uma cobertura multicelular em torno de cada célula ovo, no megagametófito (Williams, 2009). De acordo com Rogge-Renner et al

(2017), a fertilização ocorre cerca de um ano após a polinização, nos meses de setembro e outubro. Segundo Shimoya (1962), em Minas Gerais a fertilização ocorre entre os meses de outubro e dezembro.

2.1.2. Embriogênese zigótica

O termo embriogênese corresponde as fases de desenvolvimento compreendidas entre a fertilização até o início da dormência do embrião zigótico (Harada et al., 2010). Em *A. angustifolia* o estágio inicial da embriogênese, após a fusão dos gametas, é marcado por divisões nucleares formando de 32 a 64 núcleos livres antes da formação das paredes celulares (Guerra et al., 2008, 2012). Depois da completa formação da parede celular, ocorre a elongação celular, de forma simultânea, nos grupos de células superiores e inferiores. O grupo de células inferiores forma as células de capa (Cc), o grupo de células superiores forma as células do suspensor (Cs) e o grupo central de células constitui um grupo de células embriogênicas (Ce) (Burlingame, 1914; Guerra et al., 2008, 2012; Johansen, 1950).

Após a polinização, transcorrem 14 meses até a identificação dos poliembriões, caracterizando a poliembrionia, ou seja, a formação de mais de um embrião nos estágios iniciais de desenvolvimento da semente (Guerra et al; 2008, 2012). Dois tipos de poliembrionia são relatas: (i) poliembrionia polizigótica: onde duas ou mais arquegônias são fecundadas em um mesmo megagametófito, produzindo separadamente proembriões e (ii) poliembrionia por clivagem: dentro da arquegônia, cada proembrião formado pode se dividir em até oito proembriões (Steeves; Sussex, 1989; Williams, 2009). Em espécies do gênero *Araucaria* foi observada apenas poliembrionia polizigótica (Shimoya, 1962; Gifford; Foster, 1989). No entanto, um estudo que analisou parâmetros morfológicos com marcadores moleculares relatou a primeira evidência de poliembrionia por clivagem em *A. angustifolia* (Agapito-Tenfen et al., 2011). Contudo, apenas um embrião permanece na semente matura (Haines; Prakash, 1980; Bozhkov; Filonova et al., 2000; Filonova; Suarez, 2005), e a regressão dos embriões subordinados ocorre entre os meses de novembro e dezembro (Mantovani; Morellato; Reis, 2004; Guerra et et al., 2008, 2012)

Coníferas em geral, o embrião se desenvolve de acordo com o estabelecido por Singh (1978), onde três fases podem ser reconhecidas: i) pró-embrionária: englobando os eventos entre a fertilização até o

rompimento do arquegônio pelo proembrião; ii) embrionária inicial: estádios após o alongamento do suspensor e antes do estabelecimento dos meristemas; e iii) embrionária final: com intensa histogênese, onde a protoderme e o procâmbio são diferenciados e os meristemas apical e radicular são estabelecidos (Figura 2) (Arnold et al., 2002; Williams, 2009).

Em *A. angustifolia* e outras espécies pertencente à família Araucariaceae o proembrião foi caracterizado pela presença de células da capa, células embrionárias e células do suspensor. Na região central do embrião, as células embrionárias permanecem inativas até o completo desenvolvimento do suspensor. Durante a transição do proembrião para o embrião inicial, células de capa sofrem degeneração e células embrionárias dividem-se longitudinalmente para aumentar em cerca de 20 vezes o número de células (Burlingame, 1914, 1915; Johansen, 1950; Haines; Prakash, 1980; Haines, 1983; Guerra et al. 2008; 2012). A embriogenia tardia em coníferas é iniciada com a formação meristemática e histogênese intensiva, que resultam na diferenciação da protoderme e procâmbio, e no estabelecimento dos meristemas apical caulinar e radicular. A região proximal do embrião forma a coifa e o meristema radicular, que é responsável por todo o desenvolvimento posterior da raiz. A região distal forma o eixo hipocótilo-caulinar e cotilédones (Haines, 1980; Singh, 1978; Owens, 2004).

O desenvolvimento do embrião dominante de *A. angustifolia* ocorre entre os meses de dezembro e junho (Guerra et al., 2002, 2008). No entanto, no mês de março sementes maturas já são encontradas. Após a germinação, a planta leva em torno de 15 anos para entrar novamente em idade reprodutiva (Carvalho, 2003).

Cabe ressaltar que sementes de *A. angustifolia* perdem a viabilidade ao serem desidratadas, dificultando a sua conservação por longos períodos, uma vez que são classificadas como recalcitrantes (Tompsett, 1984; Farrant; Pammenter; Berjak, 1989; Espindola et al., 1994; Farias-Soares et al., 2013; Shibata et al., 2016). Alguns estudos investigaram aspectos bioquímicos e fisiológicos do desenvolvimento do embrião zigótico (Panza et al., 2002; Astarita; Handro; Floh, 2003; Amarante et al., 2007; Balbuena et al., 2009, 2011a; Farias-Soares et al., 2013; Shibata et al., 2016; Shibata; Maria; Coelho, 2016). Porém, trabalhos morfológicos dos eventos que ocorrem desde a pré-fertilização até a formação de embriões zigóticos maturos de *A. angustifolia* são antigos, ferramentas e técnicas de microscopia mais refinadas eram de

difícil acesso, impossibilitando a obtenção de imagens mais detalhadas (Burlingame, 1914; Johansen, 1950; Shimoya, 1962). Neste contexto, estudos das alterações celulares das estruturas envolvidas nos processos de prefertilização até a formação do embrião maturo foram realizados. Além disso, o perfil de carboidratos e o acúmulo de proteínas do tipo dehidrinas foram monitorados durante o desenvolvimento embrionário.

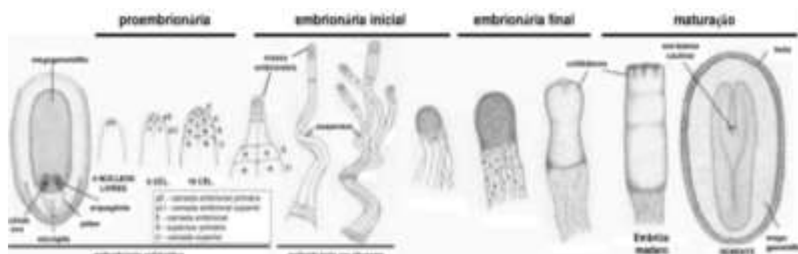

Figura 2: Desenvolvimento do embrião zigótico de *Picea abies* (Adptado von-Arnold et al., 2002)

2.3 CARBOIDRATOS E DEHIDRINAS

O desenvolvimento das sementes de gimnospermas, como *A. angustifolia* pode ser dividido em duas fases principais: a embriogênese e a maturação (Singh; Johri, 1972). As principais substâncias de reserva durante a formação das sementes das coníferas, são as proteínas, carboidratos e lipídios (Bewley; Black, 1994; Panza et al., 2002). Entre esses compostos de reserva, os carboidratos agem como fonte de energia, proteção proteica, regulação osmótica e também como moléculas sinalizadoras (Weber; Borisjuk; Wobus, 2005; Rolland; Baena-Gonzalez; Sheen, 2006; Winkelmann, 2016). Análises de transcriptoma e proteomica sugerem que vários genes e proteínas envolvidos no metabolismo de carboidratos são altamente expressos durante o desenvolvimento do embrião (Dos Santos et al., 2016; Elbl et al., 2014; Navarro et al., 2017). Recentemente, um estudo comparando os sistemas de embriogênese zigótica e somática em *A. angustifolia,* analisou o conteúdo de carboidratos e a expressão de genes envolvidos na síntese destes (Navarro et al., 2017). No entanto, esses autores consideram somente três estágios de desenvolvimento do embrião zigótico o *globular*, *cotiledona*r e *maturo*, os quais não correspondem respectivamente os mesmos estágios de desenvolvimento do embrião somático. Neste

sentido, a priori, o presente estudo visou caracterizar os estágios de desenvolvimento do embrião zigótico de Araucária evitando que equívocos referentes aos estágios de desenvolvimento e durante a comparação com estágios de desenvolvimento dos embriões somáticos não ocorram. Além disso, estudamos o comportamento dos carboidratos, durante os três estágios de desenvolvimento do embrião zigótico *proembriogênico*, *embriogenico iniacial* e *embriogenico avançada* seus respectivos megagametófitos, e nas sementes maturas, comportamento não registrado na literatura para esta espécie.

As fases iniciais do desenvolvimento são caracterizadas pelo crescimento devido a intensa divisão celular. Após, ocorre a histodiferenciação onde as células se diferenciam para dar forma ao plano básico do corpo do embrião (Singh; Johri, 1972; Williams, 2009). De maneira geral, durante desenvolvimento embrionário uma tendência comum em coníferas é a diminuição no conteúdo de hexose com um acúmulo transitório de sacarose (Gösslová et al., 2001; Pullman; Buchanan, 2008; Oliveira et al., 2016). Níveis elevados de glicose e frutose no início do desenvolvimento estão relacionados aos processos de divisão celular (Wang; Ruan, 2013). O aumento na relação sacarose:hexose, em estágios finais da embriogênese, se relaciona com o armazenamento e a diferenciação por meio da regulação na expressão de enzimas metabólicas (Gutierrez et al., 2007). O conteúdo de hexoses pode ser diminuído pela atividade da sacarose fosfato sintase, que é expressa em células do parênquima na maturação (Weber et al., 1996). Essa enzima pode sintetizar a sacarose ao final da fase de divisão celular, diminuindo assim a razão entre hexoses e sacarose. Essas mudanças podem estar relacionadas com o crescimento celular, já que para esse processo ocorrer é necessária a o acúmulo de substâncias e a entrada de água na célula, resultando na expansão celular (Cosgrove, 1997). Assim, os carboidratos podem atuar como osmólitos fornecendo a força motriz para o influxo de água para aumentar o turgor. Em sementes de leguminosas a sacarose está associada à expansão celular e à síntese de amido, o que é evidenciado por teores mais elevados de transcritos de sacarose sintase e ADP-Glc pirofosforilase (Borisjuk et al., 2002, 2003). Além disso, durante a maturação embrionária, a sacarose é um fator-chave para a aquisição da tolerância à dessecação, auxilia na estabilização das membranas, já que a desintegração da membrana é um dos primeiros danos da dessecação. Assim, a sacarose pode atuar como um protetor na "hipótese da reposição de água", onde os grupos hidroxila substituem a água e fornecem as

interações hidrofílicas necessárias para a estabilização da membrana e proteína (Leprince; Bronchart; Deltour, 1990). Além do acúmulo de sacarose, os estágios finais da embriogênse são caracterizados pela presença de maltose. Sugere-se que este carboidrato esteja relacionado a hidrólise lenta, também denominada teoria do "déficit de carboidratos", reduzindo a disponibilidade de hexose e como consequência as células diminuem a divisão e se diferenciam (Scott; Lyne; Rees, 1995; Blanc et al., 2002; Pullman; Buchanan, 2008).

Diferente das sementes ortodoxas, que terminam seu desenvolvimento com uma fase de dessecação as sementes de *A. angutifolia* são classificadas como recalcitrantes, não tolerando dessecação e o armazenamento a longos períodos (Farrant; Pammenter; Berjak, 1989; Farrant et al., 1996; Panza et al., 2002; Farias-Soares et al., 2013). A ausência de tolerância à desidratação é uma desvantagem em termos de adaptação das sementes limitando a viabilidade em condições ambientais severas e em longos períodos de armazenamento. Estudos apontam que a germinação é reduzida quando o teor de umidade cai abaixo de 37%, enquanto um nível de dessecação de 25% leva à perda de germinabilidade (Tompsett, 1984). Na maturação de sementes de *A. angustifolia* foram encontradas proteínas proteínas LEA "*Iate embryogenesis abundant"* (Farrant; Pammenter; Berjak, 1989; Farias-Soares et al., 2013), sabendo-se que o ABA está envolvido na expressão dos genes que codificam essas proteínas, as quais são sintetizadas nas sementes após a histodiferenciação do embrião (Farrant; Pammenter; Berjak, 1989; Finch-Savage; Pramanik; Bewley, 1994). Na desidratação as LEA fornecem uma camada de seus próprios resíduos hidroxilados para interagir com os grupos de superfície de outras proteínas, atuando como "água de reposição" (Figura 3) (Hoekstra; Golovina; Buitink, 2001). As proteínas do tipo dehidrinas, fazem parte do Grupo 2 (originalmente chamado de D-11) da classe de proteínas descritas como *LEA* ((Finch-Savage; Pramanik; Bewley, 1994; Campbell et al., 1997; Close, 1996, 1997; Gumilevskaya; Azarkovich, 2010). Essas proteínas são hidrofílicas, termoestáveis e caracterizadas por sequências altamente conservadas, incluindo um segmento K rico em lisina (EKKGIMDKIKEKLPG), um segmento S de poliserina, um segmento Y e um segmento U (Close, 1996, 1997; Campbell et al., 1997). Durante os estágios finais, a perda de água diminui o volume celular causa o adensamento dos componentes citoplasmáticos, aumentando a chance de interações moleculares, resultando em desnaturação de proteínas e fusão

de membrana. (Finch-Savage; Pramanik; Bewley, 1994; Hoekstra; Golovina; Buitink, 2001; Battaglia et al., 2008; Kleinwachter et al., 2014). Para evitar esses danos causado pela dessecação, as dehidrinas formam uma hélice anfipática, essa estrutura, permite que interações hidrofílicas e hidrofóbicas estabilizem proteínas e as membranas em ambientes de estresse hídrico (Dure, 1993; Hoekstra; Golovina; Buitink, 2001).

Em sementes recalcitrantes, diferentes estratégias de armazenamento precisam ser adotadas visando a sua conservação por maiores períodos, uma vez que estas não toleram a desidratação e permanecem com o metabolismo ativo. Estudos recentes mostraram diferenças fisiológicas e bioquímicas durante a fase de maturação de sementes de *A. angustifolia* (Shibata et al., 2016; Shibata; Maria; Coelho, 2016). Observa-se que estudos sobre o perfil de dehidrinas durante os estágios tardios do desenvolvimento embrionário para esta espécie são escassos, se limitando ao estágio de maturação. O estudo sobre o perfil de dehidrinas e carboidratos associados aos aspectos morfológicos durante o desenvolvimento das sementes de *A. angutifolia* pode aprimorar as técnicas de uso e conservação desta espécie, auxiliando em estratégias para o seu armazenamento.

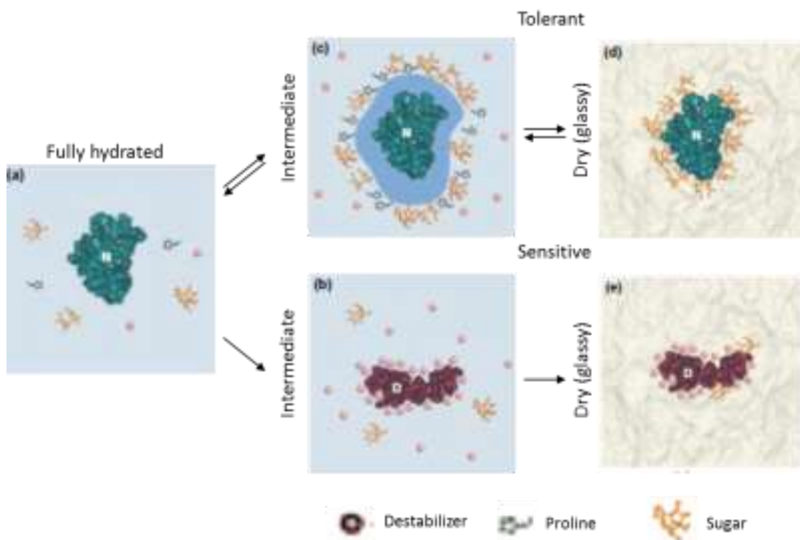

Figura 3: Mecanismos de estabilização da estrutura proteica (Hoekstraet al., 2001)

3 REFERÊNCIAS

AGAPITO-TENFEN, Sarah Z. et al. Patterns of polyembryony and frequency of surviving multiple embryos of the Brazilian pine *Araucaria angustifolia*. **Australian Journal of Botany**, [s. l.], n. 59, p. 749–755, 2011.

AMARANTE, Cassandro Vidal Talamini Do et al. Conservação pós-colheita de pinhões [sementes de *Araucaria angustifolia* (Bertoloni) Otto Kuntze] armazenados em diferentes temperaturas. **Ciência Rural**, [s. l.], v. 37, n. 2, p. 346–351, 2007.

ANSELMINI, Justina Inês; ZANETTE, Flávio. Development and growth curve of the pine cones of *Araucaria angustifolia* (Bert.) O . Ktze, in the Region of Curitiba – PR. **Brazilian archives of biology and technology aninternational journal**, [s. l.], v. 51, n. 4, p. 665–669, 2008.

ANSELMINI, Justina Inês; ZANETTE, Flávio; BONA, Cleusa. Reproductive phenology of the *Araucaria angustifolia* (Bert.) O . Ktze, in region of Curitiba – PR. **Floresta e Ambiente**, [s. l.], v. 13, n. 1, p. 44–52, 2006.

ARNOLD, Sara Von et al. Developmental pathways of somatic embryogenesis. **Plant Cell, Tissue and Organ Culture**, [s. l.], n. 69, p. 233–249, 2002.

ASTARITA, Leandro V; HANDRO, Walter; FLOH, Eny S. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O . Ktze . **Revista Brasileira Botanica**, [s. l.], v. 26, n. 2, p. 163–168, 2003.

AULER, Neiva Maria Frizon et al. The genetics and conservation of *Araucaria angustifolia* : I . Genetic structure and diversity of natural populations by means of non-adaptive variation in the state of Santa Catarina , Brazil. **Genetics and Molecular Biology**, [s. l.], v. 25, n. 3, p. 329–338, 2002.

BALBUENA, Tiago S. et al. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*).

Journal of Proteomics, [s. l.], v. 72, n. 3, p. 337–352, 2009. Disponível em: <http://dx.doi.org/10.1016/j.jprot.2009.01.011>

BALBUENA, Tiago S. et al. Phytochemistry Differential proteome analysis of mature and germinated embryos of *Araucaria angustifolia*. **Phytochemistry**, [s. l.], v. 72, n. 4–5, p. 302–311, 2011. a. Disponível em: <http://dx.doi.org/10.1016/j.phytochem.2010.12.007>

BALBUENA, Tiago S. et al. Differential proteome analysis of mature and germinated embryos of *Araucaria angustifolia*. **Phytochemistry**, [s. l.], v. 72, n. 4–5, p. 302–311, 2011. b. Disponível em: <http://dx.doi.org/10.1016/j.phytochem.2010.12.007>

BARBEDO, Claudio José; MARCOS, Filho Júlio. Tolerância a dessecação em sementes. **Acta Botanica Brasileira**, [s. l.], v. 12, n. 2, p. 145–164, 1998.

BATE, Nicholas J. et al. An invertase inhibitor from maize localizes to the embryo surrounding region during early kernel development. **Plant Physiology**, [s. l.], v. 134, n. January, p. 246–254, 2004.

BATTAGLIA, Mariana et al. The enigmatic *LEA* proteins and other hydrophilins. **Plant Physiology**, [s. l.], v. 148, n. 1, p. 6–24, 2008. Disponível em: <http://www.plantphysiol.org/cgi/doi/10.1104/pp.108.120725>

BLANC, G. et al. Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of Hevea brasiliensis (Mull. Arg.). **Journal of Experimental Botany**, [s. l.], v. 53, n. 373, p. 1453–1462, 2002.

BORISJUK, Ljudmilla et al. Spatial analysis of plant metabolism: sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns. **Plant Journal**, [s. l.], v. 29, n. 4, p. 521–530, 2002.

BORISJUK, Ljudmilla et al. Differentiation of legume cotyledons as related to metabolic gradients and assimilate transport into seeds. **Journal of Experimental Botany**, [s. l.], v. 54, n. 382, p. 503–512, 2003.

BOZHKOV, Peter V; FILONOVA, Lada H.; SUAREZ, Maria F. Programmed cell death in plant embryogenesis. **Current Topics in Developmental Biology**, [s. l.], v. 67, n. 04, p. 135–179, 2005.

BURLINGAME, L. Lancelot. The morphology of *Araucaria brasiliensis* . II . The ovulate cone and female gametophyte. **The University Chicago Press Journals**, [s. l.], v. 57, n. 6, p. 490–508, 1914.

BURLINGAME, L. Lancelot. The morphology of *Araucaria brasiliensis*. **Botanical Gazette**, [s. l.], v. LIX, p. 42, 1915.

BURRIEZA, Hernán P. et al. High salinity induces dehydrin accumulation in *Chenopodium quinoa* Willd. cv. Hualhuas embryos. **Plant and Soil**, [s. l.], v. 354, p. 69–79, 2012.

CAMPBELL, Scott A. et al. Dehydrins : genes , proteins , and associations with phenotypic traits. **New Phytopogist**, [s. l.], v. 137, n. 1, p. 61–74, 1997.

CLOSE, Timothy J. et al. An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. **Plant Physiology** [s. l.], v. 101, n. 3, p. 773–779, 1993. Disponível em : <https://www.jstor.org/stable/4275035>

CLOSE, Timothy J. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. **Physiologia Plantarum**, [s. l.], v. 97, n. 4, p. 795–803, 1996.

CLOSE, Timothy J. Dehydrins: A commonalty in the response of plants to dehydration and low temperature. **Physiologia Plantarum**, [s. l.], v. 100, n. 2, p. 291–296, 1997.

COSGROVE, D. J. Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. **The Plant Cell Online**, [s. l.], v. 9, n. 7, p. 1031–1041, 1997. Disponível em: <http://www.plantcell.org/cgi/doi/10.1105/tpc.9.7.1031>

DOS SANTOS, André Luis Wendt et al. Protein expression during seed

development in *Araucaria angustifolia* : transient accumulation of class IV chitinases and arabinogalactan proteins. **Physiologia Plantarum**, [s. l.], p. 138–148, 2006.

DOS SANTOS, André Luis Wendt et al. Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential. **Journal of Proteomics**, [s. l.], v. 130, p. 180–189, 2016. Disponível em: <http://dx.doi.org/10.1016/j.jprot.2015.09.027>

DURZAN, D. J. Process control in somatic polyembryogenesis. In:HÄLLGREN, J. (Ed.). **Proceedings of Frans Symposium Department of Forest Genetics and Plant Physiology'**. Swedish: University of Agricultural Sciences, 1988. p. 8–147.

ELBL, Paula et al. Comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine, *Araucaria angustifolia* (Bertol.) Kuntze. **Plant Cell, Tissue and Organ Culture**, [s. l.], v. 120, n. 3, p. 903–915, 2014.

ESPINDOLA, L. Salmen et al. Cellular and metabolic damage induced by desiccation in recalcitrant *Araucaria angustifolia* embryos. **Seed Science Research**, [s. l.], v. 4, n. 2, p. 193–201, 1994.

FARIAS-SOARES, Francine L. et al. The transition of Pro-embryogenic masses to somatic embryos in *Araucaria angustifolia* (Bertol.) Kuntze is related to the endogenous contents of IAA , ABA and polyamines. **Acta Physiologiae Plantarum**, [s. l.], n. 36, p. 1853–1865, 2014.

FARRANT, J. M.; PAMMENTER, N. W.; BERJAK, P. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. **Planta**, [s. l.], p. 189–198, 1989.

FARRANT, Jill M. et al. Presence of dehydrin - like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may be related to habitat. **Seed**, [s. l.], v. 6, n. 4, p. 175–182, 1996.

FERGUSON, M. **Contributions to the knowledge of the life history of Pinus with special reference to development of the gametophytes**

and fertilization. 6. ed. Washington: Proceedings of the Washington Academy of Sciences, 1904.

FILONOVA, L. H. et al. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, *Norway spruce*. **Journal of cell science**, [s. l.], v. 113, n. 24, p. 4399–4411, 2000.

FILONOVA, L. H. et al. Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. **Cell Death and Differentiation**, [s. l.], v. 9, n. 10, p. 1057–1062, 2002.

