Ana Paula Lando

Fisiologia da germinação de sementes de *Trichocline catharinensis* **Cabrera**

Tese apresentada ao Programa de Pós-Graduação em Recursos Genéticos Vegetais, Departamento de Fitotecnia, do Centro de Ciências Agrárias da Universidade Federal de Santa Catarina, como requisito para obtenção do Título de Doutora em Ciências.

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Fisiologia da germinação de sementes de *Trichocline catharinensis* Cabrera

por

Ana Paula Lando

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Florianópolis, fevereiro de 2019

“Se não houver frutos

Valeu a beleza das flores

Se não houver flores

Valeu a sombra das folhas

Se não houver folhas

Valeu a intenção da semente”

Henfil

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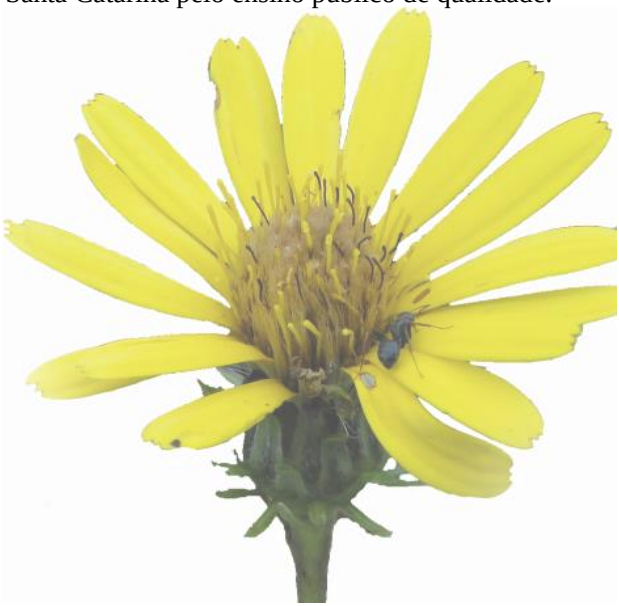

RESUMO

O aprimoramento do conhecimento fisiológico sobre qualidade e controle da germinação de sementes é uma maneira eficaz de contribuir para o uso e conservação de espécies nativas com valor ecológico e econômico, como a *Trichocline catharinensis* Cabrera, uma Asteraceae endêmica dos Campos de altitude do sul do Brasil. Esse estudo teve por objetivo caracterizar a fisiologia da germinação de sementes de *T. catharinensis*, sob a influência dos hormônios GA_3 e ABA. Para tanto foi investigado os efeitos das soluções de giberelina (GA_3), ácido abscisíco (ABA) e seus inibidores, paclobutrazol (PAC) e fluridone (FLU) na germinação e sua relação com poliaminas (PAs) e enzimas antioxidantes (SOD, CAT, APX, GR). Também, foram caracterizadas as mudanças estruturais e no ciclo celular antes da protrusão radicular, associado a isso as variações na metilação global do DNA. Ainda, analisamos as alterações celulares e a abundância proteica diferencial em resposta ao GA_3 e ABA, PAC e FLU na fase II da germinação. Os resultados mostraram que FLU e GA_3 promovem aumento na velocidade de germinação, assim como alta porcentagem de germinação ao contrário do ABA que apresentou atraso no início da germinação, e também baixa porcentagem de germinação (58,75%). O tratamento com PAC inibiu a germinação das sementes (7,5%). O efeito estimulante de GA_3 e FLU favoreceu o equilíbrio de putrescina (PUT) e espermidina (SPD) sobre espermina (SPM). O balanço de PAs (alta razão (PUT + SPD / SPM)) com as enzimas SOD, CAT e APX medeiam a germinação de *T. catharinensis*, uma vez que estas biomoléculas mostraram um padrão de acumulação oposto em baixa porcentagem de germinação (PAC e ABA) e alta porcentagem (FLU, GA_3 e H_2O). Aplicação de todas as PAs exógenas (200 μM) promoveram um aumento na atividade oxidativa. Sugere-se que há uma relação direta entre PAs e sistema antioxidante com o mecanismo fisiológico da germinação de sementes. A conclusão da germinação é indicada pelo aparecimento da radícula através do endosperma micropilar e exotesta. O alongamento celular é iniciado pelo aumento da vacuolização nas células do eixo hipocótilo-radicular, quando os vacúolos de armazenamento de proteínas fusionam-se e transformam-se em vacúolos típicos. Antes da conclusão da germinação observamos células com conteúdo de DNA 4C (fase G2 do ciclo celular). Isso relacionou-se com a redução dos níveis de metilação global do DNA (GDM) observada após 72 horas de embebição, quando a replicação do DNA é possivelmente iniciada. Na semente matura observamos a cromatina compactada em regiões elétron-densas (heterocromatina), e durante a germinação descondensada (eucromatina). A análise dos componentes

principais (PCA) explicou 84% da variação na expressão das proteínas e revelou classes distintas de proteínas associadas aos efeitos da FLU, GA_3, PAC, ABA e H_2O. FLU e GA_3 estimulam a germinação, observada por intensa atividade celular e mobilização de reservas. Percebe-se que as sementes submetidas ao GA_3, recuperam rapidamente a atividade metabólica, em comparação com o H_2O. GA_3 up-accumulated proteínas associadas ao metabolismo energético, regulação redox, proteólise e sinalização. Com efeito oposto PAC, down-accumulated proteínas, associadas ao metabolismo de energia, proteólise, estrutura celular e biossíntese de proteínas. Isso é confirmado, quando observamos as células com poucas organelas e plasmodesmos, associados à baixa ativação do metabolismo, e nenhuma mobilização de reservas. Além disso, no tratamento com PAC, observou-se poucos espaços intercelulares no eixo hipocótilo-radicular, na qual pode resultar em menor alongamento e crescimento. Interessante, que as sementes embebidas em ABA apresentaram osmotolerância, observada pela retração do plasmalema, assim como a coalescência de vacúolos, cercada de reservas proteicas (PSVs) enquanto os corpos lipídicos estão próximos à membrana celular. O ABA altera a abundância de proteínas relacionadas a sinalização, metabolismo de energia e metabolismo de nucleotídeos. O ABA influencia o alongamento do eixo hipocótilo-radicular, através do achatamento das células. Essas proteínas diferencialmente acumuladas associadas às alterações celulares são críticas para a transição da absorção de água para a protrusão da radícula. Nossos resultados fornecem informações sobre a fisiologia da germinação, a nível celular e bioquímico de uma Asteraceae selvagem do sul do Brasil, com potencialidades para uso como planta ornamental. Isto é importante no desenvolvimento de práticas de conservação *ex situ* e *in situ* para sementes, considerando especialmente as espécies silvestres de famílias biodiversas.

Palavras-chave: Campos de altitude, Asteraceae, planta ornamental, fisiologia da germinação, histologia, proteômica, enzimas antioxidantes, conservação.

