Marilia Shibata

MODIFICAÇÕES BIOQUÍMICAS, FISIOLÓGICAS E MORFOLÓGICAS NO *CONTINUUM* MATURAÇÃO-GERMINAÇÃO DE SEMENTES DE *Araucaria angustifolia*

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Modificações bioquímicas, fisiológicas e morfológicas no *continuum* maturação-germinação de sementes de *Araucaria angustifolia*

por

Marilia Shibata

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“Debaixo de uma aparente desordem e confusão, tudo é ordem e harmonia, na terra entre os viventes, como nos céus entre as estrelas.”

Marquês de Maricá

RESUMO

Araucaria angustifolia é uma das espécies de maior importância para o sul do Brasil. Suas sementes apresentam valor alimentício, econômico, social e cultural para as comunidades coletoras. O desenvolvimento das sementes de gimnospermas, como *A. angustifolia*, pode ser dividida em duas fases principais: a embriogênese zigótica e a maturação das sementes. Diversos estudos têm sido conduzidos durante a embriogênese zigótica de *A. angustifolia*, contudo pouco se conhece sobre o processo de maturação das sementes, que ocorre entre os meses de abril até julho. Assim, o presente estudo objetivou analisar as mudanças bioquímicas, fisiológicas, morfológicas e no ciclo celular durante a maturação das sementes de *A. angustifolia* e o efeito de diferentes estádios de desenvolvimento no armazenamento. Sementes de *A. angustifolia* foram coletadas nos estádios de desenvolvimento cotiledonar, II, III e IV nos anos de 2014 e 2015 e, em seguida, armazenadas em refrigerador por até 360 dias, com intervalos de 30, 60, 90 e 180 dias. Foram realizadas análises morfológicas pela análise de microscopia de luz e eletrônica de transmissão; análises bioquímicas com o uso da espectroscopia de infravermelho com transformada de Fourier (FTIR), atividade enzimática e níveis hormonais; e análises fisiológicas pelos testes de germinação, tetrazólio, condutividade elétrica e índice de velocidade de germinação. Os resultados indicaram uma intensa atividade metabólica durante todo o período de coleta das sementes. Nos estádios III e IV foram observadas mitocôndrias com uma morfologia diferenciada, uma diferenciação do procâmbio e a formação de canais resiníferos. Nestes estádios também foram observados um aumento nas células na fase G2 do ciclo celular e a presença de algumas células em processo de divisão celular pela microscopia de luz. Tais mudanças parecem indicar uma mudança durante o desenvolvimento das sementes para o metabolismo germinativo. Estas mudanças associadas a uma queda nos níveis de ácido abscísico e um aumento nos níveis de ácido indolacético podem ter colaborado para o incremento na qualidade fisiológica nestes estádios. Tais resultados são inéditos e tornam-se evidentes as mudanças morfológicas, hormonais e fisiológicas das sementes, sendo que claramente pode-se observar uma divisão dos estádios cotiledonar e II dos estádios III e IV. As diferenças das sementes em cada estádio de desenvolvimento refletiram no potencial de armazenamento, com um incremento na qualidade fisiológica após o armazenamento, quando as

sementes foram coletadas no estádio cotiledonar. As modificações ultraestruturais e enzimáticas indicaram que até os 60 dias de armazenamento as características são semelhantes ao processo germinativo e aos 90 dias de armazenamento, as sementes parecem iniciar o processo de deterioração com um incremento na atividade das enzimas antioxidantes e desorganização celular. Assim, os resultados destacaram a importância do detalhamento do processo de maturação das sementes de *A. angustifolia* e elucidaram algumas mudanças que podem ocorrer neste período.

Palavras-chave: desenvolvimento de sementes; araucária; microscopia; germinação; conservação.

ABSTRACT

Araucaria. angustifolia is one of the most important plant species of southern Brazil. Its seeds are of dietary, economic, social and cultural value to the communities that gather them. Seed development of gymnosperms, such as *A. angustifolia*, can be divided into two main phases: embryogenesis and seed maturation. Several studies have investigated zygotic embryogenesis of *A. angustifolia*, but little is known about the process of seed maturation, which occurs between April and July. Therefore, the present study aimed to analyze the biochemical, physiological, morphological and cell-cycle changes during the maturation of seeds of *A. angustifolia*, and the effect of different stages of development on storage. Seeds of *A. angustifolia* were collected in the cotyledonary developmental stages II, III and IV in the years of 2014 and 2015, and then stored in a refrigerator for up to 360 days at intervals of 30, 60, 90 and 180 days. Morphological analyses were performed using light and transmission elctron microscopy; biochemical analyses were performed using fourier transform infrared spectroscopy (FTIR), enzymatic activity and hormone levels; and physiological analyses were done using tests for germination, tetrazolium, electrical conductivity and germination speed index. The results indicated an intense metabolic during the period of see collection. Observations in stages III and IV, revealed mitochondria with a differenciated morphology, a differentiated procambium and the formation of resiniferous channels. These stages also revealed an increase in the number of cells in the G2 phase of the cell cycle and the presence of some cells under going division by light microscopy. Such changes seem to indicate an early change during seed development for germinative metabolism. These changes, along with the associated drop in abscisic acid levels, and an increase in indoleacitic acid levels, may have contributed to an increase in physiological quality during these stages. The results of the present study are unprecedented, and the morphological, hormonal and physiological changes of seeds become evident, and a division between cotyledonary stage and stage II, and stages III and IV are clearly observed. The differences among seeds at each stage of development reflect the storage potential, with an increase in physiological quality after storage, when the seeds were collected at the cotylendary stage. The ultrastructural and enzymatic modifications indicated that until 60 days of storage the changes are similar to the germination process, and at the 90 days of storage, the seeds seem to initiate the deterioration process with an increase in the activity of

antioxidant enzymes and cellular disorganization. The results of this study highlight the importance of detailing the maturation process of *A. angustifolia*, and elucidating changes that may occur during this period.

Keywords: seed development; araucaria; Microscopy; Germination; Conservation

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INTRODUÇÃO E JUSTIFICATIVA

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LISTA DE ABREVIATURAS E SIGLAS

ABA – Ácido abscísico
AIA – Ácido indolacético
Am – Altered mitochodria
APX – Ascorbato peroxidase
A.S – Ácido salicílico
CAT – Catalase
CBB – Coomassie Brilliant Blue
CE – Condutividade elétrica
CW – Cell wall
EC – Electrical conductivity
ER – Endoplasmic reticulum
FOM – Floresta Ombrófila Mista
FTIR – Fourier transform infrared spectroscopy
GB – Golgi body
GSI – Germination speed index
IUCN – União Internacional para a Conservação da Natureza e dos Recursos Naturais
IVG – Índice de Velocidade de Germinação
Lb – Lipid bodies
LEA – "Late embryogenesis abundant"
LM – Light microscopy
Mt – Mitochodria
N - nucleus
PAS – Periodic acid-Schiff
PCA – Principal component analysis
RAS – Regras para Análise de Sementes
RAM - Root apical meristem
RER - Rough endoplasmic reticulum
SAM – Shoot apical meristem
SOD – Superoxido dismutase
S – Starch
TB-O - Toluidine blue
TEM - Transmission electron microscopy
V – Vacuole

SUMÁRIO

1. INTRODUÇÃO E JUSTIFICATIVA

A espécie *Araucaria angustifolia* (Bert.) Kuntze é uma conífera nativa do Sul do Brasil que apresenta importância social, econômica e ecológica (WENDLING; BRONDANI, 2015). É encontrada na Floresta Ombrófila Mista (FOM), com ocorrência nos estados do Paraná, Santa Catarina e Rio Grande do Sul com algumas manchas esparsas em São Paulo, Minas Gerais e Rio de Janeiro (MATTOS, 2011).

Esta espécie sofreu intensa exploração madeireira, e, além disso, em sua região de ocorrência houve grande expansão da agricultura (WREGE et al., 2009), tornando-a criticamente em perigo de extinção (The World Conservation Union) (IUCN, 2016) e na Lista Oficial das Espécies da Flora Brasileira Ameaçadas de Extinção (BRASIL, 2008). Atualmente, no sul do Brasil foi observado menos de 25% da área original de ocorrência (VIBRANS et al., 2013) e há uma grande necessidade de preservar estas áreas, pois são consideradas prioritárias para a conservação (MYERS et al., 2000; REZENDE et al., 2014).

Com o esgotamento das reservas naturais e com incentivos para plantios de espécies exóticas com potencial madeireiro, novas preocupações surgiram no intuito de conservar *A. angustifolia* (ZECHINI et al., 2012), assim como surgiram preocupações em relação aos recursos dela provenientes, dentre estes suas sementes (Figura 1).

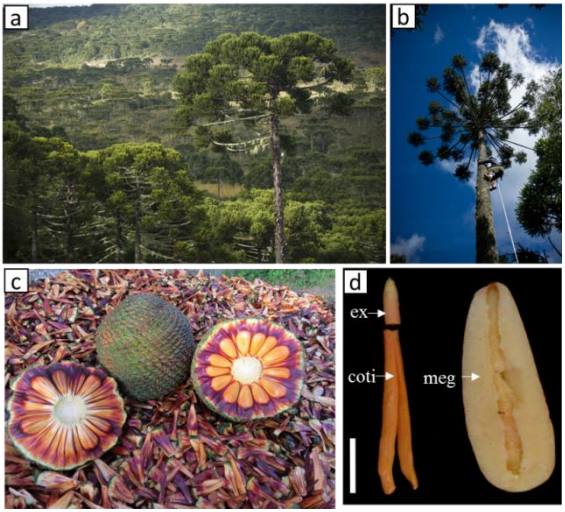

Figura 1 - Áreas remanescentes de *A. angustifolia* no município de Painel – SC. Fonte: (a, b)Valdemir Cunha; (c, d) autoria própria. (a), agricultor escalando um

exemplar de *A. angustifolia* (b) para a coleta das pinhas (c); partes da semente: ex: eixo embrionário, coti: cotilédones, meg: megagametófito (d). Barra: 1 cm

As sementes de *A. angustifolia* são, atualmente, fonte de alimento e renda para muitas propriedades de agricultores familiares e para pessoas de baixa renda (ZECHINI et al., 2012). Não somente um recurso alimentício e econômico, as sementes desta espécie possuem importante valor social e cultural para comunidades coletoras (ADAN et al., 2016; SILVA; REIS, 2009). Por isso, diversos estudos têm sido realizados envolvendo a fenologia reprodutiva desta espécie com a descrição dos períodos de cada estádio reprodutivo, desde a dispersão do pólen até as sementes estarem maduras, ocorrendo em média em dois anos (ANSELMINI; ZANETTE, 2008; ANSELMINI; ZANETTE; BONA, 2006; GUERRA et al., 2008; LATORRE; ALARCÓN; FASSOLA, 2013; MANTOVANI; MORELLATO; REIS, 2004; SANT'ANNA et al., 2013; SOUSA; HATTEMER, 2003) ou a descrição morfológica das estruturas reprodutivas e das sementes maduras (ADAN, 2013; KUHN; MARIATH, 2014; PANZA et al., 2002; ROGGE-RENNER, 2014; SOUZA et al., 2010).

Nos últimos anos, os principais avanços têm sido obtidos na elucidação das rotas metabólicas do desenvolvimento das sementes de *A. angustifolia* (BALBUENA et al., 2011; FARIAS-SOARES et al., 2013; OLIVEIRA et al., 2016) e maior ênfase para a definição de um protocolo adequado e entendimento dos mecanismos que envolvem a embriogênese somática (DIAS, 2016; DOS SANTOS et al., 2016; DOUÉTTS-PERES et al., 2016; ELBL et al., 2015; FARIAS-SOARES et al., 2014; FRAGA et al., 2016a, 2016b; JO et al., 2014; SCHLÖGL et al., 2012; STEINER et al., 2015). E também um importante avanço na técnica de criopreservação para a conservação a longo prazo desta espécie (FRAGA et al., 2016c). Contudo, pouco se conhece sobre as alterações das sementes de *A. angustifolia* durante o processo de maturação. Em geral, este período é denominado apenas de maduro pois é observado principalmente a morfologia do embrião que encontram-se maduro a partir de abril/maio. Entretanto, o período de maturação e de coleta das sementes compreende os meses de abril até junho/julho, observando-se diferenças fisiológicas e bioquímicas neste período (SHIBATA, 2013).

O desenvolvimento das sementes de gimnospermas, como *A. angustifolia*, pode ser dividido em duas fases principais: a embriogênese zigótica e a maturação das sementes (SINGH; JOHRI, 1972). Inicialmente, a embriogênese zigótica de *A. angustifolia* é caracterizada

por uma fase de núcleos livres, seguida por divisões celulares assimétricas até a formação dos proembriões (BURLINGAME, 1915; GUERRA et al., 2008; ROGGE-RENNER, 2014). Posteriormente, há a formação de mais de um embrião (poliembrionia), porém apenas um embrião permanece na semente madura (AGAPITO-TENFEN et al., 2012; BURLINGAME, 1915; GUERRA et al., 2008; ROGGE-RENNER, 2014). O desenvolvimento do embrião dominante inicia no mês de dezembro, seguindo pelos estádios iniciais (pró-embrião) e tardios (globular e torpedo), e por fim, o embrião encontra-se no estádio cotiledonar e maduro (Steiner, 2005).

No estádio cotiledonar, as sementes iniciam a maturação e adquirem a capacidade de germinação. Apesar do embrião já estar formado, estudos anteriores relataram uma baixa germinação, que não permanece no final da maturação (estádio IV) (SHIBATA; COELHO, 2016), e até o momento não se conhece qual a causa deste resultado. Em geral, o desenvolvimento da semente é influenciado pelos níveis de fitormônios, principalmente o ácido abscísico (ABA) e também por fatores ambientais, como precipitação e temperatura (MARCOS FILHO, 2015). Durante a embriogênese zigótica de *A. angustifolia*, foram observados um aumento níveis de ABA do estádio torpedo para o estádio pré-cotiledonar, seguido por um decréscimo no estádio cotiledonar e maduro (SILVEIRA et al., 2008). Contudo, não há relatos dos níveis deste hormônio durante a maturação das sementes, e também sua relação com as respostas fisiológicas e variações entre anos de produção de sementes.

Alterações na morfologia das pinhas e das sementes de *A. angustifolia* permitiram classificar em quatro estádios (Figura 2):

- Cotiledonar: pinhas com superfície de coloração verde, sementes com coloração creme/amarela, e maioria dos embriões no estádio cotiledonar com massa fresca menor que 150 mg;
- Estádio II: pinhas com superfície verde com algumas pontuações de coloração marrom, sementes de coloração creme/amarela e extremidade de coloração marrom/avermelhada, embriões com massa fresca de até 150 mg;
- Estádio III: pinhas de coloração verde com menos de 50% de manchas de coloração marrom, sementes de coloração marrom/avermelhada, embriões com massa fresca acima de 150 mg e início da dispersão das sementes e;

- Estádio IV: pinhas com superfície marrom e pequenas partes ainda verdes, sementes de coloração marrom/avermelhada e embriões com massa fresca acima de 150 mg.

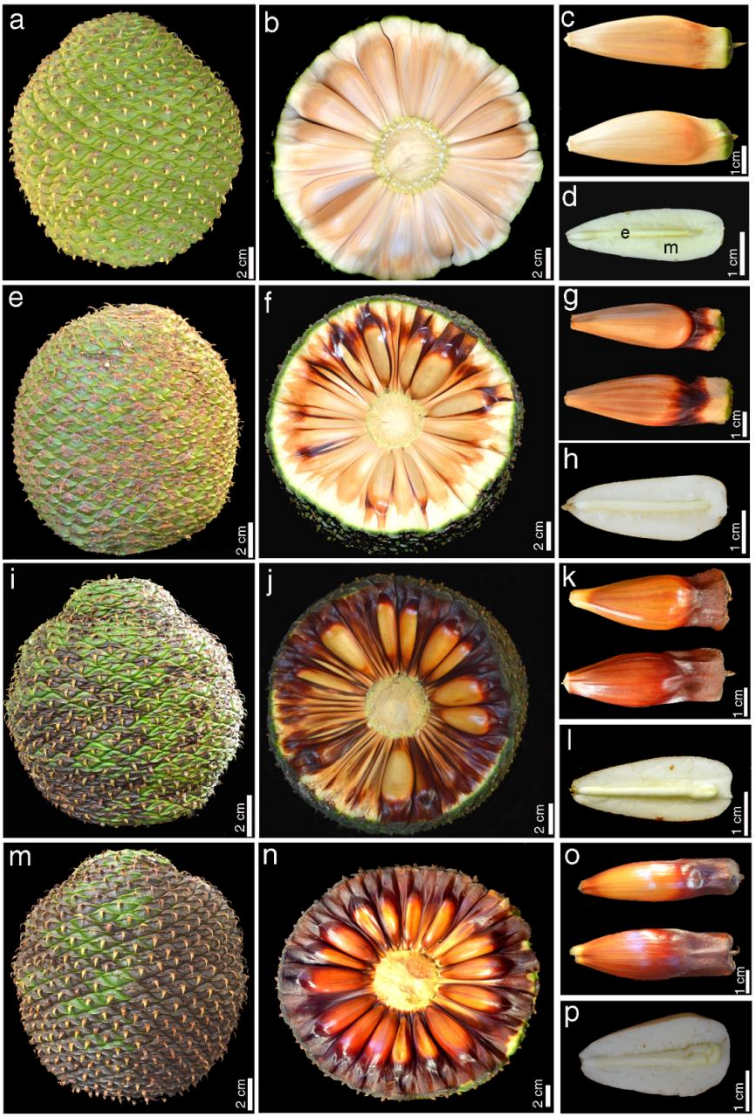

Figura 2 - Alterações morfológicas em diferentes estádios de desenvolvimento. As figuras a, b, c e d referem-se as características do estádio cotiledonar: pinhas

com superfície externa verde (a), secção transversal da pinha (b); sementes com coloração creme/amarela (c) e secção transversal da semente (d) demonstrando o embrião (e) e o megagametófito (m). As figuras e, f, g e h referem-se as características do estádio II: pinhas com superfície externa verde com algumas pontuações de coloração marrom (e), secção transversal da pinha (f); sementes com coloração creme/amarela e extremidade de coloração marrom/avermelhada (g) e secção transversal da semente (h). As figuras i, j, k e l referem-se as características do estádio III: pinhas com superfície externa verde com menos de 50% de manchas de coloração marrom (i), secção transversal da pinha (j); sementes com coloração marrom/avermelhada (k) e secção transversal da semente (l). As figuras m, n, o e p referem-se as características do estádio IV: pinhas com superfície externa marrom e pequenas partes ainda verdes (i), secção transversal da pinha (j); sementes com coloração marrom/avermelhada (k) e secção transversal da semente (l).