FILSON, Paul B.; BENJAMIN, E. Dawson-Andoh. Characterization of sugars from model and enzyme-mediated pulp hydrolyzates using high-performance liquid chromatography coupled to evaporative light scattering detection. **Bioresource Technology**, [s. l.], v. 100, n. 24, p. 6661–6664, 2009. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2008.12.067>

FINCH-SAVAGE, W. E.; PRAMANIK, S. K.; BEWLEY, J. D. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. **Planta**, [s. l.], v. 193, n. 4, p. 478–485, 1994.

FRAGA, Hugo P. F. et al. Time-lapse cell tracking reveals morphohistological features in somatic embryogenesis of *Araucaria angustifolia* (Bert.). **Trees**, [s. l.], v. 29, n. 5, p. 1613–1623, 2015.

GAHAN, Peter G. **Plant histochemistry and cytochemistry: an introduction**. Califórnia: Academic Press, 1984.

GORDON, E; MCCANDLESS, L. Ultrastructure and histochemistry of *Chondrus crispus* Stackhouse. In: HARVEY, M. J.; MCLACHLAN, J. (Eds.). *Chondrus Crispus*: **TNScot Inst Sci**. Halifax. p. 111–113.

GÖSSLOVÁ, Monika et al. Comparing carbohydrate status during *Norway spruce* seed development and somatic embryo formation. **In vitro Cell. Dev. Biol**, [s. l.], v. 37, n. February, p. 24–28, 2001.

GUERRA, Miguel Pedro et al. Somatic embryogenesis in *Araucaria*

angustifolia (Bert) O. Ktze. In: JAIN, S. Mohan; GUPTA, Pramod K.; NEWTON, Ronald J. (Eds.). **Somatic embryogenesis in woody plants.** Dordrecht: Kluwer Academic Publishers, 2000. p. 457–478.

GUERRA, Miguel Pedro et al. Exploração, manejo e conservação da araucária (Araucaria. In: SIMÕES, Luciana Lopes; LINO, Clayton Ferreira (Eds.). **Sustentável Mata Atlântica a exploração de seus recursos florestais Organizadores**. São Paulo: Senac São Paulo, 2002. p. 2015.

GUERRA, Miguel Pedro et al. Evolução, ontogênese e diversidade genética em *Araucaria angustifolia*. In: BARBIERI, Rosa Lía; STUMPF, Elisabeth Regina Tempel (Eds.). **Origem e evoluçao de plantas cultivadas.** Brasília DF: Embrapa, 2008. p. 149–184.

GUERRA, Miguel Pedro et al. Araucaria Evolution, ontogenesis and genetic diversity. In: BARBIERI, R.; STUMPF, E. R. (Eds.). **Origin and evolution of cultivated plants.** 1. ed. Brasília DF: Embrapa, 2012. p. 151–186.

GUMILEVSKAYA, N. A.; AZARKOVICH, M. I. Identification and characterization of dehydrins in horse chestnut recalcitrant seeds. **Russian Journal of Plant Physiology**, [s. l.], v. 57, n. 6, p. 918–924, 2010. Disponível em: <http://link.springer.com/10.1134/S1021443710060154

GUTIERREZ, Laurent et al. Combined networks regulating seed maturation. **Trends in Plant Science**, [s. l.], v. 12, n. 7, p. 294–300, 2007.

HAINES, R. J. **The embryology of** *Araucaria juss***.** 1981. University of New England, Armidale, New South Wales, [s. l.], 1981.

HAINES, R. J. Embryo development and anatomy in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 31, n. 2, p. 125–140, 1983.

HAINES, R. J.; PRAKASH, N. Pro-embryo development and suspensor elongation in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 28, n. 6, p. 511–522, 1980.

HOEKSTRA, Folkert A.; GOLOVINA, Elena A.; BUITINK, Julia. Mechanism of plant desiccation tolerance. **Trends in Plant Science**, [s. l.], v. 6, n. 9, p. 431–438, 2001.

JOHANSEN, Donald Alexander. **Plant embryology: embryology of the spermatophyta.** Massachusetts: Waltham: Chronica Botanica Company, 1950.

KING, Janice E.; GIFFORD, David J. Amino acid utilization in seeds of *Loblolly pine* during germination and early seedling growth. **Plant Physiol**, [s. l.], n. 113, p. 1125–1135, 1997.

KLEINWACHTER, Maik et al. Dehydrin expression in seeds: an issue of maturation drying. **Frontiers in Plant Science**, [s. l.], v. 5, n. August, p. 1–3, 2014. Disponível em: <http://journal.frontiersin.org/article/10.3389/fpls.2014.00402/abstract>

KOROL, A.; PREYGEL, I.; PREYGEL, S. **Recombination Variability and Evolution**. 1. ed. London: Chapman Hall, 1994.

KUHN, Sofia Aumond; MARIATH, Jorge Ernesto de Araujo. Reproductive biology of the “Brazilian pine” (*Araucaria angustifolia* – Araucariaceae): Development of microspores and microgametophytes. **Flora: Morphology, Distribution, Functional Ecology of Plants**, [s. l.], v. 209, n. 5–6, p. 290–298, 2014. Disponível em: <http://dx.doi.org/10.1016/j.flora.2014.02.009>

LAEMMLI, U. K. Nature Publishing Group. **Nature**, [s. l.], v. 227, n. 15, p. 227–231, 1970.

LEPRINCE, O.; BRONCHART, R.; DELTOUR, R. Changes in starch and soluble sugars in relation to the acquisition of desiccation tolerance during maturation of Brassica campestris seed. **Plant Cell and Environment**, [s. l.], n. 13, p. 539–549, 1990.

LIPAVSKA, Helena; KONRÁDOVÁ, Hana. Invited review: Somatic embryogenesis in conifers: the role of carbohydrate metabolism. **In vitro Cell. Dev. Biol**, [s. l.], n. February, p. 23–30, 2004.

MANTOVANI, Adelar; MORELLATO, L. Patrícia C.; REIS, Maurício Sedrez Dos. Fenologia reprodutiva e produção de sementes em *Araucaria angustifolia* (Bert.) O. Kuntze 1. **Revista Brasil. Bot**, [s. l.], v. 3, n. 27, p. 787–796, 2004.

MATTOS, João Rodrigues. **O Pinheiro Brasileiro**. 2º ed ed. Lages - SC: Artes gráficas Princesa Ltda., 1994.

MATTOS, João Rodrigues. **O pinheiro brasileiro**. Ed. da UFS ed. Florianópolis.

NAVARRO, Bruno V et al. Carbohydrate-mediated responses during zygotic and early somatic embryogenesis in the endangered conifer , *Araucaria angustifolia*. **Plos one**, [s. l.], p. 1–20, 2017.

OLIVEIRA, Leandro F. De et al. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. **Tree Physiology**, [s. l.], n. 37, p. 116–130, 2016.

OURIQUES, L.; BOUZON, Z. Organização estrutural e ultra-estrutural das células vegetativas e da estrutura plurilocular de *Hincksia mitchellia* (Harvey). P.C Silva (Ectocarpales, Phaeophyceae). **Rodriguese**, [s. l.], v. 56, p. 435–477, 2008.

OWENS, John N. et al. The reproductive biology of kauri (*Agathis australis*). 1. pollination and prefertilization development. **International Journal of Plant Sciences**, [s. l.], v. 156, n. 3, p. 257, 1995.

OWENS, John N. **The Reproductive biology of western** *White pine*. 4. ed. Columbia: Prepared for Forest Genetics Council of British Columbia., 2004.

PALUDO, Giovani F. et al. Inferring population trends of *Araucaria angustifolia* (Araucariaceae) using a transition matrix model in an old-growth forest. **Southern Forests: a Journal of Forest Science**, [s. l.], p. 1–7, 2016.

PANZA, Víctor et al. Storage reserves and cellular water in mature seeds of *Araucaria angustifolia*. **Botanical Journal of the Linnean Society**, [s. l.], n. 140, p. 273–281, 2002.

PULLMAN, Gerald S.; BUCHANAN, Mike. Identification and quantitative analysis of stage-specific carbohydrates in loblolly pine (*Pinus taeda*) zygotic embryo and female gametophyte tissues. **Tree Physiology**, [s. l.], v. 28, n. August, p. 985–996, 2008.

REITZ, Raulino; KLEIN, Roberto M. **Araucariáceas. Flora Ilustrada Catarinense**. Itajai: Herbário Barbosa Rodrigues, 1966.

RIBEIRO, Milton Cezar et al. The Brazilian Atlantic Forest : How much is left , and how is the remaining forest distributed? Implications for conservation. **Biological Conservation**, [s. l.], v. 142, n. 6, p. 1141–1153, 2009. Disponível em: <http://dx.doi.org/10.1016/j.biocon.2009.02.021>

ROGGE-RENNER, Gladys Daniela et al. Ontogenia de megaestróbilos de *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae) Ontogeny. **Acta biologica catarinese**, [s. l.], v. 4, n. 2, p. 30–41, 2017.

ROLLAND, Filip; BAENA-GONZALEZ, Elena; SHEEN, Jen. Sugar Sensing and Signaling in Plants : Conserved and Novel Mechanisms. **Annu Rev Plant Biol**, [s. l.], v. 57, p. 675–712, 2006.

SANT'ANNA, Cristina S. et al. Realized pollen and seed dispersal within a continuous population of the dioecious coniferous Brazilian pine [*Araucaria angustifolia* (Bertol.) Kuntze]. **Conservation Genetics**, [s. l.], v. 14, n. 3, p. 601–613, 2013.

SCHMIDT, Éder C. et al. Effects of UVB radiation on the agarophyte *Gracilaria domingensis* (Rhodophyta, Gracilariales): Changes in cell organization, growth and photosynthetic performance. **Micron**, [s. l.], v. 41, n. 8, p. 919–930, 2010.

SCHMIDT, Éder Carlos et al. Changes in ultrastructure and histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta , Gigartinales), as a consequence of ultraviolet B radiation

exposure. **Micron**, [s. l.], v. 40, p. 860–869, 2009.

SCOTT, P.; LYNE, R. L.; REES, T. Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L .). **Planta**, [s. l.], v. 44, p. 435–441, 1995.

SETOGUCHI, Hiroaki et al. Phylogenetic relationships within Araucariaceae based on rbcl gene sequences. **American Journal of Botany**, [s. l.], v. 85, n. 11, p. 1507–1516, 1998.

SHIBATA, Marília et al. Physiological and physical quality of local *Araucaria angustifolia* seed variety. **Acta Scientiarum**, [s. l.], v. 38, n. 2, p. 249–256, 2016.

SHIBATA, Marília; MARIA, Cileide; COELHO, Medeiros. Early harvest increases post-harvest physiological quality of *Araucaria angustifolia* (Araucariaceae) seeds. **Biología Tropical**, [s. l.], v. 64, n. June, p. 885–896, 2016.

SHIMOYA, C. **Contribuição ao estudo do ciclo biológico de** *Araucaria angustifolia* **(Bert.) O. Ktze**. 2. ed. Viçosa: Experimentiae, 1962.

SILVA, Camila Vieira Da; REIS, Maurício Sedrez Dos. Brazilian pine nuts' production in Caçador's region, SC: aspects of the attainment and its importance for local communities. **Ciência Florestal**, [s. l.], v. 19, n. 4, p. 363–374, 2009.

SILVEIRA, Vanildo et al. Effect of plant growth regulators on the cellular growth and levels of intracellular protein , starch and polyamines in suspension cultures of *Pinus taeda*. **Plant Cell, Tissue and Organ Culture**, [s. l.], v. 76, p. 53–60, 2004.

SINGH, H. Embryology of gymnosperms. In: ZIMMERMAN, W. et al. (Eds.). **Handbuch der Pflanzenanatomie**. Berlim, Stuttgar. p. 187–241.

STEFENON, Marcos Valdir et al. Integrating approaches towards the conservation of forest genetic resources: a case study of *Araucaria angustifolia*. **Biodiversity Conservation**, [s. l.], v. 18, p. 2433–2448,

2009.

STEINER, Neusa et al. Effect of carbon source on morphology and histodifferentiation of *Angustifolia angustifolia* embryogenic cultures. **Brazilian Archives of Biology and Technology**, [s. l.], v. 48, n. November, p. 895–903, 2005.

STEINER, Neusa et al. Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. **Plant Cell, Tissue and Organ Culture**, [s. l.], v. 89, n. 1, p. 55–62, 2007.

STEINER, Neusa et al. Toward establishing a morphological and ultrastructural characterization of Pro-embryogenic masses and early somatic embryos of *Araucaria angustifolia* (Bert .) O . Kuntze. **Protoplasma**, [s. l.], 2015. Disponível em: <http://dx.doi.org/10.1007/s00709-015-0827-0>

STOCKEY, Ruth A.; KO, Helen. Cuticle micrimorphology of *Araucaria juss*. **Botanical Gazete**, [s. l.], v. 147, n. 4, p. 508–548, 1986.

THOMAS, P. *Araucaria angustifolia*. 2013. Disponível em: <https://www.iucnredlist.org/species/32975/2829141>.

TOMPSETT, P. Desiccation studies in relation to the storage of Araucaria seed. **Annals of applied Biology**, [s. l.], v. 105, n. 3, p. 581–586, 1984.

WANG, Lu; RUAN, Yong-ling. Regulation of cell division and expansion by sugar and auxin signaling. **Plant science**, [s. l.], v. 4, n. May, p. 1–9, 2013.

WEBER, Hans et al. Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: Expression patterns, metabolic regulation and implications for seed development. **Plant Journal**, [s. l.], v. 9, n. 6, p. 841–850, 1996.

WEBER, Hans; BORISJUK, Ljudmilla; WOBUS, Ulrich. Molecular physiology of legume seed development. **Annual Review of Plant Biology**, [s. l.], v. 56, p. 253–279, 2005.

WILLIAMS, Claire G. **Conifer Reproductive Biology**. New York: Springer, 2009.

WINKELMANN, Traud. **Somatic Versus Zygotic Embryogenesis: Learning from Seeds: In Vitro Embryogenesis in Higher Plants**. New York: Humana Press, 2016.

ZANETTE, Flávio et al. Particularidades e biologia reprodutiva de *Araucaria angustifolia*. In: WENDLING, Ivar; ZANETTE, Flávio (Eds.). **Araucária particularidades, propagação e manejo de plantios**. 1. ed. Brasília DF: Embrapa, 2017. p. 15–39.

ZECHINI, Aleex Anderson et al. Genetic Conservation of Brazilian Pine (*Araucaria angustifolia*) Through Traditional Land Use. **Economic Botany**, [s. l.], n. 10, p. 1–14, 2018.

ZECHINI, Alex Anderson et al. Produção, comercialização e identificação de variedades de pinhão no entorno da Floresta Nacional de Três Barras–SC. **Revista Biodiversidade Brasileira**, [s. l.], v. 2, n. 2, p. 74–82, 2012. Disponível em: http://www.icmbio.gov.br/revistaeletronica/index.php/BioBR/article/view/275>

4 OBJETIVOS

4.1 OBJETIVO GERAL

Estudar e caracterizar aspectos morfo-anatômicos, o perfil de carboidratos e o acúmulo de dehidrinas ao longo do desenvolvimento dos embriões zigóticos de *Araucaria angustifolia*, visando gerar subsídios para a conservação das sementes desta espécie.

4.2 OBJETIVOS ESPECÍFICOS

- Caracterizar aspectos morfo-anatômicos e ultra-estruturais da pré-fertilização e do desenvolvimento de embriões zigóticos de *A. angustifolia.*

- Estudar a presença das proteínas *LEA*, especificamente as dehidrinas e o perfil dos carboidratos em estágios específicos do desenvolvimento dos embriões zigóticos de *A. angustifolia*.

Esta dissertação foi organizada em dois capítulos.

Neste capítulo, as imagens da Figura 1 a 5, foram reestruturadas e reinterpretadas a partir da tese de doutorado de Gladys Rogge-Renner com a autorização da devida autora. Este capítulo retoma estes dados e finaliza o estudo do desenvolvimento do embrião zigótico de *Araucaria angustifolia* resultando em um artigo no qual Daniela Goeten e Gladys Daniela Rogge-Renner se configuram como as primeiras autoras.

5 CAPÍTULO I

SEED ONTOGENY OF BRAZILIAN PINE *Araucaria angustifolia* **(ARAUCARIACEAE): PREFERTILIZATION AND EMBRYO DEVELOPMENT**

ABSTRACT

Zygotic embryogenesis of Brazilian pine *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae) native from Brazil is not yet fully understood. The present work aimed at to study its seed ontogenesis including those events occurring between pre-fertilization and the late embryo formation, characterizing the different cell types, by means of histochemistry and microscopy analyses. The megastrobili in development were collected monthly from August 2011 to July 2013, on a natural population in the southern Brazil. The Brazilian pine has a two - year reproductive cycle to produce mature seeds, the pollination occurring from August to September of the first year of the cycle, fertilization about one year after pollination, and mature seeds from March to July of the second reproductive year. The mother cell of the megaspore is observed in the nucellus from fertile scale collected during the month of November of the first year of the reproductive cycle. A free nuclear megagametophyte is observed in plant material collected from December to July of the first year of the reproductive cycle. During these seven months, nucellus central region cells organized in layers in the ellipsoidal shape, indicating free nuclear division to formation initial megagametophyte. After this period, from August to September, the megagametophyte develops rapidly, and in October archegonia matured and fertilization occurred. In November it was identified supernumerary pro-embryos on the same seed, indicating the occurrence of polyembryony, however only one pro-embryo completes its development. At pro-embryonic stage, three different cell types were noted: cap cells (cc), embryonic cells (EC) and suspensor cells (SC). In the middle of December pro-embryo through a stage transition in which cap cells degenerating an early embryo was observed in February. From March, twenty-one days after pollination late embryo was found. This undergone intense differentiation and a hypocotyl axis whit meristem apical meristem (SAM) and root apical meristem (RAM) and two cotyledons were present in the months of June to July. Our data describe for the first time the complete zygotic embryo development and our findings contribute to evolutionary knowledge of this species.

ADDITIONAL KEYWORDS: Nucellus, Megagametophyte, Archegonium.

ABREVIATIONS

CBB	Coomassie brilliant blue
CLSM	Confocal laser scanning microscopy
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
PI	Propidium iodide
LM	Light microscopy
PAS	Periodic acid-schiff
RAM	Root apical meristem
SAM	Shoot apical meristem
TB-O	Toluidine blue
TEM	Transmission electron microscopy

5.1 INTRODUCTION

Araucariaceae family is a Southern Hemisphere conifer family with a restricted distribution that holds thirty species distributed in three genera, *Agathis* Salisbury, *Araucaria* Juss. and *Wollemia* Jones, is a Southern Hemisphere conifer family with a restricted distribution (Setoguchi et al., 1998). The *Araucaria* genus has eighteen species (Stockey; Ko, 1986) distributed throughout South America and New Caledonia, New Guinea, Australia and New Zealand (Setoguchi et al., 1998). *Araucaria angustifolia* (Bertol.) Kuntze, also known as Brazilian pine, is concentrated in South and Southeastern Brazil, with small spots in the northwest of Argentina and Paraguay (Farjon; Filer, 2013; Guerra et al., 2008, 2012). It is a dioecious native conifer species, rarely monoecious after trauma or disease, with unisexual structures (Reitz; Klein, 1966). The reproductive biology of the Araucariaceae family is not well understood, and most studies have focused on one or two species of each genus (Singh, 1978). Some previous studies in *A. angustifolia* reported that it takes almost two years from pollination to seed maturity, similar to other members of the Araucariaceae family (Singh, 1978; Haines, 1981; Owens et al., 1995; Mantovani; Morellato; Reis, 2004; Anselmini; Zanette; Bona, 2006). *A. angustifolia* is considered a dioecious species and the adult male or female plants produce microstrobili or megastrobili, respectively, which are the reproductive structures. These structures consist of fertile and infertile scales, attached to a central axis. Microsporophylls are male fertile scale that store microsporangia and megasporophylls are female fertile scales, which store prothalial tissue, also called megasporangia (Reitz; Klein, 1966; Guerra et al., 2008, 2012; Mattos, 2011; Williams, 2009; Kuhn; Mariath, 2014). In conifers, microsporogenesis starts from meiosis of a microsporocyte or pollen mother cells, that are located inside microsporangia, producing four haploid microspores and each one develop a cell wall and form a pollen grain (Ferguson, 1904; Korol; Preygel; Preygel, 1994; Williams, 2009; Kuhn; Mariath, 2014). Megasporogenesis in conifers takes place on prothalial tissue, whose nucellar tissue form sporogenous cells, giving rise to a megasporocyte or megaspore mother cell that undergoes meiosis. Cell division results in four megaspores and the one closest to the chalazal end survives and becames a multicellular, translucent megagametophyte, with one or more archegonia inside, as was described for *A. australis* (Owens et al., 1995),

Pinus sp. (Williams, 2009) and *A. angustifolia* (Burlingame, 1915; Johansen, 1950; Shimoya, 1962; Mattos, 2011). Despite these previous studies, the prefertilization, fertilization and cellular features embryo development was not described yet for *A. angustifolia*. Seed production (pinion) highlights the economic, ecological and cultural importance of *A. angustifolia* (Guerra et al., 2002, 2008, 2012; Silva; Reis, 2009). As a result of wide exploitation not only of seed but also wood, this species was included as a vulnerable species and more recently classified as critically endangered by the International Union for Conservation of Nature (Thomas, 2013). In *A. angustifolia*, many studies about genetic diversity and seed harvest have been reported addressing to identify priorities of populations and conservation zones (Auler et al., 2002; Mantovani; Morellato; Reis, 2004; Ribeiro et al., 2009; Paludo et al., 2016; Zechini et al., 2018). However, the conservation of *A. angustifolia* seeds is difficult because they are recalcitrant, losing viability when dried, making storage for long periods difficult (Espindola et al., 1994; Farias-Soares et al., 2013; Tompsett, 1984).

In gymnosperms, the fertilization process it is complex and megagametophyte starts developing before fertilization and represents a significant investment of maternal resources, which are wasted if fertilization does not occur (Steeves; Sussex, 1989). In this group of seeds the development of multiple embryos does occur, however only one embryo survives, while the subordinate embryos are eliminated by programmed cell death (Steeves; Sussex, 1989; Filonova et al., 2000, 2002; Bozhkov; Filonova; Suarez, 2005; Williams, 2009; Agapito-Tenfen et al., 2011). The overall embryo development pathway can be divided into three phases, (i) Pro-embryogeny: includes the stages before the elongation of the suspensor system, (ii) early embryogeny: initiates with the elongation of the suspensor system and terminates before establishment of root meristem and (iii) late embryogeny: culminates with the maturation of the embryo (Singh, 1978). Embryos grow and develop within the corrosion cavity of the megagametophyte, which houses the majority of the storage reserves of the seed (King; Gifford, 1997). In particular, little is known about cellular biology during *A. angustifolia* zygotic embryo development. The available literature on sexual reproductive biology of *Araucaria* genus is old, and some aspects have never been described or illustrated. Most studies have investigated biochemical and physiological aspects of *A. angustifolia* zygotic embryo development (Panza et al. 2002; Astarita; Handro; Floh, 2003; Agapito-

Tenfen et al. 2011; Balbuena et al. 2009, 2011; Farias-Soares et al. 2013; Shibata et al. 2016). However, few studies have reported on the cellular changes which occur from prefertilization to the late zygotic embryo of *A. angustifolia* (Shimoya, 1962; Guerra et al., 2008, 2012), and the fertilization time was not characterized. The lack of this information could be associated to the difficulty to access deep tissues in the seeds as well as the fact that the reproductive cycle of all Araucariaceae species to have a two-year course (Owens et al., 1995).

Frequently, in conifers, in the reproductive biology as well in biochemistry studies, embryo development stage has been erroneously reported, and has been associated to the nomenclature of angiosperm embryo development. Thus far, we are not aware of another study that has reported cellular, histochemical and ultrastructural alterations that occur during the complete embryo development and female reproductive structures of *A. angustifolia*. On the other hand, in the somatic embryo development of this species, structural and biochemical aspects has been abundantly reported (Silveira et al. 2004; Steiner et al. 2005, 2007, 2015; Farias-Soares et al. 2014; Fraga et al. 2015). Based on this and considering the ecological and economic importance of this species, as well the vulnerability once it is classified as "critically endangered" by the International Union for Conservation of Nature, we elucidate the fate map of *A. angustifolia* seed biology development. The present study described the morphological, histological and ultrastructural characteristics from the pre-fertilization until development of late embryo. Some processes of which are shown here for the first time using optical, electronic and confocal microscopy, as well as histochemical analysis.