RESUMO EXPANDIDO

Introdução

O aprimoramento do conhecimento fisiológico sobre qualidade e controle da germinação de sementes pode contribuir para o uso e conservação de espécies nativas com valor ecológico e econômico, como a *Trichocline catharinensis* Cabrera, uma Asteraceae endêmica dos Campos de altitude do sul do Brasil. Poucos estudos têm investigado a germinação de sementes de espécies brasileiras de Asteraceae. A germinação das sementes é um processo fisiológico complexo, que inicia com a absorção de água e comumente compreende três fases. A fase I com uma rápida embebição pela semente seca, iniciando a fase II, na qual as principais alterações morfológicas e fisiológicas resultam em ativação do crescimento do embrião, através de um rigoroso controle bioquímico e molecular, e terminando com o surgimento da radícula e início da fase III. Este processo é geralmente regulado pelo antagonismo entre os hormônios ABA e GA. Para fornecer informações de como esses hormônios atuam, é utilizado inibidores de biossíntese, para ABA é utilizado Fluridone, e para GA, Paclobutrazol. O uso de inibidores da síntese de GA e ABA fornece informações sobre como o conteúdo relativo desses hormônios, podem induzir mudanças em outras biomoléculas, como proteínas, poliaminas e enzimas antioxidantes durante a germinação de sementes.

Objetivos

Cada capitulo consistiu de objetivos específicos, no primeiro capítulo procuramos investigar a influência de GA_3, ABA e seus inibidores sintéticos PAC e FLU ao longo das três fases de germinação de sementes, relacionando ao conteúdo de poliaminas e atividade de enzimas antioxidantes (SOD, CAT, APX, GR). Ainda, avaliar os efeitos de poliaminas exógenos na germinação e na atividade das enzimas antioxidantes. No segundo capítulo analisamos as mudanças estruturais e a atividade do ciclo celular associado à protrusão da radícula. Além disso, a fim de obter uma visão epigenética associada à germinação de sementes, investigamos a dinâmica da variação global da metilação do DNA, considerando três fases de germinação, em resposta às soluções exógenas de GA_3, ABA e seus inibidores PAC e FLU. Além disso, observamos a ultraestrutura da cromatina na semente matura e na fase II da germinação. No capítulo três, procuramos compreender a transição da captação de água e protrusão radicular, analisamos as alterações celulares e a abundância

proteica diferencial em resposta a GA_3 e ABA e seus inibidores PAC e FLU na fase II da germinação.

Metodologia

Sementes de *T. catharinensis* foram coletadas em duas populações naturais localizadas em Curitibanos, Santa Catarina. As sementes foram separadas manualmente e desinfestadas. Após foi realizada curva de embebição, aonde as sementes foram pesadas antes da embebição, em intervalos de 30 minutos por 6h, 1h de intervalo até 12h, a cada 12h até 6 dias e, finalmente, intervalos de 24h até que 50% das sementes apresentassem protrusão da radícula. Foi calculado o incremento de massa ao longo do tempo e ajustado uma curva de 3º grau ao ciclo trifásico da germinação. Determinamos a raiz quadrada da equação derivada de 2º grau e as curvas de inflexão expressas como porcentagem do incremento de massa por hora (% $.h^{-1}$). Para a germinação das sementes foram realizadas aplicações de GA_3, ABA, FLU e PAC (200µM) e o controle (H_2O). As soluções foram preparadas de acordo com instruções do fabricante e diluídas em H_2O, após foram aplicadas em papel germitest, em caixas gerbox. As caixas foram colocadas em BOD. Todas as soluções e suas concentrações foram testadas previamente. Também foi realizada aplicação de poliaminas exógenas (200 µM). Foi avaliado índice de velocidade de germinação e porcentagem diária de sementes germinadas. Para todos os tratamentos com hormônios, seus inibidores e poliaminas, amostras foram coletadas considerando as três fases, e acondicionadas em freezer para posterior análises. Foi realizada quantificação endógena de poliaminas pelo método de HPLC. Extração e ensaio de enzimas antioxidantes- catalase, superóxido dismutase, ascorbato peroxidase e glutationa redutase. Atividade das enzimas foi obtida através de espectrofotômetro para microplacas. Para as análises estruturais foi utilizado microscopia de luz e microscopia eletrônica de transmissão após aplicação de ABA, GA_3, PAC e FLU e H_2O. Após 150 hs de embebição nas soluções de ABA, GA_3, PAC e FLU e H_2O, foi dissecado a parte do eixo hipocótilo radicular para análise em citometria de fluxo. Histogramas foram analisados usando software Flowing. Para análise de metilação global do DNA, foram consideradas as três fases. Após a extração do DNA e digestão de ácidos nucleicos, as amostras foram colocadas em HPLC e analisadas pelo software LC Solution. Para a análise proteômica comparativa foi utilizado o método livre de gel (shotgun), as amostras foram separadas em cromatografia líquida (UPLC) de ultra-alta pressão acoplada a um espectrômetro de massas. Para esta análise foi considerado apenas a fase II. Foram realizadas análises estatísticas pelo programa R (teste de normalidade e SNK $p < 0.05$), médias e intervalos de confiança. Análise de PCA pelo software MVSP.

Resultados e Discussão

A germinação de sementes consistiu de três fases fisiologicamente distintas, similar a outras espécies de Asteraceae. Os tratamentos com FLU e GA_3 promovem aumento na velocidade de germinação, assim como alta porcentagem de germinação ao contrário do ABA que apresentou atraso no início da germinação, e também baixa porcentagem de germinação (58,75%). O tratamento com PAC inibiu a germinação das sementes (7,5%). O efeito estimulante de GA_3 e FLU favoreceu o equilíbrio de putrescina (PUT) e espermidina (SPD) sobre espermina (SPM). O balanço de PAs (alta razão (PUT + SPD / SPM)) com as enzimas SOD, CAT e APX medeiam a germinação de *T. catharinensis*, uma vez que estas biomoléculas mostraram um padrão de acumulação oposto em baixa porcentagem de germinação (PAC e ABA) e alta porcentagem (FLU, GA_3 e H_2O). Aplicação de todas as PAs exógenas (200 µM) promoveram um aumento na atividade oxidativa. A conclusão da germinação em *Trichocline* é marcado pelo aparecimento da radícula através do endosperma micropilar e exotesta. Na análise ultraestrutural observamos aumento da vacuolização nas células do eixo hipocótilo-radicular, devido à transformação de vacúolos de armazenamento de proteína em vacúolos líticos ocorridos antes do início do alongamento celular. Na análise de citometria observamos células com conteúdo de DNA 4C (fase G2 do ciclo celular) antes da conclusão da germinação. Os níveis de metilação global na semente matura é 50%, associado com alta compactação da cromatina (heterocromatina). Após embebição, os níveis de metilação global do DNA diminuem, possivelmente relacionados ao aumento da atividade metabólica. Com isso, a cromatina se solta, em regiões de eucromatina. Essa diminuição do DNA metilado, pode estar antecedendo a replicação do DNA, observado na análise de citometria. Quando a germinação é inibida pelo PAC, inibidor da síntese de GA_3, os níveis de metilação global diminuem drasticamente na fase III. Na análise proteômica comparativa em PCA, observamos que 84% da variação na abundância das proteínas foram explicadas. A análise revelou distintas classes de proteínas associadas aos efeitos da FLU, GA_3, PAC, ABA e H_2O. O tratamento com GA_3 estimula a germinação, comprovado por intensa atividade celular e mobilização de reservas. Estas respostas referem-se ao acúmulo de proteínas associadas ao metabolismo energético e à regulação redox. As sementes embebidas em PAC diminuem a acumulação de proteínas relacionadas ao metabolismo energético e à regulação redox, associadas a isso, apresentaram poucas organelas e plasmodesmos, o que resulta em baixa ativação do metabolismo, e nenhuma mobilização de reservas. Além disso, no tratamento com PAC, observamos poucos espaços intercelulares