Ao final do processo de maturação, as sementes de *A. angustifolia* não reduzem seu metabolismo e não toleram perda de água abaixo de 30% (EIRA et al., 1994; GASPARIN et al., 2016; SALMEN ESPINDOLA et al., 1994; TOMPSETT, 1984). Devido a intensa atividade metabólica, outras espécies com sementes recalcitrantes iniciam uma mudança no metabolismo do desenvolvimento para a germinação, ainda presa a planta-mãe, como em sementes de *Inga vera* (CACCERE et al., 2013), *Quercus ilex* (SGHAIER-HAMMAMI et al., 2016) e *Durio zibethinus* (BROWN; HOR; GREENWOOD, 2001). Contudo em sementes de *A. angustifolia* até o momento não há relatos da ocorrência de tais mudanças ao final do desenvolvimento das sementes.

Algumas modificações podem sugerir a mudança no metabolismo do desenvolvimento para a germinação a nível anatômico e no ciclo celular. As sementes recalcitrantes ao final do desenvolvimento apresentam maior conteúdo de DNA 2C, indicando células na fase G1 do ciclo celular (BINO et al., 1993; FARIA et al., 2006; GASPARIN et al., 2016) e maior conteúdo de DNA 4C (fase G2 do ciclo celular) durante a germinação (FINCH-SAVAGE, 1998). Esse aumento no conteúdo de DNA 4C é uma fase preliminar para que ocorra a divisão celular, sendo necessário a duplicação do material genético para a formação de novas células e consequentemente a protrusão e crescimento da radícula. Para *A. angustifolia*, sementes recém-colhidas apresentaram 15% de DNA 2C e quando mais de 50% das sementes apresentaram radícula durante o processo de embebição, o conteúdo de DNA 2C foi de 13 a 19% (GASPARIN et al., 2016) (Tabela 1).

Enquanto a nível anatômico, a presença, quantidade e a distribuição dos canais de resina e feixes vasculares são consideradas as principais características para *A. angustifolia* (FERREIRA, 1981; HAINES, 1983; PANZA et al., 2002). Em sementes maduras desta espécie, as células são compactas com grandes núcleos e abundantes reservas incluindo amido, proteínas e lípidios (FARRANT, PAMMENTER E BERJAK, 1989; PANZA et al., 2006; ROGGE-RENNER et al., 2013).

Estas alterações que ocorrem durante a maturação das sementes, podem refletir na capacidade de conservação da qualidade fisiológica durante o armazenamento. Sementes maduras iniciam a perda da viabilidade a partir de 30 dias de armazenamento em temperaturas entre 4 e 5 °C, (AQUILA; FERREIRA, 1984; FERREIRA; HANDRO, 1979; FONTES; DAVIDE; DAVIDE, 2001; GARCIA et al., 2014). Contudo, quando as sementes são coletadas no estádio cotiledonar, com pinhas com superfície externa verde, a qualidade fisiológicas das sementes é incrementada após o armazenamento por até 120 dias de armazenamento a 8 °C (SHIBATA; COELHO, 2016), uma vez que, para sementes recalcitrantes é recomendado armazenar as sementes com elevados teores de água e evitar a perda de água durante esse período (EIRA et al., 1994) ou até mesmo, sementes maduras apresentaram um aumento na germinação após o armazenamento por até 180 dias (CAÇOLA et al., 2006; PIRIZ-CARRILLO et al., 2003). Tal incremento tem sido atribuido ao inicio do processo germinativo durante o armazenamento (FARRANT; PAMMENTER; BERJAK, 1989). Estes diferentes resultados da qualidade fisiológica das sementes durante o armazenamento, podem ser causados pelo período de maturação em que as sementes foram coletadas, pois os estudos já realizados descrevem apenas que as sementes foram coletadas maduras, sem o detalhamento de quais características foram avaliadas ou o períod/mês das coletas. Além disso, a conservação das sementes também podem ser influenciada pelas variações ambientais durante o desenvolvimento das sementes. Assim, estudos com sementes coletadas em diferentes estádios de maturação e posterior armazenamento em diferentes anos de produção são necessários, porém até o momento não foram realizados.

Outro fator que pode favorecer a conservação da viabilidade durante o armazenamento das sementes recalcitrantes é a eficiência das enzimas antioxidantes, através da remoção das espécies reativas de oxigênio (HENDRY et al., 1992; TOMMASI et al., 2006). Nos embriões de *A. angustifolia* tem sido relatados aumento da atividade enzimática da superóxido dismutase, ascorbato peroxidase e catalase

(ARALDI et al., 2016; GARCIA et al., 2015). No entanto, não se conhece a atividade destas enzimas durante o armazenamento quando as sementes são coletadas em diferentes estádios de desenvolvimento (Tabela 1).

Um resumo sobre os estudos já realizados e o que até o momento não se conhece foram apresentados na Tabela 1, com a descrição das principais variavéis estudadas no presente estudo e quais estádios do desenvolvimento ou no armazenamento já foram estudadas.

Tabela 1 – Coloração das pinhas, germinação, vigor, alterações anatômicas, ciclo celular, compostos de reservas, níveis de ácido abscísico (ABA), ácido indolacético (AIA) e ácido salicílico (A.S) e atividade das enzimas antioxidantes durante o desenvolvimento das sementes de *A. angustifolia* e no armazenamento (armaz.) já descritas (x) e que ainda não foram descritas (?) na literatura. As letras sublinhadas: x ou ? referem-se as análises realizadas no presente estudo.

Variavéis	Embriogênese zigótica		Maturação das sementes			Dispersão das sementes		
	Inicial	Tardia	Cot	II	III	IV	armaz.	Referência
Coloração das pinhas	verde	verde	verde	verde/ marrom	verde /marrom	verde/ marrom	-	1, 2
Germinação	-	-	x	x	x	x	x	2, 3, 4
Vigor	-	-	x	x	x	x	x	2, 3, 4
Alterações anatômicas	x	?	x	x	**?**	**?**	**?**	5, 6
Ciclo celular	?	?	?	x	**?**	**?**	**?**	7
Compostos de reservas	x	x	**x**	**x**	**x**	**x**	**x**	8, 9, 10, 11
Níveis de ABA	x	x	x	**x**	**?**	**?**	?	8, 11
Níveis de AIA	x	x	**x**	**x**	**?**	**?**	?	8, 12
Níveis de A.S	x	x	x	x	**?**	**?**	?	13
Enzimas antioxidantes	?	?	**?**	**?**	**?**	**?**	**x**	14

Referências: 1: MATTOS, (2011); 2: SHIBATA; COELHO; STEINER, (2013); 3: SHIBATA; COELHO, (2016); 4: GARCIA et al., (2014) 5: ROGGE-RENNER et al., (2013); 6: PANZA et al., (2002); 7: GASPARIN et al., (2016); 8: STEINER, (2005); 9: SHIBATA, (2013); 10: ARALDI; COELHO; MARASCHIN, (2016); 11: SILVEIRA et al., (2008); 12: ASTARITA; FLOH; HANDRO, (2003); 13: (BUENO, 2014); 14: ARALDI et al., (2016).

Durante o desenvolvimento de sementes de *A. angustifolia*, as sementes adquirem a capacidade de germinar a partir do estádio cotiledonar e durante a maturação há um aumento da germinação e vigor até o estádio IV (SHIBATA; COELHO, 2016; SHIBATA; COELHO; STEINER, 2013). Contudo a relação entre a qualidade fisiológica e os niveis de ABA, AIA e A.S. durante a maturação ainda não é conhecido. Alguns estudos tem reportado a presença de ABA e AIA durante a embriogênse zigótica de *A. angustifolia*, com um aumento nos níveis de ABA até o estádio pré-cotiledonar e decréscimo quando o embrião está maduro (SILVEIRA et al., 2008). Os maiores valores de AIA ocorreram nas fases iniciais de desenvolvimento, seguido por um decréscimo na fase em que ocorreu um alongamento dos cotilédones (ASTARITA; FLOH; HANDRO, 2003). Enquanto, o conteúdo de AS nos embriões zigóticos apresentaram valores mais elevados na embriogênese inicial, seguida pela matura e por último pela tardia (BUENO, 2014) (Tabela 1).

Assim, diante do exposto o presente estudo foi desenvolvimento em três capítulos: primeiramente foram realizadas análises de microscopia de luz, microscopia eletrônica de transmissão, citometria de fluxo e espectrofotometria de Infravermelho por Transformada de Fourier em diferentes estádios de desenvolvimento, com o intuito de verificar se ocorre uma modificação ao final do desenvolvimento das sementes de *A. angustifolia* para o metabolismo germinativo. No segundo capítulo, foram analisados os níveis de ácido abscísico, ácido indolacético, ácido salicílico, giberelina e a qualidade fisiológica durante o desenvolvimento das sementes em dois anos de produção para verificar as diferenças destes hormônios vegetais na qualidade . No terceiro capítulo, verificou-se se as sementes continuam seu metabolismo germinativo ou iniciavam o processo deteriorativo durante o armazenamento através das alterações enzimáticas, fisiológicas e ultraestruturais das sementes.

1.1 HIPÓTESES

- O início do processo germinativo ocorre durante a maturação das sementes de *A. angustifolia*, e as alterações anatômicas, bioquímicas e no ciclo celular dos eixos embrionários podem indicar essa mudança.
- A maturidade fisiológica das sementes de *A. angustifolia* ocorre anteriormente ao máximo acumulo de massa seca das sementes.

- Os níveis dos hormônios vegetais ABA, AIA, S.A e GA_4 são diferentes conforme as condições climáticas durante o desenvolvimento das sementes e estão relacionados com a qualidade fisiológica.
- Quando as sementes são mantidas sob refrigeração a 8 °C continuam o metabolismo germinativo iniciado durante a maturação das sementes.

1.2 OBJETIVOS

1.2.1 **Objetivo geral**

Avaliar a continuidade do processo de maturação com a germinação quando as sementes de *Araucaria angustifolia* ainda estão presas a planta mãe e a redução do metabolismo das sementes quando mantidas em baixas temperaturas.

1.2.2 Objetivos específicos

- Verificar as alterações morfológicas, bioquímicas e no ciclo celular dos eixos embrionários de *A. angustifolia* nos estádios cotiledonar, II, III e IV;
- Quantificar os níveis de ABA, AIA e A.S no eixo embrionário e nos cotiledones e sua relação com a qualidade fisiológica durante dois anos de produção de sementes;
- Avaliar o efeito de diferentes épocas de coleta na conservação da qualidade fisiológica por até 360 dias de armazenamento em refrigerador;
- Analisar as alterações ultraestruturais, fisiológicas e enzimáticas nos estádios de desenvolvimento cotiledonar, II e III durante o armazenamento por 30, 60 e 90 dias.

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CAPÍTULO 1

ANATOMICAL AND BIOCHEMICAL CHANGES ASSOCIATED WITH DEVELOPMENT AND GERMINATION OF *Araucaria angustifolia* EMBRYONIC AXIS

ABSTRACT

Recalcitrant seeds do not decrease metabolic activity at the end of development, nor do they exhibit a readily identifiable switch from developmental metabolism to germinative metabolism. This study aimed to assess the cell cycle, and anatomical and biochemical changes that embryonic axis of *Araucaria angustifolia* undergo during development, with a focus on the maturation stage. Embryonic axis were analyzed by light microscopy (LM), transmission electron microscopy (TEM), flow cytometry and Fourier transform infrared spectroscopy (FTIR). During development, cells exhibited intense metabolic activity with an abundance mitochondria, lipid bodies and vacuolated cells. The continued accumulation of starch and protein was observed by LM and TEM, and indicated by spectra of FTIR, which also indicated the presence of lipids and phenolic compounds in all of the samples. Cell differentiation of the procambium was observed with a thickening of cell wall and the formation of resiniferous ducts. At Stage III and IV, cells exhibited structural changes such as altered or elongated mitochondria, the presence of plastoglobules and cellular division. The majority of cells contained 2C DNA with a reduction from 82.58% to 77.35% from the Cotyledonary Stage to Stage IV. These results suggest that seeds of *A. angustifolia* lack the ability to switch-off their metabolism, as occurs in orthodox seeds, and so there is a gradual transition from developmental metabolism to germination metabolism. Such changes can be contributed to the rapid germination of seeds soon after their dispersion, making it an ecological strategy to reduce post-dispersal exposure to predators and to avoid damage from reduced moisture.

Keywords*:* maturation, recalcitrant seed, light microscopy, transmission electron microscopy, flow cytometry.

1. INTRODUCTION

Plants with recalcitrant seeds form a group of unrelated species whose seeds do not undergo maturation drying, and remain hydrated and desiccation sensitive through-out development and post-shedding (PAMMENTER; BERJAK, 2014; ROBERTS, 1973). Such seeds are considered to have a minimum threshold of water content for survival (WALTERS, 2015), with most, if not all, being killed by desiccation to 15-20% moisture content (HONG; ELLIS, 1996). Several mechanisms have been considered relevant to the acquisition of desiccation tolerance such as intracellular physical characteristics, like a reduction in the degree of vacuolization, accumulation of insoluble reserves and increased integrity of the cytoskeleton; "switching off" of metabolism; presence and efficient operation of antioxidant systems; and accumulation of protective molecules such as late embryogenic abundant proteins and oligosaccharides (BARBEDO; CENTENO; RIBEIRO, 2013; BERJAK; PAMMENTER, 2000, 2002, 2008, 2013; MELLO et al., 2010; PAMMENTER; BERJAK, 1999) among others.

Over the short life span of mature recalcitrant seeds, high water content is maintained as a prerequisite for high metabolic activity (OBROUCHEVA; SINKEVICH; LITYAGINA, 2016), and there is no readily identifiable switch from developmental metabolism to germinative metabolism (BERJAK; DINI; PAMMENTER, 1984), with these two processes being more or less continuous (SCHMIDT, 2007). Species with recalcitrant seeds, such as *Inga vera* Willd. subsp. *affinis* (DC.) T. D. Penn., have be found to experience rapid temporary metabolic shifts during maturation with highly vacuolated cells, which suggest a continuum from maturation to germination prior to seed dispersion (CACCERE et al., 2013). The embryonic axis of *Quercus ilex* L. has also shown the ability to carry out signal transduction for seed germination during maturation (SGHAIER-HAMMAMI et al., 2016). Others species have shown similar behaviors, such as *Avicennia marina* (Forssk.) Vierh. (FARRANT; PAMMENTER; BERJAK, 1992) and *Durio zibethinus* Rumph. ex Murray (BROWN; HOR; GREENWOOD, 2001). The cell cycle, a process in which DNA synthesis and cell division occurs, has been indicated as a marker during the development and germination of seeds (KOZEKO; TROYAN, 2000; SLIWINSKA, 2009). Later developmental stages of recalcitrant seeds were found to possess higher 2C DNA content (BINO et al., 1993;

FARIA; VAN LAMMEREN; HILHORST, 2004), and higher 4C DNA content during germination (FINCH-SAVAGE, 1998).

Among species with recalcitrant seeds in Brazil, *Araucaria angustifolia* (Bertol.) Kuntze is considered one of the most important since it is a native conifer with social, economic and ecological significance (WENDLING; BRONDANI, 2015). The maturation period for seeds of *A. angustifolia* is between April and August (MATTOS, 2011). Until now, the majority of studies have been undertaken during April, when seeds begin the process of maturation. Thus, we intended to investigate the changes these seeds experience from April until the time they are dispersed in the months of June/July, depending on climatic conditions. We also wanted to investigate whether seeds of *A. angustifolia* exhibit recalcitrant behavior, such as undergoing changes in anatomic, biochemistry and/or DNA-content at the end of development that facilitate rapid germination after dispersion. Thus, light microscopy, transmission electron microscopy, flow cytometry and infrared vibrational analyses were performed in order to assess changes in anatomy, chemical composition and the cell cycle of the embryonic axis of *A. angustifolia* during the maturation period of seed development.

2. MATERIALS AND METHODS

Megastrobili of *A. angustifolia* var. indehicensis (ADAN et al., 2016) were collected from a natural population located in Santa Catarina, Brazil (27°55' 30" S and 49° 58' 35" W, altitude 1300 m) during March – June of 2014 and 2015. Megastrobili were chosen based on the main color changes they experience during seed maturation: green (megastrobilus with predominantly green surface – Cotyledonary Stage), green-brownish (megastrobilus with green surface or with small brown spots for less than 50% of the total surface area – Stage II), brown-green (megastrobilus with green surface and large brown spots for more than 50% of the total surface area and exhibiting the start of dehiscence – Stage III) and brown (megastrobilus with a predominantly brown surface and undergoing dehiscence – Stage IV) (Fig.1).

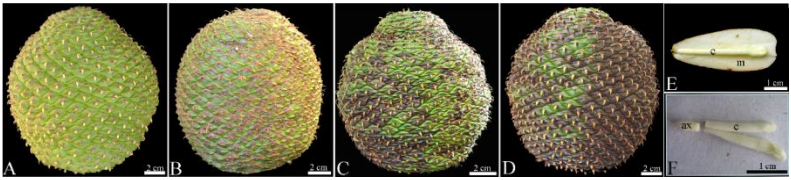

Figure 1 - Alterations of megastrobilus of *A. angustifolia.* Cotyledonary Stage: megastrobili with green surface (A); Stage II: megastrobilus with green surface of with brown small spots (B); Stage III: megastrobili with green surface and brown large spots (C); Stage IV: megastrobilus with surface predominantly brown. Cross section of *A. angustifolia* seeds (E) showing the embryo (e) and megagametophyte (m). An embryo (F) showing the two cotyledons (c) and embryonic axis (ax).

After seeds were collected, their embryonic axis were extracted (Fig. 1F) and submitted to light microscopy, transmission electron microscopy, DNA-content and infrared vibrational analyses.

Transverse sections of shoot apical meristems in the four development stages of *A. angustifolia* seeds collected in 2014 were used for the anatomical analyses. Embryonic axis were separated in shoot (SAM) and root (RAM) apical meristems and fixed overnight in phosphate buffer 0.1 M (pH 7.2) containing 2.5 % formaldehyde at 4 °C. Subsequently, the samples were dehydrated in an increasing series of aqueous ethanol solutions and then infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Semi-thin sections (4 μm thick) containing both SAM and RAM were treated with different

histochemical techniques. Periodic acid-Schiff (PAS) was used to identify neutral polysaccharides (GAHAN, 1984), 0.5 % toluidine blue (TB-O) pH 3.0 (Merck Darmstadt, Germany) to identify acidic polysaccharides (GORDON; MCCANDLESS, 1973) and 0.4 % Coomassie Brilliant Blue (CBB) in Clarke's solution (Serva, Heidelberg, Germany) to identify proteins (GAHAN, 1984). Sections were analyzed using an Olympus BX 41 light microscope equipped with Image Q Capture Pro 5.1 Software (QImaging Corporation, Austin, TX, USA).