5.2 MATERIAL AND METHODS

5.2.1 Plant material

A. angustifolia megastrobili from the first and the second reproductive cycle year, were collected monthly from August 2011 to July 2013, preselected from an open-pollinated trees in a natural population located in the county of Curitibanos (960 m altitude, 27º18’11” south latitude and 50°38’12” west longitude), Santa Catarina State, Brazil. Until processing, megastrobili were kept at 4ºC. Then fertile

scales were selected, and the ovules were excised to process for different microscopy techniques.

5.2.2 Morphological analyses

Pro-embryo morphology was monitored in stereomicroscope monthly by double staining with Evans blue (0.1% w/v) and acetocarmine (2% w/v) using procedures described by Durzan (1988)The observations were performed at the Laboratory of Developmental Physiology and Plant Genetics (LDPPG) using an Olympus BX 40 microscope equipped with Olympus DP71 capture images with the DP Controller software system.

5.2.3 Light microscope (LM)

Light microscopy analyses were carried out according to (Schmidt et al. 2009). Ovules in different developmental phases (3 mm length or more) and megagametophytes without integuments (7 mm in length or more) were fixed in 2.5 % paraformaldehyde in 0.1 M (pH 7.2) phosphate buffer overnight. Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions. After dehydration, the samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Sections (4 µm) were stained with different histochemical techniques: Periodic Acid-Schiff (PAS) to identify neutral polysaccharides (Gahan, 1984); Toluidine Blue (TB-O) 0.5 %, pH 3.0 (Merck Darmstadt, Germany) to identify acid polysaccharides (Gordon; Mccandless, 1973), and Coomassie Brilliant Blue (CBB) 0.4 % in Clarke's solution (Serva, Heidelberg, Germany) to identify proteins (Gahan, 1984; Schmidt et al., 2010) . Some sections were double stained with PAS+CBB (Schmidt et al., 2012). Controls consisted of applying solutions to sections without the staining component (e.g., omission of periodic acid application in the PAS reaction). LM sections were analyzed with an Epifluorescence (Olympus BX 41) microscope equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA).

5.2.4 Confocal laser scanning microscopy (CLSM)

Immature embryos, also called Pro-embryos, were carefully obtained by making longitudinal cuts on megagametophytes collected

from November to December 2011 of the second reproductive cycle year and placed on glass slides. To study Pro-embryos nuclei, two drops of 0.5 mg. mL^{-1} DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, St. Louis, MO) were added on plant material on glass slides for 50 minutes (Ouriques; Bouzon, 2008) or Pro-embryos were treated with 1 mM of the fluorochrome propidium iodide (PI) (Sigma-Aldirch, St Louis, MO, USA) for 30 min on glass slides. Colorless nail polish was used to seal the glass slides. Fluorescence in the nuclei was observed in UV-diode laser with a 405 nm excitation wavelength for DAPI visualization and laser emission spectrum between 510-566 nm. After 30 min with PI, the samples were excited at 532 nm with detection between 595 and 730nm and analysis by CLSM. A confocal laser scanning microscope (Leica TCS SP-5, Wetzlar, Germany) was used with the Leica HCX PLAPO lambda 63×/1.4–0.6 oil immersion objective was fitted on the inverted fluorescent microscope. The LAS-AF Lite program (Leica) was used for final processing of the confocal images.

5.2.5 Fluorescence microscopy (FM)

Pro-embryos were obtained from plant material collected from November to December 2011 of the second reproductive cycle year and prepared with DAPI or PI, as previously described for CSLM procedures. DAPI-stained nuclei were observed with the U-MWU2 (Olympus Ultraviolet Mirror Blue Wide Unit) blue filter, with excitation wavelength between 330 and 385 nm and emission spectrum at 420nm. Nuclei stained with PI were observed with a U-MGW2 (Olympus Ultraviolet Mirror Green Wide) red filter, with excitation wavelength between 510 and 550 nm and emission at 590nm. All fluorescence analyses were performed with an Olympus BX 41 microscope equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA).

5.2.6 Transmission electron microscope (TEM)

For observation under the transmission electron microscope (TEM), Pro-embryos that were obtained from plant material collected from November to December 2011 of the second reproductive cycle year were embedded in agarose (6g/L, Sigma-Aldrich; low gelling temperature, as described for light microscopy) and fixed in 4 % paraformaldehyde with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate

buffer (pH 7.2) overnight. Pro-embryo samples were post-fixed with 1% osmium tetroxide for four hours, dehydrated in a graded acetone series, and then embedded in Spurr's resin (Schmidt et al., 2009). Thin sections (70 nm) were stained with aqueous uranyl acetate followed by lead citrate. Two grids were then examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV) for each Pro-embryo sample.

5.3 RESULTS

5.3.1 Pre-fertilization

At the end of October 2012, approximately one month after pollination, which occurred from August to September in 2011, during the first year of the reproductive cycle, green megastrobili (seed cone) had an average diameter of 6 cm, length of 9 cm and weight of 20 g (Figure 1a). The longitudinal megastrobili section showed a central axis and scales with light beige color (Figure 1b). At this stage, the scales were 1 cm in length and the fertile scales at their ends were beige color indicating the onset of the development of the ovule (Figure 1c) the micropyles could be seen (Figure 1d). In light microscopy, fertile scale section when stained with TB-O, showed an orthochromatic reaction and indicated the presence ovule as a lump (Figure 1e). In November, integument and nucellus were observed under light microscopy longitudinal sections, with sporogenous cells emerging on the nucellus (Figures 1f, g). Ovule and nucellus regions when subjected to PAS showed a positive reaction with neutral polysaccharides (e.g., cellulose and starch), especially the central region of the nucellus (Figure 1g). In the distal half of the nucellus, an enlarged megaspore mother cell (megasporocyte) was surrounded by numerous darkly stained sporogenous cells (Figure 1h). Megaspore mother cell highlighted a blue central nucleus and slightly stained cytoplasm while sporogenous cells reacted strongly to PAS when double staining was used (Figure 1h). Fertile scale section collected in December 2011 showed an early free nuclear megagametophyte cell in place of a megaspore mother cell. This initial cell was blue colored on double staining indicating protein presence (Figures 1i, j). From January to July 2012 fertile scale sections showed in nucellus central region with cells organized in layers in the ellipsoidal shape, indicating free nuclear division to formation initial megagametophyte (Figure 1k). Cell wall formation of each free nucleus

was observed only in August 2012. Section stained with TB-O showed that cell division and elongation occurred filling the entire space of the central cavity with prothallial cells, which would give rise to archegonia (Figure 1l).

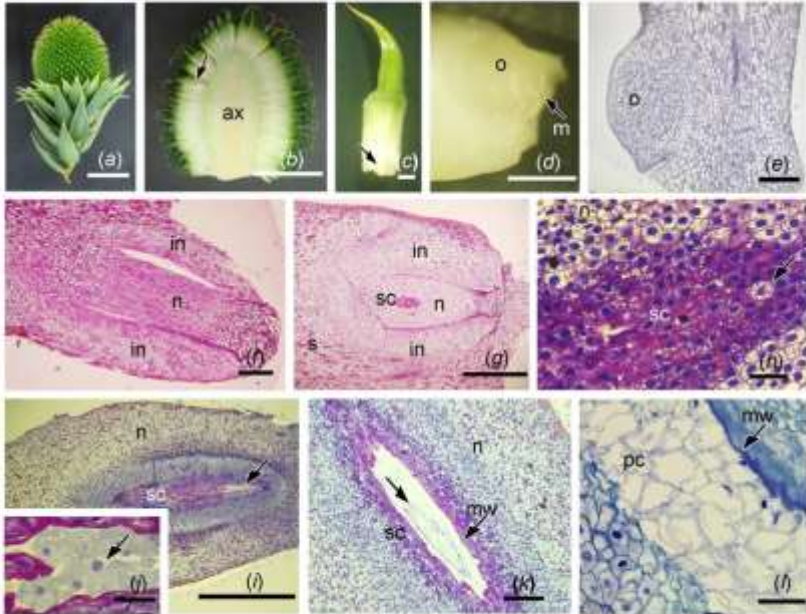

Figure 1: General features of *A. angustifolia* megastrobili (*a-d*) and light microscopy (*e-l*). Megastrobili (*a*) and in longitudinal section (*b*) showing axis (ax), fertile scale (arrow) (*c*) and ovule (o) (*d*) with micropyle (m) (arrow). Light microscopy of median longitudinal fertile scale (*e*), showing ovule (o). Ovule with PAS staining (*f-g*) showing integument (in), nucellus (n) (*f-g*) and sporogenous cells (sc) (*g*). Megaspore mother cell (arrow) surrounded by sporogenous cells (sc) and nucellus (n) (*h*). Free nuclear megagametophyte (arrow) surrounded by sporogenous cells (sc), nucellus (n) (*i*) and in detail (arrow) (*j*). Free nuclei with ellipsoidal form (*k*) (arrow) surrounded by megaspore wall (mw with arrow), sporogenous cells (sc) and nucellus (n). Prothallial cells (pc) and megaspore wall (mw with arrow) (*l*). Scale bars = 3 cm (*a-b*), 1 mm (*c, d, g, i*), 200 µm (*e-f, h, k*), 500 µm (*j,l*).

5.3.2 Archegonial development

Archegonial development began to be observed approximately

thirteen months after pollination, from September 2012 during second reproductive cycle year in *A. angustifolia*. At archegonial stage, green megastrobili had an average diameter of 8 cm, length of 11 cm and average weight of 40 g (Figure 2a). Scale and nucellus were beige (Figures 2b-c). Megagametophyte sections in early September showed an archegonium surrounded by jacket cells (Figures 2d-e) and in October, several archegonia and pollen tubes were observed on the nucellus (Figure 2f). Mature archegonia surrounded by one or two layers of jacket cells stained darkly showing dense cytoplasm when compared to megagametophytes cells (Figure 2e-g). In archegonia cells blue color indicated the presence of proteins (Figures 2d-f). Up to six archegonia were observed in the same *A. angustifolia* megagametophyte (Figure 2f). On the micropylar nucellus region, PAS and CBB double-stained cells showed blue and pink colors, indicating the presence of total proteins and starch grains (Figure 2f). The archegonial egg nucleus was not centrally located, and vacuoles were observed in the cytoplasm (Figure 2g). When stained by TB-O, jacket cells, archegonial egg nucleus and megagametophyte nucleus cells showed orthochromatic reaction, indicating the absence of acidic polysaccharides (Figure 2g). Archegonial cell wall, jacket cell walls, neck cell walls and megagametophyte cell walls were all stained pink with positive PAS reaction, indicating the presence of cellulose and many starch grains in the neck cells cytoplasm (Figure 2*h*). Jacket and neck cells were observed at the same time in mature archegonium, although they were not found in all same sectional plane when subjected to different histochemical techniques. Archegonia three-dimensional forms difficults nuclei observation in the same histological plans sections which were made (Figure 2*h*).

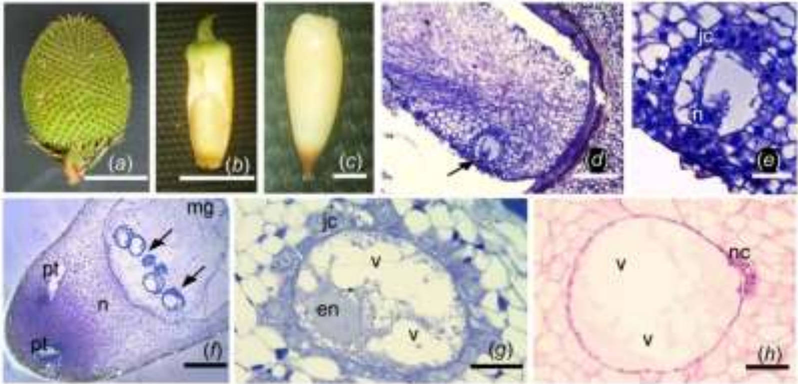

Figure 2: General features of *A. angustifolia* megastrobili (*a-c*) and light microscopy (*d-h*). Megastrobili (*a*), fertile scale (*b*) and nucellus (*c*). Longitudinal sections of megagametophyte (*d*) with one archegonium (arrow) stained with double coloration (*d-e*). Megagametophyte (mg) double stained with six archegonia (arrows) on nucellus (n) and two pollen tubes (pt) (*f*). Archegonium stained with TB-O with egg nucleus (en), vacuoles (v), jacket cells (jc) (*g*) and stained with PAS showing vacuoles (v) and neck cells (nc) (*h*). Scale bars = 5 cm (*a*), 1 cm (*b*), 1 mm (*c*), 200 µm (*d*), 500 µm (*f*), 50 µm (*e, g-h*).

5.3.3 Fertilization and pro-embryogenesis

Fifteen months after pollination, in November 2012, pro-embryos were observed, indicating that the fertilization has just happened, in the second year of the reproductive cycle, at the end of October 2012. At this stage green megastrobilus had an average diameter of 25 cm, length of 13 cm, and weight of 300 g (Figure 3a). Individual seeds were 4 cm in length and beige color (Figure 3b). Megagametophyte was translucent presenting opaque white coloration on the micropylar end (Figure 3c). At this stage it was identified supernumerary Pro-embryos on the same seed indicating the occurrence of polyembryony (Figure 3d). However only a single embryo was identified from each archegonium, which indicate polyzygotic polyembryony. Apical-basal polarity was identified and the proembyos were characterized by apical region reactive to Acetocarmine and stained in red (Figures 3d-e) and a long tail formed by elongated cells stained with Evan's blue (Figure 3e). Under confocal microscopy, DAPI staining showed globular nuclei on apical region (Figures 3f-h) and a deformed nucleus in elongated cells, suggesting nuclear dismantling

(Figures 3f-g). In reaction to PI, which is an indicator of cell death; these cells reacted positively, most likely indicating a Pro-embryo regression (Figures 3i-j).

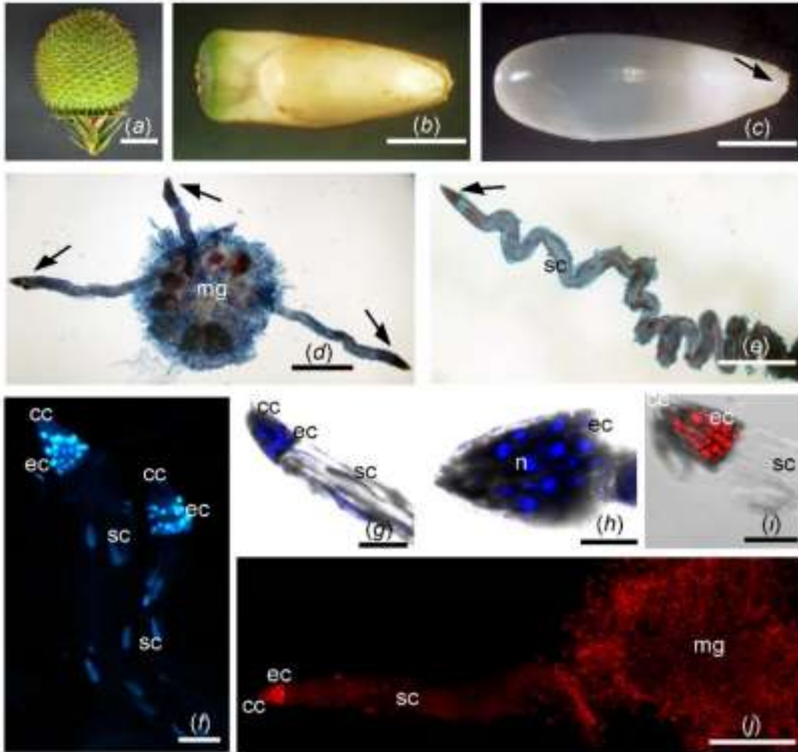

Figure 3: General features (*a-c*) and morphological analyses of *A. angustifolia* with stereomicroscope (*d-e*), fluorescence (*f* and *j*) and confocal microscopy (*g-i*). Megastrobili (*a*), developing seed (*b*) and megagametophyte without teguments showing micropylar end (arrow) (*c*) on opaque white region. Three pro-embryos (arrows) (*d*) leaving the megagametophyte (mg) and one pro-embryo (e) with embryonary region (arrow) and long suspensor (sc) stained with acetocarmine and Evans Blue. Pro-embryos under fluorescence (*f, j*) and confocal microscopy (*g-i*) with DAPI (*f-h*) and with PI (*i-j*), showing cap cells (cc), suspensor cells (sc) and embryonic cells (ec) (*f-j*) with nuclei (n) (*h*) and megagametophyte (mg) (*j*). Scale bars = 5 cm (*a*), 1 cm (*b*), 3 mm (*c*), 500 µm (*d*), 1 mm (*e*), 100 µm (*f-g,j*), 200 µm (*i*), 50 µm (*h*).

At pro-embryonic stage, three different cell types were noted: cap cells (cc), embryonic cells (ec) (Figures 4a-e) and suspensor cells (sc) (Figures 4f-h). In the apical region, the arrangement of cap cells reveals a triangular morphology (Figures 4a-e). Central cap cells were elongated with a narrow base (Figures 4b, c). Lateral cap cells were shorter, with wide base and narrow apex, and nuclei were compressed and small (Figure 4b). Vacuoles were present in the cytoplasm of apical and lateral cap cells and appeared white with TB-O stain (Figures 4b, c). Embryonic cells were polyhedral bearing a large central nuclei showing active chromatin (visible phases of mitosis) and small vacuoles in the cytoplasm (Figure 4b). In response to PAS or double staining plus CBB (Figures 4d-e, respectively), the cell wall of cap cells and embryonic cells appeared thick and pink indicate the presence of polysaccharides. Also, starch grains could be seen in the cytoplasm of embryonic cells (Figure 4d). Basal pro-embryo region was formed by long and vacuolated suspensor cells showing a peripheral and compacted nucleus, not always present, in a degenerating cytoplasm that appear slightly stained with CBB (Figure 4f). Irregular cell wall thickness was detected with PAS (Figure 4g) and with double staining PAS plus CBB, purple cell wall indicates a mixture of polysaccharides and protein while blue cytoplasm showed mainly proteins (Figure 4h).

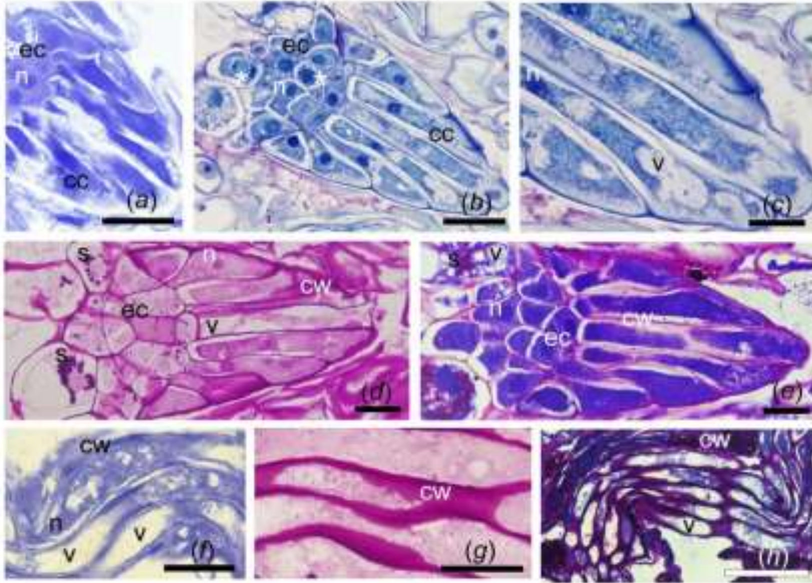

Figure 4: Light microscopy of *A. angustiolia* Pro-embryonic cells. Section of a dominant embryo stained with CBB (*a*) and TB-O (*b-c*) showing cap cells (cc) and embryonic cells (ec) (*a, b*), detail of cap cells with nuclei (n) and vacuoles (v) (c). Staining with PAS (d) and double staining (PAS + CBB) (e) showed starch grains (s), nuclei (n) and vacuoles (v) in embryo cells, and cell wall (cw) marked in pink. Section of suspensor cells stained with CBB (f) PAS (g) and double staining (PAS + CBB) (h) showed many vacuoles (v), nuclei (n) and protein content in blue (f, h) and irregular shape of the cell wall (cw) intensely stained in pink (g). This figure represents events that occur approximately fifteen months after pollination. Scale bars = 50 µm *(b, d, e),* 20 µm *(a, c, h)*, 10 µm *(f, g).*

Ultrastructural analysis showed cap cells (Figures 5a-c) with thin cell wall and dense cytoplasm in the central region (Figure 5a), including starch grains, large and elongated central nucleus with nucleoli (Figure 5b). In the peripheral region close to the cell wall, the cytoplasm appeared disorganized with vacuolated cytoplasm (Figures 5b-c), but it was possible to identify large numbers of mitochondria, vacuoles and Golgi bodies (Figure 5c). Embryonic cells (Figures 5d-f) appeared to be meristematic; with large nucleoli in central nuclei (Figure 5d) where euchromatin and heterochromatin could be distinguished (Figure 5e). Thin cell walls with plasmodesm and the cytoplasm with many

mitochondria and lipid bodies were identified (Figures 5e, f). Suspensor cells (Figures 5g-i) presented a thick cell wall, when compared to cap and embryonic cells (Figure 5g). The cytoplasmic content appeared disorganized and highly vacuolated (Figure 5h). It was possible to identify Golgi bodies, rough endoplasmic reticulum and autophagy vacuole encompassing mitochondria (Figures 5 h,i).

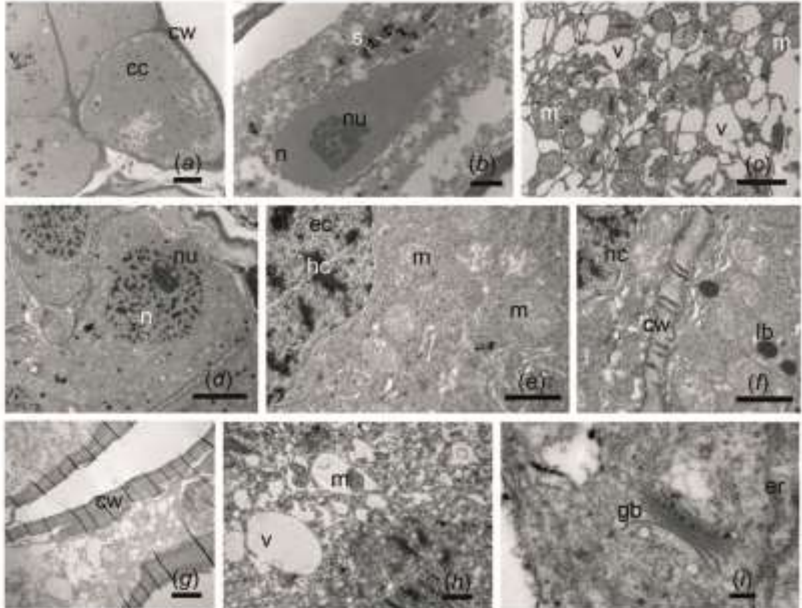

Figure 5: Transmission electron microscopy images of *A. angustifolia* Pro-embryonic cells. Flank cap cells (cc) (*a-c*) showing thick cell wall (cw) (*a*), nuclei (n) with nucleoli (nu) and starch grains (s) (*b*), vacuoles (v) and mitochondria (m) (*c*). Embryonic cells (*d-f*), showing nucleus (n), nucleolus (nu) (*d*), euchromatin (ec), heterochromatin (hc), and mitochondria (m) (*e*), cell wall (cw), lipid bodies (lb) and heterochromatin (hc) (*f*). Suspensor cells (*g-i*) with cell wall (cw) (*g*) vacuoles (v), mitochondria (m) (*h*), rough endoplasmic reticulum (er) and Golgi bodies (gb) (*i*). Scale bars = 5 µm (*a-b,d-e,g*), 2 µm (*c*), 1 µm (*f,h*), 0.2 µm (*i*).