no eixo hipocótilo-radicular, o que influencia o alongamento e o crescimento. As proteínas acumuladas pelo ABA, relacionam-se à resposta ao estresse osmótico. As células apresentaram osmotolerância observada pela retração do plasmalema, coalescência de vacúolos. Além disso, o ABA diminuiu a acumulação de proteínas relacionadas à estrutura celular e crescimento, e isso pode estar associado à interrupção da extensão do eixo hipocótilo-radicular, que mostrou células achatadas. Em resposta a isso, o ABA retarda e diminui a porcentagem de germinação.

Considerações Finais

Quando aplicamos GA, ABA, PAC e FLU observamos que os resultados apresentados na germinação, condizem com as observações ultraestruturais e análise proteômica. E as mudanças observadas estão associadas à transição da absorção de água pela semente seca quiescente para a protrusão da radícula. Sugerimos que há uma relação direta entre poliaminas e sistema antioxidante com o mecanismo fisiológico da germinação de sementes. O tratamento com GA_3 pareceu estar envolvido no controle da abundância de várias proteínas associadas à protrusão da radícula, uma vez que quando inibimos a biossíntese de GA com PAC, as células permanecem na fase II. ABA parece dificultar a absorção de água entre a fase II e III, mostrando células com características de osmotolerância. Como observamos, existem poucos estudos que correlacionam alterações ultraestruturais com processos moleculares antes da protrusão da radícula. Com estes resultados conseguimos fornecer informações sobre a fisiologia da germinação, a nível celular e bioquímico de uma Asteraceae selvagem do sul do Brasil, em relação aos papéis de GA e ABA na germinação das sementes.

Palavras-chave: Campos de altitude, Asteraceae, planta ornamental, fisiologia da germinação, histologia, proteômica, enzimas antioxidantes, conservação.

ABSTRACT

Improving physiological knowledge on seed germination quality and control is an effective way to contribute to the use and conservation of neglected native species of ecological and economic value, such as the *Trichocline catharinensis* Cabrera, an endemic Asteraceae from the Southern Highlands Brazil. The objective of this study was to characterize the germination physiology of *T. catharinensis*, under the influence of the GA_3 and ABA hormones. In order to do so, we investigated the effects of gibberellin (GA_3), abscisic acid (ABA) and its inhibitors, paclobutrazol (PAC) and fluridone (FLU) on germination and its relationship with polyamines (PAs) and antioxidant enzymes, GR). Also, we characterized the structural and cell cycle changes prior to radicle protrusion, associated with this variation in the global DNA methylation. Also, we analyzed the cellular alterations and the differential protein abundance in response to GA_3 and ABA, PAC and FLU in phase II of the germination. The results showed that FLU and GA_3 promoted an increase in the germination speed, as well as a high percentage of germination, as opposed to the ABA, which presented a delay at the beginning of germination, and also a low percentage of germination (58.75%). PAC treatment completely inhibited seed germination (7.5%). The stimulatory effect of GA_3 and FLU favored the balance of putrescine (PUT) and spermidine (SPD) on spermine (SPM). The balance of PAs (high ratio (PUT + SPD / SPM) with the SOD, CAT and APX enzymes mediate the germination of *T. catharinensis*, since these biomolecules showed an opposite accumulation pattern in a low percentage of germination (PAC and ABA) and a high percentage (FLU, GA_3, and H_2O). Application of all exogenous PAs (200 μM) promoted an increase in oxidative activity. It is suggested that there is a direct relationship between PAs and antioxidant system with the physiological mechanism of seed germination. The conclusion of the germination is indicated by the appearance of the radicle through the micropylar endosperm and exotesta. Cellular elongation is initiated by increased vacuolation in the hypocotyl-radicular axis cells when the protein storage vacuoles fuse and turn into typical vacuoles. Before completion of germination, we observed cells with 4C DNA content (G2 phase of the cell cycle). This was related to the reduction of DNA global methylation (GDM) levels observed after 72 hours of imbibition when DNA replication is possibly initiated. In the mature seed, we observed the compact chromatin in electron-dense regions (heterochromatin), and during decondensed germination (euchromatin). The major components analysis (PCA) explained 80% of the variation in protein expression and revealed distinct classes

of proteins associated with the effects of FLU, GA_3, PAC, ABA, and H_2O. FLU and GA_3 stimulate germination, observed by intense cellular activity and mobilization of reserves. It is noticed that the seeds submitted to GA_3, quickly recovered metabolic activity, compared to the H_2O. GA_3 up-accumulated proteins associated with energy metabolism, redox regulation, proteolysis, and signaling. With oppositely capped, down-accumulated proteins, associated with energy metabolism, proteolysis, cell structure, and protein biosynthesis. This is confirmed when we observe cells with few organelles and plasmodesmata, associated with low activation of metabolism, and no mobilization of reserves. In addition, in the treatment with PAC, few intercellular spaces were observed in the hypocotyl-radicular axis, in which it can result in smaller elongation and growth. Interestingly, the ABA-imbibed seeds showed osmotolerance, observed by plasmalemma retraction, as well as the coalescence of vacuoles, surrounded by protein reserves (PSVs) while the lipid bodies are close to the cell membrane. ABA alters the abundance of proteins related to signaling, energy metabolism, and nucleotide metabolism. The ABA influences the elongation of the hypocotyl-radicular axis, through the flattening of the cells. These differentially accumulated proteins associated with cell changes are critical for the transition from water absorption to radicle protrusion. Our results provide information on the physiology of germination, at the cellular and biochemical level of a wild Asteraceae from the south of Brazil, with potentialities for use as an ornamental plant. This is important in the development of *ex situ* and *in situ* conservation practices for seeds, especially considering the biodiverse wild plant families.

Keywords: Altitude fields, Asteraceae, ornamental plant, germination physiology, histology, proteomics, antioxidant enzymes, conservation.