Semi-thin sections (2 µm) were made of samples collected in 2015 to compare with the sections from samples of 2014. Due the similarly of the samples from the two years, those collected in 2015 were used for transmission electron microscopy (TEM). Samples of shoot and root apical meristems were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4 % formaldehyde and 2.5 % glutaraldehyde for 12 h (SCHMIDT et al., 2009). The material was post-fixed with 0.1 M sodium cacodylate buffer containing 1 % osmium tetroxide for 4 h, dehydrated in an increasing series of aqueous acetone solutions, and then embedded in Spurr's resin (SPURR, 1969). Ultra-thin sections (70 nm thick) were collected on grids and stained with aqueous uranyl acetate followed by lead citrate. Two grids were then examined using a JEM 1011 TEM (JEOL Ltd., Tokyo, Japan) at 80 kV.

To analyze the cell cycle, the apex of the embryonic axis was macerated on a Petri dish containing 1 mL of cold LB01 buffer using a scalpel blade to release the nuclei into suspension (DOLEZEL; GREILHUBER; SUDA, 2007). The chopped tissue was aspirated through two layers of cheesecloth with a plastic pipette, filtered through a 50 mm nylon filter, and collected in a polystyrene tube. The nuclei were stained by adding 50µL of propidium iodide (1mg mL^{-1}) and 5µL of RNase (100µg mL^{-1}) to each sample. The analysis was performed using a FACSCanto II (Becton, Dickinson and Company, USA) flow cytometer and histograms created with Cell Quest software.

Samples of *A. angustifolia* (15 embryos/sample) were submitted to Fourier transform infrared spectroscopy (FTIR) in a Bruker IFS 55 spectrophotometer with a glycerin-sulphate detector (DTGS) and attenuated total reflectance accessories (ATR, Golden Gate). One hundred and twenty-eight scans per sample were collected in spectral windows of 4000–500 waves cm^{-1} at a resolution of 4 waves cm^{-1}. Three spectra were collected for each sample. Spectra were normalized, baseline corrected in the region of interest (3000 to 600 cm^{-1}) and processed with the aid of Essential Ftir software. The FTIR dataset was

subjected principal components analysis (PCA) in R software (R DEVELOPMENT CORE TEAM, 2011).

The experimental design of the flow cytometry analysis was a completely randomized 2 x 4 factorial design (two years of harvest and four development stages) with three replicates of each treatment. The data were tested for normality and subjected to ANOVA. The means were compared by SNK's test at 5% significance.

3. RESULTS

3.1 ANATOMICAL CHANGES TO SEEDS

Cells of shoot apical meristems showed intense development when stained with TB-O due to cellular differentiation of the procambium and formation of resin ducts (Fig. 2). A greenish-blue color shift was observed at Stage IV, indicating a thickening of the walls of tracheid cells containing phenolic and/or lignin compounds (Fig. 2J). At Stage III, cells undergoing mitosis were recognized by the presence of two nuclei, thin cell walls between cells and separation of sister chromatids (Fig. 2K).

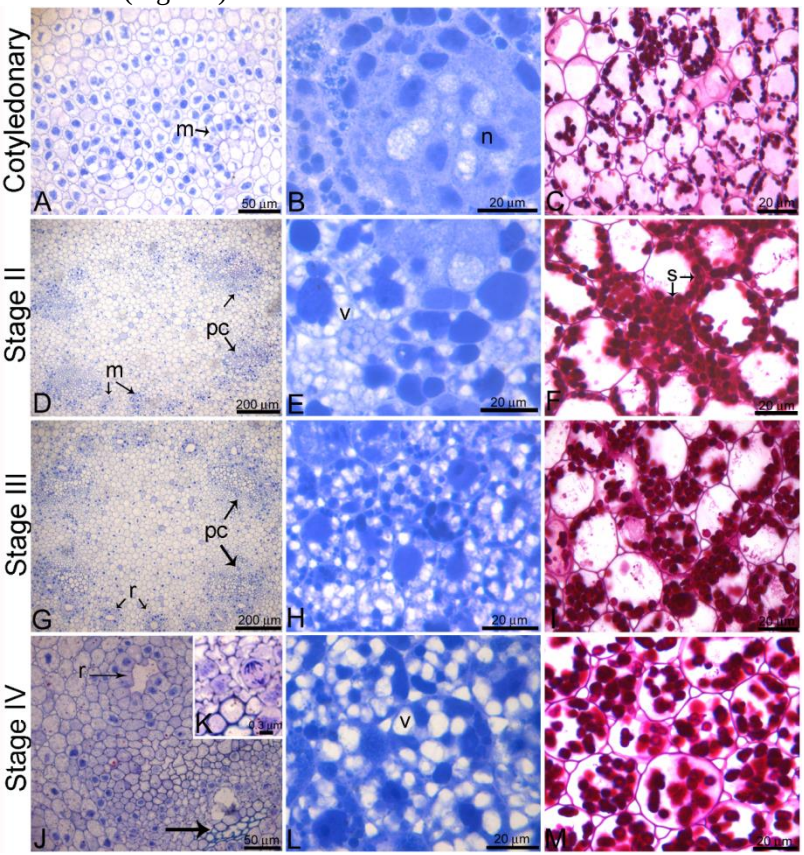

Figure 2 - Light microscopy of *A. angustifolia* SAM. Transversal sections stained with Toluidine Blue TB-O (a, d, g, j, k), CBB (b, e, h, l) and PAS (c, f, I,

m). Note the sections stained with TB-O staining enhanced procambium (pc), the resins ducts (r) got bigger at Stages III (g) and IV (j) and a greenish-blue color shift at stage IV (arrow). CBB staining showing vacuoles (V), nucleus (n) and protein in blue (b, e, h, l). PAS staining, note the increased of starch grains (S) from Cotyledonary Stage (c) to others stages (f, i, l).

PAS staining showed a positive reaction, indicating the presence of neutral polysaccharides. Such compounds were found primarily as starch grains and cell wall constituents. During development, starch grains and cytoplasm density increased from Stage II onward (Fig. 2F). CBB staining revealed a large amount of protein throughout the cell cytoplasm and many white-appearing vacuoles, which increased both in number and size from Stage I onwards (Fig. 2E). Root apical meristems showed few changes throughout seed development (supplementary material).

TEM analysis of ultrastructure revealed a nucleus, secretory vesicles, abundant storage reserves, mitochondria and some Golgi bodies throughout development. The storage reserves of both root and shoot apical meristems included starch inside plastids and lipid bodies (Fig. 3).

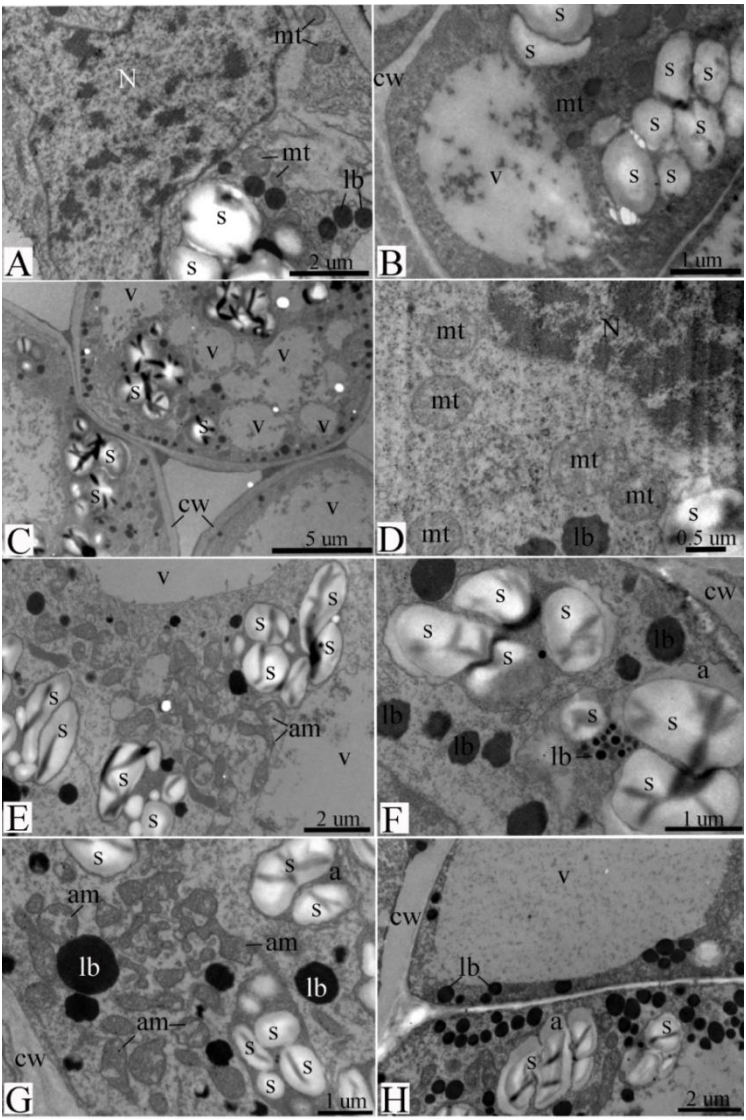

Figure 3 - Transmission electron microscopy of *A. angustifolia.*
Shoot apical meristem (A, C, E,G) and root apical meristem (B, D, F, H) at Cotyledonary Stage (A, B), Stage II (C, D), Stage III (E, F) and Stage IV (G, H). Abbreviations: amyloplast (A); altered mitochondria (am); cell wall (CW); lipid body (lb); mitochondria (Mt); nucleus (N); starch granule (S); and vacuole (V).

In Stages III and IV, the cells possessed relatively large vacuoles and a change in the appearance of mitochondria, with them being elongated and undergoing fission or fussion (Fig. 3E and 3G). The mitochondrial matrices became increasingly devoid of internal detail with electron-transparent regions (Fig. 3E). Plastoglobules were observed inside amyloplasts (Fig. 3F).

3.2 FLOW CYTOMETRY ANALYSIS

Flow cytometry determined that most of the nuclei of the embryonic axis of *A. angustifolia* contained 2C DNA, indicating that most of the cells were in the G1 phase of the cell cycle. Year of collection and stage of development did not exhibit interactions with 2C and 4C DNA values. A decrease in the content of 2C DNA from 82.58% to 77.35% was, however, observed from the Cotyledonary Stage to Stage IV. However, the content of 4C DNA (G2 phase of the cell cycle) did not differ significantly between the Cotyledonary Stage and Stage IV, with it being 8.44% and 10.35%, respectively (Fig. 4).

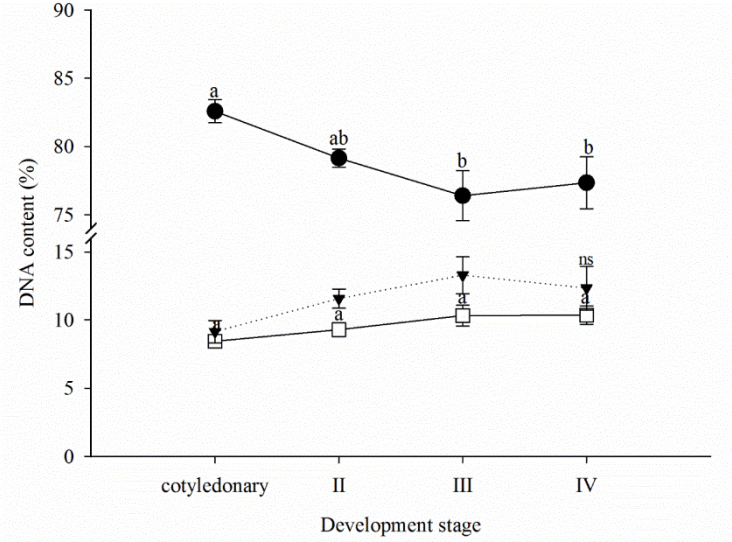

Figure 4 - Cell cycle of *A. angustifolia* embryonic axis.
G1 phase (●): 2C DNA; S phase (▼): 2C/4C DNA and G2 phase (□): 4C DNA content at Cotyledonary Stage, II, III and IV. Bars represent standard error. The letters refer to the SNK's test ($P < 0.05$).

3.3 FTIR SPECTROSCOPY ANALYSES

FTIR spectra of *A. angustifolia* showed vibrational band assignments for the major groups of biochemical components, with most of the absorption peaks being detected in the 3000–600 cm^{-1} spectral window, indicating the presence of lipids (2924, 2854, and 1740 cm^{-1}), proteins (1650–1500 cm^{-1}), starch (1200–800 cm^{-1}) and phenolic compounds (900-690 cm^{-1}) at different developmental stages of seeds.

Three prominent areas in the lipid region were found at different development stages of *A. angustifolia* seeds: 2927, 2856, and 1745 cm^{-1}. Carbocyclic groups, associated with the axial deformation of functional group C=O, are typically found in fatty acids and can be detected in the regions of 3000-2800 cm^{-1} (LAHLALI et al., 2014) and 1740 cm^{-1} (KUHNEN et al., 2010).

The two major vibrational bands of protein backbone are the Amide I and Amide II bands (GARIDEL; SCHOTT, 2006) in the region 1500–1700 cm^{-1} (BAKER et al., 2014), which are mainly associated with the stretching vibration of C=O (LAHLALI et al., 2014; SCHULZ; BARANSKA, 2007). At least one main absorption peak was observed at 1648 cm^{-1} (Fig. 5) in the Amide I region.

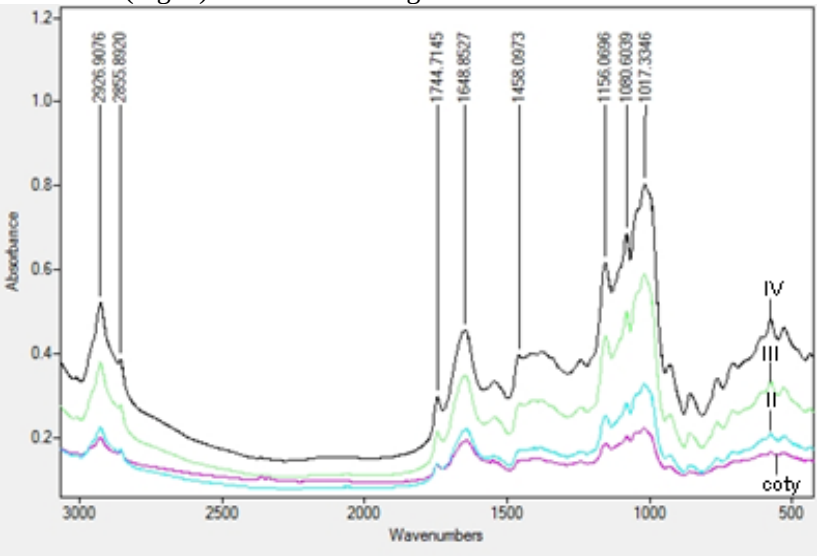

Figure 5 - FTIR spectra of *A. angustifolia* embryonic axis. Absorption peaks being detected in the 3000–600 cm^{-1} spectral window at Cotyledonary Stage (coty), Stage II, III and IV.

The peaks observed in the 800-1200 cm^{-1} spectral window indicated the presence of starch associated with the axial deformation of functional groups C-O, C-C and C-O-H and angular deformation of C-O-H (ČERNÁ et al., 2003; WARREN; GIDLEY; FLANAGAN, 2016).

The first two components of the principle components analysis (PCA) revealed a clear discrimination of samples and explained 99.8% of the total variance of the FTIR dataset. The samples were dispersed along the first axis (PC1; 98% variance) and formed two groups with Cotyledonary Stage and Stage II in PC1-, and Stages III and IV in PC1+ (Fig. 6).

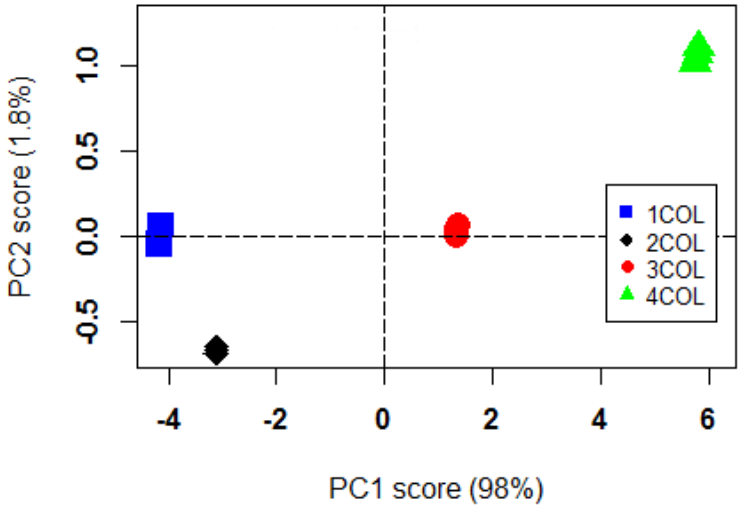

Figure 6 - PCA of FTIR dataset of *A. angustifolia* embryonic axis at different development stages. 1COL: Cotyledonary Stage; 2COL: Stage II; 3COL: Stage III and 4COL: Stage IV.

4. DISCUSSION

During the beginning of seed development there is intense cell-cycle activity in the meristematic cells. This cycle comprises the events necessary for cell division and is divided into two stages: interphase – subdivided into G1, S and G2 phases when there is intense metabolic activity due to DNA replication, growth and synthesis; and mitosis – the period wherein the genetic material is divided into two identical cells (BEWLEY et al., 2013). Intense cellular activity occurs in the initial phases and at the end of seed development, with recalcitrant seeds being dispersed with the majority of their cells in the G1 phase, such as is seen in *Castanea sativa* Mill. (BINO et al., 1993), *Acer pseudoplatanus* L. (FINCH-SAVAGE, 1998), and *Inga vera* (FARIA; VAN LAMMEREN; HILHORST, 2004). Resumption of mitotic activity occurs during germination or during a later germination event with the protrusion of the radicle (BOUBRIAK et al., 2000; FINCH-SAVAGE, 1998; SLIWINSKA, 2009). The majority of the nuclei of the embryonic axis of *A. angustifolia* were in the G1 phase (DNA content 2C), as determined by flow cytometry analysis, while a decrease in the proportion of cells in the G1 phase was observed in Stages III and IV. Light microscopy found that in these stages some meristematic cells exhibited mitotic activity, as evidenced by the occurrence of nuclei being divided into two within the same cell (Fig. 2-K). These cells comprised part of the percentage of cells in the G2 phase as observed by flow cytometry (8 to 10%). There have been no previous reports regarding DNA content during *A. angustifolia* seed development; however, freshly harvested and germinated mature seeds have been found not to differ in 2C DNA content (GASPARIN et al., 2016). These observations demonstrate that the seeds of *A. angustifolia*, even in the process of germination, seem to modify their DNA content late, probably after protrusion of the radicle.