After the regression of the subordinate polyembryos, between early and mid-December 2012, green megastrobilus had an average diameter of 30 cm, length of 17 cm, and weight of 500 g (Figure 6a). Individual seeds are 4 cm in length and beige color (Figure 6b). Megagametophyte were translucent and presented opaque white

coloration on the micropylar region (Figure 6c). In early December, the dominant pro-embryo was formed by cap cells, embryonic and suspensor cells and still was presented a triangular shape in the apical region (Figure 6 d-e). In response to PAS staining cap cells had a thicker cell wall when compared to embryonic cells (Figure 6 e-f). In addition, larger amounts of starch grains in the cytoplasm of cells located most basally in the small embryogenic mass were observed (Figure 6 f). In the basal region of the pro-embryo, the suspensor cells suffered an elongation, showing starch grains dispersed in their cytoplasm (Figure 6 g). At this stage, approximately thirteen months after pollination, occurred transition from pro-embryogenic stage to early embryogenic stage. The transition stage was marked by the of cap cells degeneration (Figure 6 h). In parallel with cap cells degeneration the true embryonal group divided increasing the size embryogenic mass, in function of embryo development by apical cell growth (Figure 6 i). Embryogenic cells in response to CBB reaction showed dense cytoplasm with large nucleus central and presence of proteins (Figure 6 i).

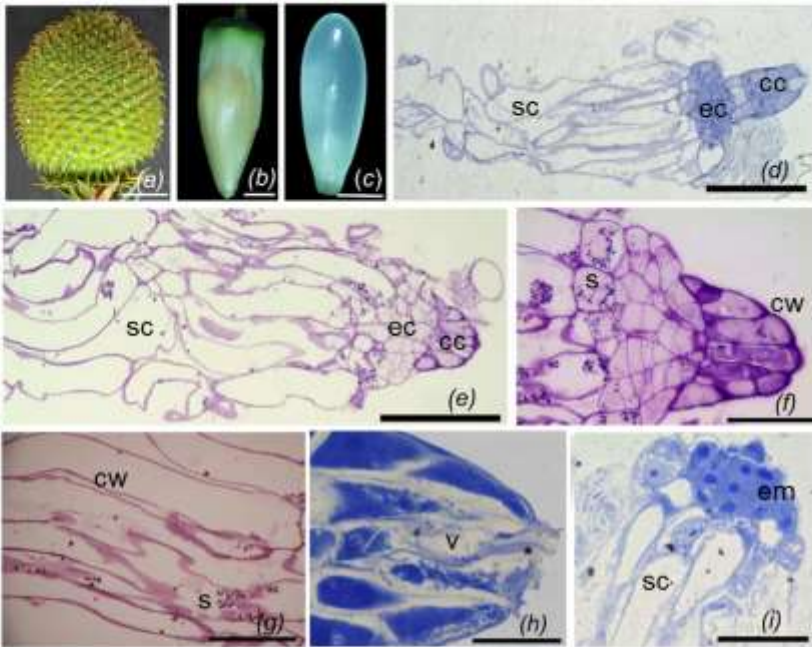

Figure 6 General features of *A. angustifolia* megastrobili *(a-c)* and light microscopy of pro-embryo *(d-j)***.** Megastrobili (*a*), developing seed (*b*), and megagametophyte without teguments (*c*); dominant pro-embryo section stained to AT-O *(d)* and PAS *(e)* showing cap cells (cc) embryonic cells (ec) and suspensor cells (sc). Detail of cap cells (cc), embryonic cells (ec) showed starch grains (s) and cellulosic compounds in the cell wall (cw) *(f)*; detail of elongated suspensor cells (sc) with starch grains (s) and cell wall (cw) *(g)*; section stained with CBB *(h-i)*, cap cells in process of degeneration showing vacuole (v), and cell wall rupture (asterisks) *(h)*; death of the cap cells and increase embryogenic mass (em) composed of cells by embryogenic cells (ec) with evident nucleus (n) *(i)*. Scale bars = 5 cm *(a)*, 1 cm *(b, c)*, 100 µm *(d, e),* 50 µm *(f, i)*, 20 µm *(g, h).*

5.3.4 Early and late embryogenesis - dominant embryo development

After complete degeneration of cap cells, between January and the end of February 2013 the, embryo showed a significant growth, giving rise to an embryo in early embryogenic stage. In this stage, Green megastrobilus had an average diameter of 47 cm length of 23 cm, and weight of 1.464,00 g (Figure 7a). Individual seeds were 4 cm in length and beige color (Figure 7b). Megagametophyte was translucent but presented opaque white coloration on the micropylar (Figure 7c). A mass of embryogenic cells organized into an elongate cylindrical body with a smooth apical region and suspensor system was observed (Figure 7d). In the apical region, embryogenic cells, showed an intense reaction to CBB and PAS and this allowed us to identify a nucleus with nucleoli, dense cytoplasm with starch grain, vacuoles and a thin cell wall with the presence of neutral polysaccharides (Figure 7e). Also, in these cells, TBO-O staining showed intense mitotic activity with anticline and periclinal cell divisions contributing to the axial and radial growth of the embryo proper (Figure 7f). These cellular characteristics become less intense along of the embryo body (Figure 7g), where cells in the central region of the embryo differentiated to form suspensor cells. In response staining double (PAS CBB) these cells showed involved nuclei by starch grain and start of vacuolization (Figure 7h). While in the basal region of embryo, suspensor cells differentiated were elongated and vacuolated with peripheral and compacted nucleus, not always present, in a degenerating cytoplasm where appear slightly stained with CBB (Figure 7i). In reaction with AT-O observed degenerate of primary suspensor cell (Figure 7j).

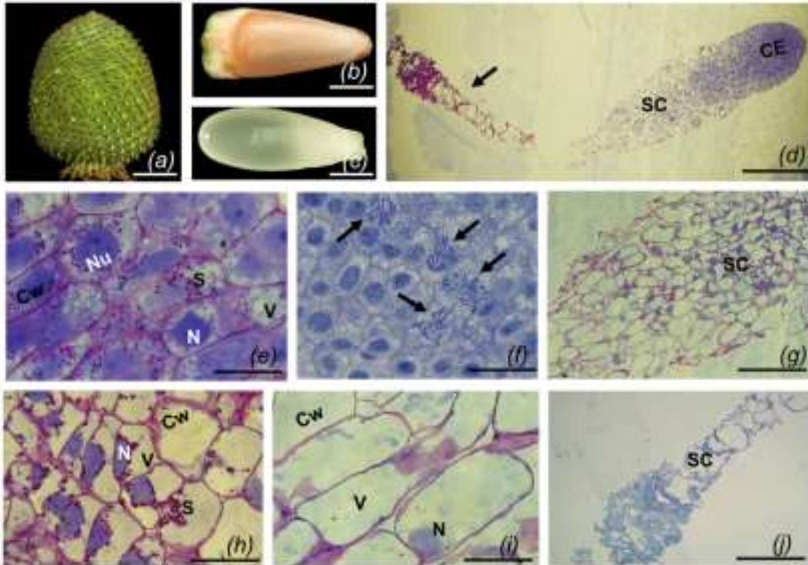

Figure 7: General features of *A. angustifolia* megastrobili (*a-c*) and light microscopy of early embryo (d-j**)**. Megastrobili (*a*), developing seed (*b*) and megagametophyte without teguments (*c*). Early embryo section with double staining (PAS+CBB), showing embryonic cells (EC), and suspensor cells (SC) *(d)*, detail of embryogenic cells with large nucleus (N) with nucleoli (Nu), vacule (V), starch grains (S) and cellulosic compounds in the cell wall (Cw) *(e),* staining AT-O showing process of high mitotic activity, indicating division mitotic (arrow) *(f),* cells region that stay between the embryogenic and suspensors cells *(g)*; detail of the central cells of the embryo, showing involved nuclei by starch grain and vacuole evident *(h);* detail of suspensor cells with degenerative cytoplasm with absence of nucleus in some cells, presence of large vacuoles (v), nucleus (n) in periphery region of cell wall (cw) *(i)*; death of primary suspensors cells *(j)*. Scale bars = 5 cm *(a)*, 1 cm *(b, c)*, 100 µm *(d),* 50 µm *(g, j),* 20 µm *(f, h)*, *e)*, 10 µm *(e, i).*

In the months of March to May of 2013, megastrobilus was green had an average diameter of 55 cm, length of 23 cm, and weight of 2.130 g (Figure 8a). Individual seeds are 7 cm in length and beige color (Figure 8b). Megagametophyte also was beige color (Figure 8c). A remarkable event of early to late embryo transition was the differentiation of the major tissue regions of the embryo. In the apical region, the distal and proximal meristematic zones become recognizable (Figure 8d). Cells of

the proximal meristematic zone divide predominantly in a direction perpendicular to the axis of the embryo arranged in vertical rows, with evident nucleus and vacuolated (Fig 8e). Cells of distal meristematic zone were less dense citoplasmatic with large and compacted nucleus and divisions in several directions (Figure 8f). The layer of cells more external in the embryogenic mass, with anticlinally divisions, was flattened and distinct of the adjacent cells in the distal meristematic zone (Figure 8g). In the subsequent development the differentiation starts of shoot apical meristem (SAM) as well the emerging cotyledons enlargement has become apparent (Figures 8h, i). At this stage the suspensor cells were enucleate, elongated and showed a deformed cell wall (Figure 8j). The collapse of suspensor cells system forming an abscission zone between the apical and suspensor regions was observed (Figure 8k). In the months June and July of 2013, the mature embryo was observed, formed by an elongated hypocotyl-radicle axis (h) with two cotyledons (ct) and a root cap (rc) well developed (Figure 8l). The hypocotile axis region showed in the inner part a group of procambial cells close to the ground tissue and protoderm (Figure 8m). Cotyledons enlargement were observed was formation, filled by tissue similar to that of the cortex and procambial tissue has become continuous between cotyledons and hypocotyl (Figure 8m). In the basal pole of the embryo, it was identified a long root cap over to the root apical meristem. In the central region of root cap, the columella (co) is well evident, once that differ from peripheral region (pr) cells that was takes an oblique orientation of division (Figure 8n).

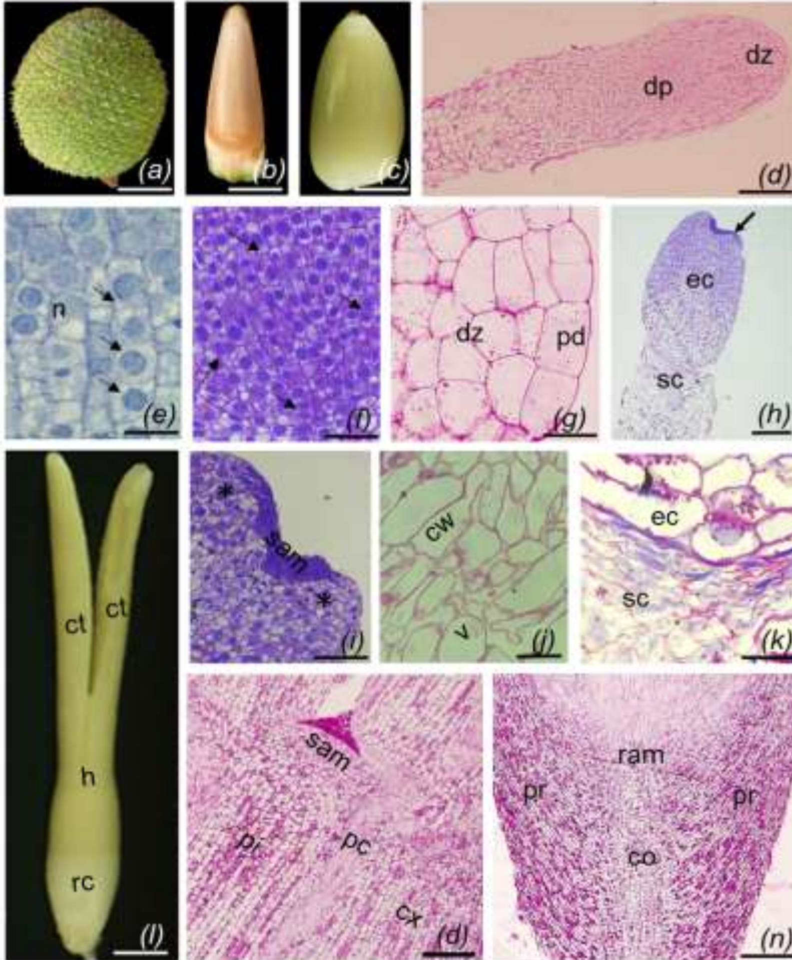

Figure 8: General features of *A. angustifolia* megastrobili and late embryo (*a-c* and *l*) with stereomicroscope and morphological analyses of late embryo *(d-k* and *n-m)*. Section of late embryo stained with PAS showing distal meristematic zone region (dz) and proximal meristematic zone region (pz) *(d)*; detail proximal meristematic zone, stained with AT-O, vertical arranged cells with evident nucleus (n) *(e)*; detail distal meristematic zone double staining (PAS+CBB) showing detail of distal meristematic zone with dense citoplasmatic cell *(f);* protoderm (pr) *(e)*; beginning of differentiation shoot apical meristem (sam) *(h)*; detail of the shoot apical meristem establishment (sam) and emerging cotyledons (asterisks) *(i)*; suspensor cells enucleate, elongated and with deformed cell wall

(j); abscission zone between the apical and suspensor *(k);* late embryo with two cotyledon (ct), hypocotyl (h) and root cap (rc) *(l)*; shoot apical meristem (sam), pith (p), procambium (pc), cortex (cx) *(m),* root apical meristem (ram), collumela (co) and peripheral (pr) *(n)*. Scale bars = 5 cm *(a)*, 1 cm *(b, c)*, 100 µm *(d, h, l)*. 50 µm *(i, m, n),* 20 µm *(j, j, k)*, 10 µm *(e, g).*

At the late embryogenesis stages, ultrastructural characterization of suspensor cells by transmission electron microscopy allowed the identification of different degrees of cellular disassembly (Figures 9). Autolytic vacuole arises through engulfment of organelles and portions of cytoplasm that progressively destroy the cytosol and organelles (Figure 9a). Vacuoles increase in size toward the cell periphery and cytoplasm occupies a narrow layer confined between tonoplast plasma membrane andcell wall deformed also was observed (Figure 9b). A plastolysome-like structure (Pl) was revealed as a portion of cytoplasm, surrounded by one or several double membranes arising from a plastid-like leucoplast. Next to these structures, an autophagic vacuole engulfment of myelin structure was observed (Figure 9c). At the same time, many leucoplasts completely differentiate into amyloplasts (Figure 9d). Dismantling of the nuclear envelope was detected by the large clusters of nuclear pore complexes (NPCs), and contents of chromatin leaking into the cytoplasm (Figure 9e). At the end of cellular dismantling, the autolytic vacuole occupies the entire cellular extension, and the protoplasm disappears, resulting in a cellular corpse represented by only the cell wall (Figure 9f).

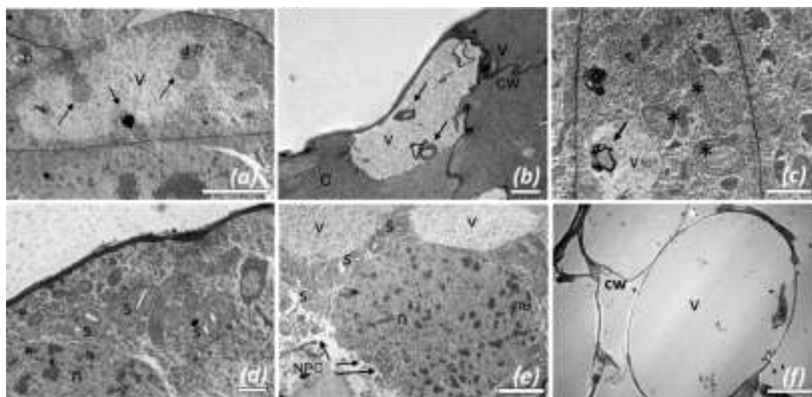

Figure 9 Transmission electron microscopy images of *A. angustifolia* suspensor cells. Atolytic vacuole *(a),* central large autolytic vacuole (v) pushing the

cytoplasm and deformed cell wall *(b),* Plastid-like leucoplast becoming a plastolysome-like structure, note utophagic vacuoles with myelin structures *(c),* Detail of a small part of cytoplasm with number large number of amyloplasts with starch grains (s) *(d),* dismantling of nuclear envelope (ne), presence of nuclear pore complexes (NPCs,arrows) in cytoplasm *(e),* cellular corpse represented by only the cell wall *(f)*. Scale bars = 5 µm (a), 1 µm (b,d), 2 µm (c,e), 10 µm (f).

5.4 DISCUSSION

In the present work the pollination event in *A. angustifolia* occurred before to the archegonial formation in the fertile scale. Only, three months after pollination, integument, sporogenous cells emerging on the nucellus and megaspore mother cell were observed. In most terrestrial plants and all conifers, pollination is the beginning of sexual reproduction. To reach megastrobili, conifers pollen grains are carried by the wind (Williams, 2009) and deposited anywhere on the fertile scale of the megastrobili and starts germinating *in situ* (Guerra et al., 2008, 2012). According to Shimoya (1962), the onset of pollination in Viçosa, State of Minas Gerais, in Brazil, occurred at the end of September, Mantovani et al. (2004) observed in the State of São Paulo, released of pollen grains in August and September. In southern Brazil, this phenomenon occurs between the months from August to October Anselmini e Zanette (2008), however this period for *A. angustifolia* can be longer, depending on climatic conditions (Guerra et al., 2008, 2012). In our work, the pollination occurred in august and the germination of pollen tubes was observed only thirteen months after pollination simultaneously with archegonial formation. Previous work with this same species indicates the occurrence of this event simultaneous to early free nuclear megagaethophyte period Shimoya (1962). However, our results are similar to the observed in *A. australis*, on what pollen tubes penetrate the nucellar tip and remain there without penetrating more deeply during free nuclear period (Owens et al., 1995). It was being reported that the pollen tubes grow in the nucellus and cause cell destruction, which stimulate the production of some substances, probably hormonal, which seems to be determinant of cell division, resulting in the formation of prothallial cells and archegonia differentiation (Shimoya, 1962; Owens et al., 1995). The association between the formation of archegonia and the presence of pollen tubes in the nucellus was also observed in the genus *Larix* (Mill.) (Ferguson, 1904), *Pinus* sp (Owens et al., 1995) and *A. australis* (Shimoya, 1962). Based on this findings, the slight time difference

observed in the present work in relation to the reported by Shimoya (1962) could be due the pollination period or the lack of microscope techniques and image quality that prevented detailed analysis. In the present work, we observed an early free nuclear megagametophyte cell four months after pollination, that remained dormant for seven months. Megagametophyte formation in *A. australis* takes five to eight months' period of free nuclear multiplication (Owens et al., 1995). Moreover, our results, indicate that subsequent formation of prothallial cells which give rise to archegonia was observed only twelve months, after pollination. Shimoya (1962) reported for *A. angustifolia* megastrobili, a state of dormancy from May to July, a period of only two months. However, megastrobili became dormant also in *A. australis*, pausing nuclear division (Owens et al., 1995) similar what we observed in *A. angustifolia*. After the dormancy period reported for *A. australis*, the authors observe cell walls formation on free nuclei, divisions cells return, give rise to prothallial cells and mature megagametophyte (Owens et al., 1995). Besides the different time scale development between the species, the sequential structural alteration is quite similar in conifers. In *A. australis* the initial stage of megagametophyte within the nucellus is marked by a free nuclei stage, ellipsoid form and cell wall formation for each free nucleus and subsequent formation of prothalial cells and archegonial (Owens et al., 1995). All these characteristics are also observed in *A. angustifolia* and additionally we observed the hydrolysis of starch grains on nucellar cells occurred before the pollen tube growth and this also was observed in *A. australis* (Owens et al., 1995). It was reported that nucellar cells and micropyle seem to produce the same chemotropic substances that promote the growth of the pollen tube in their direction, irrespective of the position of the pollen grain Singh (1978).

In *A. angustifolia*, has not yet been accurately determined the time of fertilization (Mantovani; Morellato; Reis, 2004; Guerra et al., 2008, 2012). Rogge-Renner et al. (2017), reported the occurrence of fertilization, approximately one year after pollination. In the present work, we observed the occurrence fertilization process in October, of the second year of the reproductive cycle, that is fourteen months after pollination. In Viçosa (Minas Gerais State, Brazil) fertilization was reported to occurs from October to December, fifteen months after pollination (Shimoya, 1962). In *Pinus* sp. a period of about thirteen months between pollination and fertilization was reported (Ferguson, 1904) and in *A. australis* an interval of approximately one year occurs

between pollination and fertilization (Owens et al., 1995). In the present work, fifteen months was the interval from pollination to polyembryos presence and for the first time by the histological analysis of the fertilization event in *A. angustifolia* was characterized.

The process of syngamy was only partially described for *A. angustifolia* (Burlingame, 1915; Johansen, 1950; Shimoya, 1962; Williams, 2009; Mattos, 2011). Our results showed that archegonia were fertilized give rise a group of polyembryos. This also was reported in other conifers as *A. australis* (Owens et al., 1995) and *Pinus* sp. (Williams, 2009) on what translucent mature megagametophyte showed one or more archegonia fertilized or not. The zygotic embryogenesis in conifers is characterized by the presence of polyembryos in the early seed developmental stages (Steeves; Sussex, 1989; Williams, 2009). In our work, around six archegonia were identified, but only three embryos were observed, and it was not possible to indicate if some archegonia were not fertilized or an abort occurred in pro-embryonal stages. However, identified polyzygotic polyembryony once more than one archegonia were fertilized in the same megagametophytes, and each archegonium produces a single Pro-embryo (Gifford; Foster, 1989; Williams, 2009). In conifers polyembryony by cleavage is also a common feature in which each pro-embryo may suffer cleavage, divided into up to eight Pro-embryos (Steeves; Sussex, 1989). In *Araucaria* sp. it was reported only polyzygotic polyembryony (Gifford; Foster, 1989), but, a study that analyzed morphological parameters with molecular markers reported the first evidence of late cleavage polyembryony in *A. angustifolia* (Agapito-Tenfen et al., 2011). Besides that, we identified only a single embryo from each archegonium and the number was similar to the observed by Shimoya (1962) which found up to four polyembryos in developing *A. angustifolia* seeds. In the present work, three polyembryos were imaged, although up to five of them were found when developing seeds were opened (unpublished data). The identification of fully formed polyembryos in *A. angustifolia* becomes possible only fifteen months after pollination, shortly before the onset of regression of subordinate embryos (Mantovani; Morellato; Reis, 2004; Anselmini; Zanette, 2008; Guerra et al., 2008, 2012). Usually, the three pro-embryo start development at the same time in search of a position in the center of the endosperm, when they have reached the center, compete for nutrients and only one pro-embryo survives (Burlingame, 1915; Owens; Simpson; Molder, 1982). In our work, it was identified that only one dominant

embryo develops while, the other pro-embryos degenerate through programmed cell death (PCD), as evidenced by the positive reaction to PI. It was quite well described in conifer that the major mechanism responsible for the elimination of subordinate embryos in seeds is PCD, which was reported in *Norway spruce* and *P. sylvestris* (Bozhkov et al 2005; Filonova et al. 2002). Simultaneously to embryonic development the achegonium collapses, creating a small cavity in the megagametophyte on micropylar end of the seed (Owens, 2004; Cairney; Pullman, 2007). At the same time whit the suspensor development the Pro-embryo is push out of the archegonium until the corrosion region in the megagametophyte (Burlingame, 1915; Haines; Prakash, 1980; Owens; Simpson; Molder, 1982; Williams, 2009). These features to visible in the present work at the moment when polyembryos were identified. The development of a dominant embryo in conifers follows the classic model established for Conyferophyta Singh (1978), where three phases can be distinguished: a) Pro-embryogeny – before suspensor elongation; b) early embryogeny – before establishment of root meristems and c) late embryogeny - procambium and protoderm differentiation, root and shoot meristems establishment (Filonova; Bozhkov; Arnold, 2001; Guerra et al., 2008, 2012; Williams, 2009).