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LISTA DE ABREVIATURAS E SIGLAS

ABA- ácido abscisíco
ADC- Arginina descarboxilase
APX- Ascorbato peroxidase
ATP- Adenosina trifosfato
CAT- Catalase
DAI- Dias após embebição
DAO- diamina oxidase
FLU- Fluridone
GA – Giberelina
GL- Glioxissomos
GR- Glutationa redutase
GSI- Índice velocidade germinação
HCL- ácido clorídrico
HPLC- do inglês “high-performance liquid chromatography”
HRA- eixo hipocótilo-radicular
HSP- Proteínas de choque térmico
IAA- ácido Indol-3-acetico
LB- Corpos lipídicos
LM- Microscopia de luz
LV- Vacúolos líticos
M- Mitocôndria
NBT- Azul de nitrotetrazólio
ODC- Ornitina decarboxylase
PAC- Paclobutrazol
PAO- Poliamina oxidase
PAs- poliaminas
PCA- análise dos componentes principais
PGRs- Reguladores de crescimento vegetal
POD- Peroxidase
PSV- vacúolos de armazenamento de proteínas
PUT- Putrescina
R- Ribossomos
RER- reticulo endoplásmatico rugoso
ROS- Espécies reativas de oxigênio
SOD- Superóxido dismutase
SPD- Espermidina
SPM- Espermina
TCA- ciclo do ácido tricarboxílico
TEM- microscopia eletrônica de transmissão

SUMÁRIO

1 INTRODUÇÃO, ANTECEDENTES E JUSTIFICATIVA

O Brasil é considerado o país da megadiversidade, com 15 a 20% das espécies do planeta, apresentando a maior riqueza de espécies da flora, além dos maiores remanescentes de ecossistemas tropicais (Myers *et al.*, 2000; Lewinsohn e Prado, 2005; Forzza *et al.*, 2012). Dentre estes ecossistemas, a Mata Atlântica no sul do Brasil apresenta formação campestre denominada Campos de Altitude, em que destacamos uma diversidade significativa de plantas (Vasconcelos, 2011). Estudar esta biodiversidade dos campos do sul do país tem sido apontada como uma necessidade crescente, pois além de sua flora ser pouco conhecida, são considerados ecossistemas únicos e ímpares, uma vez que possuem peculiaridades, fragilidades e potencialidades ecológicas, as quais são importantes para manter o equilíbrio ambiental (Vasconcelos, 2011).

Pertencente a essa biodiversidade presente nos campos do sul do Brasil, está a família Asteraceae. Asteraceae destaca-se mundialmente pela diversidade e importância econômica, ornamental e medicinal, sendo comumente encontrada em áreas abertas e menos frequente em áreas florestais. A diversidade da família está estimada entre 24.000–30.000 espécies distribuídas em 1.600–1.700 gêneros, 12 subfamílias e 43 tribos (Funk *et al.*, 2009). A família é constituída de ervas perenes, subarbustos e arbustos, mas também ocorrem ervas anuais, lianas e árvores. Possuem alta adaptação ambiental, e são encontradas nos mais variados habitats e condições climáticas (Cancelli *et al.*, 2007), um dos fatores importantes para seu sucesso biológico deve-se a sua grande capacidade de dispersão devido à presença de sementes com pápus plumosos, apêndices, estruturas de aderência e metabólitos secundários (Venable e Levin, 1983). No Brasil, a família é representada por 300 gêneros e 2.000 espécies (Souza e Lorenzi, 2005) 17 tribos, entre elas a Mustisieae, que possui 25 gêneros (Cancelli *et al.*, 2007), sendo um destes o gênero *Trichocline*. *Trichocline,* do grego trix = cabelo e cline = cama (devido à superfície fimbriada do eixo da inflorescência), foi descrito em 1817 por Henri Cassini. O gênero compreende 22 espécies com distribuição desde o sul do Peru até a região central da Argentina e Chile (Katinas *et al.*, 2008), com exceção de uma espécie disjunta ocorrente no oeste da Austrália (*Trichocline spathulata* (D. Don) Hemsl). Na América do Sul o gênero ocorre em duas regiões bem definidas, a andina abrangendo o oeste da Bolívia, norte do Chile e oeste da Argentina e o leste da América do Sul, abrangendo as regiões Sudeste e Sul do Brasil e áreas limítrofes com o Uruguai, Argentina e Paraguai (Zardini, 1975). No Brasil ocorrem oito

espécies do gênero e dessas, anteriormente, cinco foram registradas para o estado do Rio Grande do Sul (Zardini, 1975; Mondin, 1996). As espécies do gênero são herbáceas e perenes, o rizoma é grosso e as folhas geralmente arrosetadas, com o receptáculo plano possuindo flores do disco hermafroditas (dimorfas) (Cabrera e Klein, 1973).

Entre as espécies do gênero, destaca-se a *T. catharinensis* considerada uma espécie endêmica dos campos de altitude do sul do Brasil em altitudes entre 750 e 1500 m (Santa Catarina e Rio Grande do Sul) com vasta e expressiva dispersão em Santa Catarina, ocorrendo nas zonas dos campos de Lages, São Joaquim, Campos Novos, Curitibanos, Caçador e Lebon Régis (Cabrera e Klein, 1973). Trata-se de uma espécie heliófita e seletiva xerófila, formando agrupamentos densos no meio das pastagens e campos naturais em solos rasos e pedregosos (Fig. 1a) (Cabrera e Klein, 1973). Possui folhas inteiras com 6-16 cm de comp. verdes, oblanceolada ou espatulada. As flores estão reunidas num capítulo solitário, sobre um escapo robusto de 5 a 17cm de altura, abaixo deste capítulo encontramos brácteas involucrais dispostas em três ou quatro séries, formando uma estrutura firme e destacada (Fig.1b), flores dimórficas; flores do raio pistiladas e do disco andróginas, corola amarela a amarelo-alaranjada, (Fig.1c), cipsela cilíndrica a obovada, tricomas arredondados no ápice, translúcidos, esparsamente distribuídos, pápus com cerdas barbeladas (Cabrera e Klein, 1973; Pasini e Ritter, 2012).

Fig. 1: *T. catharinensis*. a. Habitat. b. Detalhe Inflorescência e brácteas involucrais. c. Inflorescência em capitulo.