The LM analysis observed cells stained with TB-O indicating the cellular differentiation of the procambium with a thickening of cell wall and greater accumulation of lignin and/or phenolic compounds (Fig. 2J). Meristematic cells were observed in the early stages (Fig. 2A and 2D), and the formation of resiniferous ducts in other stages (Fig. 2G and 2J). The formation of resin is a defense strategy for plants (MITHÖFER; BOLAND, 2012) and their initial formation can be considered preparation for defense of the seed and its subsequent germination

During all stages of development, cells of *A. angustifolia* seeds seemed to have intense metabolic activity, which could be deduced from

the occurrence and frequency of mitochondria, Golgi bodies and lipid bodies. This elevated metabolism is frequently reported for recalcitrant seeds and is related to desiccation intolerance, such as with *A. angustifolia* (BALBUENA et al., 2009; ROGGE-RENNER et al., 2013; SILVEIRA et al., 2008), *Inga vera* (CACCERE et al., 2013), *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (BONOME et al., 2011) and *Aesculus chinensis* Bge. (YU; CHEN, 2011).

The samples stained with CBB and PAS demonstrate the accumulation of proteins and starch during the stages analyzed (Fig. 2L and 2M). These reserves were also observed using FTIR spectrophotometry (Fig. 4), which has been used previously to analyze the composition of seeds (AMIR et al., 2013; ARALDI; COELHO; MARASCHIN, 2016; BARSBERG; RASMUSSEN; KODAHL, 2013; KUHNEN et al., 2010; PHILIPPE et al., 2006). Lipids, starch, proteins and phenolic compounds were present in all seed samples. Principal components analysis of FTIR demonstrated that the most important variables for PC1 were starch and proteins, which discriminated a group to the left formed by Cotyledonary Stage and Stage II, and a group to the right formed by Stage III and IV. These groups also showed similar features by LM and TEM analyses.

In addition, different mitochondrial morphologies seemed to occur more frequently in Stages III and IV (Fig. 3C and 3D). In addition to the standard mitochondrial morphology with a typically cylindrical-shape and well-packed and organized cristae, these elongated or altered mitochondria were observed often with matrices increasingly devoid of internal detail and with a modified electron-transparent region. These changes in mitochondria have been reported for *A. angustifolia* in response to stress (FRAGA et al., 2015). Thus, the presence of plastoglobules and altered mitochondria appears to be a response to a stress to which the seeds had been subjected. These events, along with the cellular differentiation and division observed by LM analysis, appear to be responses to stress imposed on seeds by dispersion or in preparation to change metabolism.

Species with recalcitrant seeds, such as *Inga vera*, have exhibited rapid temporary metabolic shifts during maturation, moving directly from maturation to germination (CACCERE et al., 2013). Other species have shown similar behaviors, such as *Avicennia marina* (FARRANT; PAMMENTER; BERJAK, 1992) and *Durio zibethinus* (BROWN; HOR; GREENWOOD, 2001). Early germination after being shed has been reported previously for *A. angustifolia* seeds, as well as its continuance during storage (ARALDI; COELHO, 2015; FARRANT;

PAMMENTER; BERJAK, 1989; GARCIA et al., 2014). This germination is rapid and non-homogeneous, plus the seeds exhibit different post-harvest early-developmental categories (ARALDI; COELHO, 2015). However, the seeds are able to germinate even when conditions were unfavorable.

The period of imbibition required by recalcitrant seeds at the onset of germination might be shorter than that of orthodox seeds, and it may be that they can germinate rapidly due to both having a high moisture content and an active metabolism, and so limited or no imbibition is required to initiate germination (BERJAK; PAMMENTER, 1995; DAWS; GARWOOD; PRITCHARD, 2005; PRITCHARD et al., 2004), as has been observed in *A. angustifolia* seeds (BALBUENA et al., 2011). Thus, in latter development stages, *A. angustifolia* seeds could initiate germination without the requirement of additional water.

Recalcitrant seeds transition quickly from development to germination, and they lack the ability switch-off their metabolism after seed dispersal. This strategy is associated with their short lifespan (OBROUCHEVA; SINKEVICH; LITYAGINA, 2016). However, some recalcitrant seeds modify their metabolism for germination even before dispersion (i.e., during seed development; (BROWN; HOR; GREENWOOD, 2001; CACCERE et al., 2013; SGHAIER-HAMMAMI et al., 2016). These changes can be contributed to the rapid germination of seeds soon after their dispersion, making it an ecological strategy to reduce post-dispersal exposure to predators and to avoid damage from reduced moisture.

5. CONCLUSION

Seeds of *A. angustifolia* seem to lack the ability to reduce their metabolism at the end of seed development and the onset of changes in anatomic, biochemistry and DNA-content for germination seems to occur during development.

6. ACKNOWLEDGEMENTS

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8. SUPPLEMENTARY MATERIAL

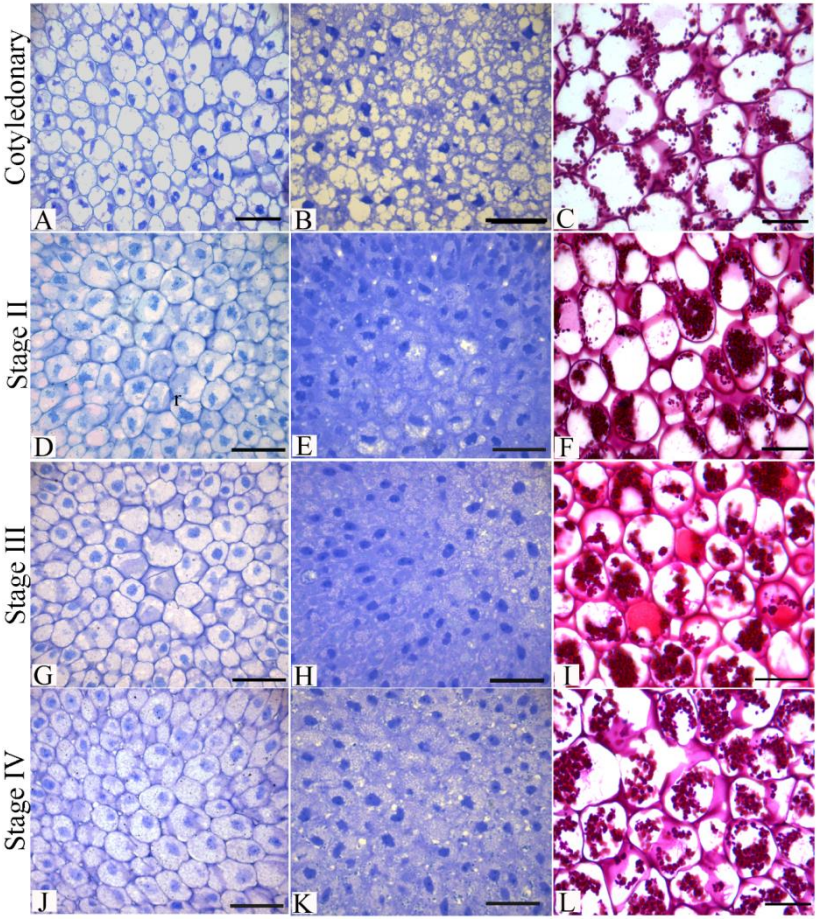


Figure S1 – Transversal sections of root apical meristem of *Araucaria angustifolia* stained with TB-O: Toluidine Blue (A, D, G, J), CBB: Coomassie Brilliant Blue (B, E, H, K) and PAS: Periodic acid-Schiff (C, F, I, L).

CAPÍTULO 2

PHYSIOLOGICAL RESPONSE TO ENVIROMENTAL VARIATION DURING SEED DEVELOPMENT OF *Araucaria angustifolia*

ABSTRACT

During development, plant hormones are involved in processes such as the accumulation of reserves, cellular activity and physiological responses. The present study aimed to analyze ABA, IAA and SA levels and their role in the physiological responses of *A. angustifolia* seeds. Seeds were collected in the cotyledonary stage and stages II, III and IV during two years of production and submitted to germination, tetrazolium, germination velocity index and electrical conductivity tests. The embryonic axes and the cotyledons were extracted from the seeds and submitted to ABA, GA_4,IAA and SA analyses. The highest levels of ABA were observed in the cotyledonary stage and stage II, and were about three-times higher in the year of low precipitation. Similar the values of GA_4, a decrease was observed during seed development in the year of low precipitation and cold hours. addition, a negative correlation between ABA levels and germination was observed. In contrast to ABA, there was a positive correlation between IAA and GSI, and higher levels of SA during stages III and IV. Cluster analysis performed with physiological and hormonal data from both years revealed clusters according to each stage of development. These results highlight the antagonistic relationship between ABA and IAA, which acts as a regulatory factor for faster germination and, in addition, high levels of ABA did not affect the final germination of *A. angustifolia* seeds.

Key words*:* seed maturation; germination; physiological quality; araucaria.

1. INTRODUCTION

Seed development is a complex process that requires the coordinated integration of genetic, metabolic and physiological pathways, as well as the involvement of strict hormonal control and exchange of signals between maternal tissues and the seed. (LOCASCIO et al., 2014). This process begins with the formation of the embryo, which is followed by the accumulation of reserves and ultimately the acquisition of desiccation tolerance (BEWLEY et al., 2013). Seeds with the ability to lose water at the end of development are known as orthodox seeds, but there is a group of species, known as recalcitrants, that lose viability as water level decreases (ROBERTS, 1973).

In both types of seeds, the main hormones that appear are abscisic acid (ABA), gibberellins (GA), auxins and cytokinins (BEWLEY et al., 2013). The natural form of auxin is indole-3-acetic acid (IAA), which plays an important role in zygotic embryogenesis by determining the structure and size of the embryo (CHIWOCHA; VON ADERKAS, 2002; LOCASCIO et al., 2014). In seed germination, this hormone is clearly involved in vascular differentiation, lateral root initiation and regulation of meristem elongation (Davies, 2010). Whereas, ABA plays crucial roles during seed development, avoiding the early germination of the embryo and inducing the expression of genes associated with the accumulation of reserve products (BORISJUK et al., 2004; FINKELSTEIN; GAMPALA; ROCK, 2002) and biologically active GAs are involved in cell expansion and they are known to be present during early embryogenesis in some species (BORISJUK et al., 2004; WHITE et al., 2000).

Despite the importance and regulatory role of these hormones, their actions in physiological responses during seed maturation remain unknown for some species, such as *A. angustifolia.* Some studies have reported the presence of ABA and IAA during zygotic embryogenesis of *A. angustifolia*, with an increase in the levels of ABA up to the pre-cotyledonary stage, and decrease when the embryo is mature (SILVEIRA et al., 2008). The highest values of IAA occur in the early stages of development, followed by a decrease in the phase when the cotyledons are elongated (ASTARITA; FLOH; HANDRO, 2003). Furthermore, to date there is no information about GA in *A. angustifolia* seeds.

This species is one of the most important in southern Brazil, where it possesses recalcitrant seeds with loss of viability with humidity near 30%, preventing its conservation for long periods (EIRA et al.,

1994; GASPARIN et al., 2016). In addition, *A. angustifolia* is a critically endangered species according to the IUCN Red List of Threatened Species (IUCN, 2016). Previous studies of different stages of seed development have shown low-levels or germination at the cotyledonary stage (SHIBATA; COELHO, 2016) even though the embryo is already formed; the component or mechanism is related to this physiological response remains unknown.

Thus, the objective of the present study was to analyze alterations to the free forms of ABA, IAA and SA during the development of *A. angustifolia* seeds, in order to understand their role in physiological responses. Therefore, seeds were collected at different stages of development during two years of production and germination, GSI, and electrical conductivity tests and hormonal analyses were carried out on the embryonic axis and cotyledons of *A. angustifolia*.

2. MATERIAL AND METHODS

2.1 PLANT MATERIAL

Megastrobilus of *A. angustifolia* were collected in a natural population located in Painel - Santa Catarina, Brazil (27°55' 30" S and 49° 58' 35" W, altitude 1300 m) during March until July of 2014 and 2015.

Seeds collects were realized at cotyledonary stage: megastrobilus with green surface, cream/yellow seeds and embryos with ~150 mg fresh mass. Stage II: megastrobilus with green surface and with small brown spots for less than 50% of the total surface area, cream/yellow seeds and seed tip with brown/reddish color and embryo with up 150 mg fresh mass. Stage III: megastrobilus with green surface and large brown spots for more than 50% of the total surface area, red/orange seeds and embryos from 150 to 200 mg fresh mass. Stage IV: megastrobilus with predominantly brown surface and undergoing dehiscence, red/orange seeds and embryos from 150 to 200 mg fresh mass (Figure 1).

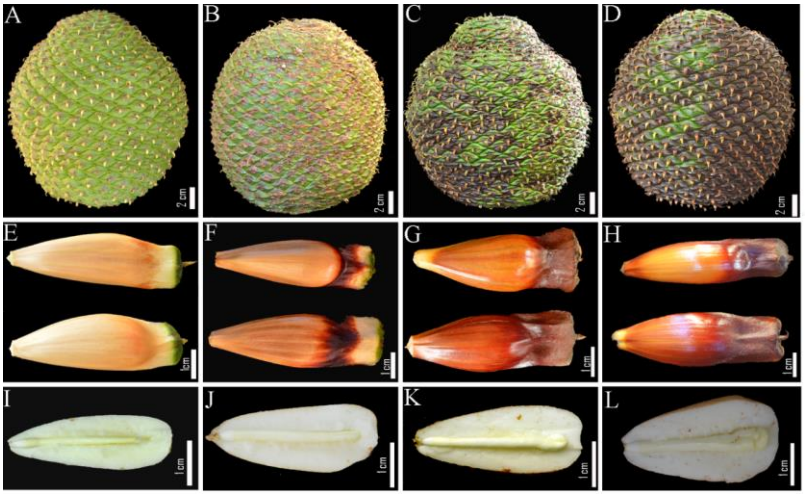


Figure 1 - Morphological changes during different development stages. Cotyledonary stage showed megastrobili with green surface, seeds with yellow color (I) and embryos with fresh mass ~150 mg (I); stage II: megastrobilus with green surface of with brown small spots (B), seeds with yellow-red color (F) and embryo with fresh mass ~150 mg (J); stage III: megastrobili with green surface and brown large spots (C), seeds with red/orage color (G) and embryo

with 150-200 mg fresh mass (K); stage IV: megastrobilus with surface predominantly brown (D), seeds with red/orange color and embryo with 150-200 mg fresh mass(L).

Painel is located at an altitude of 987 m and the variations in temperature minimum, maximum and precipitation monthly from fertilization to the maturation of the seeds of the two years of collections are showed in figure 2, according to Epagri/Ciram (2017). The exact period of fertilization of *A. angustifolia* is not easily identifiable and therefore period was based on study of Rogge-Renner (2014) using microscopy techniques.

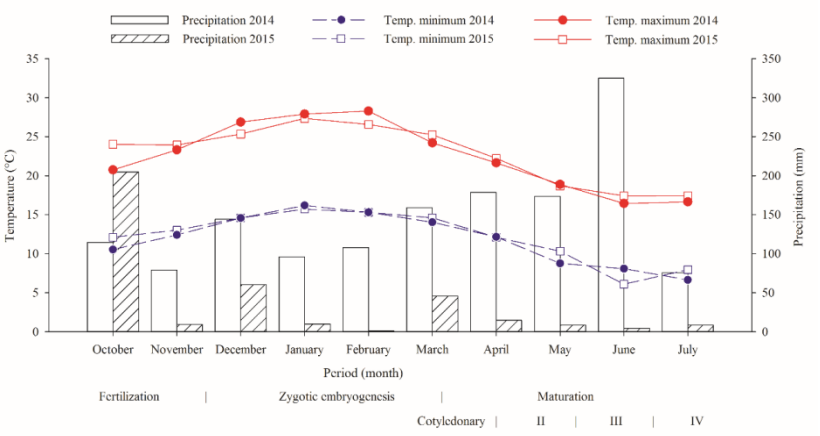


Figure 2 – Average monthly minimum (----) and maximum (——) temperature and precipitation (bars) monthly in Painel – Santa Catarina during the period between fertilization and collect of *Araucaria angustifolia* seeds in the years 2014 and 2015 (EPAGRI/CIRAM, 2017).

Sunlight, cold hours (below 7.2 ° C), growth degree-day (GDD) were calculated with daily data from fertilization to stage IV. The GDD (°C day), according to the equations proposed by Villa Nova (1972), considering 10°C as base temperature.

Table 1 –Sunlight, cold hours, growth degree-day (GDD) and relative humidity (RH) from fertilization to development stage of *A. angustifolia* seeds in 2014 and 2015.

	Sunlight		Cold (hours)		GDD		RH (%)	
	2014	2015	2014	2015	2014	2015	2014	2015
cotyledonary	1128	1024	10	1	1573,05	1555,00	84,63	85,14
II	1253	1172	17	1	1885,70	1790,76	85,22	85,58
III	1384	1279	49	24	2002,99	1941,03	85,77	86,29
IV	1470	1406	120	110	2156,92	1961,88	86,18	86,39

2.2 MEASUREMENT OF DRY MASS AND MOISTURE CONTENT

Four replicates of three seeds in each development stage were cut transversally, then weighed (wet weight), dried at 105°C ± 3°C for 24 hours and reweighed to determine dry weight and moisture content (BRASIL, 2009).

2.3 PHYSIOLOGICAL ANALYSIS

Four replicates of 25 seeds were surface-decontaminated with sodium hypochlorite solution (2%, v/v) for three minutes for germination test. After, seeds each were sown in trays with vermiculite and placed in a germination chamber at 25°C (KISSMANN; HABERMANN, 2014) with a photoperiod of 12 hours. Seeds were monitored for 70 days.

Germination counts were made every three days from the beginning of germination and germination speed index (GSI) calculated using the formula proposed by Maguire (1962): GSI = G1/N1 + G2/N2 + …Gn/Nn, where G1, G2, Gn are the number of germinated seeds, and N1, N2, Nn the number of days in the test.

Tetrazolium test was realized with four replicates of 25 seeds were soaked in water for 18 hours; then the embryo was separated from seed coat and the nutritive tissue with a stylet, for later immersion in 0.1% tetrazolium solution at 25°C for one hour (OLIVEIRA et al., 2014). Embryos were classified as viable or non-viable according to colour and appearance of the tissues, extent of damage and location of the colour patches.