In the present work, three cells types characterized pro-embryonal stage: cap cells (cc) in the upper region, embryonic cells (ec) in the central region and suspensor cells (sc) in the basal region of the embryo. In the pro-embryogenic stage, cap cells divide and elongate to for produce the cap region, which is formed by long central cells and shorter lateral cells, both deposited in a hemispherical plane involving the true embryonal group, giving a triangular morphology to the Pro-embryo. This morphological organization was described for *A. cunninghamii*, *A. bidwillii* and *A. heterophylla,* and incipiently for *A. angustifolia* (Burlingame, 1915; Johansen, 1950; Haines; Prakash, 1980). Central cap cells are composed of a dense cytoplasm with large and elongated nuclei, while in lateral cap cells, small and compressed nuclei were observed. It was previously described that in the central part of the pro-embryo, the embryonic cells group, remains inactive until the development of the suspensor (Burlingame, 1914; Johansen, 1950).

The presence of cap cells in Araucariaceae family have multiple functions, as the prevention of polyembryony by cleavage (Johansen, 1950), secretion of enzymes that digest megagametophyte cells at the same time that embryo orientation is determined (Shimoya, 1962), and

protection of embryo cells in *A. australis* (Owens et al., 1995). In the present work the transition of Pro-embryogenic stage for early embryonic stage is accompanied by cap cells death. Similar to our work it was report that concomitant to the development of the dominant embryo in *Araucaria* spp, the cap cells are crushed and degenerated (Eames, 1913; Burlingame, 1915; Haines; Prakash, 1980). We identified some features of cell death in cap cells, where a unorganized group of organelles in the cytoplasm remained only in the peripheral region close to the cell wall. While a large number of provacuoles were observed this provacuoles merged and formed central vacuole that phagocytes all the cell content. In sequence the rupture of cell wall and leaking of the cellular content does occur. However, in this work were not able to unravel the precise mechanisms of death that occurs in cap cells once additional caspases analyses and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) should be done. After cap cell degeneration, a globular mass connected to a suspensor system gives rise a cylindrical body at the upper embryo region which characterize the early embryo stage, similar what was described by Haines and Prakash (1980). Early embryos were observed, sixteen months after pollination, during mid-December until February 2012. This stage is limited to divisions in the embryogenic cells, that display nuclear enlargement and acquire more densely staining cytoplasm (Burlingame, 1915; Owens; Simpson; Molder, 1982; Haines, 1983). This also was confirmed in our work by the histochemistry analyzes, at where embryogenic cells had a large central nucleus, small vacuoles, a large amount of proteins and active chromatin. In other members of the Araucariaceae family described for Haines (1983) no organized meristematic regions characterized early embryo, as well as those observed in our work. At this stage, suspensor cells elongate acquire numerous starch grains and no cellular division are observed. According to Haines e Prakash (1980) suspensor cells remain attached to one another as a column, although there are spaces between them. In our work, double staining (CBB+PAS) showed vacuolated suspensor cell with peripheral and compacted nucleus, not always present, in a degenerating cytoplasm. Simultaneously to early embryo development, the megagametophyte cells around the embryo collapse and forming the cavity of corrosion which has been associated to the function of providing storage substances that are absorbed by the developing embryo (Buchholz, 1920). In this study with *A. angustifolia* we identified the presence of starch and proteins in the cells surrounding the developing Pro-embryo which leads

us also to associate it with the early embryo nutrition. Approximately, nineteen months after pollinization, the late embryo was observed, cellular divisions became less random, and cells begin to differentiate. Distal and proximal meristematic zone become visible and, this cellular organization was also observed for *Pinus monticola, Pinus contorta*, *Pinus strobus*, and other members of the Araucareceae family such as *A. cunninghamii*, *A. heterophylla* and *A. bidwillii* (Spurr, 1949; Haines, 1983; Owens; Simpson; Molder, 1982; Owens, 2004). In our work, we observed that cells of the proximal zone are arranged in vertical rows and this type of cell organization have become well defined since immediately above this limit, cellular divisions in various directions occur, indicating distal meristematic zone. Spurr (1949) and Haines (1983) described that, in the distal meristematic zone, cellular divisions occur in vertical or oblique planes, although in an organized way to produce a symmetrical shape, while cells of the proximal meristematic zone divide in a direction perpendicular to the axis of the embryo (Spurr 1949; Haines 1983). In the subsequent development the proximal region will originate the root cap; and forms root meristem, while distal region forms the hypocotyl-shoot axis (Haines, 1983; Spurr, 1949; Owens; Simpson; Molder, 1982; Owens, 2004). In this work, the first sign of the histodifferentiation was the protoderm formation, in followed by shoot apical meristem (SAM) differentiation and emerging of cotyledons. Similar to our results, in *A. bidwillii*, the cotyledonary primordium forms at an earlier stage to differentiation of central meristem, while in *A. cunninghamii* and *A. heterophylla*, central cells, in the distal region, were then were the first to show signs of differentiation (Haines, 1983). In *A. angustifolia* mature embryo Rogge-Renner et al. (2013) characterized the meristems cells as pluripotent stem cells, isodiametric with a spherically shaped nucleus containing one or more nucleoli and dense cytoplasm. Root initials appeared in the base of the distal region, Brumfield (1943) and Owens (2004) suggest that the entire root, including the root cap, develops from a few, possibly only three, initial cells. In our work the root cap, cells in its outer region assume an oblique orientation that gradually decreased as the center. Spurr (1949) denominated the central region of root cap with column and cells that surrounding the column as the peripheral region. In our work also we refer this as with collumn and peripheral region; however Hams (1983) described the peripheral region as “pericolumn”. Differentiation of the root cap is a feature of many conifers, *Pseudotsuga menziesii* (Allen 1947), *Pinus strobus* (Spurr 1949), *Picea smithiana*

(Venkataratnam et al. 1975), *Pinus contorta* (Owens 1982). In our work we also observe the suspensor cell system degenerate and, according to Burligame (1915) in *A. angustifolia* the suspensor was not present in the mature embryo. In our study, in the subsequent late embryo development of A. *angustifolia*, the presence of only two cotyledons a procambium, pith and cortex, we observed. The number of cotyledons was variable, *A. hunsteinii*, *A. araucana* and *A. mirabilis* embryos had two cotyledons, while *A. cunninghamii* and *A. heterophylla* exhibit four (Seward and Ford 1906; Haines, 1983). After differentiation of the tissue described above, the growth extends over a period of about two months, and include the deposition of storage materials (Haines, 1983).

5.5 CONCLUSION

Morphological studies of Pro-embryo development in Araucaria sp. suggested that this genus is the most primitive, when compared to Cupressaceae, Pinaceae and Taxodiaceae. The occurrence of (i) a prolonged free nuclei phase, (ii) presence of cap cells and (iii) the absence of Pro-embryo cleavage supports primitiveness (Haines e Prakash 1980). In the present work, it was possible to identify all of them by morphohystological analyses. Moreover, we characterized pre-fertilization stages, mature megagamethofyte formation as well the fertilization time by the identification of presence of archegonia and polyembryos. Indicated a time of fourteen months between between pollination and pollen tube penetration on the nucellus, about thirteen months to the mature archegonia formation and free nuclei stage in the megagametophyte formation. Moreover, for the first time it was characterized the three stages of zygotic embryo development for this species: Pro-embryogenesis, early and late embryogenesis. During these stages histochemical and ultra-structural analyses of embryo cells suggest the occurrence of cell death in suspensor and cap cell, and the differentiation of tissue in late embryo (meristems, procambium, hypocotyl axis and cotyledons). Our data describe for the first time the complete zygotic embryo development and which take twenty-three months between pre-fertilization until mature embryo from a native and endangered conifer of South of Brazil. Partial seeds tissues development was also here described and these data become more and more relevant take in account the conservation status of this species, the seeds

recalcitrant behavior as well as the fact of this species has been considered a primitive conifer in evolutionary context (Haines e Prakash 1980). Based on the morphological characteristics, our results allow us to propose adaptations in the reproductive cycle of *A. angustifolia* proposed by Guerra et al., 2008;2012, as shown in Figure 10.

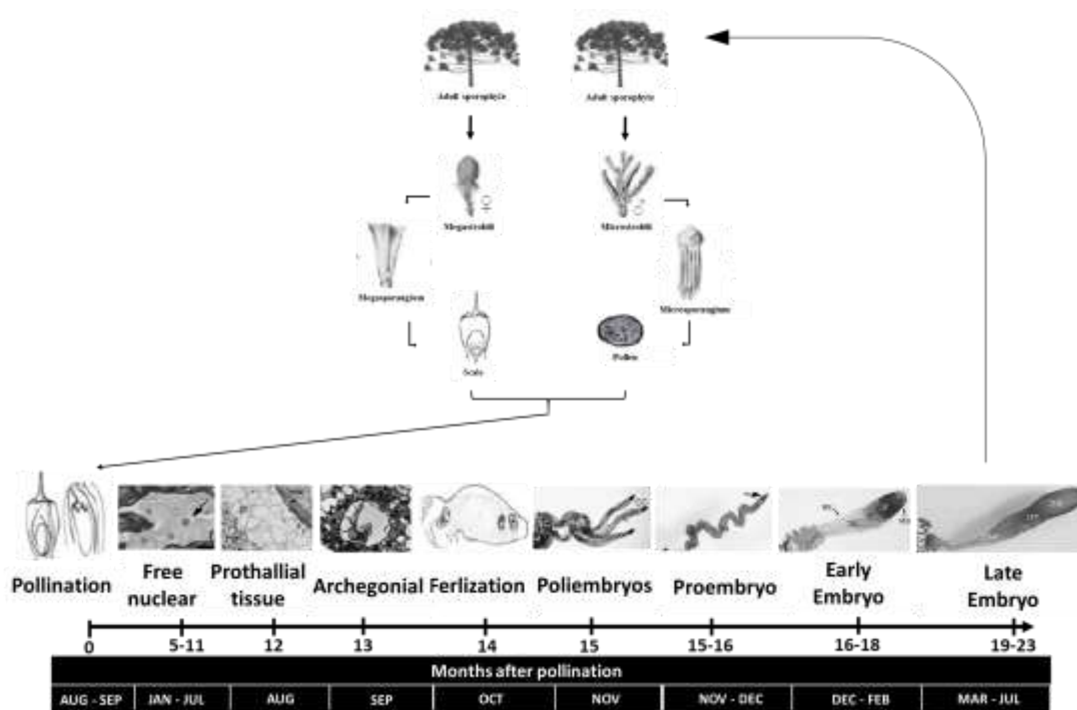
Pollination
Free nuclear
Prothallial tissue
Archegonial
Ferlization
Poliembryos
Proembryo
Early Embryo
Late Embryo
0
5-11
12
13
14
15
15-16
16-18
19-23
Months after pollination
AUG - SEP
JAN - JUL
AUG
SEP
OCT
NOV
NOV - DEC
DEC - FEB
MAR - JUL

Figure 10: Schematic representation of the life cycle of *Araucaria angustifolia.* It is a dioecious species with male reproductive structure (microstrobili), constituted by microsporophyll and microsporangium, which opens leaving the pollen free to be carried by the wind. The female reproductive structure (megastrobili) consists of numerous coriaceous bracts that contain the ovuliferous scale. The pollination period occurs in the months of August and September of the first year of the reproductive cycle. The pollen is deposited anywhere on the megastrobili scales and initiates germination *in situ*. After pollination, the formation of a cavity with free nuclei begins. This free nuclear stage lasts about six months. Approximately twelve months after pollination the free nuclei give rise to a homogeneous tissue composed of prothallial cells filling the cavity. Archegonia are formed thirteen months after pollination, while fertilization occurs after fourteen months. One month after fertilization, the presence of polyembryos with cap cells, embryonic cells and the suspensor cells was observed. In the subsequent development stage, regression of the subordinate embryos occurs and only the dominant embryo remains in the seed. The development of the dominant *A. angustifolia* embryo occurs between December and June, through *proembryogenic* development, *early* and *late embryogenesis* stages.

5.6 ACKNOWLEDGMENTS

The authors acknowledge the staff of the Central Laboratory of Electron Microscopy (LCME) of the Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil. The authors also acknowledge Leandro Fuck Camargo and Leandro Dill for providing the immature seeds. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and the Fundação de Apoio à Pesquisa Cientifica e Inovação Tecnológica do Estado de Santa Catarina (FAPESC). This study is part of the Ph.D. thesis of the Gladys Daniela Rogger-Renner.

5.7 REFERENCES

AGAPITO-TENFEN, Sarah Z. et al. Patterns of polyembryony and frequency of surviving multiple embryos of the Brazilian pine *Araucaria angustifolia*. **Australian Journal of Botany**, [s. l.], n. 59, p. 749–755, 2011.

ANSELMINI, Justina Inês; ZANETTE, Flávio. Development and growth curve of the pine cones of *Araucaria angustifolia* (Bert.) O . Ktze, in the region of Curitiba – PR. **Brazilian archives of biology and technology aninternational journal**, [s. l.], v. 51, n. 4, p. 665–669, 2008.

ANSELMINI, Justina Inês; ZANETTE, Flávio; BONA, Cleusa. Reproductive phenology of the *Araucaria angustifolia* (Bert.) O . Ktze, in region of Curitiba – PR. **Floresta e Ambiente**, [s. l.], v. 13, n. 1, p. 44–52, 2006.

ASTARITA, Leandro V; HANDRO, Walter; FLOH, Eny S. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O . Ktze . **Revista Brasileira Botanica**, [s. l.], v. 26, n. 2, p. 163–168, 2003.

AULER, Neiva Maria Frizon et al. The genetics and conservation of *Araucaria angustifolia*: I . Genetic structure and diversity of natural populations by means of non-adaptive variation in the state of Santa Catarina , Brazil. **Genetics and Molecular Biology**, [s. l.], v. 25, n. 3, p. 329–338, 2002.

BALBUENA, Tiago S. et al. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*). **Journal of Proteomics**, [s. l.], v. 72, n. 3, p. 337–352, 2009. Disponível em: <http://dx.doi.org/10.1016/j.jprot.2009.01.011>

BALBUENA, Tiago S. et al. Phytochemistry Differential proteome analysis of mature and germinated embryos of *Araucaria angustifolia*. **Phytochemistry**, [s. l.], v. 72, n. 4–5, p. 302–311, 2011. Disponível em: <http://dx.doi.org/10.1016/j.phytochem.2010.12.007>.

BOZHKOV, Peter V; FILONOVA, Lada H.; SUAREZ, Maria F. Programmed cell death in plant embryogenesis. **Current Topics in Developmental Biology**, [s. l.], v. 67, n. 04, p. 135–179, 2005.

BUCHHOLZ, John T. Embryo development and polyembryony in relation to the phylogeny of conifers. **American Journal of Botany**, [s. l.], v. 7, n. 4, p. 125–145, 1920.

BURLINGAME, L. Lancelot. The morphology of *Araucaria brasiliensis*. II. The ovulate cone and female gametophyte. **The University Chicago Press Journals**, [s. l.], v. 57, n. 6, p. 490–508, 1914.

BURLINGAME, L. Lancelot. The morphology of *Araucaria brasiliensis*. **Botanical Gazette**, [s. l.], v. LIX, p. 42, 1915.

CAIRNEY, John; PULLMAN, Gerald S. The cellular and molecular biology of conifer embryogenesis. **New Phytopogist**, [s. l.], v. 176, p. 511–536, 2007.

DURZAN, D. J. Process control in somatic polyembryogenesis. In:HÄLLGREN, J. (Ed.). **Proceedings of Frans Symposium Department of Forest Genetics and Plant Physiology'**. Swedish: University of Agricultural Sciences, 1988. p. 8–147.

EAMES, Arthur J. The morphology of *Agathis australis*. **Annals of Botany**, [s. l.], v. 27, n. 1, p. 1–38, 1913.

ESPINDOLA, L. Salmen et al. Cellular and metabolic damage induced by desiccation in recalcitrant *Araucaria angustifolia* embryos. **Seed Science Research**, [s. l.], v. 4, n. 2, p. 193–201, 1994.

FARIAS-SOARES, Francine L. et al. The transition of Pro-embryogenic masses to somatic embryos in *Araucaria angustifolia* (Bertol .) Kuntze is related to the endogenous contents of IAA , ABA and polyamines. **Acta Physiologiae Plantarum**, [s. l.], n. 36, p. 1853–1865, 2014.

FARIAS-SOARES, Francine Lunardi et al. Immunoanalysis of dehydrins in *Araucaria angustifolia* embryos. **Protoplasma**, [s. l.], v. 250, p. 911–918, 2013.

FARJON Aljos; FILER, Denis. **An atlas of the world's conifers: an analysis of their distribution, biogeography, diversity, and conservation status**. 1. ed. Boston: EJ Brill, 2013

FARRANT, J. M.; PAMMENTER, N. W.; BERJAK, P. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a

study on three unrelated species. **Planta**, [s. l.], p. 189–198, 1989.

FARRANT, Jill M. et al. Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may b. **Seed**, [s. l.], v. 6, n. 4, p. 175–182, 1996.

FERGUSON, M. **Contributions to the knowledge of the life history of Pinus with special reference to development of the gametophytes and fertilization.** 6. ed. Washington: Proceedings of the Washington Academy of Sciences, 1904.

FILONOVA, L. H. et al. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, *Norway spruce*. **Journal of cell science**, [s. l.], v. 113, n. 24, p. 4399–4411, 2000.

FILONOVA, L. H. et al. Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. **Cell Death and Differentiation**, [s. l.], v. 9, n. 10, p. 1057–1062, 2002.

FILONOVA, Lada H.; BOZHKOV, Peter V; ARNOLD, Sara Von. Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. **Jounal of Experimental Botany**, [s. l.], v. 51, n. 343, p. 249–264, 2001.

FRAGA, Hugo P. F. et al. Time-lapse cell tracking reveals morphohistological features in somatic embryogenesis of *Araucaria angustifolia* (Bert). **Trees**, [s. l.], v. 29, n. 5, p. 1613–1623, 2015.

GAHAN, Peter G. **Plant histochemistry and cytochemistry: an introduction**. Califórnia: Academic Press, 1984.

GIFFORD, Ernest M.; FOSTER, Adriance S. **Morphology and Evolution of Vascular Plants**. 3. ed. [s.l.] : WH Freeman, 1989.

GORDON, E.; MCCANDLESS, L. Ultrastructure and histochemistry of *Chondrus crispus* Stackhouse. In: HARVEY, M. J.; MCLACHLAN, J. (Eds.). **Chondrus Crispus: TNScot Inst Sci**. Halifax. p. 111–113.

GUERRA, Miguel Pedro et al. Exploração, manejo e conservação da araucária (Araucaria. In: SIMÕES, Luciana Lopes; LINO, Clayton Ferreira (Eds.). **Sustentável Mata Atlântica A exploração de seus recursos florestais Organizadores**. São Paulo: Senac São Paulo, 2002. p. 2015.

GUERRA, Miguel Pedro et al. Evolução, ontogênese e diversidade genética em *Araucaria angustifolia*. In: BARBIERI, Rosa Lía; STUMPF, Elisabeth Regina Tempel (Eds.). **Origem e evoluçao de plantas cultivadas.** Brasília DF: Embrapa, 2008. p. 149–184

GUERRA, Miguel Pedro et al. Araucaria Evolution, ontogenesis and genetic diversity. In: BARBIERI, R.; STUMPF, E. R. (Eds.). **Origin and evolution of cultivated plants.** 1. ed. Brasília DF: Embrapa, 2012. p. 151–186.

HAINES, R. J. **The embryology of *Araucaria juss*.** 1981. University of New England, Armidale, New South Wales, [s. l.], 1981.

HAINES, R. J. Embryo development and anatomy in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 31, n. 2, p. 125–140, 1983.

HAINES, R. J.; PRAKASH, N. Pro-embryo development and suspensor elongation in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 28, n. 6, p. 511–522, 1980.

JOHANSEN, Donald Alexander. **Plant embryology: embryology of the spermatophyta.** Massachusetts: Waltham: Chronica Botanica Company, 1950

KING, Janice E.; GIFFORD, David J. Amino Acid Utilization in Seeds of *Loblolly pine* during Germination and Early Seedling Growth'. **Plant Physiol**, [s. l.], n. 113, p. 1125–1135, 1997.

KOROL, A.; PREYGEL, I.; PREYGEL, S. **Recombination Variability and Evolution**. 1. ed. London: Chapman Hall, 1994.

KUHN, Sofia Aumond; MARIATH, Jorge Ernesto de Araujo. Reproductive biology of the "Brazilian pine" (*Araucaria angustifolia* –

Araucariaceae): Development of microspores and microgametophytes. **Flora: Morphology, Distribution, Functional Ecology of Plants**, [s. l.], v. 209, n. 5–6, p. 290–298, 2014. Disponível em: <http://dx.doi.org/10.1016/j.flora.2014.02.009>

MANTOVANI, Adelar; MORELLATO, L. Patrícia C.; REIS, Maurício Sedrez Dos. Fenologia reprodutiva e produção de sementes em *Araucaria angustifolia* (Bert.) O . Kuntze 1. **Revista Brasil. Bot**, [s. l.], v. 3, n. 27, p. 787–796, 2004.

MATTOS, João Rodrigues. **O Pinheiro Brasileiro**. 2º ed ed. Lages - SC: Artes gráficas Princesa Ltda., 1994.

MATTOS, João Rodrigues. **O pinheiro brasileiro**. Ed. da UFS ed. Florianópolis.

OURIQUES, L.; BOUZON, Z. Organização estrutural e ultra-estrutural das células vegetativas e da estrutura plurilocular de *Hincksia mitchellia* (Harvey) P C Silva (Ectocarpales, Phaeophyceae). **Rodriguese**, [s. l.], v. 56, p. 435–477, 2008.

OWENS, John N. et al. The reproductive biology of kauri (*Agathis australis*). 1. pollination and prefertilization development. **International Journal of Plant Sciences**, [s. l.], v. 156, n. 3, p. 257, 1995.

OWENS, John N. **The Reproductive biology of western White pine**. 4. ed. Columbia: Prepared for Forest Genetics Council of British Columbia., 2004.

OWENS, John N.; SIMPSON, Sheila J.; MOLDER, Marje. Sexual reproduction of Pinus contorta . II. Postdormancy ovule, embryo, and seed development. **Canadian Journal of Botany**, [s. l.], v. 60, n. 10, p. 2071–2083, 1982. Disponível em: <http://www.nrcresearchpress.com/doi/10.1139/b82-254>

PALUDO, Giovani F. et al. Inferring population trends of *Araucaria angustifolia* (Araucariaceae) using a transition matrix model in an old-growth forest. **Southern Forests: a Journal of Forest Science**, [s. l.],

p. 1–7, 2016.

PANZA, Víctor et al. Storage reserves and cellular water in mature seeds of *Araucaria angustifolia.* **Botanical Journal of the Linnean Society**, [s. l.], n. 140, p. 273–281, 2002.

REITZ, Raulino; KLEIN, Roberto M. **Araucariáceas. Flora Ilustrada Catarinense**. Itajai: Herbário Barbosa Rodrigues, 1966.

RIBEIRO, Milton Cezar et al. The Brazilian Atlantic Forest : How much is left , and how is the remaining forest distributed ? Implications for conservation. **Biological Conservation**, [s. l.], v. 142, n. 6, p. 1141–1153, 2009. Disponível em: <http://dx.doi.org/10.1016/j.biocon.2009.02.021>

ROGGE-RENNER, Gladys D. et al. Structural and component characterization of meristem cells in *Araucaria angustifolia* (Bert.) O. Kuntze zygotic embryo. **Protoplasma**, [s. l.], v. 250, n. 3, p. 731–739, 2013.

ROGGE-RENNER, Gladys Daniela et al. Ontogenia de megaestróbilos de *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae) Ontogeny. **Acta biologica catarinese**, [s. l.], v. 4, n. 2, p. 30–41, 2017.

ROLLAND, Filip; BAENA-GONZALEZ, Elena; SHEEN, Jen. Sugar sensing and signaling in plants : conserved and novel mechanisms. **Annu Rev Plant Biol**, [s. l.], v. 57, p. 675–712, 2006.

SCHMIDT, Éder C. et al. Effects of UVB radiation on the agarophyte *Gracilaria domingensis* (Rhodophyta, Gracilariales): Changes in cell organization, growth and photosynthetic performance. **Micron**, [s. l.], v. 41, n. 8, p. 919–930, 2010.

SCHMIDT, Éder C. et al. Responses of the macroalgae *Hypnea musciformis* after in vitro exposure to UV-B. **Aquatic Botany**, [s. l.], v. 100, p. 8–17, 2012. Disponível em: <http://dx.doi.org/10.1016/j.aquabot.2012.03.004>

SCHMIDT, Éder Carlos et al. Changes in ultrastructure and

histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta , Gigartinales), as a consequence of ultraviolet B radiation exposure. **Micron**, [s. l.], v. 40, p. 860–869, 2009.