Popularmente, a espécie *T. catharinensis* é conhecida como cravo-do-campo-catarinense, cravo-do-campo-amarelo (Cabrera e Klein, 1973). A espécie foi encontrada pela primeira vez em Santa Catarina, por isso o epíteto específico *catharinensis* (Cabrera e Klein, 1973). A floração predominante começa no final de novembro, estendendo-se até março ou abril. A espécie se propaga por sementes e divisão de touceiras. As sementes viáveis devem ser retiradas dos capítulos antes da dispersão pelo vento, sendo a quantidade variável por capítulo, com alguns apresentando apenas sementes inviáveis (Cabrera e Klein, 1973; Coradin *et al.*, 2011). *Trichocline* apresenta semelhança com o gênero Gerbera,

que já é cultivada comercialmente. A *Trichocline speciosa* Less., *Trichocline macrocephala* Less., *e Trichocline catharinensis* Cabrera são as espécies mais promissoras em termos de potencial ornamental, destacando-se o tamanho e cor das flores (Coradin *et al.*, 2011) . Devido a estas características *T. catharinensis* foi incluída como espécie ornamental na lista de plantas para o futuro da Região Sul (Coradin *et al.*, 2011).

Apesar do Brasil apresentar grande diversidade de espécies com potencial ornamental, a floricultura brasileira está voltada para a exploração das espécies tradicionalmente cultivadas, que são essencialmente exóticas (Coradin *et al.*, 2011). Neste sentido, há grande oportunidade de diversificação deste ramo, introduzindo espécies nativas. Esta diversificação pode ser encontrada nos ecossistemas brasileiros, onde se encontram espécies com potencial ornamental, como é o caso da *T. catharinensis.* Entretanto, há ausência de conhecimento disponível sobre a propagação e manejo da espécie, de modo a fornecer subsídios visando o cultivo e o aproveitamento adequado de suas potencialidades (Kainer *et al.*, 1999). Da mesma forma ainda existe um escasso conhecimento sobre a germinação de sementes de espécies nativas para os ecossistemas brasileiros, considerando-se o grande potencial biológico existente (Coradin *et al.*, 2011). Somente poucos estudos tem investigado a germinação de sementes de espécies brasileiras de Asteraceae (Ferreira *et al.*, 2001; Gomes e Fernandes, 2002; Cury *et al.*, 2010; Bombo *et al.*, 2015). O estudo da biologia de sementes está entre os tópicos mais relevantes de pesquisa para as plantas de ecossistemas tropicais uma vez que tem um papel central no entendimento de como as plantas são estabelecidas e como ocorre a sucessão e a regeneração natural (Varela and Albornoz 2013). Da mesma forma segundo estes autores este conhecimento é requerido também para a conservação, restauração e cultivo comercial de espécies vegetais. Do ponto de vista biológico a semente pode ser definida como a estrutura na qual o embrião é disperso e está hábil a sobreviver entre o período de maturação da semente e estabelecimento da plântula garantindo o início da próxima geração (Debeaujon and Koornneef 2000; Bewley et al. 2013).

O desenvolvimento das sementes é dividido em três fases: A primeira fase é marcada pelo crescimento inicial, com intensa divisão celular e aumento da massa fresca. Nesta fase, as sementes são constituídas principalmente de água e não tem a capacidade de tolerar a dessecação. A histodiferenciação e a morfogênese da semente acontecem à medida que o zigoto unicelular se submete a divisões mitóticas e as células resultantes se diferenciam para dar forma ao plano básico do corpo do embrião. Simultaneamente, há a formação do

endosperma (Goldberg et al. 1994; Bove et al. 2001). Na segunda fase ocorre a expansão celular, com acúmulo de reservas, principalmente proteínas e carboidratos, ocasionando aumento substancial da massa seca e redução do teor de água (Bove et al. 2001). A partir desta fase, as sementes ortodoxas adquirem a capacidade de tolerar a secagem e conservar o potencial de germinação durante longos períodos de armazenamento (Bewley *et al.*, 2013). Por outro lado, nas sementes denominadas recalcitrantes não há a etapa de dessecação ao final da maturação, sendo mantidas com elevado teor de água até a dispersão, iniciando a germinação imediatamente após a fase de maturação (Pammenter e Berjak, 1999; Bewley *et al.*, 2013) (Fig.2)

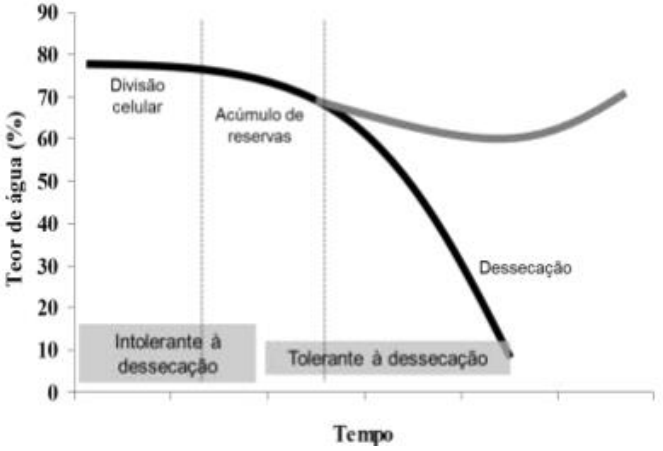

Fig. 2: Esquema geral trifásico dos eventos que ocorrem durante o desenvolvimento de sementes (linha preta – sementes ortodoxas, linha cinza – sementes recalcitrantes (De Castro e Hilhorst, 2004)

A germinação das sementes é um processo fisiológico complexo, no qual as alterações morfológicas e fisiológicas resultam em ativação do crescimento do embrião, através de um rigoroso controle bioquímico e molecular (Catusse et al. 2008). Por definição, a germinação das sementes começa com a absorção de água pela semente seca e quiescente e é completada com o surgimento da radícula a partir das estruturas circundantes (Bewley et al., 2013). Morfologicamente, o início do crescimento da plântula corresponde à

emergência da radícula, sendo a fase subsequente geralmente definida como estabelecimento da plântula (Bewley *et al*. 2013).

O processo germinativo engloba geralmente três fases: a embebição (fase I), fase lag ("*stricto sensu*") e crescimento pós-germinação de plântulas (fase III) (Fig.3) (Bewley *et al*. 2013). Uma descrição do progresso da germinação evoluiu em torno do curso de tempo de absorção de água por uma semente em germinação (Fig.3), seguindo um padrão trifásico. Inicialmente há uma rápida embebição, determinada principalmente pelo potencial matricial (Ψm) extremamente baixo da semente seca (Fase I) estendendo-se até que todas as matrizes e os conteúdos celulares sejam completamente hidratados. Seguido por um período de absorção de água limitada (Fase II), pelo fato das forças matriciais se equilibrarem e não mais serem significantes. O aumento na absorção de água associada com a Fase III é inicialmente, e brevemente relacionado com a conclusão da germinação, embora esta seja uma quantidade proporcionalmente baixa. O ligeiro aumento no conteúdo de água é seguido por uma absorção muito maior à medida que as células da radícula crescem, e subsequentemente o resto da plântula, aumenta devido às divisões mitóticas e a expansão das células (Nonogaki *et al.*, 2010; Bewley *et al.*, 2013).