Embryos (4 replicates of 10) were soaked in 75 ml distilled water at 25 °C as described by Medeiros and Abreu (2007). After 12 hours imbibition, the electrical conductivity of the solution was measured by a conductivity meter (Quimis – Q795), expressed as μS cm-1 g-1 of seeds.

2.4 HORMONE ANALYSIS

Embryos were extracted (n = 40), and separated in embryonic axis and cotyledons. Samples (~500 mg) were grinded, added to 1 ml extraction solution (methanol:water:formic acid 75:20:5, v/v) and kept at -20°C for 1 hour. Next, the tubes were extracted for 25 min at 4°C in an ultrasound bath (40kHz frequency), followed by centrifugation at

1,750 g for 30 min at 4ºC. The pellet was re-extracted more 3 times with 1 mL of extraction solvent, kept for 2 hours (second extraction), 4 hours (third extraction) and 12 hours (fourfy) at -20°C and sonicated at the same earlier condition. After the last centrifugation, supernatants were combined, dried in a vacuum concentrator, re-suspended in 2 mL of water. The ressuspended samples were applied in Oasis MCX, the eluted were combined, dried in a vacuum concentrator, re-suspended in 75 µL of methanol and filtered through a 0.22 µm PTFE filter. Samples were then analyzed by LC-MS/MS

The SPE cartridge was wetted with 4 mL mL methanol fallowed by a conditioning with 6 mL water, then samples were applied (after dried and reconstituted in 2 mL water), washed with 3 mL 1 M formic acid and eluted with 5 mL methanol, 1.5 mL water and then with 5 mL 5% ammonium hydroxide in methanol:water (4:1, v:v). The obtained eluates were dried and then reconstituted in 75 µL of methanol for LC–ESI-MS/MS analysis. Prior to analysis, each sample extract was filtered through a 0.22-µm PTFE filter.

The detection system consisted of an Acquity UPLC™ System (Waters, USA) quaternary pump equipped with an autosampler. An Acquity UPLC BEH C18 (Waters, USA) column (2.1 × 50 mm, 1.7 µm) and an Acquity UPLC BEH C18 (Waters, USA) pre-column (2.1 × 5 mm, 1.7 µm) was used. During analysis the samples extracts were kept at 4°C and column at 40°C. The mobile phase in the chromatographic separation consisted of a binary mixture of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The gradient was from 1% to 100% B in 2.2 min, and kept at 100% B up to 2.8 min at the flow rate of 0.3 mL.min^{-1}. An injection volume of 5 µL was applied in all analyses. The tandem MS detection of hormones were carried out on a Waters Xevo™ triple quadrupole mass spectrometer system (MS/MS) equipped with an ESI interface and adjusted with the following conditions: capillary voltage, 2.5 kV; source temperature, 150 °C, desolvation temperature, 500 °C; desolvation gas flow, 600 L.h^{-1}; cone gas flow, 100 L.h^{-1}, collision gas flow 0.5 ml.min^{-1}. The multiple reaction monitoring (MRM) mode was used and the parameters of MS/MS detection were optimized to each hormone: ABA (263 > 153 m/z, cone 40 V, collision 20 V), SA (137 > 93 m/z, cone 34 V, collision 18 V), IAA (174 > 130 m/z, cone 30, collision 15 V), GA_4 (345 > 142 m/z, cone 35 V, collision 23 V) which were performed in negative ionization (ESI−).

Accurately weighed solid portions of ABA (A1049), SA (S5922), GA4 (G7276) and IAA (I2886) standards (Sigma-Aldrich) were

dissolved in methanol to prepare 1 mg.mL^{-1} of stock solutions. All solutions were stored under darkness at -20°C and the working solutions were prepared from these stock solutions and were serially diluted in methanol immediately before use. The standard curve, with the concentration sequence of 5, 10, 25, 50, 100, 200, 400, 800, 1600 ng.ml-1, were prepared in three independents dilutions in the sample matrix (38% of sample extract in methanol) with analysis/quantification in LC-MS/MS in triplicate. To quantify, the limit of detection (LOD), greater than 3, and the limit of quantification (LOQ), greater than 10, were respected, using the TargetLynx™ software (Waters, USA). To determine the recovery efficiency and matrix effect, as described by Trufelli et al. (2011), 100 ng.mL-1 of standards were spiked in each sample at the beginning of extraction. In parallel, samples were extracted without spiking. The recovery and the matrix effect were determined by comparing the peak areas of the analytical standard spiked before and after extraction.

2.5 STATISTICAL ANALYSIS

The experimental design, to physiological quality was completely randomised design with four replicates for each treatment. First, the data were tested for normality (Lilliefors test) and the results obtained in percentage terms, such as germination and tetrazolium were arcsine transformed and the means were compared by SNK's test at 5% significance.

The exploratory techniques of multivariate statistics were applied through the Principal Component Analysis. Variables were standardized, and analysis was performed in R software (R Core Team, 2011).

3. RESULTS

3.1 PHYSIOLOGICAL AND PHYSICAL CHANGES OF SEEDS

The highest values of moisture content were observed at cotyledonary stage for both years, with 67.63% (2014) and 68.27% (2015). At the other stages, the seeds did not change from stage II to stage IV in 2015, however in 2014, an increased from the stage III (47.38%) to stage IV (50.62%) were reported.

In 2014, seeds dry mass undergo double increased from the cotyledonary stage to stage III with raised from 2.56 to 5.67 g / seed. However in 2015, the maximum accumulation of dry mass occurred at Stage II with 5.11 g / seed. After these periods, a dry mass remained constant (2015) or decreased (2014) to stage IV. While, the higher dry mass of the embryos were observed at stages III and IV for both years.

Table 2 – Weight of 100 seeds (W100), moisture content of seeds and embryo, dry mass of seeds and embryo of *A. angustifolia* during two years (2014 and 2015) and different development stages.

Seeds characteristics	Year	Development stage							
		Cotyledonary		II		III		IV	
W100 (g)	2014	764.13	b	897.8	a	974.79	a	881.17	a
	2015	872.32	b	945.35	ab	953.56	ab	1033.67	a
Moisture content of seed (%)	2014	67.63	a	47.82	c	47.38	c	50.62	b
	2015	68.27	a	52.62	b	50.43	b	50.65	b
Moisture content of embryo (%)	2014	67.98	a	55.82	b	57.61	b	56.92	b
	2015	73.29	a	63.52	b	60.45	c	60.76	c
Dry mass of seed ($g.seed^{-1}$)	2014	2.56	c	4.56	b	5.67	a	4.3	b
	2015	2.81	b	5.11	a	4.62	a	4.65	a
Dry mass of embryo ($mg.embryo^{-1}$)	2014	37.07	c	61.05	b	80.3	a	78.27	a
	2015	27.27	c	58.12	b	62.72	ab	69.45	a

The letters refer to the SNK's test ($P < 0.05$).

The percentage of germination increased up to the stage III (96%) in 2014. However, in 2015 the results showed no significant difference among stages. Viability by tetrazolium test was similar at all stages in 2014 but in the other year, the seeds showed the lower values at Cotyledonary Stage (84%). In 2014, the vigor by GSI showed an increase during development, with 0.23 at the cotyledonary stage, followed by 0.40 at stage II, then remaining stable at stage III and an increase to 0.58 at stage IV. However, in 2015 an increase until stage III (0.74) was observed, following by a decreased to 0.55 at stage IV. Electrical conductivity, at stage IV showed higher leaching of exudates than at others stages; in this stage, the values of exudates liberate were 98.50 µS/cm.g and 139.78 µS/cm.g in 2014 and 2015, respectively (Figure 3).

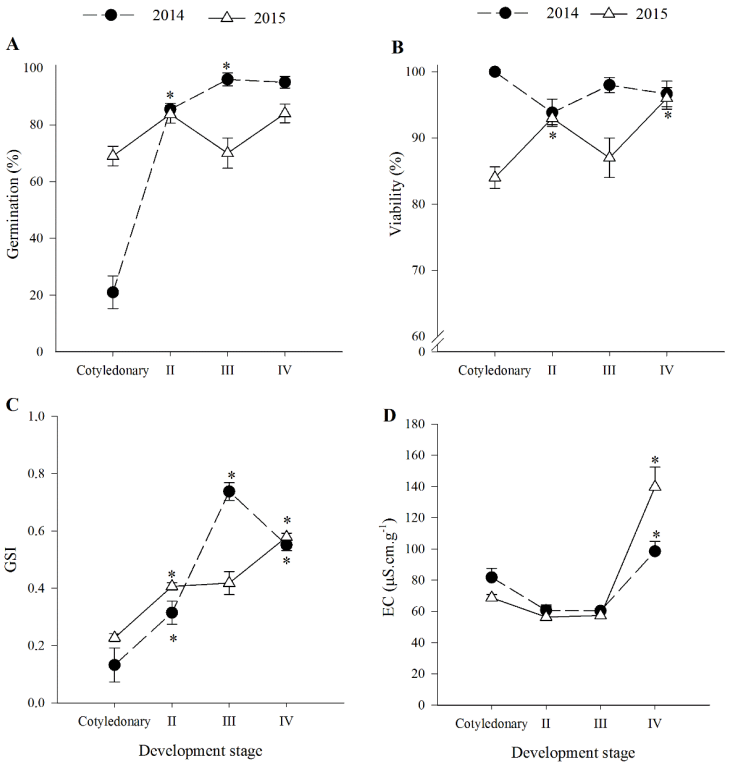


Figure 3 – Viability by germination (A) and tetrazolium (B) test, germination speed index – GSI (C) and electrical conductivity – EC (D) of *Araucaria*

angustifolia seeds at different development stages. * indicate differents between development stage by SNK's test ($P < 0.05$)

3.2 CHANGES OF HORMONES AT DIFFERENT DEVELOPMENT STAGES

Differences in free ABA levels were observed during at development stage, with the highest values at stage II in embryonic axis with 3.73 $\mu g.g^{-1}$ in 2014 and 12.63 $\mu g.g^{-1}$ in 2015. After, free ABA levels decreased until stage IV in both years. While in cotyledons were observed higher values at both cotyledonary (11.15 $\mu g.g^{-1}$) and stage II (11.58 $\mu g.g^{-1}$) in 2015 and only stage II in 2014, with 4.38 $\mu g.g^{-1}$.
GA4 levels in *A. angustifolia* cotyledons followed the same tendency of ABA in both years, with the highest levels at cotyledonary stage and stage II, with 0.1 $\mu g.g^{-1}$ and 0.14 $\mu g.g^{-1}$ respectively for 2015 and only at stage II with 0.03 $\mu g.g^{-1}$ in 2014 (Figure 4). However, GA4 levels in the embryonic axis were distinct between years,with a decreased from Stage II to Stage III, following by a decreased at stage IV in 2015. In contrast, an increase were observed only Stage IV in 2014.

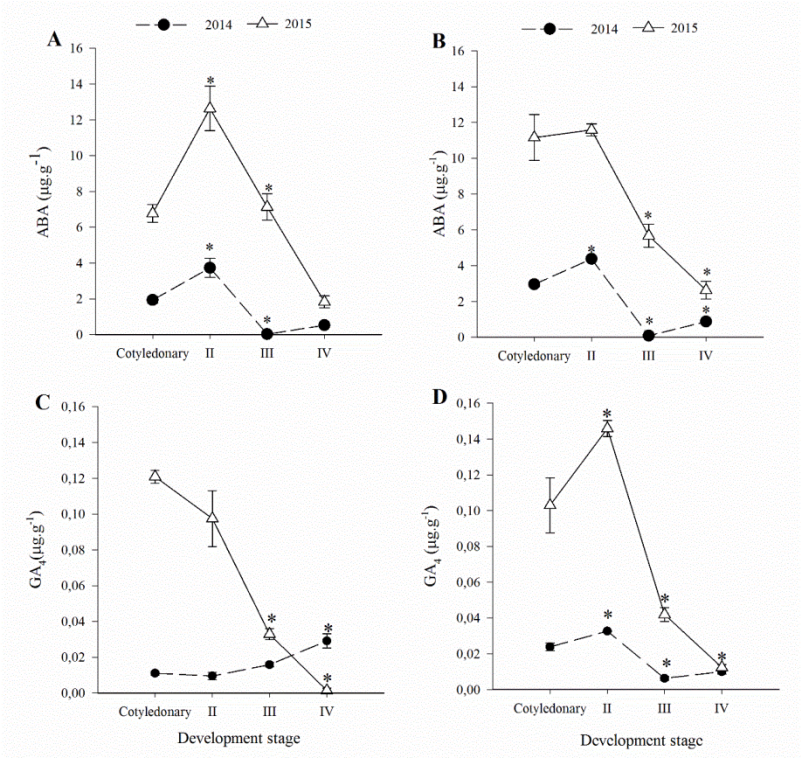


Figure 4 – ABA (A, B) e GA_4 (C, D) in embryonic axis (A, C) and cotyledons (B, D) of *Araucaria angustifolia* at different development stages during 2014 and 2015. * indicate differences between the development stages SNK's test ($P<0.05$).

During seed development, no differences were observed in salicid acid levels in the cotyledons in both years. In contrast, in the embryonic axis was observed an increase during development, i.e. at cotyledonary stage and stage II the levels remained similar with 0.42 $\mu g.g^{-1}$ and 0.38 $\mu g.g^{-1}$ respectively, followed by an increase at stage III (0.81 $\mu g.g^{-1}$) and stage remaining stable at stage IV (0.69 $\mu g.g^{-1}$).

IAA in embryonic axis revealed higher levels at stage III (15.01 $ng.g^{-1}$) in 2014 and stage II (9.83 $ng.g^{-1}$) in 2015. However, in cotyledons was observed difference only in 2015, with low levels 1.41 $ng.g^{-1}$ and an increase at stage II (11.13 $ng.g^{-1}$) and stage III (20.40 $ng.g^{-1}$) (Figure 5).

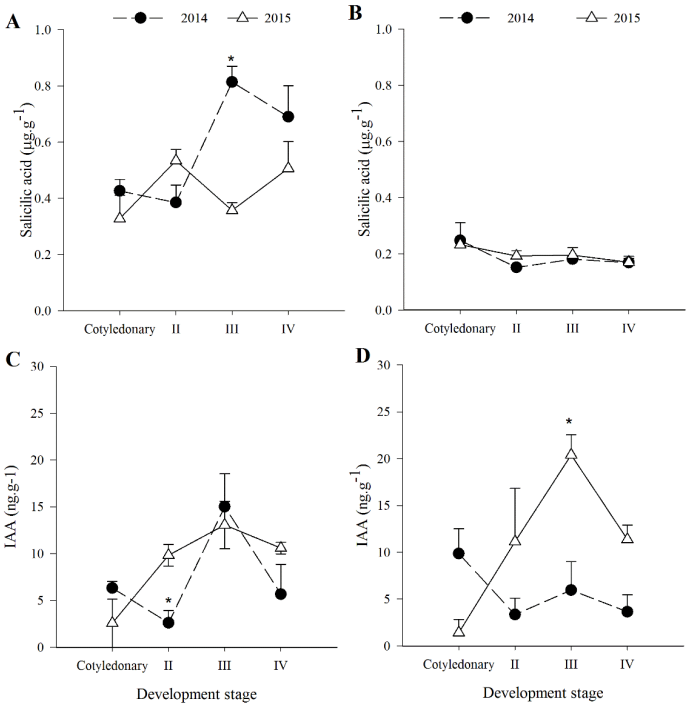
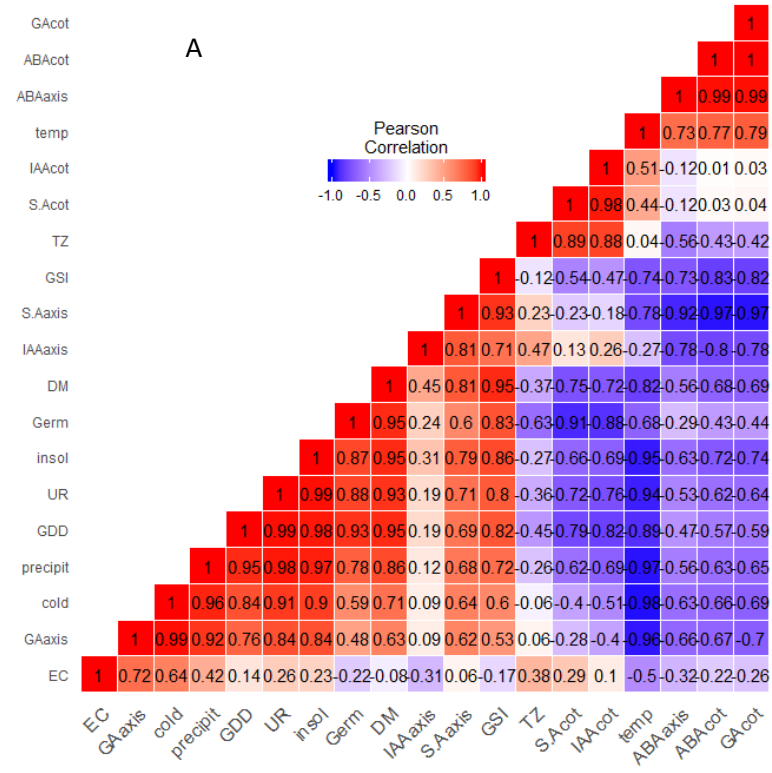


Figure 5 – Salicilic acid (A, B) and IAA (C, D) in embryonic axis (A, C) and cotyledons (B, D) of *Araucaria angustifolia* seeds at different development stages in 2014 and 2015. * indicate differents between development stage by SNK's test ($P < 0.05$)

3.3 ANNUAL CHANGES OF THE PHYSIOLOGICAL ANALYSESAND HORMONES

Seeds were exposed to different climatic conditions during development in 2014 and 2015 (Figure 2) and might have influenced in hormonal and physiological responses. Therefore, correlation analyzes were performed separately, in order to verify if the results were similar in the both years. Correlation between ABA levels in cotyledons with temperature were observed, with 0.77 (2014) and 0.96 (2015). Similarly in embryonic axis were also observed correlation 0.73 in 2014 and 0.69 in 2015.

Results indicated a negative correlation between ABA levels and GSI, with -0.73 (embryonic axis) and -0.83 (cotyledons) in 2014 and -0.83 and -0.44 in embryonic axis and cotyledons, respectively in 2015. In contrast, a positive correlation was observed between IAA in embryonic axis with GSI, with 0.71 (2014) and 0.80 (2015) (Figure 6).