SETOGUCHI, Hiroaki et al. Phylogenetic relationships within Araucariaceae based on rbcl gene sequences. **American Journal of Botany**, [s. l.], v. 85, n. 11, p. 1507–1516, 1998.

SHIBATA, Marília et al. Physiological and physical quality of local *Araucaria angustifolia* seed variety. **Acta Scientiarum**, [s. l.], v. 38, n. 2, p. 249–256, 2016.

SHIMOYA, C. **Contribuição ao estudo do ciclo biológico de** *Araucaria angustifolia* **(Bert.) O. Ktze**. 2. ed. Viçosa: Experimentiae, 1962.

SILVA, Camila Vieira Da; REIS, Maurício Sedrez Dos. Brazilian pine nuts' production in Caçador's region, SC: aspects of the attainment and its importance for local communities. **Ciência Florestal**, [s. l.], v. 19, n. 4, p. 363–374, 2009.

SILVEIRA, Vanildo et al. Effect of plant growth regulators on the cellular growth and levels of intracellular protein , starch and polyamines in suspension cultures of *Pinus taeda* . **Plant Cell, Tissue and Organ Culture**, [s. l.], v. 76, p. 53–60, 2004.

SINGH, H. Embryology of gymnosperms. In: ZIMMERMAN, W. et al. (Eds.). **Handbuch der Pflanzenanatomie**. Berlim, Stuttgar. p. 187–241.

SPURR, R. Histogenesis and Organization of the Embryo in Pinus Strobus L. **American Journal of Botany**, [s. l.], v. 36, n. 9, p. 629–641, 1949.

STEEVES, Taylor. A.; SUSSEX, Ian. M. **Patterns in plant development.** 2. ed. New York: Cambridge University Press, 1989.

STEINER, Neusa et al. Effect of carbon source on morphology and histodifferentiation of *Araucaria angustifolia* embryogenic cultures. **Brazilian Archives of Biology and Technology**, [s. l.], v. 48, n.

November, p. 895–903, 2005.

STEINER, Neusa et al. Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. **Plant Cell, Tissue and Organ Culture**, [s. l.], v. 89, n. 1, p. 55–62, 2007.

STEINER, Neusa et al. Araucaria angustifolia Biotechnology *Araucaria angustifolia* Biotechnology. **Global Science Books**, [s. l.], v. 2, n. 1, p. 20–28, 2008.

STEINER, Neusa et al. Toward establishing a morphological and ultrastructural characterization of Pro-embryogenic masses and early somatic embryos of *Araucaria angustifolia* (Bert.) O . Kuntze. **Protoplasma**, [s. l.], 2015. Disponível em: <http://dx.doi.org/10.1007/s00709-015-0827-0>

STOCKEY, Ruth A.; KO, Helen. Cuticle micrimorphology of Araucaria de jussieu. **Botanical Gazete**, [s. l.], v. 147, n. 4, p. 508–548, 1986.

THOMAS, P. *Araucaria angustifolia*. 2013. Disponível em: <https://www.iucnredlist.org/species/32975/2829141>.

TOMPSETT, P. Desiccation studies in relation to the storage of Araucaria seed. **Annals of applied Biology**, [s. l.], v. 105, n. 3, p. 581–586, 1984.

WILLIAMS, Claire G. **Conifer Reproductive Biology**. New York: Springer, 2009.

ZECHINI, Aleex Anderson et al. Genetic Conservation of Brazilian Pine (*Araucaria angustifolia*) Through Traditional Land Use. **Economic Botany**, [s. l.], n. 10, p. 1–14, 2018.

6 CAPÍTULO II

CARBOHYDRATES AND DEHYDRINS PROFILE DURING ZYGOTIC EMBRYOGENESIS OF *Araucaria angustifolia* (BERT.) O. KUNTZE

ABSTRACT

Araucaria angustifolia is a native conifer from the Atlantic rainforest, characteristic of the Mixed Ombrophilous Forest typology. Due its importance economically, ecologic and social has a high potential for studies that aiming its conservation, since this species is also endangered. Knowledge of the physiological and biochemical aspects that regulate embryogenesis is crucial for defining appropriate strategies of conservation for this recalcitrant specie. Thus, we investigated the carbohydrate and dehydrins profile during embryo developmental and seed maturation of *A. angustifolia*. Early development stages of embryo and their respective megagametophyte were characterized by high contents of fructose and glucose. As the embryo develops towards physiological maturity, there is an increase in the sucrose contents at the same time that hexose contents decrease and maltose beocomes detected. Although concentrations were not always similar, embryo and megagametophyte tissues generally showed similar patterns of changing concentrations over time. Dehydrins were studied by western blot analysis and were not detected in the early embryogenesis stages. The heat-sensitive protein fraction, as estimated by SDS-PAGE followed by western-blotting, revealed four main bands of approximately 21,5; 23; 26 and 28kDa in axis and cotyledons, during the transition from the early stage to the late embryogenesis. Two heat-stable bands, with molecular mass of approximately 26 and 45 kDa, were present in the embryo axis only and during the late embryogenesis stage. Maturation was characterized by main for two heat-sensitive fractions 21,5 and 26kDa. Studies of carbohydrate and dehydrins behavior at specific stages of development of the zygotic embryos and during seeds maturation of *A. angustifolia*, may generate insights to improve conservation strategies.

ADDITIONAL KEYWORDS: Seeds, Recalcitrant, LEA and Carbohydrates.

6.1 INTRODUCTION

Araucariaceae is restricted to the Southern Hemisphere, distributed by South America, Australia, New Guinea, New Caledonia, Norfolk Island, and other Pacific Islands (Setoguchi et al., 1998). This family comprises three genera: Araucaria, Agathis, and Wollemia. In the genus Araucaria the *Araucaria angustifolia* (Bertol.) Kuntze species is found a dioecious native conifer (Reitz and Klein 1966), popularly known as Brazilian pine. Its occurrence is restricted to areas of southern Brazil, eastern Paraguay and Argentina, particularly in the Province of Missiones (Carvalho, 1994; Guerra et al., 2000, 2008, 2012). *A. angustifolia* is a native gymnosperm with great ecological and economic value, due production of wood, resin and seeds (pinion) (Carvalho, 1994; Guerra et al. 2000; Guerra et al. 2008; Guerra et al. 2012). These characteristics made the species has been the target of high exploration throughout the last century, as a result, its forest remnants are estimated at 12.6% of its original extent (Ribeiro et al., 2009). The drastic population decline and habitat reduction placed *A. angustifolia* as vulnerable specie and more recently classified as "critically endangered" by the International Union for Conservation of Nature (IUCN 2018). In this context, *A. angustifolia* is one of the native species of Brazil with great potential for studies that support its genetic conservation (Guerra et al., 2008). However, the conservation of *A. angustifolia* seeds is difficult because they are classified as recalcitrant, losing viability when dried, making it difficult to store them for long periods (Tompsett, 1984; Farrant et al., 1996; Panza et al., 2002; Farias-Soares et al., 2013). Studies that improve the information about the basal and energetic metabolism, aiming to elucidate aspects involved in embryo formation, seed maturation and physiological behavior are valuable to promote their conservation, and sustainable use.

Seeds are until now the only way to successful propagate *A. angustifolia* beside of efforts to establish *in vitro* propagation (Steiner et al. 2015; Wendling and Brondani 2015). In this sense, the knowledge of seed physiological behavior and metabolism becomes essential to improve conservation strategies of this species (Tompsett, 1984; Farrant et al., 1996; Panza et al., 2002; Farias-Soares et al., 2013). Species with recalcitrant seeds, water cannot be removed at levels that allow almost complete reduction of metabolism, as they rapidly lose viability, and suffer damages when submitted to desiccation (Berjak; Pammenter, 2008). In contrast, orthodox seeds are tolerant in relatively extreme

desiccation (5 - 15%) and may survive in the dehydrated state for long periods (Hilhorst; Toorop, 1997; Barbedo and Marcos 1998; Kermode; Finch-Savage, 2002; Berjak and Pammenter 2008). Desiccant-tolerant seeds depends on the presence of a system that guarantees membrane integrity as presence of antioxidative mechanisms, protective molecules, such as LEA proteins, heat-resistant proteins, and non-reducing sugars (Close, 1997; Pammenter; Berjak, 1999; Bewley, Hilhorst; Nonogaki, 2013).

Developmental stage in which seeds are collected can affect the desiccation sensibility and this that may be increased with storage (Farrant; Pammenter; Berjak, 1989). In *A. angustifolia* the early harvesting favors the physiological quality in terms of germination (Shibata; Maria; Coelho, 2016). Thus, the characterization of biochemical compounds, helps in the compression of the best time of harvest of the seeds aiming its conservation. Carbohydrates had the energy source and also can act as regulators that control gene expression during embryonic development (Iraqi e Tremblay 2001; Rolland, Baena-gonzalez, e Sheen 2006; Weber, Borisjuk, e Wobus 2005). According to Winkelmann (2016) carbohydrates are involved in the osmotic potential adjustment during seed desiccation and in the proteins synthesis. These events, are characteristic of late embryogenesis stages and seeds maturation. Dehydrins are proteins that accumulate in seeds when the embryos acquire desiccation tolerance (Battaglia et al., 2008). Dehydrins are part of the second group (originally called D-11) of Late embryogenesis abundant (LEA) proteins (Battaglia et al., 2008; Bewley; Hilhorst; Nonogaki, 2013). These proteins contain the K segment conserved with lysine-rich 15-residue repeat - KKGIMDKIKEKLPG (Galau; Close 1992). An important feature of dehydrins is their extreme hydrophilicity due to a high content of lysine and glycine and virtual absence of hydrophobic amino acids. This gives them high solubility and the ability to bind water, possibly forming a stabilizing envelope of ordered water in association with membranes and macromolecules under conditions of cell water deficit, perhaps in conjunction with compatible solutes (Close, 1996, 1997; Campbell et al., 1997; Kermode, 1997; Bewley; Hoekstra; Golovina; Buitink, 2001; Hilhorst; Nonogaki, 2013; Radwan et al., 2014).

In *A. angustifolia*, advances have been obtained in elucidating some metabolic routes of seed development (Balbuena et al, 2009; Farias-Soares et al., 2013; Oliveira et al., 2016). However, the screening of carbohydrates profile associated to the dehydrins synthesis during the

complete embryo development of this species was not performed. Considering that carbohydrates and dehydrins are closely associated to the seed desiccation tolerance and sensibility, this study amplifies our physiological knowledge about it and give us the precise idea of how much these biomolecules are associated to the seed recalcitrance in this species. Moreover, the knowledge of the physiological aspects that regulate the zygotic embryogenesis is crucial to understand the seed storage behavior and germination. In the present work we studied carbohydrate and dehydrins profiles at specific stages of *A. angustifolia* seed development (embryo and megagamethophyte) looking at to deepen on the physiological and biochemical aspects underlying the seed development and conservation in this species.

6.2 MATERIAL AND METHODS

6.2.1 Plant material

Female cones of *A. angustifolia* were collected monthly from November 2011 to August 2012, preselected from open-pollinated trees in a natural population located in the county of Curitibanos (960 m altitude, 27º18'11" south latitude and 50°38'12" west longitude), Santa Catarina State, Brazil. The seeds were separated from the megastrobilos and embryos were excised from seeds to morpho-cytochemical and biochemical analyses.

6.2.2 Morphological analyses

Zygotic embryo development was monitored in stereomicroscope monthly by double staining with Evans blue (0.1% w/v) and acetocarmine (2% w/v) using procedures described by Durzan (1988). The analyzes were performed at the Laboratory of Plant Developmental Physiology and Genetics (LFDGV) of the Federal University of Santa Catarina using an Olympus BX 40 microscope equipped with Olympus DP71 capture images with the DP Controller software system.

6.2.3 Soluble carbohydrates

Soluble carbohydrates were determined according to the method of Filson e Benjamin (2009) with modifications. Samples (300 mg FM each) were macerated in 1 mL of extraction solution containing 80 % ethanol (Merck, Darmstadt, Germany), 3 % polyvinylpolypyrrolidone (Sigma-Aldrich, St. Louis, MO, USA) and 1 % ascorbic acid (Sigma-Aldrich), at 4 °C. The extracts were then incubated at 70 °C for 90 min. Following centrifugation at 20,000×g for 10 min, the supernatant was removed, and the pellets were re-extracted with 1 mL of extraction solution and centrifuged again at 20,000×g for 10 min. The supernatants were combined and filtered through a 20 μm membrane and stored at − 20 °C until carbohydrate analysis. Carbohydrates were identified and quantified using a high performance liquid chromatograph system (HPLC) (Shimadzu, Kyoto, Japan) with an evaporative light scattering detector ELSD-LT II (Shimadzu) at 40 °C with a nitrogen gas pressure of 350 kPa. A Prevail Carbohydrate ES (Alltech Associates, Deerfield, IL, USA) column (5 μm—250 × 4.6 mm) and a pre-column (5 μm—7.5 × 4.6 mm) were used. The gradient was achieved by mixing decreasing proportions of absolute acetonitrile (Merck) with water. The acetonitrile gradient was programmed as follows: 80 % during the first 16 min, 80–70 % between 16 and 23 min and 70 % from 23 to 30 min. The flow rate was 1 mL min^{-1} at 25 °C. A 5-μL sample was injected, and the peak areas and retention times were measured by comparison with known quantities of carbohydrate standards containing arabinose, fructose, glucose, maltose and sucrose (Sigma-Aldrich).

6.2.4 Proteins and Western Blot assay

Embryos excised from seeds in early embryogenesis stage were kept intact, while late embryos and mature were cut to separate axis and cotyledon samples. Protein extraction was carried out according to Farias-Soares et al. (2013). Samples were lyophilized and homogenized with extraction buffer, and centrifugation at 10,000g (two times), the pellet was discarded and the supernatant divided into two fractions. Fraction 1 was prepared according to version described by Close et al. (1993) and with modification of Farias-Soares et al. (2013). Fraction 2 was not heated to conserve heat-sensitive proteins. Protein contents of both fractions

were quantified using a Qubit Quantitation Platform Protein Assay Kit (Invitrogen, Carlsbad, CA, USA). Proteins (30 µg) were resolved by 17.5 % SDS-PAGE (LAEMMLI, 1970) at a constant voltage of 100 V for 120 min using a Mini-PROTEAN II Electrophoresis Cell II (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose membrane (Osmonics Inc., Minnetonka, MN, USA) at 100 V for 60 min using a Mini Trans-Blot Electrophoretic Transfer Cell II (Bio-Rad Laboratories, Hercules, CA, USA). The western blot assays were carried out according to Burrieza et al. (2012). Protein molecular masses were calculated by means the MW Analysis tools of Image Lab software version 6.0.1 (Bio-Rad CA, USA).

6.2.5 Statistical analysis

Data normality was evaluated using the Shapiro-Wilk test. Data that did not follow a normal distribution were transformed using the log (x+1) function. Analysis of variance (ANOVA) was performed on all data set using variance analysis with two crossed fixed factors, followed by the Student–Newman–Keuls (SNK) ($P < 0.05$) using the "R" statistical software (R Core Team 2014).

6.3 RESULTS

6.3.1 Zygotic embryo development and morphology

During zygotic embryo development three distinct stages were identified: Pro-embryogenic, early embryogenesis and late embryogenesis stage. In immature seeds, the Pro-embryogenic stage, corresponding to the months of November to the middle of December, was characterized by the presence of multiple polarized zygotic Pro-embryos, indicating the occurrence of polyembryony (Figure 1a). Pro-embryonal apical region Pro-embryo was constituted by embryogenic cell (EC), and cap cells (CC), both of them reactive to Acetocarmine (Figure 1b). Cap cells were deposited in a hemispherical plane involving the embryogenic cells and give a triangular morphology to the Pro-embryo. Cap cells are elongated while embryogenic cells are isodiametric with a dense cytoplasm. These groups of cells are linked to basal region of pro-embryo which consisted in a long tail of suspensor cells, (complex system

of elongated cells), reactive to Evan's Blue (Figure 1b). In middle of December until February the embryo goes through early embryogenesis stage. Cap cells degenerate and the triangular morphology of embryo becomes globular by the presence of a globular embryonic mass (EM) in apical region attached to suspensor region (SR) (Figure 1c). It was observed an intense embryogenic cells division (Figure 1d). At this stage, a transitional region was observed between embryogenic mass and suspensor region, which was composed by elongated tube cells (TC) (Figure 1e). Besides being reactive to Acetocarmine we observed that the embryogenic mass presented a smooth surface, formed by dense cytoplasmic cells (CE) while suspensor region was formed by elongated and vacuolated cells (SC) observed by the positive reaction to Evan's Blue (Figure 1 f). We observed in the late embryogenic stage, during March and to late May months, the presence of EC, TC, SC and the onset of cotyledon formation (Fig 1g). Due to the impermeability of Acetocarmine and Evan's Blue in differentiated cells, the mature embryo, found in the June to July months, was observed in directly in stereomicroscope. In the hypocotyl-radicle axis the formation of two cotyledons and the root cap, were observed (Figure 1h).

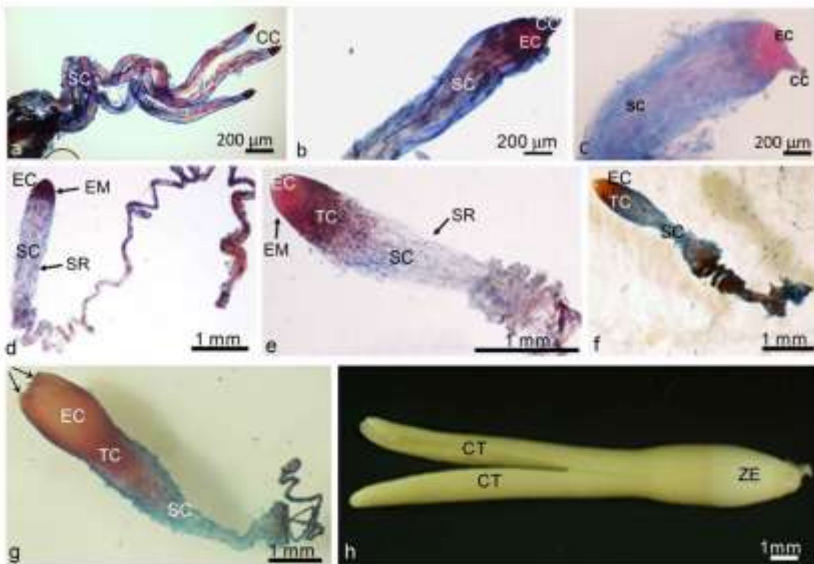

Figure 1 Light microscopy and morpho-cytochemical analysis of *Araucaria angustifolia* zygotic embryo in response to Acetocarmine and Evan's Blue. Supernumerary pro-embryos (*a*) Dominant pro-embryo with cap cells (CC), embryonic cells (EC) and suspensor cells (SC) (*b*). Transition of pro-embryogenic stage for early embryogenesis stage, showing CC degeneration (*c*). Early embryo with an embryonic mass (EM) and suspensor region (SR) demonstrate the presence of the embryogenic cells (EC) stained red and suspensor cells (SC) reactive to Evan's Blue (*d*). Between EC and SC note the presence of embryonic tube cells (TC) *(e-f)*. Late embryo, with EC, TC, SC and cotyledons formation start (arrow) (*g)*. Mature embryo with two cotyledons and well established cap root (*h*).

6.3.2 Soluble carbohydrate profile during development of zygotic embryo

Soluble carbohydrates profile in the embryo and their respective megagametophytes were analyzed during pro-embryogenesis, early embryogenesis and late embryogenesis. In the pro-embryogenic and early embryogenesis stages, the embryo and megagametophyte were analyzed together due to the small size and fresh weight of the embryos at these stages. Concentrations of fructose and glucose were higher than sucrose and arabinose, during pro-embryogenic and early embryogenesis stages. Glucose levels showed a similar pattern to fructose, but always at lower concentrations. Sucrose contents were lower than those found for arabinose and remained constant until the end of the early embryogenesis stage. The beginning of the pro-embryogenic stage was marked for high arabinose content (4.92 $mg.g_{DW}^{-1}$) that showed decreased in the subsequent stages. Maltose was not detected in the early stages of development (Figure 2). The transition of early embryogenesis stage for late embryogenesis stage, corresponding the period between February and March, was marked by an increase in contents of arabinose and sucrose and a decrease in the hexose contents (fructose and glucose) (Figure 2, 3).

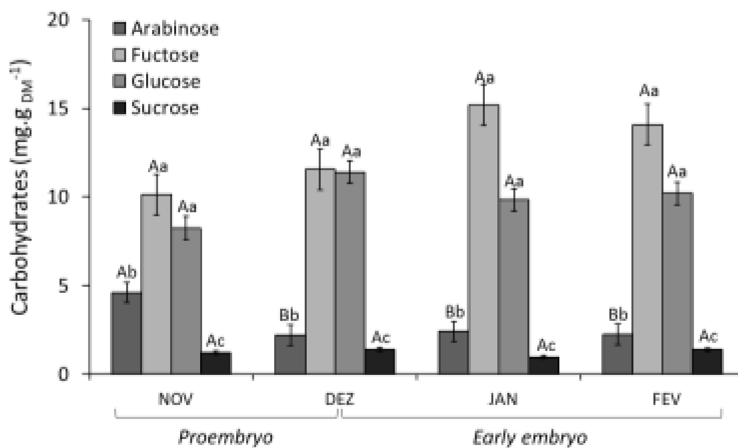

Figure 2 Endogenous carbohydrates contents (mg. g_{DW}^{-1}) in megagametophytes of *Araucaria angustifolia* zygotic embryos along pro-embryogenic and early embryogenesis stages. Data means ± SD (n = 3). Small letters compare different carbohydrates at the same month and capital letters compare each carbohydrate in distinct months. Means followed by the same letter are not significantly different by the SNK test ($p < 0.05$).

During the late embryogenesis, maltose contents became detectable. In the embryo the maximum content of arabinose was found in the onset of late embryogenesis stage (March). In April. arabinose content suffered a reduction that oscillated a little until maturation of embryo (Figure 3a). A similar behavior was observed for fructose (Figure 3b), and maltose (Figure 3e), which reduced its contents by approximately 70%, between March and April. In the sequence the fructose content suffered variations until embryo maturation, while maltose levels remained constant. In the late embryogenesis stage (April) high glucose content was found that during the transitions for maturation stage; their contents decreasing to the point of not being detected (Figure 3c) while an opposite behavior was observed for sucrose (Figure 3d). Concomitant the reduction of fructose and glucose we observed an accumulation of sucrose, presenting high contents in the mature embryo (Figure 3d). In the megagametophyte during the late embryogenesis stage and in the maturation, the contents of arabinose were higher than those found in the embryo (Figure 3a). The arabinose content underwent oscillations during these stage with a slightly increase in the end of each stage of

development. A similar pattern was found regarding to fructose and arabinose, with a reduction in their contents in the April and a subsequent small increment at the end of each stage of development. In the embryo, although drastically reduced, glucose was found during all in the late embryogenesis stages, already in the tissue of megagametophyte was found only in the month of March (Figure 3c). The opposite behavior was observed for sucrose, in parallel to the reduction of the hexoses, the content of sucrose had an accumulation gradient in the megagametophyte, being higher concentrations found at the end of the maturation stage of the embryo (Figure 3d). In the megagametophyte different of embryo, maltose content was not detected in March. However, in the April an increment was observed and it remained constant until the embryo maturation (Figure 3e).