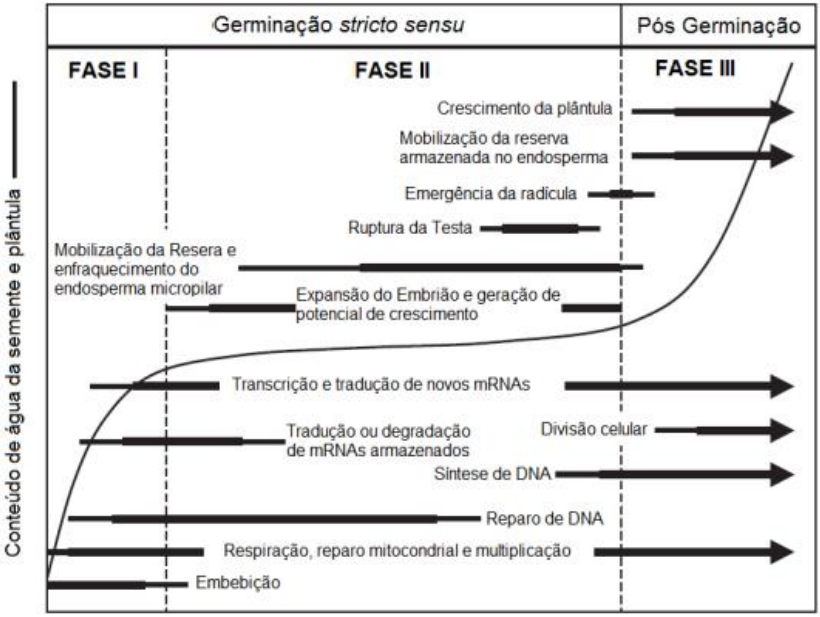

Fig. 3 : Curso de tempo de eventos físicos e metabólicos que ocorrem durante a germinação (fases I e II) e crescimento inicial da plântula (Fase III). O tempo necessário para esses eventos ocorrerem varia entre espécies e é influenciada por condições de germinação. A curva mostra um curso de tempo de absorção de água estilizado (Nonogaki *et al.*, 2010).

A reidratação por meio de embebição (fase I) é o início do processo, é essencial para ativação metabólica. Nesta fase há retomada da atividade respiratória para fornecer energia metabólica para os processos, tradução de mRNAs armazenados, ativação de enzimas, início do desdobramento dos materiais de reserva em substâncias menores, capazes de serem transportadas até o embrião, e início da reorganização das membranas fosfolipídicas (Nonogaki *et al.*, 2010; Bewley *et al.*, 2013). A fase II é relacionada a vários acontecimentos celulares e bioquímicos, sendo considerada uma fase longa. Atividade respiratória se estabiliza ou cresce de maneira muito lenta em relação a fase anterior. Ocorre reparação mitocondrial. É verificado aumento no reticulo endoplasmático, ribossomos, preparo das células para quebra, transporte e

síntese de substâncias utilizadas no processo germinativo. Inicialmente, a síntese de proteínas depende do mRNA que foi formado durante o desenvolvimento e ter permanecido no período seco. Mobilização de reservas para crescimento do embrião. Reforma do citoesqueleto celular e reparação de danos ao DNA (Nonogaki *et al.*, 2010; Bewley *et al.*, 2013). Em algumas sementes, pode ter início divisões mitóticas, antes da protrusão radicular (Masubelele *et al.*, 2005). Ativação das enzimas é iniciada pela ação de giberelinas (GA), que atuam na síntese de enzimas hidrolíticas como α- amilase, ribonucleases e fosfatases resultando na degradação do endosperma e suas paredes celulares (Bewley *et al.*, 2013). Na fase III, há retomada da absorção de água auxiliada pelo crescimento da radícula. A expansão celular da radícula é facilitada pela redução da força exercida pelas paredes celulares e aumento de turgor através de absorção de água (começando assim a fase III) (Nonogaki *et al*. 2010).

A transição da maturação da semente para germinação reflete uma mudança no equilíbrio hormonal. Os dois hormônios centrais na germinação das sementes: ácido abscisíco (ABA) e giberelinas (GA) (Bewley et al. 2013). Na semente matura, onde a germinação é reprimida, os níveis de ABA são elevados e os níveis de GA são baixos. Sob condições normais de germinação (isto é, umidade e luz), a síntese de GA começa a aumentar aproximadamente 16 horas após a embebição das sementes, o que é essencial para a ruptura de ambos testa e endosperma (Debeaujon and Koornneef 2000; Lee et al. 2002). Ao mesmo tempo, os níveis de ABA caem rapidamente e o papel do ABA torna-se facultativo: após embebição, um estresse osmótico repentino ou aplicação direta de ABA (que sinaliza tensões osmóticas) evita eficientemente a ruptura do endosperma, atrasa a ruptura da testa e confere osmotolerância ao embrião (Lopez-Molina et al. 2001; Bethke et al. 2007; Muller et al. 2009). Consistente com o aumento nos níveis de GA, a embebição desencadeia um aumento na expressão de alguns genes da biossíntese de GA como *KO, GA20OX1, GA20OX2, GA20OX3, GA3OX1 E GA3OX2* (Ogawa and Hanada 2003). GA promove a germinação das sementes, aumentando a destruição mediada por proteossomo de RGL2, umas das proteínas do domínio-DELLA (Piskurewicz and Lopez-Molina 2009). As proteínas DELLA são rapidamente degradadas em resposta ao GA e são, portanto, consideradas como repressoras da germinação de sementes na ausência de GA (Tyler *et al.*, 2004).

Para a diminuição rápida dos níveis de ABA durante a germinação das sementes, a análise de expressão revelou que o gene catabólico *CYP707A2* é responsável pela diminuição (Matakiadis *et al.*, 2009). A expressão do gene

CYP707A2, baixa na semente seca, aumenta após a embebição nas primeiras 6 horas, o que corresponde ao momento em que os níveis ABA se deterioram rapidamente (Piskurewicz et al. 2008; Finkelstein 2013). A regulação ABA da germinação de sementes também requer fatores de transcrição responsivos a ABA. O ÁCIDO ABSCÍSICO INSENSITIVE3 (ABI3), ABI4 e ABI5, são altamente expressos em sementes e estão envolvidos na regulação da sensibilidade ao ABA (Finkelstein 2013). ABI4 aumenta a biossíntese de ABA enquanto diminuí a biossíntese de GA (Shu *et al.*, 2013). ABI4 interage diretamente com as regiões promotoras do *NCED6*, um gene da biossíntese do ABA, e do *GA2OX7*, um gene inativador do GA (Shu *et al.*, 2016). Resumidamente as condições que favorecem a germinação estão associadas a maior expressão de genes biossintéticos de GA, como *GA3OX1 E GA3OX2* e menor de genes catabólicos de GA, como *GA2OX2*. Associado a isso, menor expressão de genes da biossíntese do ABA tais como *ABA1, NCED6, NCED9*. Abaixo será apresentado sucintamente as vias de biossíntese da GA e ABA, considerando ação dos seus inibidores (PAC) e (FLU).