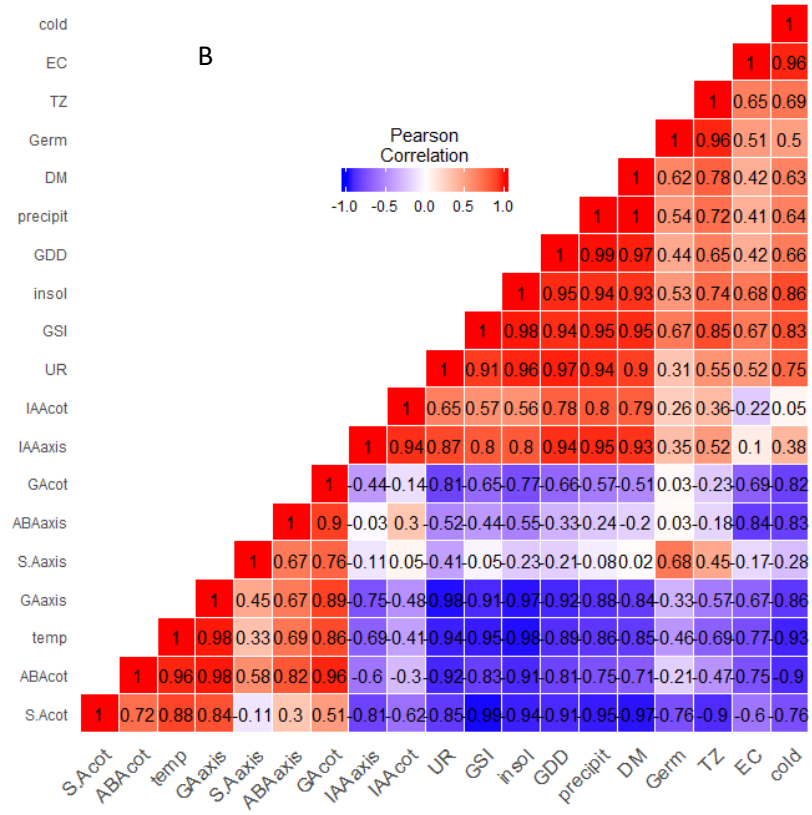


Figure 6 – Pearson correlation between environmental, physiological and hormonal variables during the development of *A. angustifolia* seeds in 2014 (A) and 2015 (B).

The first two components of principal components analysis represented 55.7% of the original variability. Although some differences between 2014 and 2015 were observed, the clusters were formed according to development stages. In Component 1, a group to the right of the axis, formed by stages III and IV were highlighted, and also on the left by the stages cotyledonary and II (Figure 7). For component 2, samples at stage cotyledonary showed higher distances between the years 2014 (upper corner) and 2015 (lower part). At stages II and IV, the sample were close in both years. Thus, it was possible to observe groups formed by stage cotyledonary, II, III and IV.

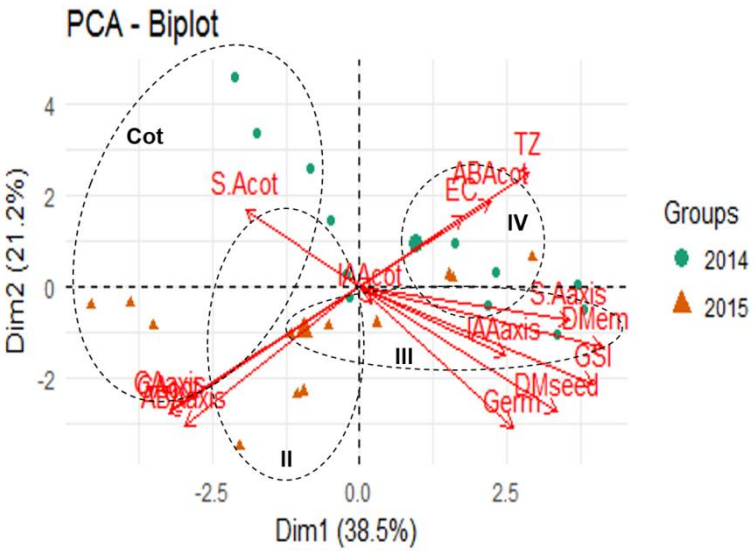


Figure 7- Ordination diagram based on axes 1 and 2 obtained through Principal Component Analysis, representing 59.7% of the original variability of physiological and hormones analysis at stage cotyledonary (Cot), II, III and IV of Araucaria angustifolia seeds. Dim1: first dimension (PC1); Dim2: second dimension (PC2); ABAcot: ABA leves in cotyledons; ABAaxis: ABA in embryonic axis; DMem: dry mass of embryo; DMseed: dry mass of seeds; EC: electrical conductivity; GAaxis: GA_4 in embryonic axis; GAcot: GA_4 in cotyledons; Germ: germination; IAAcot: IAA in cotyledons; IAAaxis: IAA in embryonic axis; GSI: germination speed index;; S.Acot: S.A in cotyledons; S.Aaxis: S.A in embryonic axis; TZ: viability by tetrazolium test.

4. DISCUSSION

The period of maximum dry matter accumulation for *A. angustifolia* seeds differed between the two years of the study. The results indicated that the seeds reached physiological maturity at stage III in 2014 and at stage II in 2015. Differences in time of maturity may be associated with different environmental conditions, with longer cycles occurring when rainfall is higher and temperature lower during seed development (Lamarca et al., 2013; Mata et al., 2013). For example, the maturation period of seeds of *Eugenia pyriformis* was longer in years with lower temperatures or longer rainy periods (Lamarca et al., 2013). Likewise, the maturation time of seeds of *Hopea hainanensis* differed by one month in different years of production, with seeds maturing faster in the dry years (Lan et al., 2012, 2007). Our results also showed that in 2014, the period from fertilization to stage II experienced abundant precipitation (879 mm cumulative precipitation), compared to 2015 (345 mm) (EPAGRI / CIRAM, 2017), and consequently the seeds matured more slowly.

When analyzing other climatic factors, a positive correlation between dry mass and GDD, *UR*, sunlight in both years were observed. In other studies, the accumulation of GDD during seed maturation affected some characteristics such as seed size, water content and dry mass, dormancy and sensitivity to desiccation (DAWS et al., 2004; LAMARCA et al., 2013; LAMARCA; SILVA; BARBEDO, 2011). In Inga vera seeds, the year with higher accumulation of degrees day and higher precipitation showed longest maturation period. In addition, our results also showed that the climatic conditions influenced the hormonal levels, mainly ABA, GA4 and IAA.

In the sample of 2015, ABA levels were found to be near three times higher than in 2014. These levels may have been influenced by the low precipitation during the entire development of the seeds, and especially in the months of collection (Figure 1). ABA is involved in plastic responses to changing water potential and nutrient availability; in which it increases considerably in stressed tissues due to drought, salinity or heat (FARNSWORTH, 2004; LATA; PRASAD, 2011), and plays an important role in stress response and plant tolerance (NAKASHIMA; YAMAGUCHI-SHINOZAKI, 2013). Other studies also found differences among five years of sampling recalcitrant seeds of *Quercus robur*, and a correlation between ABA and dry mass accumulation rate were also observed (FINCH-SAVAGE; FARRANT, 1997). Differences were also observed for fruit species during two years

of production, but a clear understanding of the observed differences between the years was not reached (OIKAWA et al., 2015).

In general, when seeds reach physiological maturity, their corresponding ABA peaks may correlate with low seed germinability (Finkelstein et al., 2002). Unlike other species, the germination capacity of *A. angustifolia* seeds does not seem to have a direct relationship with ABA, since the highest levels of this hormone were recorded in stage II with 3.73 μg.g-1 in 2014 and 12.63 μg.g-1 in 2015, and 85% germination in both years. High levels of ABA were also observed on the second day after sowing of *A. angustifolia* seeds (PIERUZZI et al., 2011). Thus, the lower germination in the cotyledonary stage in 2014 could be associated with other factors, such as lower accumulation of dry mass and consequently lower levels of enzymes and stored reserves or adverse climatic conditions during the development of morphologically immature seeds and/or embryos. These factors, isolated or in combination, could influence the physiological response observed at this stage.

Although no correlation was observed between ABA and the final percentage of germination, a negative correlation between ABA and GSI was observed for both years and tissues. The seed germination period of *A. angustifolia* is around 70 to 80 days, during which ABA levels may have decreased and, consequently, no change in final germination was observed, since this hormone is involved in plastic responses and its levels may change within hours (Farnsworth, 2004).

After stage II, an increase in embryo dry mass, high levels of IAA and a continuous accumulation of starch and histochemically detected proteins were observed (Chapter 1). This hormone may have stimulated differentiation of meristematic tissues for the formation of resin canals and the onset of the cell division process due to an increase of cells in the G2 phase of the cell cycle observed in the embryonic axes of *A. angustifolia* in stages III and IV (Chapter 1). It is accepted that much of the growth of a plant depends on the division, expansion and cellular differentiation induced by auxin (PIERUZZI et al., 2011). Previous studies have found high levels of IAA in the initial stages of *A. angustifolia* embryo formation, which decreased during development (ASTARITA; FLOH; HANDRO, 2003) and increased again on the second day after sowing (PIERUZZI et al., 2011). Our results reported an antagonistic balance between ABA and IAA, which may contribute to the germination of seeds soon after their dispersion.

An association between IAA and SA was reported during zygotic embryogenesis of *A. angustifolia* (BUENO, 2014). In the present study,

IAA and SA in the embryonic axis showed a correlation of 0.81 (2014) and 0.4 (2015), with higher levels of SA being recorded in stages III and IV in 2015. The effect of this hormone during seed development is not clear, however, several studies have associated an increase in SA levels in seed germination, radicle protrusion and tolerance to abiotic and biotic stresses, such as salinity, ozone, radiation, extreme temperatures and drought (Khan et al., 2015; Klessig et al., 2016, Lotfi and Ghassemi-Golezani, 2015, Rivas-San Vicente e Plasencia, 2011).

In summary, seeds reached physiological maturity faster in the year with lower precipitation, cold hours and GDD. ABA levels showed a negative correlation with germination velocity rather than final germination. Although differences in hormone levels and physiological responses were observed in both years, it was possible to observe a clustering of each stage of development by principal component analysis. Thus, these results are evidence of changes in the levels of ABA, GA_4, SA and IAA and their role in physiological responses during seed development, as well as changes in the levels of these hormones in different years of production. However, seed development is a complex process and elevated levels of ABA and GA_4 may imply prominent physiological roles during the development and maturation of *A. angustifolia* seeds. New approaches for future studies may be conducted with seeds at the cotyledonary stage and stage II in order to elucidate the role of hormones in the synthesis of proteins, carbohydrates or antioxidant enzymes that could contribute to the desiccation tolerance of *A. angustifolia* seeds.

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CAPÍTULO 3

STORAGE OF *Araucaria angustifolia* SEEDS: DETERIORATION OR GERMINATION EVENTS

ABSTRACT

During storage of *Araucaria angustifolia* seeds, it still not clear if the seeds begin germination or deterioration process, which can lead to a faster loss of viability. This study aimed to evaluate enzymatic and ultrastructural changes in *A. angustifolia* seeds collected at different developmental stages and its linked to physiological quality on storage. Seeds were collected from two crop seasons at stages Cotyledonary, II and III, and stored in a refrigerator for up to 360 days. During storage periods, physiological quality, and ultrastructural and enzymatic changes were evaluated. Physiological quality of *A. angustifolia* seeds during storage was influenced by developmental stage in both years evaluated, showing an increase in germination and vigor, mainly when the seeds were collected at cotyledonary stage and stored for 60 days. An increased germination and Germination Speed Index (GSI) were observed at 30 and 60 days of storage at all development stages, followed by mitochondria development, and an increase of endoplasmic reticulum and starch inside plastids. By contrast, at 90 days of storage, accumulation of cellular abnormalities was observed, such as alteration in mitochondrial structure and loss of cytoplasm integrity, following by the lower protein content and higher APX and SOD activities. These changes seem to collaborate for a decline of 16% in germination and 0.21 in GSI an increase from 65.25 to 121.90 µS.cm-1.g-1 in electrical conductivity. Thus, *A. angustifolia* seeds seem to start germination process until 60 days of storage, however, no additional water was supplied to continue this process, leading gradually to deterioration process at 90 days storage.

Key words*:* developmental stage; antioxidant enzymes; recalcitrant seed; seed conservation; transmission electron microscopy.

1. INTRODUCTION

Araucaria angustifolia (Bert.) Kuntze seeds are very important both as a food and economic resource and have a social and cultural value for many smallholders and local communities (ADAN et al., 2016; REIS; LADIO; PERONI, 2014; SILVA; REIS, 2009; ZECHINI et al., 2012). However, remnant populations are estimated between 4% and 12% (GUERRA et al., 2002; RIBEIRO et al., 2009), leading this species to be included in the list in IUCN Red List of Threatened Species as "critically endangered" (IUCN, 2016), and in the Brazilian List of Endangered Species (MMA, 2008). Its seeds are considerate recalcitrant (EIRA et al., 1994; GASPARIN et al., 2016) and short natural longevity under natural conditions, with at least a 60% loss of viability within four months of harvest (GARCIA et al., 2014). For long term conservation of recalcitrant seeds is recommended the cryopreservation, usually in liquid nitrogen (WALTERS, 2015). However, the application of this technique is far from small farmers or seedlings producers due to the high cost and level of technology required, so that generally, the seeds storage is done in refrigerator or cold chamber even though for short period.

The period when the seeds remain viable in storage is determined by genetic and physio- logical factors, by the seed development stage, and by any deteriorating events or damage prior to or during storage (BARBEDO; CENTENO; RIBEIRO, 2013; LAMARCA et al., 2013; SCHMIDT, 2007). For recalcitrant seeds, the effect of developmental status on seed viability during storage is known, as described for *Avicennia marina* (FARRANT; PAMMENTER; BERJAK, 1989; PAMMENTER; FARRANT; BERJAK, 1984), *Hopea hainanensis* (LAN et al., 2012) and *Inga vera* (PARISI et al., 2013). When *A. angustifolia* seeds are collected at cotyledonary stage, an increase in physiological quality after 120 days storage was observed, unlike seeds at stage II and III had undergone a decrease in germination after storage (SHIBATA; COELHO, 2016). In general, after seeds reached the maximum dry mass at the end of seed development, deterioration process is initiated (SCHMIDT, 2007). However, the enhanced germination during storage seems to be a consequence of the initiation of subcellular germinate events (FARRANT; PAMMENTER; BERJAK, 1989), that could already initiated during seed development (Brown et al. 2001, Caccere et al. 2013). Therefore, recalcitrant seeds present a continuous development between formation and germination.

In recalcitrant seeds, some ultrastructural changes have suggested germination process during short-term storage, such as an increased mitochondrial organization, indicating increased respiratory activity; cell division; reserve mobilization and appearance of Golgi bodies (BERJAK; DINI; PAMMENTER, 1984; FARRANT; PAMMENTER; BERJAK, 1989). While in deterioration process, damage cellular is indicated by the accumulation of cellular abnormalities, as altered mitochondrial; fragmentation or loss of endoplasmic reticulum; an increase and coalescence of lipid droplets; breaks in the plasmalemma structure and its contraction from the cell wall and a loss of cytoplasm integrity (MAHJABIN; ABIDI, 2015; SMITH; BERJAK, 1995). Ultrastructural studies with *A. angustifolia* seeds have been conducted until 45 days of storage and showed germinated-events associated (FARRANT; PAMMENTER; BERJAK, 1989). However, until this date, it is not known if different development stages may cause loss or enhancement on viability after storage, considering that knowledge of seeds maturity is essential to the maintenance of viability during storage and this might depend on the period when seeds reach maturity and of environment conditions during seed formation.

Furthermore, recent studies of *A. angustifolia* embryos have demonstrated that antioxidant enzymes play an essential role of metabolism during storage, with an increase SOD (superoxide dismutase), CAT (catalase) and APX (ascorbate peroxidase) activities during storage (ARALDI et al., 2016). Reactive (active) oxygen species (ROS) mainly comprise of superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) which are produced during deterioration or germination processes (SHARMA et al., 2012; SINGH; CHAUDHURI; KAR, 2014; TOMMASI et al., 2001). SOD acts as the first line of defense against O_2^- and APX and CAT have been reported as efficient destruction of hydrogen peroxide (BAILLY, 2004; DE GARA et al., 2003; GOEL; GOEL; SHEORAN, 2003; MITTLER, 2002; VAN DOORN; KETSA, 2014).

However, there is no report showing how the collection of *A. angustifolia* seeds at different development stages might reflect in changes in ultrastructure and SOD, APX and CAT activity on storage and what their link to physiological changes. Thus, this study aimed new insight about enzymatic, physiological, and ultrastructural changes of *A. angustifolia* seeds during storage. Changes on germination and vigor were evaluated is seeds collected from two crop seasons and stored by 360 days. Ultrastructural and enzimatic assays were performed until 90 days of storage and the results were linked to physilogical quality loss.

2. MATERIAL AND METHODS

Megastrobili of *A. angustifolia* were collected from a natural population located in Santa Catarina State, southern Brazil (27° 55' 30" S and 49° 58' 35" W, altitude 1300 m) during March – June of 2014 and 2015. Megastrobili were chosen based on the main color changes they experience during seed maturation: green (megastrobilus with predominantly green surface – Cotyledonary Stage), green-brownish (megastrobilus with green surface and with small brown spots for less than 50% of the total surface area – Stage II) and brown-green (megastrobilus with green surface and large brown spots for more than 50% of the total surface area and exhibiting the start of dehiscence – Stage III) (Fig.1).

Twenty cones were collected at each stage and they were manually shelled, and the seeds from different cones were combined and were stored in a refrigerator (8±2 °C) for 60, 90, 180 and 360 days, in semipermeable plastic bags for samples collected in 2014. Fresh and stored seeds were evaluated in moisture content and the physiological quality by germination, tetrazolium, germination speed index (GSI) and electrical conductivity.

In 2015, seeds were collected again from the same population at cotyledonary stage, stage II and III, and stored for 30, 60 and 90 days in the refrigerator and submitted to physiological tests mentioned above, and antioxidant enzymes activities and transmission electron microscopy (TEM) were realized.

Moisture content: Four replicates of three seeds in each development stage and storage period were cut transversally, then weighed (wet weight), dried at 105°C ± 3°C for 24 hours and reweighed to determine dry weight and moisture content (BRASIL, 2009).

2.1 PHYSIOLOGICAL ANALYSIS

Germination test: seeds were treated with 2 % sodium hypochlorite solution for 3 min and then were sown in trays with vermiculite and placed in a germination chamber at 25±2 °C (Kissmann and Habermann 2014) with a photoperiod of 12 hours. Seeds were monitored for 70 days. Germination counts were made every three days from the beginning of germination and germination speed index (GSI) were calculated (MAGUIRE, 1962).