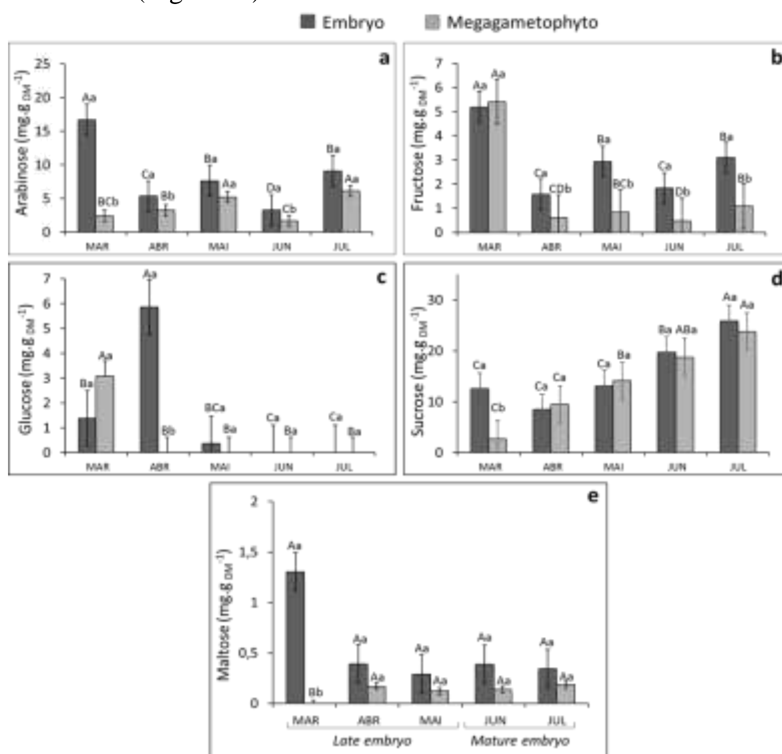

Figure 3 Endogenous contents (mg. g_{DW}^{-1}) of Arabinose (a), Fructose (b), Glucose (c), Sucrose (d) and Maltose (e) in *Araucaria angustifolia* during the late embryogenesis and mature embryo and their respective megagametophytes. Data

means ± SD (n = 3). Small letters compare carbohydrate contents between embryo with megagametophyte the same month and capital letters compare carbohydrate in embryo and megagametophyte in distinct months. Means followed by the same letter are not significantly different by the SNK test ($p < 0.05$).

6.3.3 Dehydrin western blot analysis in the development embryos

In the dehydrin-like proteins profile analysis, four major bands of 21,5; 23; 26 and 27kDa were identified in *A. angustofolia* cotyledons and embryos axis of. In early embryo developmental stage, collected in the month of February, dehydrin was not detected (Figure 4a, lanes 1TP, 1HS and 2TP, 2HS). In the transition of early embryogenesis stage for late embryogenesis (February), in the embryo axis the bands of 21,5; 23; 26 and 27-kDa were detected (Figure 4b, lane 3TP) while in cotyledons a similar profile, except the absence of the band 23kDa (Figure 4b, lane 4TP) was observed. In both tissues heat-stable protein was not detected (Figure 4b, lane 3HS and 4HS). Dehydrin of 21,5KDa was present throughout the during all late embryogenesis stage and maturation seeds, in both on the axis and in cotyledons (Figure 4b-c). However, a greater abundance of the protein fraction of 21.5KDa was observed during mid-late embryogenesis (March) (Figure 4b, lane 6TP and 7TP), followed by a decrease in the end late embryogenesis (April) (Figure 4b, lane 8TP and 9TP). In this stage, heat-stable fractions protein of around 25 and 26 kDa was detected in the axis (Figure 4b, lane 6HS). Band of 23kDa were only present in the axis of embryo (Figure 4b, lane 3TP, 6TP and 8TP). The dehydrins contents decreased at the end of late embryogenesis and at the beginning of maturation (Figure 4c, lane 12TP, 13TP, 14TP, 15TP and 16TP). The maturation was marked by the presence of dehydrins of 21.5 and 23KDa (Figure 4c). Band of 21,5kDa was present in the axis and cotyledons, accumulating, especially at the end of the maturation stage (Figure 4dc, lane 12TP, 13TP, 14TP, 15TP and 16TP).

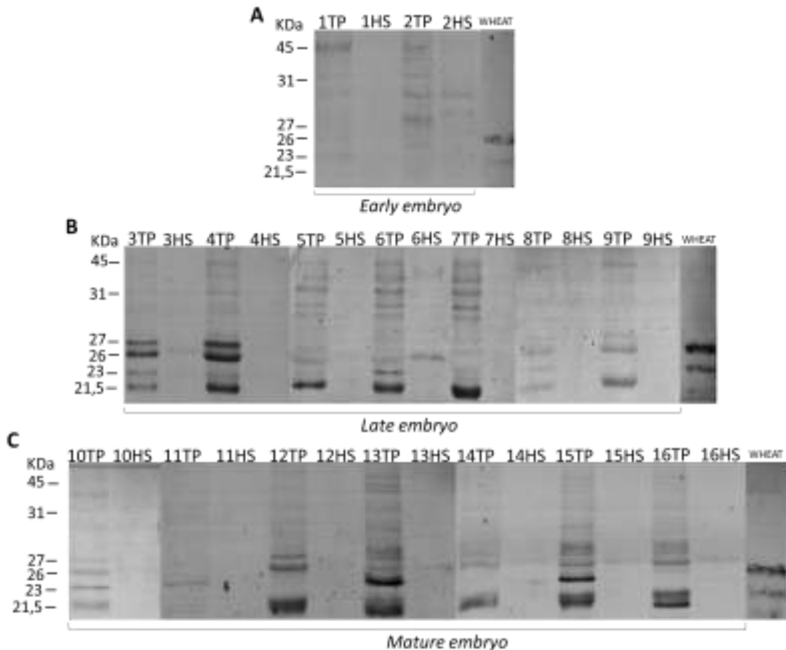

Figure 4 Western blot analyses of dehydrin-like proteins present in the development of *Araucaria angustifolia* zygotic embryos. The proteins were separated in 17,5% SDS-PAGE, and electroblotted onto nitrocellulose membranes. Samples were treated whit a purified anti-dehydrin antiserum directed against the K domain and then an antiserum composed alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. Lanes: *Early embryo*: 1 and 2. *Late embryo*: 3, 6 and 8 *axis* 4, 5, 7 and 9 cotyledons. *Mature embryo* (adhered seed to the mother plant): 10, 12, 14 and 16 *axis* 11, 13 and 15 *cotyledons*. *Mature embryo* (disperse seeds): 16 *axis* and 17 *cotyledons*. Heat-sensitive (TP), heat-stable fractions (HS). Protein molecular masses were calculated by the Image Lab software version 6.0.1.

6.4 DISCUSSION

Embryogenesis is part of seed development and describes the process of growth and differentiation that begins with a fertilized egg and culminates in a newly germinated plant (Gifford; Foster, 1989). In gymnosperms, during embryo development three phases can be distinguished: (i) Pro-embryogeny - before the elongation of the

suspensor; (ii) early embryogenic - after elongation of the suspensor and before establishment of meristems; (iii) late embryogeny - intensive histogenesis, establishment of the root and shoot meristems (Singh,1978; Raghavan; Sharma, 1995; Filonova; Bozhkov; Arnold, 2001 Williams, 2009). Zygotic embryogenesis in conifers is characterized by the presence of polyembryos, which is the presence of more than one embryo in the early stages of seed development (Filonova et al., 2002; Owens, 2004; Guerra et al., 2008, 2012; Williams, 2009). There are two basic types of polyembryony, the polyzygotic polyembryony where two or more archegonia are fertilized in the same megagametophyte, , archegonium producing a single Pro-embryo (Gifford; Foster, 1989; Steeves; Sussex, 1989; Williams, 2009) and cleavage polyembryony in which each Pro-embryo suffer divisions Pro-embryogenerating others Pro-embryo (Steeves; Sussex, 1989). In *Araucaria* sp. only polyzygotic polyembryony was reported (Shimoya, 1962). However, a study analyzing morphological parameters with molecular markers reported also cleavage polyembryony in *A. angustifolia* (Agapito-Tenfen et al., 2011). In the present work, three polyembryos were observed in early November, however only one embryo was present in the mature seed. The identification of fully formed Pro-embryos becomes possible fifteen months after fertilization, shortly before the onset of regression of subordinate embryos similarly to what was observed by Anselmini e Zanette (2008); Burlingame (1914); Guerra et al. (2008), (2012); Mantovani, Morellato, e Reis (2004); Rogge-Renner et al. (2017); Steiner et al. (2005). In this work, Pro-embryos were found between mid-November to December, and constituted for cap cells (cc), embryonic cells (ec) and suspensor cells (sc). In apical region of embryo, cap cells are elongate and deposited in a hemispherical plane involving the embryogenic cells, gives a pointed shape to Pro-embryo. Embryogenic cells are attached to vacuolated cells, termed suspensor cells, which make up the basal region of embryo. These structures and organization were described also by Burlingame (1915); Johansen (1950); Haines and Prakash (1980); Guerra et al. (2008) and Guerra et al. (2012). A positive reaction to Acetocarmine in the cap cells, and embryogenic cells revealed a dense cytoplasm. In contrast, the elongated and vacuolated suspensor cells were permeable to Evans blue. The transition of pro-embryogenic stage to early embryogenesis, include the cap cells degeneration. The embryo assumes globular shape with a cylindrical body, giving origin an early embryo, from December to February. In the apical region,

embryogenic cells suffer intense division forming an embryogenic mass (EM), which will give rise to the meristematic regions. In the basal region, cells cease to divide and elongate producing the suspensor, that pushes the Pro-embryo still farther down into the endosperm (Burlingame, 1915; Johansen, 1950). A remarkable event of early to late embryo transition was the differentiation of the major tissue regions of the embryo. In our study, late embryo was observed in the period of March until May. In *A. angustifolia*, the embryogenic mass delimitates of three meristems, one that will form hypocotyl and root and other two develop produce the two cotyledons (Burlingame, 1915; Johansen, 1950; Guerra et al., 2008, 2012; Rogge-Renner et al., 2013). After the differentiation of meristematic zones, storage compounds are deposited (Haines, 1983) and the growth extends over a period of about two months, mainly in the cotyledons, which are exceptionally large in comparison to the remainder of the embryo (Burlingame, 1915; Johansen, 1950). In our work in the June month was possible to find mature embryos composed for two cotyledons and a hypocotyl-radicle with root and shoot meristems the were well distinguishable. Similar structures have been in *A. cunninghamii*, *A. heterophylla* and *A. bidwillii* (Haines, 1983).

Apart from morphological alteration during embryo development of *A. angustifolia* the in carbohydrate type and concentration. During Pro-embryogenic and early embryogenesis stage low content of sucrose were found, while fructose and glucose were elevated and presented similar behavior, although glucose always was being in lowers concentrations. This pattern changed during development, in the end of late embryogenesis and in the maturation, a decrease in hexose content with an associated accumulation of sucrose was observed. Navarro et al. (2017) characterized the globular stage in *A. angustifolia*, by as presenting high levels of hexose, reducing by about 98% during seed development. Similar pattern was also observed in other conifers, such as *Picea abies*, *Pinus taeda* (Gösslová et al., 2001; Pullman; Buchanan, 2008). In somatic embryos of *Pinus pinaster*, *Picea abies* and *A. angustifolia* presented same patterns when they passed from the stage of proliferation to maturation (Downie; Bewley, 2000; Konrádová et al., 2002; Navarro et al., 2017). Early embryogenesis is characterized by intense mitotic activity, principally in the embryogenic cells, in function of embryo development by apical cell growth. Studies show the role of hexoses in regulating of cell division promoting embryonic growth (Bate et al., 2004; Wang; Ruan, 2013). Invertase is the enzyme responsible for hydrolyzing

sucrose into glucose and fructose, increasing the content of hexose in the cells. In somatic embryos of *P. abies* high activities of invertases and low activity of sucrose synthase were determined in the proliferation stage (Konrádová et al., 2002; Lipavska; Konrádová, 2004), which is analogous to the Pro-embryogenic stage, in the zygotic embryo. The cell cycle, responsible for cell division and growth, is divided into four phases: DNA replication (S), mitosis (M), and two Gap phases (G1 and G2) (Perrot-Rechenmann, 2010), and in addition to auxin, it has recently been found that the glucose signal activates the expression of key enzymes in the cell cycle initiating the G2/M transition (Wang; Ruan, 2013). Thus, we suggest that in the early stages of development, glucose may act mainly as a signal in the rate of cell division, rather than providing nutrients. In *A. angutifolifa* seeds, the zygotic embryo grows and develops within the female megagametophyte where nuclear divisions occur earlier and faster than the embryo cell proliferation. In *Zea mays* seeds, genes inhibitory of invertase was localized is a region of the cells within the endosperm possibly reducing hexose flow to young embryo, ensuring nuclear division in endosperm (Bate et al., 2004). Cheng; Tallcercio; Chourey (1996) observed that mutations in the cell wall invertase gene resulted in miniature kernels and with endosperm severely reduced. When the embryo expands to the point where it begins to touch these cells, they die, and the invertase activity disappears. As a consequence, the levels of hexoses fall and sucrose becomes the main endosperm sugar. This induces a change of gene expression in favor of storage product accumulation. A change from predominantly hexose to sucrose content in the endosperm during development has also been reported for *Pisum sativum* (Borisjuk et al., 2002). Arabinose was present at all stages of embryonic development, both in the embryo and in megagametophyte tissue. After intense mitotic activity, in the early embryogenesis stage, the divisions are less random and the cells begin to differentiate give origin to late embryo. During the event of early to late embryo transition the maximum content of arabinose was found. In *P. taeda* arabinose concentrations were high during the earliest developmental stages and decreased to a constant concentration during late maturation (Pullman; Buchanan, 2008). These authors related concentrations about five-fold higher in embryo tissue than in megagametophyte tissue, similar to what we observed in the month of March at the beginning of late embryogenesis *in A. angustifolia*. Arabinose is an branching residues in storage polysaccharides have as xyloglucans and galactans (Buckeridge

et al., 2000). In a studies with seeds of Arabidopsis the arabinose was responsible for approximately 40% monosaccharide that composite the from the non-cellulosic polysaccharides of cell wall in embryos (Gomez et al., 2009). In zygotic embryo of *A. angustifolia* arabinose residues were abundant in arabinogalactan proteins, that predominantly accumulated during early and late embryogenesis of seed development characterized by intensive cell proliferation and root and shoot meristem differentiation, reducing the content at maturation (Dos Santos et al., 2006). In our work, we did not detect maltose contents during the Pro-embryogenic phase and in the early embryogenesis, but was present from in the late embryogenesis, higher levels of maltose were found in at the beginning of late embryogenesis. In seeds of *P. taeda* maltose contents presented an increasing concentration during the early stages of embryonic development that peaked in mid-development and then declined (Pullman; Buchanan, 2008). It was suggested that maltose has an effect in the morphology and histodifferentiation, since higher concentrations are found in the late embryogenesis, and this could be ascribed to the low supply of hexoses (Blanc et al., 2002; Steiner et al., 2005). This explanation tallies well with ‘low-nutrient stress’ or the ‘carbohydrate-deficit hypothesis’(Scott et al., 1995; Blanc et al, 2002; Pullman; Buchanan, 2008) which activate proteins synthesis of late embryo development. Maltose is often used during the late embryogenesis of somatic embryogenesis of *A. angustifolia*, aiming at to improve maturation of somatic embryos (Dos Santos et al., 2002; Steiner et al., 2005, 2008; Farias-Soares et al., 2014). Moreover, it was observed in *Vicia faba* seeds that cellular differentiation is initiated when hexose decreases and sucrose increases (Weber et al., 1998). In addition, has been reported that the transition of the division to cellular expansion and storage is usually associated with a decrease in invertase activity and an increase in Sucrose-syntase activity (Weber et al, 1998; Wang; Ruan, 2013). These findings may explain the high contents of sucrose in mature seeds of *A. angustifolia*. Kermode (1997) explains that the sucrose together with other disaccharides (oligosaccharides, raffinose and stachyose), may play a key protective role by accumulating under water deficit conditions, functioning to replace water and thus stabilizing membranes and other sensitive systems. Bryant, Koster, and Wolfe (2001); Halperin and Koster (2006) suggest that sucrose hindering the close approach of membranes to one another, and this proximity promotes phase transition of some phospholipids and even the demixing of

membrane components which is accompanied by exclusion of integral proteins.

Together with sucrose, LEAs have been the focus of much recent attention in the context of the acquisition of seed desiccation tolerance (Berjak; Pammenter, 2008). The dehydrin analysis during the early embryonic development of *A. angustifolia* was not yet reported in the literature. The studies are restricted to the mature embryo (Farias-Soares et al., 2013; Farrant et al., 1996). In the present work dehydrins screnned by the Western blot analysis did not reveal bands in early embryogenesic stages. Dehydrins are part of Group 2 (D11) of *LEA* proteins (Battaglia et al., 2008; Bewley; Hilhorst; Nonogaki, 2013), as the name itself suggests, they are produced in abundance during the late development of the embryo, thus justifying the absence of bands during the early embryogenesis. The transition of early to late embryogenic stage was marked by the presence of four bands of 21,5; 23; 26 and 27kDa. Only during the late embryogenesis heat-stable proteins of 23 and 45kDa were observed in axis embryonic. Sallandrouze; Faurobert; El Maâtaoui (2002) observed that *LEA* proteins are synthesized after the histodifferentiation and Battaglia et al. (2008) reported that accumulation of dehydrins occur when the embryos acquire desiccation tolerance. The desiccation induce reactions related to water stress, such as accumulation of dehydrins, while in desiccation sensitive seeds to protection mechanisms are not activated and lose their viability when stored for longer (KLEINWACHTER et al., 2014). Dehydrins do not denature upon drying or heating, and form a disordered structure that may prevent physical collapse of associated proteins during dehydration (Close, 1996, 1997; Hoekstra; Golovina; Buitink, 2001; Bewley; Hilhorst; Nonogaki, 2013). Studies reported that dehydrins are part of the process of developing orthodox seeds (Kermode, 1997; Radwan et al., 2014). However, dehydrin-related proteins were detected in intermediate seeds as *Fagus sylvatica* (Kalemba; Pukacka, 2012; Kalemba; Bagniewska-Zadworna; Ratajczak, 2015), and also in recalcitrant seeds as *Quercus robur, Castanea sativa, Acer psuedoplatanus*, *Acer saccharinum*, *Aesculus hippocastanum*, *Euterpe edulis*, *Barrmgtonia raceme, Castanospermum austral, Camellia sinensis, A. angustifolia, Poncirus trifohat, Zizania palustri*, (Finch-Savage; Pramanik; Bewley, 1994; Farrant et al, 1996; Panza et al, 2007; Farias-Soares et al., 2013). It been reported that *A. angustifolia* seeds keep its active metabolism at the end of the maturation, and germination is reduced when the moisture content is below 37%, while desiccation level

of 25% leads to complete loss of germinability (Tompsett, 1984; Espindola et al., 1994).

Despite these physiological characteristics, at the mature seed dehydrins were identified in nuclei, specifically in chromatin and in the matrix of the protein bodies (Farias-Soares et al. 2013). These authors identified the presence of six bands of heat-stable proteins between 18 to 29kDa, while Farrant et al. (1996) report three bands of 23, 26, and 28kDa. However, in the present work work, at the seed mature stage, we did not find bands of heat-stable protein fractions, but two heat-sensitive proteins bands of 21,5 and 23kDa were detected. *A. angustifolia* is a native and not domesticated species and differences in seed maturation characterize two or more varieties, that can be found in the same region (Reitz and Klein 1966). Mattos (2011) described five varieties of according to the seed maturation period, in south Brazil and recently in Santa Catarina state, four varieties were found (Zechini et al, 2012; Tagliari; Peroni, 2018). In our work the seed were collected from different plants from natural populations and could be from different varieties. Moreover, our work indicates that the dehydrin protein synthesis starts during embryogenesis until seed maturation. According to Radwan et al. (2014), atypical recalcitrant seeds seem to undergo a slight maturation, with a little water loss, sufficient to induce related stress reactions, such as dehydrin expression, but any longer storage time for these recalcitrant seeds causes a loss of viability. However, the dehydrins found in the present work refers to heat-sensitive fractions and Borovskii et al. (2002) propose that heat-sensitive dehydrins are not involved in the stress reactions, once that their accumulation not occurred in mitochondria of wheat, rye and maize, in response to cold, freezing, drought and ABA treatment, this results can justify the reduction of the viability of *A. angustifolia* seeds.

6.5 CONCLUSION

In *A. angustifolia*, the stages of zygotic embryo development and seed maturation are characterized by changes in the biochemistry profiles. This is the first work that describe together carbohydrates metabolism and dehydrins state along the zygotic embryo developmental stages. Our work indicates a correlation between carbohydrates and dehydrins synthesis besides to being a recalcitrant seeds. Pro-embryogenic and early embryogenesis stages display high contents of hexoses and low contents

of sucrose. However, during late embryogenesis and maturation, an increase in the sucrose: hexose ratio is observed, and maltose is detected. Dehydrins are not sufficient to confer desiccation tolerance, once that are accumulation during the late embryogenesis stages and in the seed maturation. This study provides a better understanding of the biochemical and physiological features underlying development and maturation of *A. angustifolia* zygotic embryos.

6.6 REFERENCES

AGAPITO-TENFEN, Sarah Z. et al. Patterns of polyembryony and frequency of surviving multiple embryos of the Brazilian pine *Araucaria angustifolia*. **Australian Journal of Botany**, [s. l.], n. 59, p. 749–755, 2011.

ANSELMINI, Justina Inês; ZANETTE, Flávio. Development and growth curve of the pine cones of *Araucaria angustifolia* (Bert.) O . Ktze, in the region of Curitiba – PR. **Brazilian archives of biology and technology aninternational journal**, [s. l.], v. 51, n. 4, p. 665–669, 2008.

BALBUENA, Tiago S. et al. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*). **Journal of Proteomics**, [s. l.], v. 72, n. 3, p. 337–352, 2009. Disponível em: <http://dx.doi.org/10.1016/j.jprot.2009.01.011>

BARBEDO, Claudio José; MARCOS, Filho Júlio. Tolerância a dessecação em sementes. **Acta Botanica Brasileira**, [s. l.], v. 12, n. 2, p. 145–164, 1998.

BATE, Nicholas J. et al. An Invertase Inhibitor from Maize Localizes to the Embryo Surrounding Region during Early Kernel Development. **Plant Physiology**, [s. l.], v. 134, n. January, p. 246–254, 2004.

BATTAGLIA, Mariana et al. The Enigmatic *LEA* Proteins and Other Hydrophilins. **Plant Physiology**, [s. l.], v. 148, n. 1, p. 6–24, 2008. Disponível em: <http://www.plantphysiol.org/cgi/doi/10.1104/pp.108.120725>

BERJAK, Patricia; PAMMENTER, N. W. From Avicennia to Zizania:

Seed recalcitrance in perspective. **Annals of Botany**, [s. l.], v. 101, n. 2, p. 213–228, 2008.

BEWLEY, J. Derek; HILHORST, Kent J. Bradford Henk W. M.; NONOGAKI, Hiro. **Seeds: physiology of development, germination and dormancy**. 3. ed. New York Heidelberg Dordrecht London: Springer, 2013.

BLANC, G. et al. Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Mull. Arg .). **Journal of Experimental Botany**, [s. l.], v. 53, n. 373, p. 1453–1462, 2002.

BORISJUK, Ljudmilla et al. Spatial analysis of plant metabolism: Sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns. **Plant Journal**, [s. l.], v. 29, n. 4, p. 521–530, 2002.

BORISJUK, Ljudmilla et al. Differentiation of legume cotyledons as related to metabolic gradients and assimilate transport into seeds. **Journal of Experimental Botany**, [s. l.], v. 54, n. 382, p. 503–512, 2003.

BOROVSKII, Genadii B. et al. Accumulation of dehydrin-like proteins in the mitochondria of cereals in response to cold , freezing , drought and ABA treatment. **BMC Plant Biology**, [s. l.], v. 7, p. 1–7, 2002.

BRYANT, Gary; KOSTER, Karen L.; WOLFE, Joe. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. **Seed Science Research**, [s. l.], v. 11, n. 01, p. 17–25, 2001. Disponível em: <http://www.journals.cambridge.org/abstract_S0960258501000034>

BUCKERIDGE, Marcos S.; PESSOA DOS SANTOS, Henrique; TINÉ, Marco Aurélio S. Mobilisation of storage cell wall polysaccharides in seeds. **Plant Physiology and Biochemistry**, [s. l.], v. 38, n. 1–2, p. 141–156, 2000.

BURLINGAME, L. Lancelot. The Morphology of *Araucaria brasiliensis*

. II . The Ovulate Cone and Female Gametophyte. **The University Chicago Press Journals**, [s. l.], v. 57, n. 6, p. 490–508, 1914.