Biossíntese (GAs)

O nome Giberelinas (GAs) engloba um grande grupo de ácidos carboxílicos diterpenóides tetracíclicos, que são biosintetizados através de uma complexa via e controla diversos aspectos do crescimento e desenvolvimento (Yamaguchi and Kamiya 2001). Entre as 136 GAs que tem sido identificada até agora apenas algumas são biologicamente ativas (por exemplo GA_1, GA_3, GA_4 e GA_7). Portanto, muitas GAs não bioativas existem em plantas como precursores para formas bioativas ou metabolitos desativados. As concentrações de GAs bioativas em um determinado tecido vegetal são determinadas pelas taxas de síntese e desativação (Yamaguchi 2008). As principais GAs bioativas, incluindo GA_1, GA_3, GA_4 e GA_7, geralmente possuem um grupo hidroxila no C-3β, um grupo carboxila em C-6 e uma lactona entre C-4 e C-10.
Biossíntese de GA pode ser dividida em três estágios:

1) Síntese de ent-kaurene a partir geranil geranildifosfato (GGDP) nos plastídios.
2) Conversão de ent-kaurene a GA_{12} através do citocromo P450 monooxigenases, associadas com o reticulo endoplasmático.
3) Síntese de intermediários de GA e síntese de GAs bioativas no citoplasma (Fig. 4 quadro esquerdo).

O GGDP é um precursor comum às vias de biossíntese de GA, carotenoides e clorofilas, sendo a síntese do ent-kaurene o primeiro passo comprometido na via de síntese do GA. O GGDP é convertido em ent-kaurene por uma reação de ciclização em dois passos: o primeiro é catalizado pela ent-copalil difosfato sintase (CPS) e o segundo pela ent-kaurene sintase (KS). KO (ent-kaurene oxidase) está localizado na membrana externa do plastídio, enquanto ácido ent-kaurenóico oxidase (KAO) está presente no retículo endoplasmático. GA_{12} é convertido para GA_4, GA_1 e GA_3 as formas bioativas, através de uma série de eventos de oxidação no C-20 e C-3 catalisadas por GA-20 oxidase (*GA20OX*) e GA_3 oxidase (*GA3OX*) respectivamente (Yamaguchi and Kamiya 2001; Piskurewicz et al. 2008). A biossíntese de GAs pode ser inibida por paclobutrazol (PAC)((2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazolyl)-pentan-3-ol). PAC bloqueia especificamente os três passos na oxidação do ent-kaureno ao ácido ent- kaurenóico (Hedden and Graebe 1985), entre a membrana do plastídio e retículo endoplasmático na via de biossíntese de GAs. Na Fig.4 é resumidamente mostrado a via de biossíntese e sinalização, também ilustrado aonde ocorre a inibição por PAC.

Biossíntese (ABA)

Ácido abscisíco (ABA) é um sesquiterpeno ($C_{15}H_{20}O_4$) formado por clivagem de carotenoides que contem 40 átomos de carbono. A partir do isopentenil difosfato, as reações de condensação sequencial catalisadas por geranil geranildifosfato sintase (GGPPS: adicionam uma unidade de isopreno ao mesmo tempo para gerar sucessivamente moléculas C10, C15 e C20. A condensação secundária subsequente de dois GGPPs (geranil geranildifosfato) pela fitoeno sintase, produz o esqueleto C40 que se tornará o fitoeno, o primeiro carotenoide comprometido. A fitoeno é submetida a quatro reações consecutivas de desidrogenação que leva à formação de licopeno. Essas reações são catalisadas por fitoeno desaturase. Após em uma visão simplificada, o restante da biossíntese de ABA localiza-se no plastídio e pode ser dividida em três etapas revisado em: Nambara e Marion-Poll (2005) e (Finkelstein, R. (2013))

1) Conversão de β-caroteno para zeaxantina e depois para violaxantina, o último processo sendo catalisado por zeaxantina epoxidase (ZEP). ZEP é codificado por *ABA 1* em *Arabidopsis*.
2) Isomerização de violaxantina produzindo 9-cis-violaxantina e 9-cis-neoxantina.

3) Clivagem oxidativa de 9-*cis*-violaxantina e 9-*cis*-neoxanthina produzindo 15-C xantoxal.

Esta reação de clivagem é considerada a primeira etapa comprometida na produção de ABA. É realizada por enzima 9-*cis*-epoxicarotenóide dioxigenase (*NCED*). Genes *NCED* pertencem a uma família multigênica, e em *Arabidopsis*, cinco deles (*NCED* 2, 3, 5, 6 e 9) estão envolvidos na síntese de ABA. No citoplasma, a xantoxal é convertida em ácido abscisíco bioativo em duas reações enzimáticas. A primeira converte xantoxal em aldeído abscisíco e é catalisado por ABA2. Na segunda reação, o aldeído abscisíco é oxidado para o ácido abscisíco por aldeído oxidase AAO3 (Finkelstein, Ruth, 2013). A biossíntese de ABA pode ser inibida por Fluridone (FLU) (1-methyl-3-phenyl-5-[3-trifluoromethyl) phenyl]-4-(IH)-pyridinone), é um inibidor de biossíntese ABA que inibe a atividade da fitoeno desaturase I, que catalisa a conversão de fitoeno em fitoflueno (Fong et al., 1983). Assim, reduz a síntese de carotenoides que são os precursores da biossíntese de ABA nas plantas (Lopez-Molina *et al.*, 2002; Finkelstein, Ruth, 2013). Na Fig.4 é resumidamente mostrado a via de biossíntese e sinalização, também ilustrado aonde ocorre a inibição por FLU.

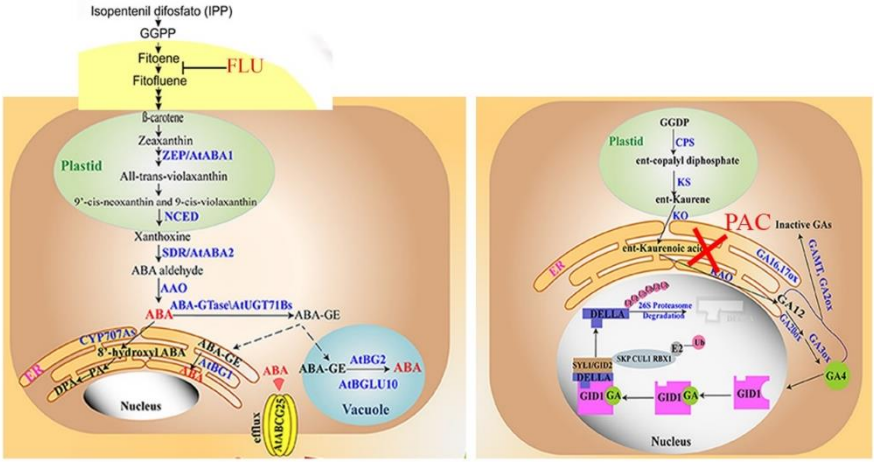

Fig. 4 : O metabolismo ABA e GA e sinalização ABA é sintetizado a partir de carotenoides em uma série de reações nos plastídios e no citoplasma (quadro esquerdo). A biossíntese de GA inicia-se a partir de GGDP no plastídio e uma parte dela é catabolizada para formas inativas (quadro direito). Na via de sinalização, GA causa a

destruição de DELLAs (regulador negativo de GA) através do proteassoma 26S (Vishal e Kumar, 2018). A biossíntese do ABA é inibida por FLU, na conversão de fitoeno em fitoflueno. (quadro esquerdo). A biossíntese do GA é inibida por PAC, que bloqueia especificamente os três passos na oxidação ent-kaureno (KO) ao ácido ent- kaurenóico (KAO) (quadro direito). Adaptado de Vishal e Kumar (2018).