Electrical conductivity: embryos (4 replicates of 10) were soaked in 75 ml distilled water at 25 °C for 12 hours (MEDEIROS; ABREU, 2007).

2.2 PROTEIN EXTRACTION AND QUANTIFICATION

Protein extraction was performed according to Azevedo et al. (1998), in a mortar with pestle containing a pool of ten embryos/replicate. Embryos were homogenized (2:1 buffer volume/macerated samples into liquid nitrogen) with 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylene diaminetetraacetic acid (EDTA), 3 mM dithiothreitol and 4 % (w/v) insoluble PVPP (Azevedo et al. 1998). Homogenate was centrifuged at 10,000 *g* for 30 min at 4 °C. Soluble protein content was assessed according to Bradford (1976) using bovine serum albumin (BSA) as standard ($y = 1{,}0752x + 0{,}0154$ and $R^2 = 0{,}99$). The assays for the determination of enzyme activities of the APX (EC 1.11.1.11), CAT (EC 1.11.1.6), and SOD (EC 1.15.1.1) were carried out with the samples prepared as described above and whose protein concentrations were determined by the Bradford method.

2.3 DETERMINATION OF ANTIOXIDANT ENZYMES ACTIVITIES

Total CAT activity was assayed spectrophotometrically at 25 °C, as described by Kraus et al. (1995) with modifications by Azevedo et al. (1998) in a reaction mixture containing 1 ml 100 mM potassium phosphate buffer (pH 7.5), and 2.5 µL H_2O_2 (30 % solution) prepared immediately before use. Activity was determined by monitoring the degradation of H_2O_2 at 240 nm over 60 s, after the addition of 20 µL embryo extract.

APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C (Nakano and Asada 1981). The reaction medium contained 80 mM phosphate buffer (pH 7.0), 5 mM ascorbate, 1 mM EDTA, 1 mM H_2O_2, and 10 µL of enzyme extract. The reaction was started by the addition of ascorbate, which was monitored by the decrease in absorbance over 120 s.

Total SOD activity was assayed by the method of Giannopolitis and Ries (1977) with modifications by Cembrowska-Lech et al. (2015) in a reaction mixture containing 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 0.1 mM EDTA, and 1.3 µM riboflavin. The reaction was conducted at 25 °C under fluorescent light, and activity was determined in a spectrophotometer at 560 nm.

2.4. TRANSMISSION ELECTRON MICROSCOPY

Samples of shoot and root apical meristems were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4 % formaldehyde and 2.5 % glutaraldehyde for 12 h (SCHMIDT et al., 2009). The material was post-fixed with osmium tetroxide, dehydrated in graded series of aqueous acetone solutions, and then embedded in Spurr's resin (SPURR, 1969). Samples were stained with 1% uranyl acetate followed by 1% lead citrate. Two grids were examined using a JEM 1011 TEM (JEOL, Tokyo, Japan) at 80 kV. The electron microscopy work has been performed with the microscope of the LCME-UFSC, Florianópolis – Brazil.

2.5 STATISTICAL ANALYSIS

The experimental design, to physiological quality was completely randomized a factorial design 3 x 5 (three development stages and five period of storage) for samples 2014, and 3 x 4 (three development stages and four period of storage) for samples 2015 with four replicates for each treatment. First, the data were tested for normality (Lilliefors test) and the results obtained in percentage terms, such as germination and tetrazolium, were arcsine transformed and the means were compared by Tukey's test at 5% significance.

3. RESULTS

The effect of different stages of development during storage was investigated, and pronounced differences in moisture content and physiological quality were observed. Samples collected in 2014, the highest moisture content has been sustained during storage in the cotyledon stage, with 59.33% after storage for 360 days. However, at stages II and III, there was a decrease after storage, with initial values around 47%, and a reduction to 29-25% moisture content at 360 days. For fresh seeds, the maximum dry mass was observed in stage III and after storage a decrease at all stages. Improved germination and GSI were observed after storage for 60 and 90 days in the cotyledonary stage. At the same stage of development, the highest germination (56%) was observed in relation to other stages after 180 days of storage. However, after 360 days, all stored seeds lost viability (Table 1).

Table 1 – Moisture content (MC), dry mass, germination, speed germination index (GSI) and electrical conductivity (EC) of *Araucaria angustifolia* seeds at cotyledonary stage (coty), stages II and III stored for 0, 60, 90, 180 and 360 days, collected in 2014.

Stage	Storage (days)	MC (%)		Dry mass ($g.seed^{-1}$)		G (%)		GSI		EC ($\mu S.cm^{-1}.g^{-1}$)*	
Coty	0	67.63	aA	2.56	cAB	21	bC	0.13	cC	81.74	B
	60	63.84	aAB	3.13	bA	75	bA	0.67	abA	72.11	B
	90	63.75	aAB	2.73	bAB	88	aA	0.78	aA	77.86	B
	180	60.94	aB	2.30	cB	56	aB	0.31	aB	106.26	B
	360	59.33	aB	1.69	bC	7	aC	0.01	aC	585.40	A
II	0	47.82	bA	4.56	bA	90	aA	0.30	bB	67.05	C
	60	49.07	bA	4.45	aAB	76	bA	0.59	bA	127.65	C
	90	42.60	bA	4.54	aA	75	aA	0.56	bA	158.14	C
	180	37.21	bB	3.92	bBC	39	bB	0.22	aB	386.21	B
	360	29.90	bC	3.80	aC	2	aC	0.02	aC	725.96	A
III	0	47.39	bA	5.67	aA	86	aA	0.86	aA	79.87	C
	60	49.42	bA	4.69	aB	90	aA	0.72	aB	135.40	C
	90	45.24	bAB	4.45	aBC	77	aA	0.70	aB	179.47	C
	180	38.94	bB	4.87	aB	43	abB	0.33	aC	320.96	B
	360	25.07	bC	3.86	aC	0	aC	0.00	aD	831.61	A

*CE no interaction between factors were observed. The letters refer to the Tukey's test ($P < 0.05$): lowercase letter - differences in the columns between the stages of development; upper letter -differences in the columns between storage times within each stage.

Samples of 2015 have not shown the same results for physiological quality as in previous years and it were also submitted lower precipitation and higher temperature during seed development. Moisture content of fresh seeds at cotyledonary stage changed from 68.27% to 72.19% at 90 days of storage. Seeds stored for 30 and 60 days in this stage, also showed an increased the germination and GSI. The leaching of exudates was different to each development stage and the highest values was dependent of storage period, i.e. stage cotyledonary after 90 days, at stage II after 60 days and at stage III after 30 days of storage. Maximum dry mass was observed at fresh seeds of stage II and a decrease after storage was observed in this stage and stage cotyledonary.

Table 2 - Moisture content (MC), dry mass, germination (G), speed germination index (GSI) and electrical conductivity (EC) of Araucaria angustifolia seeds at cotyledonary stage (coty), stages II and III stored for 0, 30, 60 and 90 days, collected in 2015.

Stage	Storage (days)	MC (%)		Dry mass ($g.seed^{-1}$)		G (%)		GSI		EC ($\mu S.cm^{-1}.g^{-1}$)	
Coty	0	68,27	aB	2,81	bAB	70	aBC	0,23	bB	68,77	aB
	30	68,88	aAB	2,93	bAB	86	abA	0,32	cA	68,67	bB
	60	71,89	aA	2,04	cBC	79	bAB	0,41	cA	65,25	cB
	90	72,19	aA	1,89	bC	53	bC	0,20	cB	121,90	aA
II	0	52,62	bA	5,11	aA	84	aAB	0,40	aC	56,41	aC
	30	53,37	bA	4,62	aA	79	bB	0,46	bC	68,36	bC
	60	55,54	bA	3,76	bB	94	aA	0,58	bB	176,54	aA
	90	53,14	bA	4,53	aAB	74	aB	0,77	aA	184,59	aB
III	0	50,43	bAB	4,62	aA	70	aC	0,44	aC	57,41	aB
	30	51,92	bA	4,40	aA	93	aAB	0,58	aB	128,43	aA
	60	51,22	cAB	4,56	aA	96	aA	0,71	aA	133,08	bA
	90	47,47	cB	4,28	aA	83	aBC	0,48	bC	134,71	aA

The letters refer to the Tukey's test ($P < 0.05$): lowercase letter - differences in the columns between the stages of development; upper letter -differences in the columns between storage times within each stage.

Protein content and activity of CAT, APX and SOD from embryos submitted to different storage period indicated diverse

accumulation dynamics. In all stages after 90 days of storage, a decrease in protein content were observed, however, at cotyledonary stage decrease was more intense reaching 2.67 $mg.g^{-1}$ at 90 days of storage. Embryos at cotyledonary stage indicated increased CAT activity after 60 days (16.88 $\mu mol.min.mg.protein^{-1}$), followed by storage for 90 days (95.33 $\mu mol.min.mg.protein^{-1}$). At stage II and III no change in CAT activity was observed during storage.

Results indicated APX activity in the fresh seeds at cotyledonary stage (3.58 µmol.min.mg.protein-1), followed by a decrease after storage for 30 and 60 days (0.55 and 0.42 $\mu mol.min.mg.protein^{-1}$) and an increase after 90 days (6.07 µmol.min.mg.protein-1). At stage II, fresh seeds and stored for 60 days showed lower APX activity (0.83 and 0.93 µmol.min.mg.protein-1, respectively) and an increase after 90 days storage was also observed (4.63 µmol.min.mg.protein-1). While at stage III, APX activity was similar at fresh seeds and stored for 30 and 60 days (0.3-0.76 µmol.min.mg.protein-1) and an increase was observed only at seeds stored for 90 days (1.66 $\mu mol.min.mg.protein^{-1}$).

Differently, SOD activity showed highest values after storage for 90 days at all stages, with 37.44 $\mu mol.min.mg.protein^{-1}$ at cotyledonary stage , followed stage II (5.62 $\mu mol.min.mg.protein^{-1}$) and stage III (5.02 $\mu mol.min.mg.protein^{-1}$).

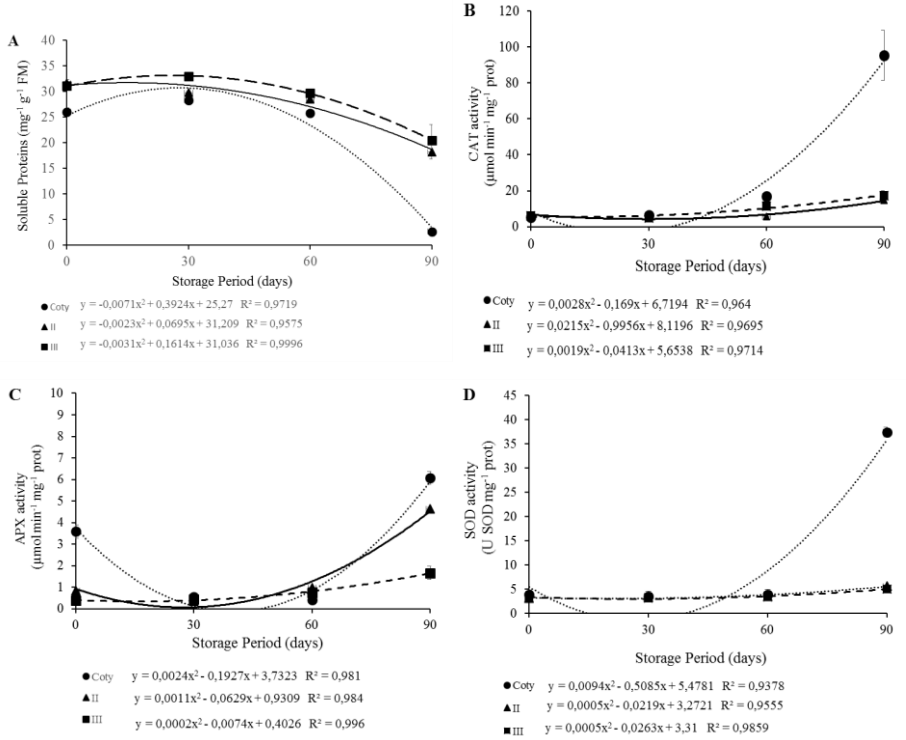

Figure 1 - Changes in soluble proteins (A), CAT (B), APX (C) and SOD (D) activities in Araucaria angustifolia embryos at cotyledonary stage, stage II and III stored for 0, 30, 60 and 90 days at refrigerator (8 °C). Values represent the mean (n = 10) of three replicates for each treatment, and vertical bars are pooled standard errors of the mean.

Ultrastructural analysis also demonstrated different changes during storage according to the development stage of seed. In fresh seeds of cotyledonary stage, stage II and III showed root cells containing large vacuoles and abundant storage reserve, in form of starch (inside the plastids) and high presence of mitochondria (Fig. 2 a, b, c). However, the mitochondria being elongated and their matrices became increasingly devoid of internal detail in fresh seeds at stage III (Fig. 2 c). After storage for 30 days, these mitochondria with altered morphological increased and were also observed at cotyledonary stage, stage II and III (Fig. 2 d, e, f). Such alterations continued of seeds stored for 60 days and also showed abundant storage reserve in form of starch and intense metabolic activity due the presence Golgi bodies, mitochondria and dense electron material (Fig. 2 g, h, i). When seeds were stored for 90 days, the cell division (Fig. 2 k) were observed, some cells showed dense electron content (Fig. 2 j, l) and loss of cytoplasm integrity suggesting subcellular deterioration and collapse of cell walls. In addition in all samples, the cells were characteristic with central nuclei with distinguishing heterochromatin and euchromatin, rough endoplasmic reticulum usually in close proximity to the plasmalemma or surrounding the amyloplast and electron dense material identified as lipid bodies in previous studies (Fraga et al., 2015; Panza et al., 2006, 2002; Rogge-Renner et al., 2013).

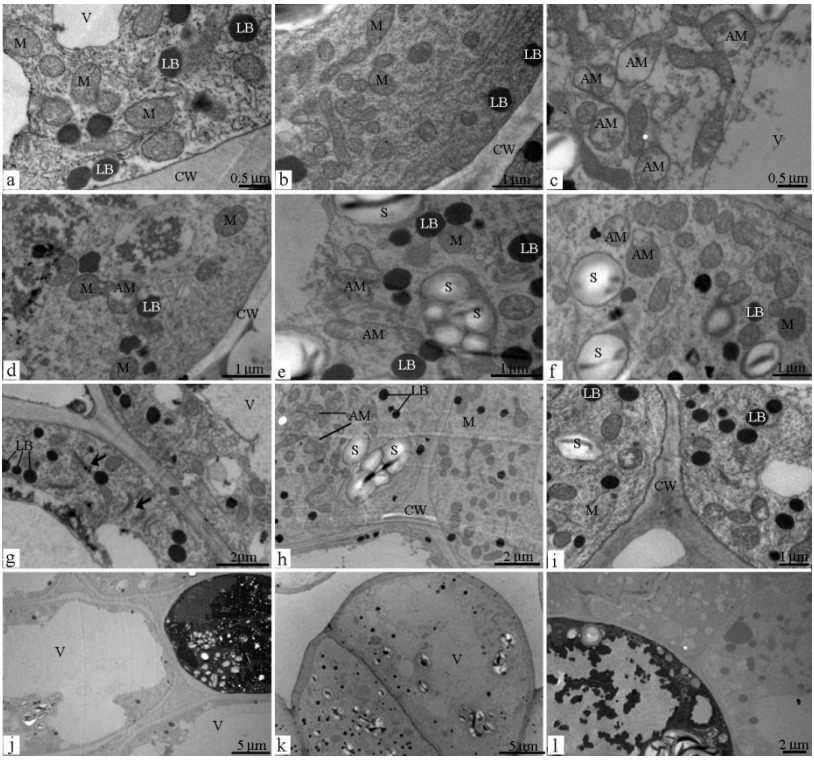

Figure 2 - Transmission electron microscopy images of *Araucaria angustifolia* root. Fresh seeds at cotyledonary stage (a) and stage II (b) showed the presence of mitochondria (M), lipid bodies (LB) and at stage III (c) was observed altered mitochondria (AM). After storage for 30 days, at cotyledonary stage (d), stage II (e) and stage III (f) was also observed cells with altered mitochondria. Intense metabolic activity was observed by mitochondria, Golgi bodies (arrow), lipid bodies (LB) and rough endoplasmic reticulum after the seeds were stored for 60 days at cotyledonary stage (g), stage II (h) and III (i). Cell division and cell with electron dense cytoplasm (j, k, l) were found after 90 days storage of all stages.

At stages II and III, the ultrastructure of shoot cells of fresh seeds was similar to that already described above for root cells, with presence amyloplast, mitochondria and lipid bodies (Fig. 3 a), although some irregularities became apparent, as occurrence of mitochondria elongated, slightly swollen and matrices with an electron-transparent regions (Fig. 3 b, c). After storage for 30 and 60 days an increase in the lipid bodies in the cytoplasm and inside amyloplast (Fig. 3 d, g) was

observed at all stages (Fig. 3 e-i). Seeds stored for 60 days at stage III showed cell division that were also observed after 90 days at all stages. In this period of storage, cells showed loss of integrative cytoplasmic and electron dense cytoplasm at stage cotyledonary (Fig. 3 j) and extensive fusion of vacuoles at stage II. In addition to different mitochondria morphologies found with appeared swollen with cristae more prominent, the root cells at stage III after 90 days of storage, electron dense bodies surrounding the mitochondria was observed (Fig. 3 l).