BURLINGAME, L. Lancelot. The morphology of *Araucaria brasiliensis*. **Botanical Gazette**, [s. l.], v. LIX, p. 42, 1915.

BURRIEZA, Hernán P. et al. High salinity induces dehydrin accumulation in *Chenopodium quinoa* Willd. cv. Hualhuas embryos. **Plant and Soil**, [s. l.], v. 354, p. 69–79, 2012.

CAMPBELL, Scott A. et al. Dehydrins : genes , proteins , and associations with phenotypic traits. **New Phytopogist**, [s. l.], v. 137, n. 1, p. 61–74, 1997.

CARVALHO, P. E. R. **Espécies florestais brasileiras: Recomendações silviculturais, potencialidades e uso da madeira**. Brasília, DF: Embrapa, 1994.

CHENG, Wan-Hsing; TALLCERCIO, Earl W.; CHOUREY, Prem S. The miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. **The Plant Cell Online**, [s. l.], v. 8, n. 6, p. 971–983, 1996. Disponível em: <http://www.plantcell.org/cgi/doi/10.1105/tpc.8.6.971>

CLOSE, Timothy J. et al. An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. **Plant Physiology**, [s. l.], v. 101, n. 3, p. 773–779, 1993. Disponível em: <https://www.jstor.org/stable/4275035>

CLOSE, Timothy J. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. **Physiologia Plantarum**, [s. l.], v. 97, n. 4, p. 795–803, 1996.

CLOSE, Timothy J. Dehydrins: A commonalty in the response of plants to dehydration and low temperature. **Physiologia Plantarum**, [s. l.], v. 100, n. 2, p. 291–296, 1997.

DOWNIE, Bruce; BEWLEY, J. Derek. Soluble sugar content of white spruce (*Picea glauca*) seeds during and after germination. **Physiologia**

Plantarum, [s. l.], v. 110, p. 1–12, 2000.

DURZAN, D. J. Process control in somatic polyembryogenesis. In: HÄLLGREN, J. (Ed.). **Proceedings of Frans Symposium Department of Forest Genetics and Plant Physiology'**. Swedish: University of Agricultural Sciences, 1988. p. 8–147.

ESPINDOLA, L. Salmen et al. Cellular and metabolic damage induced by desiccation in recalcitrant *Araucaria angustifolia* embryos. **Seed Science Research**, [s. l.], v. 4, n. 2, p. 193–201, 1994.

FARIAS-SOARES, Francine L. et al. The transition of Pro-embryogenic masses to somatic embryos in *Araucaria angustifolia* (Bertol .) Kuntze is related to the endogenous contents of IAA , ABA and polyamines. **Acta Physiologiae Plantarum**, [s. l.], n. 36, p. 1853–1865, 2014.

FARIAS-SOARES, Francine Lunardi et al. Immunoanalysis of dehydrins in *Araucaria angustifolia* embryos. **Protoplasma**, [s. l.], v. 250, p. 911–918, 2013.

FARJON, Aljos; FILER, Denis. **An atlas of the world's conifers: an analysis of their distribution, biogeography, diversity, and conservation status**. 1. ed. Boston: EJ Brill, 2013.

FARRANT, J. M.; PAMMENTER, N. W.; BERJAK, P. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. **Planta**, [s. l.], p. 189–198, 1989.

FARRANT, Jill M. et al. Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may b. **Seed**, [s. l.], v. 6, n. 4, p. 175–182, 1996.

FILONOVA, L. H. et al. Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. **Cell Death and Differentiation**, [s. l.], v. 9, n. 10, p. 1057–1062, 2002.

FILONOVA, Lada H.; BOZHKOV, Peter V; ARNOLD, Sara Von. Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. **Jounal of Experimental Botany**, [s.

l.], v. 51, n. 343, p. 249–264, 2001.

FILSON, Paul B.; BENJAMIN, E. Dawson-Andoh. Characterization of sugars from model and enzyme-mediated pulp hydrolyzates using high-performance liquid chromatography coupled to evaporative light scattering detection. **Bioresource Technology**, [s. l.], v. 100, n. 24, p. 6661–6664, 2009. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2008.12.067>

FINCH-SAVAGE, W. E.; PRAMANIK, S. K.; BEWLEY, J. D. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. **Planta**, [s. l.], v. 193, n. 4, p. 478–485, 1994.

GALAU, Glenn A.; CLOSE, Timothy J. Sequences of the cotton group 2LEA/RAB/dehydrin proteins encoded by LEA3 cDNAs. **Plant Physiology**, [s. l.], v. 98, n. 4, p. 1523–1525, 1992. Disponível em: <https://www.jstor.org/stable/4274284>

GIFFORD, Ernest M.; FOSTER, Adriance S. **Morphology and Evolution of Vascular Plants**. 3. ed. [s.l.] : WH Freeman, 1989.

GOMEZ, Leonardo D. et al. Arabinan metabolism during seed development and germination in Arabidopsis. **Molecular Plant**, [s. l.], v. 2, n. 5, p. 966–976, 2009. Disponível em: <http://dx.doi.org/10.1093/mp/ssp050>

GÖSSLOVÁ, Monika et al. Comparing carbohydrate status during norway spruce seed development and somatic embryo formation. **In vitro Cell. Dev. Biol**, [s. l.], v. 37, n. February, p. 24–28, 2001.

GUERRA, Miguel Pedro et al. Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Ktze. In: JAIN, S. Mohan; GUPTA, Pramod K.; NEWTON, Ronald J. (Eds.). **Somatic embryogenesis in woody plants.** Dordrecht: Kluwer Academic Publishers, 2000. p. 457–478.

GUERRA, Miguel Pedro et al. Evolução, ontogênese e diversidade genética em *Araucaria angustifolia*. In: BARBIERI, Rosa Lía; STUMPF, Elisabeth Regina Tempel (Eds.). **Origem e evoluçao de plantas cultivadas.** Brasília DF: Embrapa, 2008. p. 149–184.

GUERRA, Miguel Pedro et al. Araucaria Evolution, ontogenesis and genetic diversity. In: BARBIERI, R.; STUMPF, E. R. (Eds.). **Origin and evolution of cultivated plants.** 1. ed. Brasília DF: Embrapa, 2012. p. 151–186.

HAINES, R. J. Embryo development and anatomy in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 31, n. 2, p. 125–140, 1983.

HAINES, R. J.; PRAKASH, N. Pro-embryo development and suspensor elongation in *Araucaria juss*. **Australian Journal of Botany**, [s. l.], v. 28, n. 6, p. 511–522, 1980.

HALPERIN, Stephen J.; KOSTER, Karen L. Sugar effects on membrane damage during desiccation of pea embryo protoplasts. **Journal of Experimental Botany**, [s. l.], v. 57, n. 10, p. 2303–2311, 2006.

HILHORST, Henk W. M.; TOOROP, Peter E. Reviw on dormancy germinability, and germination in crop and weed seeds. **Advances in agronomy**, [s. l.], v. 61, p. 112–165, 1997.

HOEKSTRA, Folkert A.; GOLOVINA, Elena A.; BUITINK, Julia. Mechanism of plant desiccation tolerance. **Trends in Plant Science**, [s. l.], v. 6, n. 9, p. 431–438, 2001.

IRAQI, Driss; TREMBLAY, Francine M. Analysis of carbohydrate metabolism enzymes and cellular contents of sugars and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. **Jou**, [s. l.], v. 52, n. 365, p. 2301–2311, 2001.

JOHANSEN, Donald Alexander. **Plant embryology: embryology of the spermatophyta.** Massachusetts: Waltham: Chronica Botanica Company, 1950.

KALEMBA, Ewa Marzena; BAGNIEWSKA-ZADWORNA, Agnieszka; RATAJCZAK, Ewelina. Multiple Subcellular Localizations of Dehydrin-like Proteins in the Embryonic Axes of Common Beech (*Fagus sylvatica* L.) Seeds During Maturation and Dry Storage. **Journal of Plant Growth Regulation**, [s. l.], v. 34, n. 1, p. 137–149, 2015.

KALEMBA, Ewa Marzena; PUKACKA, Stanisława. Association of Protective Proteins with Dehydration and Desiccation of Orthodox and Recalcitrant Category Seeds of Three Acer Genus Species. **Journal of Plant Growth Regulation**, [s. l.], v. 31, n. 3, p. 351–362, 2012.

KERMODE, Allison R. Approaches to elucidate the basis of desiccation-tolerance in seeds. **Seed Science Research**, [s. l.], v. 7, n. 02, p. 75–95, 1997. Disponível em: <http://www.journals.cambridge.org/abstract_S0960258500003421>

KERMODE, Allison R.; FINCH-SAVAGE., Bill E. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. In: **Desiccation and survival in plants: Drying without dying,**. [s.l: s.n.]. p. 149–184.

KLEINWACHTER, Maik et al. Dehydrin expression in seeds: an issue of maturation drying. **Frontiers in Plant Science**, [s. l.], v. 5, n. August, p. 1–3, 2014. Disponível em: <http://journal.frontiersin.org/article/10.3389/fpls.2014.00402/abstract>

KONRÁDOVÁ, Hana et al. Sucrose metabolism during somatic and zygotic embryogeneses in Norway spruce : content of soluble saccharides and localisation of key enzyme activities. **Plant Physiol.**, [s. l.], v. 396, p. 387–396, 2002.

LAEMMLI, U. K. Nature Publishing Group. **Nature**, [s. l.], v. 227, n. 15, p. 227–231, 1970.

LIPAVSKA, Helena; KONRÁDOVÁ, Hana. Invited review: Somatic embryogenesis in conifers: the role of carbohydrate metabolism. **In vitro Cell. Dev. Biol**, [s. l.], n. February, p. 23–30, 2004.

MANTOVANI, Adelar; MORELLATO, L. Patrícia C.; REIS, Maurício Sedrez Dos. Fenologia reprodutiva e produção de sementes em Araucaria angustifolia (Bert .) O . Kuntze 1. **Revista Brasil. Bot**, [s. l.], v. 3, n. 27, p. 787–796, 2004.

MATTOS, João Rodrigues. **O Pinheiro Brasileiro**. 2º ed ed. Lages - SC: Artes gráficas Princesa Ltda., 1994.

MATTOS, João Rodrigues. **O pinheiro brasileiro**. Ed. da UFS ed. Florianópolis.

NAVARRO, Bruno V et al. Carbohydrate-mediated responses during zygotic and early somatic embryogenesis in the endangered conifer , Araucaria angustifolia. **Plos one**, [s. l.], p. 1–20, 2017.

OLIVEIRA, Leandro F. De et al. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (Araucaria angustifolia) seed development. **Tree Physiology**, [s. l.], n. 37, p. 116–130, 2016.

OWENS, John N. **The Reproductive biology of western White pine**. 4. ed. Columbia: Prepared for Forest Genetics Council of British Columbia., 2004.

PAMMENTER, N. W.; BERJAK, Patricia. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. **Seed Science Research**, [s. l.], v. 9, p. 13–37, 1999.

PANZA, V. et al. Detection of dehydrin-like proteins in embryos and endosperm of mature Euterpe edulis seeds. **Protoplasma**, [s. l.], v. 231, n. 1–2, p. 1–5, 2007.

PANZA, Víctor et al. Storage reserves and cellular water in mature seeds of Araucaria angustifolia. **Botanical Journal of the Linnean Society**, [s. l.], n. 140, p. 273–281, 2002.

PERROT-RECHENMANN, Catherine. Cellular responses to auxin: division versus expansion. **Cold Spring Harbor perspectives in biology**, [s. l.], v. 2, n. 5, p. 1–15, 2010.

PULLMAN, Gerald S.; BUCHANAN, Mike. Identification and quantitative analysis of stage-specific carbohydrates in loblolly pine (Pinus taeda) zygotic embryo and female gametophyte tissues. **Tree Physiology**, [s. l.], v. 28, n. August, p. 985–996, 2008.

RADWAN, A. et al. Dehydrin expression in seeds and maturation drying:

A paradigm change. **Plant Biology**, [s. l.], v. 16, n. 5, p. 853–855, 2014.

RAGHAVAN, V.; SHARMA, Kiran K. Angiosperms. In: [s.l: s.n.]. p. 73–115.

REITZ, Raulino; KLEIN, Roberto M. **Araucariáceas. Flora Ilustrada Catarinense**. Itajai: Herbário Barbosa Rodrigues, 1966.

RIBEIRO, Milton Cezar et al. The Brazilian Atlantic Forest : How much is left , and how is the remaining forest distributed ? Implications for conservation. **Biological Conservation**, [s. l.], v. 142, n. 6, p. 1141–1153, 2009. Disponível em: <http://dx.doi.org/10.1016/j.biocon.2009.02.021>

ROGGE-RENNER, Gladys D. et al. Structural and component characterization of meristem cells in Araucaria angustifolia (Bert.) O. Kuntze zygotic embryo. **Protoplasma**, [s. l.], v. 250, n. 3, p. 731–739, 2013.

ROGGE-RENNER, Gladys Daniela et al. Ontogenia de megaestróbilos de Araucaria angustifolia (Bert.) O. Kuntze (Araucariaceae) Ontogeny. **Acta biologica catarinese**, [s. l.], v. 4, n. 2, p. 30–41, 2017.

ROLLAND, Filip; BAENA-GONZALEZ, Elena; SHEEN, Jen. Sugar Sensing and Signaling in Plants : Conserved and Novel Mechanisms. **Annu Rev Plant Biol**, [s. l.], v. 57, p. 675–712, 2006.

SALLANDROUZE, Anne; FAUROBERT, Mireille; EL MAâTAOUI, Mohamed. Characterization of the developmental stages of cypress zygotic embryos by two-dimensional electrophoresis and by cytochemistry. **Physiologia Plantarum**, [s. l.], v. 114, n. 4, p. 608–618, 2002.

SCOTT, P.; LYNE, R. L.; REES, T. Metabolism of maltose and sucrose by microspores isolated from barley (Hordeum vulgare L .). **Planta**, [s. l.], v. 44, p. 435–441, 1995.

SETOGUCHI, Hiroaki et al. Phylogenetic relationships within Araucariaceae based on rbcl gene sequences. **American Journal of**

Botany, [s. l.], v. 85, n. 11, p. 1507–1516, 1998.

SHIBATA, Marília; MARIA, Cileide; COELHO, Medeiros. Early harvest increases post-harvest physiological quality of Araucaria angustifolia (Araucariaceae) seeds. **Biología Tropical**, [s. l.], v. 64, n. June, p. 885–896, 2016.

SHIMOYA, C. **Contribuição ao estudo do ciclo biológico de Araucaria angustifolia (Bert.) O. Ktze**. 2. ed. Viçosa: Experimentiae, 1962.

STEEVES, Taylor. A.; SUSSEX, Ian. M. **Patterns in plant development.** 2. ed. New York: Cambridge University Press, 1989.

STEINER, Neusa et al. Effect of Carbon Source on Morphology and Histodifferentiation of Araucaria angustifolia Embryogenic Cultures. **Brazilian Archives of Biology and Technology**, [s. l.], v. 48, n. November, p. 895–903, 2005.

STEINER, Neusa et al. Araucaria angustifolia Biotechnology Araucaria angustifolia Biotechnology. **Global Science Books**, [s. l.], v. 2, n. 1, p. 20–28, 2008.

STEINER, Neusa et al. Toward establishing a morphological and ultrastructural characterization of Pro-embryogenic masses and early somatic embryos of Araucaria angustifolia (Bert .) O . Kuntze. **Protoplasma**, [s. l.], 2015. Disponível em: <http://dx.doi.org/10.1007/s00709-015-0827-0>

TAGLIARI, Mário Muniz; PERONI, Nivaldo. Local varieties of Araucaria angustifolia (Bertol.) Kuntze (Pinales: Araucariaceae) in southern Brazil: A brief discussion about landscape domestication. **Biotemas**, [s. l.], v. 31, n. 3, p. 59–68, 2018. Disponível em: <https://periodicos.ufsc.br/index.php/biotemas/article/view/2175-7925.2018v31n3p59>

TOMPSETT, P. .. Desiccation studies in relation to the storage of Araucaria seed. **Annals of applied Biology**, [s. l.], v. 105, n. 3, p. 581–586, 1984.

WANG, Lu; RUAN, Yong-ling. Regulation of cell division and expansion by sugar and auxin signaling. **Plant science**, [s. l.], v. 4, n. May, p. 1–9, 2013.

WEBER, H. et al. Assimilate uptake and the regulation of seed development. **Seed Science Research**, [s. l.], v. 8, n. 3, p. 331–345, 1998.

WEBER, Hans; BORISJUK, Ljudmilla; WOBUS, Ulrich. Molecular Physiology of Legume Seed Development. **Annual Review of Plant Biology**, [s. l.], v. 56, p. 253–279, 2005.

WENDLING, Ivar; BRONDANI, Gilvano Ebling. Vegetative rescue and cuttings propagation of Araucaria angustifolia (Bertol.) Kuntze. **Revista Árvore**, [s. l.], v. 39, n. 1, p. 93–104, 2015.

WILLIAMS, Claire G. **Conifer Reproductive Biology**. New York: Springer, 2009.

WINKELMANN, Traud. **Somatic Versus Zygotic Embryogenesis: Learning from Seeds: In Vitro Embryogenesis in Higher Plants**. New York: Humana Press, 2016.

ZECHINI, Alex Anderson et al. Produção, comercialização e identificação de variedades de pinhão no entorno da Floresta Nacional de Três Barras–SC. **Revista Biodiversidade Brasileira**, [s. l.], v. 2, n. 2, p. 74–82, 2012. Disponível em: <http://www.icmbio.gov.br/revistaeletronica/index.php/BioBR/article/view/275>

7 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente trabalho está inserido na temática que envolve a compreensão de aspectos morfológicos e bioquímicos envolvidos durante o desenvolvimento de sementes de *A. angustifolia.* As características morfoanatômicas da pré-fertilização até a embriogênese tardia foram elucidadas, através das técnicas de microscopia de luz, de transmissão e confocal. Detalhes do desenvolvimento embrionário foram explorados, e alguns aspectos mencionados pela primeira vez, complementando informações sobre os eventos que ocorrem no ciclo reprodutivo da *A. angustifolia.* Após o evento da polinização, o qual ocorreu entre agosto a setembro do primeiro ano do ciclo reprodutivo em 2011, iniciou a formação do megagametófito, onde foi observado o nucelo com a célula mãe do megásporo rodeada pelas células esporogênicas, e a formação de uma cavidade com núcleos livres. A fase de núcleo livre durou cerca de seis meses, já que no momento da polinização as arquegônias não se encontravam diferenciadas. Entre agosto a setembro de 2012 os núcleos livres já apresentavam parede celular e estavam organizados formando um tecido homogêneo composto por células protaliais que preenchiam a cavidade. Cerca de 13 meses após a polinização, no mês de outubro de 2012, foi observado a formação de seis arquegônias. O ciclo proposto por Guerra et al., (2008;20012) e estudos realizados por Mantovani et al., (2004), não definiram o com precisão a fertilização. Neste trabalho foi observado que esta ocorre cerca de um ano após a polinização, em outubro do segundo ano do ciclo reprodutivo. Após a fecundação, em novembro, aproximadamente quatorze a quinze meses após a polinização foi observado a presença de poliembriões com suas células características (de capa, embrionárias e do suspensor). Na sequência do desenvolvimento, ocorre a regressão dos embriões subordinados e apenas o embrião dominante permanece na semente. O proembrião e se desenvolve, células suspensoras de alongam, enquanto as células de capa sofrem degeneração, e surge então uma massa de células embriogênica, dando origem ao embrião no estágio inicial. A transição do embrião inicial para o embrião tardio é marcada pela diferenciação celulardando origem a protoderme, córtex e procambio. Durante a embriogênese tardia, a diferenciação dos meristemas e apicais caulinar e radicular é evidente.

Entre os processos bioquímicos que ocorrem na embriogênese zigótica as proteínas e os carboidratos estão envolvidos no metabolismo basal e energético sendo essenciais no desenvolvimento das sementes. A

análise de carboidratos realizada durante todos os estágios de desenvolvimento do embrião zigótico e seu respectivo megagametófito, mostrou um comportamento semelhante entre os tecidos. Os estágios proembrionário e embrionário inicial, foram caracterizados por altos conteúdos de hexoses (frutose e glicose) e baixo conteúdo de sacarose. Corroborando com nossos resultados, trabalhos mostram que durante os estágios iniciais de desenvolvimento, ocorre a alta atividade de enzimas invertase, responsável pela hidrólise de sacarose em frutose e glicose, aumentado o conteúdo de hexose nas células. Estágios iniciais de desenvolvimento são marcados pela intensa atividade mitótica, visando o crescimento do embrião, e estudos apontam o papel da glicose como uma molécula de sinalização na divisão celular. Com a transição do estágio embrionário inicial para o tardio, carboidratos como maltose passaram a ser detectados, e a relação hexose:sacarose diminuiu, sendo que, o conteúdo de hexose reduziu e a sacarose passou a ser o açúcar mais abundante. Diante desse comportamento, sugerimos que sacarose e a maltose estão relacionadas com os processos de sinalização para a diferenciação celular. A suplementação de maltose em embriões somáticos de coníferas, durante a embriogênese tardia é comum e eficiente para conversão de embriões. Devido a hidrólise lenta da maltose muitos autores defendem a teoria do "déficit de carboidratos", reduzindo a disponibilidade de hexose e como consequência as células diminuem a divisão e passam a se diferenciar. O conteúdo elevado de sacarose no final do desenvolvimento pode estar relacionado ao potencial osmótico, ao crescimento celular, proteção contra dessecação além de composto de reserva. A arabinose esteve presente durante todas as fases de desenvolvimento, já que é um componente de parede celular.

Além do perfil dos carboidratos, proteínas do tipo dehidrinas foram analisadas por Western Blot durante o desenvolvimento embrionário e a maturação da *A. angustifolia*. As dehidrinas pertencem ao segundo grupo das proteínas LEA (*late embryogeneses accumulated*), as quais são acumuladas durante as fases finais da embriogênese em resposta à secagem e a sua expressão cessa rapidamente após embebição. Estas proteínas têm natureza hidrofílica, se ligam a moléculas de água impedindo a sua saída das células, consequentemente mantem a estabilização de membranas e outras proteínas. Devido a esta característica, as dehidrinas eram tidas como proteínas de sementes ortodoxas e ausentes em sementes recalcitrantes. Não foi detectada a presença dessas proteínas durante o estágio inicial de desenvolvimento, o

que é justificado, já que dehidrinas fazem parte do segundo grupo das proteínas *LEA*, as quais são abundantes durante a embriogênese tardia. Como esperado, proteínas dehidrinas passaram a ser detectadas a partir da transição da embrigênese inicial para a tardia, onde fração proteica total de 21,5, 23, 26 e 28kDa estavam presentes em eixos embrionários e nos cotilédones. Com o desenvolvimento subsequente, banda de 28kDa não foi detectada enquanto a banda de 23kDa estava presente somente no eixo. Recentemente, um estudo com sementes maturas de *A. angustifolia* usando a técnica de imunolocalizações *in situ* mostrou dehidrinas nos eixos embrionário e cotilédones, e a nível subcelular estas se encontravam associadas aos corpos proteicos, microcorpos e a cromatina no núcleo (Farias-Soares et al., 2013).

Devido à importância ecológica, econômica, social e ao risco de extinção da *A. angustifolia,* nosso trabalho fornece embasamento para a compreensão dos aspectos morfoanatômicos, bioquímicos e fisiológicos durante o desenvolvimento de sementes da *A. angustifolia.* Caracterizar estágios do desenvolvimento embrionário, em paralelo, elucidar a função dos carboidratos solúveis durante estes estágios e estudar em qual período ocorre maior acúmulo de dehidrinas, auxilia na otimização da época de coleta para armazenamento das sementes, garantindo sua viabilidade. Uma vez, que a espécie tem natureza recalcitrante, não tolera dessecação e perde a viabilidade quando armazenadas por longos períodos. Além disso, estudos durante estágios específicos do desenvolvimento embrionário das sementes de *A. angustifolia*, auxilia na compressão dos processos metabólicos envolvidos durante a embriogênese somática, gerando informações importantes para otimização do protocolo *in vitro,* o qual é uma alternativa para a conservação dessa espécie.