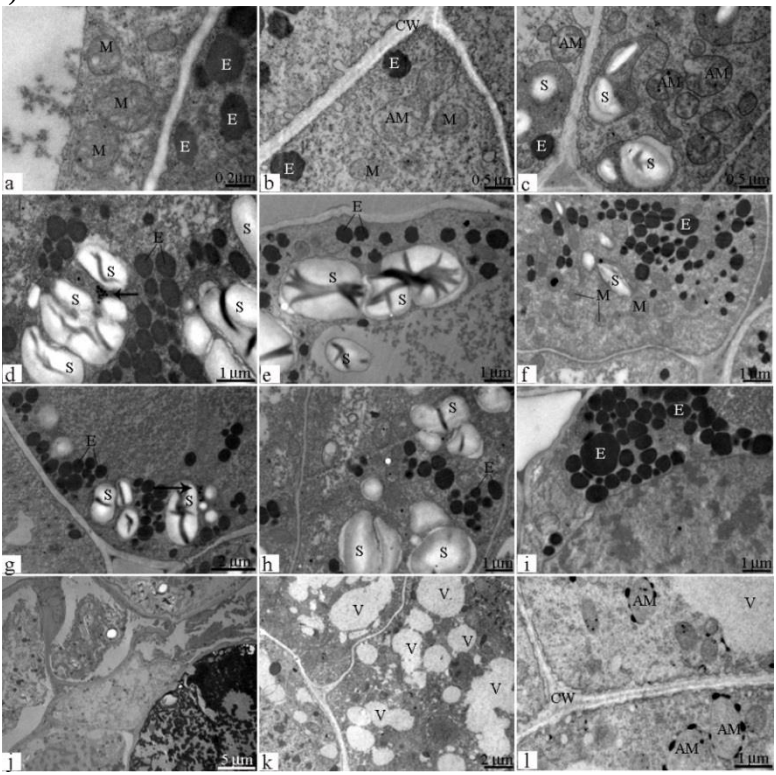

Figure 3 - Transmission electron microscopy images of *Araucaria angustifolia* shoot. Cotyledonary stage (a, d, g, j), stage ii (b, e, h, k) and stage iii (c, f, i, l) of fresh seeds (a, b, c) and stored for 30 (d, e, f), 60 (g, h, i) and 90 days (j, k, l). Abbreviations: altered mitochondria (AM); cell wall (CW); lipid body (LB); mitochondria (M); starch granule (S) and vacuole (V). Note an increase of lipid bodies from fresh seeds to seeds stored for 30 and 60 days at all development stages. When the seeds were storage for 90 days, the cells showed loss of cytoplasm integrity and black content inside cells (arrow) or mitochondria surrounded by electron dense material (l).

4. DISCUSSION

Physiological quality of *A. angustifolia* seeds during storage was influenced by development stage in both years evaluated, showing an increase in germination and vigor, mainly when seeds were collected at cotyledonary stage. Previous studies (in the year of 2013) had demonstrated an increase germination after storage at cotyledonary stage (data not shown), as well as, in other collection locations (Shibata and Coelho 2016). Thus, a probable explanation is that seeds at cotyledonary stage after-ripen during the beginning of the storage period or initiated early germination process, while mature ones are quicker to start the aging.

Seed viability at cotyledonary stage was also influenced by time of dry mass accumulation, i.e. when seeds reached maturity in a short period of time, a loss physiological quality faster during storage was observed. In 2015, seeds reached maturity at stage II and showed an increase germination after 60 days storage, whereas in 2014, maturity was reached at stage III and physiological quality was high until 90 days storage. This differences in the period of dry mass accumulation and storage potential might be influenced by climatic conditions in period of fertilization until maturity, with faster dry mass accumulation in years of lower precipitation (2015: 345 mm and 2014: 879 mm) and higher temperature (2015: 17.4 °C and 2014: 16.8 °C) (EPAGRI/CIRAM, 2017). Other recalcitrant species also showed that development stage of seeds has influenced at viability during storage, as in *Hopea hainanensis* (Lan et al. 2012) and *Inga vera* (Parisi et al. 2013). Although seeds at Cotyledonary Stage have maintained high moisture content during storage, it was not enough to avoid decline in seed viability. One of the possible explanations for this can be related to enzymatic activities and cell organization during seeds development (JYOTI; MALIK, 2013; SCHMIDT, 2007). Therefore, ultrastructural analysis and CAT, APX and SOD activities were evaluated in the 2015 collected samples and linked to physiological changes.

The changes on physiological quality, ultrastructural and enzymatic activity after storage highlights the relevance of development stage during storage of *A. angustifolia* seeds showing germination-associated changes during storage for 30 and 60 days and a more intense alteration in deterioration processes after 90 days storage, mainly when seeds were collect at cotyledonary stage. Firstly, an increase germination and GSI were observed after 30 and 60 days storage for all

development stages. This initial enhancement in germination seems to result in the initiation of metabolic events typical of early germination. Some recalcitrant seeds had shown an increase germination in short-storage, as it were already observed for seeds of *A. angustifolia* from 30 days of storage (FARRANT; PAMMENTER; BERJAK, 1989) and other recalcitrant seeds, as *Avicennia marina* (FARRANT; BERJAK; PANMENTER, 1985). Recalcitrant seeds can germinate rapidly due to both having a high moisture content and a high and active metabolism, and so limited or no imbibition is required to start of germination (BERJAK; PAMMENTER, 1995; DAWS; GARWOOD; PRITCHARD, 2005; PRITCHARD et al., 2004). Thus, when seeds were stored the germination metabolism seem to be started. In our results, changes associated with germination process were observed for all stages, with development of mitochondria, increase of endoplasmic reticulum, Golgi bodies were numerous and appeared to be active and storage material was present in plastids.

At stage II and III, the ROOT cell showed an extensive fusion of vacuoles and the occurrence of cell division (stage III) after 60 days storage. Such changes seem to have contributed for higher GSI and consequently, the faster germination. Probably, fresh seeds in this stage were more prepared to initiate germination process, mainly stage III, the seeds showed anatomic changes and higher percentage cells in phase G2 of the cell cycle that collaborates to trigger germination (chapter 1).

By contrast, after 90 days storage, a decline in both germination and GSI was observed that it was accompanied by ultrastructural, enzymatic changes and reduction protein content. These events seem to be strongly correlated with deterioration process and it were more intense at cotyledonary stage. Decline germination had already reported for *A. angustifolia* seeds at stage II and III after 120 days storage (SHIBATA; COELHO, 2016) and also observed after 4-5 days storage of *Avicennia marina* seeds (FARRANT; BERJAK; PANMENTER, 1985; PAMMENTER; FARRANT; BERJAK, 1984). The same authors stated that the reduction of germination could be interpreted as being the consequence of a requirement for additional water for the continuation of the germination and if the water is withheld, damage occurs until water is supplied (PAMMENTER; FARRANT; BERJAK, 1984). Our results at all stages corroborate this idea, with an accumulation of cellular abnormalities, as altered mitochondrial; fragmentation or loss of endoplasmic reticulum; increase lipid bodies; and a loss of cytoplasm integrity suggesting subcellular deterioration.

Another interesting change was APX, CAT, and SOD activities and protein content. Such enzymes and proteins have been associated with metabolism during storage of *A. angustifolia* seeds, with an increase in enzymatic activity and reduction of protein content (ARALDI; COELHO; MARASCHIN, 2016). Similarly at all stages, lower protein content and higher APX and SOD activities were observed in seed stored for 90 days in our study. SOD is considered a key enzyme in the regulation of intra/cellular concentrations of superoxide radical and peroxides (GOEL; GOEL; SHEORAN, 2003) and the protection against reactive forms of oxygen is conferred primarily by this enzyme and glutathione reductase in *Quercus robur* seeds (HENDRY et al., 1992). While CAT and APX play a role in eliminating hydrogen peroxide (BAILLY, 2004; DE GARA et al., 2003) and showed a strong positive correlation with the viability of *Trichilia dregeana* embryonic axes during slow drying (VARGHESE et al., 2011).

Such enzymes have a crucial role in the protection of membranes and other cell macromolecules in seeds, which are directly able to detoxify reactive oxygen species (ROS) and free radicals (PUKACKA; RATAJCZAK, 2007). ROS include free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH), as well as nonradical molecules like hydrogen peroxide (H_2O_2), singlet oxygen (O_2) (SHARMA et al., 2012). Some antioxidant and repair enzymes include superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). At stage II and III, CAT showed no differences in fresh seeds and after 30 and 60 days storage, unlike APX, which showed an increase mainly after 90 days storage in all stages. Lower CAT activity could compensate for APX activity since both enzymes act against H_2O_2. CAT is located in glyoxysomes and peroxisomes, while APX is present in all cell compartments (BAILLY, 2004) and recalcitrant seeds showed enhanced APX activity in relation orthodox seeds (TOMMASI; PACIOLLA; ARRIGONI, 1999).

In the present study, SOD activity was higher at cotyledonary stage after 90 days storage that it was combined an increase CAT and APX, as well as, ultrastructural changes and reduction on germination and GSI were observed. Such results highlighting the possible role of development stage in incident damage during storage. Probably, seeds in this stage no have accumulated reserve storage sufficient and all needed enzymes for greater longevity, that it could be caused by faster accumulation dry mass in 2015 as compared to 2014.

In summary, we described for the first time changes physiological, ultrastructural and enzymatic of *A. angustifolia* seeds

when collected at the different development stage and its effect on storage. Ultrastructural changes are characteristic of the early stage of germination at all development stages until 60 days storage. As a consequence of the initiation of these subcellular germinative events, seeds stored for these periods showed an increase in germination and GSI. At 90 days of storage, subcellular damage and an increase activity CAT, SOD and APX were observed, probably due to the lack of additional water for continuing germination process, leading the seeds to started a deterioration process that caused the decline in germination and GSI. Such changes were more intense when seeds were collected at cotyledonary stage and could be a relation to the faster accumulation of dry mass. These results showed that ultrastructural and enzymatic changes could trigger the loss of physiological quality during storage and seeds longevity at cotyledonary stage were more vulnerable the climatic changes during its development. However, seeds in this stage had showed better potential for storage in 2014 and also previous years, demonstrating that at this stage still are necessary other information due to the higher potential for storage and whilst higher vulnerability to environmental changes during development. Thus, our results highlight the importance of development stage on seed longevity and collaborate to understand the phenomenon of recalcitrant seed, and consequently developing conservation practices.

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CONSIDERAÇÕES FINAIS

Os resultados obtidos no presente estudo permitiram elucidar as mudanças bioquímicas, fisiológicas e morfológicas que ocorrem no período de coleta das sementes de *A. angustifolia*, assim como, o efeito dos estádios de desenvolvimento no potencial de armazenamento das sementes.

Os resultados apresentados no primeiro capítulo indicaram intensa atividade metabólica com presença de mitocôndrias, corpos lipídicos e proteínas, assim como, um continuo acumulo de amido e proteínas durante todo o período de coleta das sementes. Nos estádios III e IV foram observadas mitocôndrias com uma morfologia diferenciada e a presença de plastoglobulos dentro dos amiloplastos, ambas estruturas podem estar associadas com algum estresse que as sementes estavam sendo submetidas. Nestes estádios foram observados um aumento nas células na fase G2 do ciclo celular e a presença de algumas células em processo de divisão celular, seguido por uma diminuição nos níveis de ABA. Tais mudanças foram acompanhadas por uma diferenciação celular, com a formação de canais resiníferos e um aumento nos níveis de AIA estimulando a divisão e expansão celular. Provavelmente, as sementes se preparavam para sua dispersão com a formação de resina que é uma estratégia de defesa nas plantas e as mudanças nos níveis hormonais e no ciclo celular podem colaborar para que a germinação inicie rapidamente, tornando as sementes menos susceptíveis a predação. Contudo, estas mudanças podem ter colaborado para que a qualidade fisiológica aumentasse nestes estádios (Figura 1).

Nos demais estudos encontrados na literatura com sementes de *A. angustifolia* são descritas que foram coletadas sementes maduras, sem uma descrição detalhada de qual período da maturação as sementes se encontravam ou quais características visuais foram utilizadas para a determinação da maturidade. No presente estudo observou-se que diferentes mudanças ocorrem neste período, sendo que claramente pode-se observar uma divisão dos estádios cotiledonar e II com os estádios III e IV. Esta separação foi observada tanto pela análise de infravermelho (FTIR) (capítulo 1) como pela análise fisiológica e hormonal em dois anos de produção de sementes (capítulo 2). Por isso para futuros estudos, recomenda-se que a coleta das sementes deve ser feita cuidadosamente, com a descrição da coloração da superfície das pinhas e não apenas o termo sementes maduras.

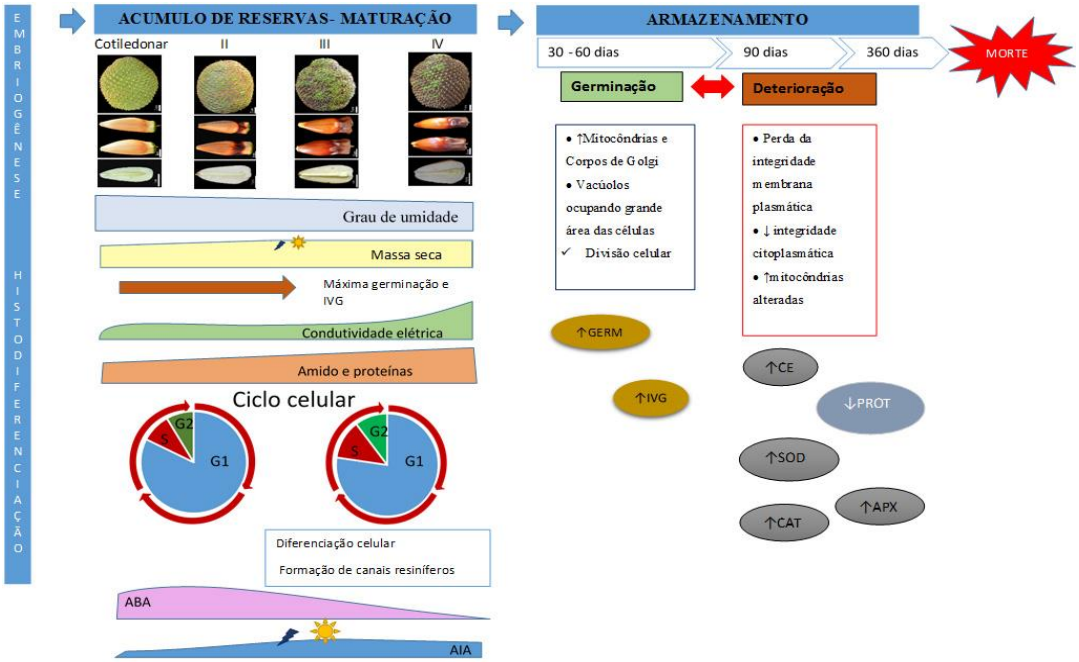

Figura 1 – Esquema ilustrativo das alterações que ocorrem durante o acumulo de reservas/maturação e armazenamento de sementes de Araucaria angustifolia. ABA: ácido abscísico; AIA: ácido indol-acético; APX: ascorbato peroxidase; CAT: catalase; CE: condutividade elétrica; GERM: germinação; IVG: índice de velocidade de germinação; PROT: proteínas; SOD: superóxido dismutase. : influência das condições climáticas: precipitação e temperatura. Note: mudança de coloração nas pinhas e sementes, acompanhada por uma diminuição no grau de umidade e aumento na massa seca das sementes durante o desenvolvimento. Nos estádios finais as sementes apresentaram

(continuação legenda Figura 1) máxima germinação e IVG, contudo maior aumento na condutividade elétrica e nos teores de amido e proteínas verificados histoquicamente. A maioria das células estavam na fase G1 do ciclo celular, contudo um aumento na fase G2 foi observado nos estádios III e IV. Os maiores níveis de ABA foram observados nos estádios cotiledonar e II e contrariamente os níveis mais elevados de AIA foram também observados nos estádios III e IV. Nestes estádios também foram observados uma diferenciação celular e a formação de canais resiníferos. Posteriormente, no inicio do período de armazenamento as sementes apresentaram alterações ultraestruturais e fisiológicas que estavam relacionadas ao processo germinativo, contudo após 90 dias de armazenamento mudanças ultraestruturais e aumento na condutividade elétrica e na atividade das enzimas APX, CAT e SOD e uma diminuição nos teores de proteínas foram observadas. Ao final de 360 dias, a maioria das sementes perderam a viabilidade.

Deve-se ressaltar que a *A. angustifolia* apresenta um certo grau de domesticação e existem diferentes variedades botânicas numa mesma população, com diferentes períodos de maturação. Por isso, para a coleta de sementes é necessário o prévio conhecimento do período de maturação das árvores-matrizes, observar atentamente as mudanças na coloração das pinhas e/ou coletar as pinhas com coloração semelhante.

As diferenças das sementes em cada estádio de desenvolvimento refletiram no potencial de armazenamento. Nos resultados obtidos no capitulo 3, a qualidade fisiológica das sementes foi diferente conforme o ano de produção e o período em que as sementes foram coletadas. Na literatura existem diferentes resultados para a qualidade fisiológica durante o armazenamento e provavelmente, estes resultados foram gerados pelas diferenças ambientais que as sementes foram submetidas durante seu desenvolvimento e/ou também, pela falta de uma clara definição em qual período de maturação as sementes foram coletadas.

Apesar das mudanças observadas nos estádios finais do desenvolvimento possibilitarem uma rápida germinação, as sementes de *A. angustifolia* demoraram longo período (70/80 dias em laboratório a 25 °C) para formarem plântulas normais com raiz e parte aérea bem formada. Estes resultados observados em laboratório também são observados a campo, provavelmente as sementes após sua dispersão, nos períodos de junho e julho, emitem a radícula mas demoram longos períodos para desenvolver a parte aérea, nos meses de setembro/outubro. Em termos ecológicos é uma estratégia das sementes para não sofrerem danos com as baixas temperaturas e geadas, pois sua dispersão ocorre durante o inverno. Contudo, as sementes ficam mais vulneráveis a

ataques de animais que consomem as sementes. Este fato associado a baixa regeneração, torna esta espécie mais susceptível a uma diminuição de sua ocorrência.

A partir dos resultados obtidos nesta tese, juntamente com os resultados obtidos em anos anteriores, permitiram concluir que as sementes coletadas no estádio cotiledonar e posteriormente armazenadas, continuam seu desenvolvimento, com posterior início de germinação. Contudo como não é fornecida condições ideais (água/substrato) durante o armazenamento, inicia-se o processo de deterioração. Enquanto nos estádios tardios, as sementes já iniciaram o processo germinativo e posteriormente, o processo de deterioração se inicia. Similarmente, ao ocorrido numa floresta, em que as sementes nos estádios iniciais continuariam seu desenvolvimento ainda presas a planta-mãe e posteriormente, nos estádios III e IV seriam dispersas e em seguida, emitiriam a radícula. Contudo apenas depois do inverno, em setembro/outubro, iniciariam o desenvolvimento da parte aérea.

Assim, apesar dos resultados obtidos durante diferentes anos de produção de sementes, *A. angustifolia* possui algumas características peculiares de sementes recalcitrantes e muitas vezes, difíceis de serem compreendidos. Tanto que o termo recalcitrante significa desobediente, difícil de lidar/operar. Por isso, muitas vezes as alterações no desenvolvimento ou armazenamento das sementes são difíceis de serem compreendidos, contudo os resultados obtidos (figura 1) colaboraram para a compreensão do comportamento recalcitrante da espécie, e poderá auxiliar futuros trabalhos para o uso e conservação desta espécie. Estudos que envolvam tolerância a dessecação das sementes nos diferentes períodos de coleta ou ainda, as possíveis proteínas que podem ser sintetizadas com os elevados níveis de ABA observados nos estádios inicias ainda são desconhecidos e podem elucidar alguns mecanismos envolvidos no comportamento das sementes.