Mediante a isso, elaboramos um desenho esquemático para ilustrar as possíveis alterações dos hormônios e seus inibidores na germinação das sementes de *T.catharinensis*, considerando as três fases da germinação (Fig.5).

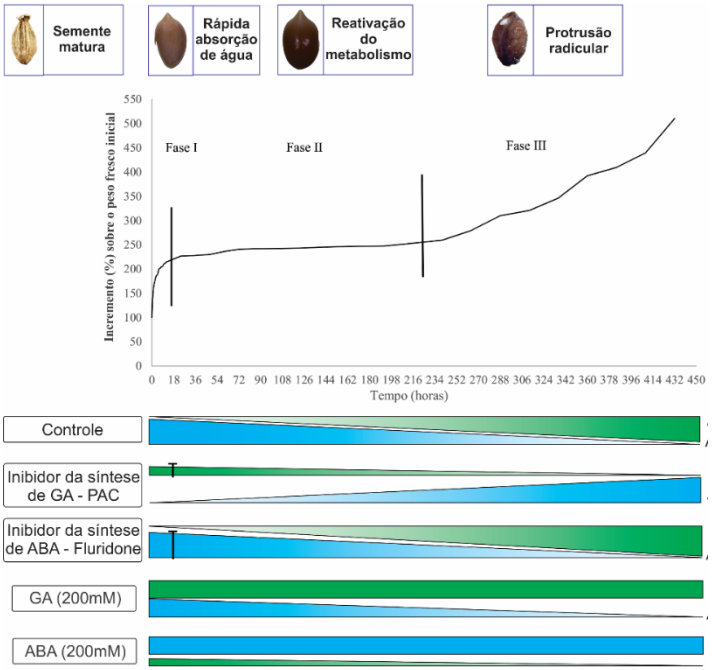

Fig. 5: Desenho esquemático das possíveis alterações hormonais em cada fase da germinação das sementes de *T. catharinensis*, submetidas aos tratamentos com H_2O (controle), soluções de GA e ABA (200µM) e inibidores da biossíntese PAC 200µM e FLU 200µM. Azul – ABA; Verde- GA.

O uso de inibidores da síntese de GA e ABA fornece informações sobre como o conteúdo relativo desses hormônios, podem induzir mudanças em outras biomoléculas, como proteínas, poliaminas e enzimas antioxidantes durante a germinação de sementes (Piskurewicz *et al.*, 2008). Tem sido proposto que as poliaminas podem atuar como mensageiros secundários na mediação dos efeitos hormonais intracelulares (Palavan e Galston, 1982). O mecanismo de ação das poliaminas durante a germinação de sementes não é bem compreendido. No entanto, pode estar intimamente relacionado com o metabolismo dos hormônios na promoção de eventos de divisão e alongamento celular, bem como na mobilização de reservas (Krasuska *et al.*, 2014). Juntamente com as poliaminas, ROS (espécies reativas de oxigênio) são vistas como essenciais dentro de uma janela oxidativa (Bailly *et al.*, 2008). Os sistemas antioxidantes celulares (superóxido dismutase (SOD), ascorbato peroxidase (APX), glutationa redutase (GR) e catalase (CAT)) mantêm a homeostase redox intracelular durante a germinação, evitando o acúmulo de quantidades tóxicas de ROS enquanto permitem a sinalização mediada por ROS (Foyer e Noctor, 2005; El-Maarouf-Bouteau e Bailly, 2008).

Estudos proteômicos consideráveis sobre a germinação de sementes em diversas espécies têm sido realizados, como em *Arabidopsis thaliana* (Gallardo *et al.*, 2001), *Oryza sativa* (Kim *et al.*, 2008; Liu *et al.*, 2015; Liu *et al.*, 2016), *Zea mays* (Huang e Song, 2013), *Pisum sativum* (Wang *et al.*, 2012), *Lactuca sativa* (Wang *et al.*, 2015), *Triticum aestivum* (He *et al.*, 2015; Liu *et al.*, 2018). No entanto, relativamente poucos estudos de abundância proteica diferencial na germinação de sementes em resposta aos hormônios GA e ABA e suas respectivas inibições por PAC e FLU foram realizados.

A germinação das sementes é acompanhada por mecanismos epigenéticos, envolvendo metilação global do DNA e modificações da cromatina que ocorrem na regulação da expressão gênica (Arıkan *et al.*, 2018). A metilação refere-se à adição de um grupo metil na quinta posição (C5) da citosina (Sahu *et al.*, 2013). Durante a germinação, as mudanças na organização da cromatina são acompanhadas por mudanças na distribuição espacial das seqüências de DNA metilado. Nas sementes secas, 5-mC são concentrados em cromocentros. Após a embebição e no processo de germinação das sementes, a

cromatina descondensa, e as seqüências de 5-mC tornam-se dispersas sobre o núcleo (Mathieu *et al.*, 2003; Van Zanten *et al.*, 2011).

Diante do exposto, e considerando poucos estudos que relacionam mudanças ultraestruturais com processos bioquímicos e moleculares esse trabalho teve como objetivo caracterizar a fisiologia da germinação de sementes de *T. catharinensis*, sob a influência dos hormônios GA_3 e ABA, visando conhecer o processo de germinação e assim contribuir com informações que além da pesquisa cientifica, proporcionem o uso e conservação da espécie, como planta ornamental, bem como a valoração do ecossistema em que ela ocorre.

1.1 OBJETIVOS

1.1.2 Objetivo Geral

O objetivo geral do trabalho é caracterizar a fisiologia da germinação de sementes de *Trichocline catharinensis* Cabr.

1.1.3 Objetivos específicos e questões norteadoras da pesquisa

Questão norteadora
- O processo de germinação nas sementes de *T. catharinensis* apresenta pelo menos três fases fisiologicamente distintas?

1) Estabelecer as condições e as etapas da germinação de sementes de *T. catharinensis.*

Questão norteadora
- Quais os efeitos da aplicação de GA_3 e ABA e seus respectivos inibidores PAC e FLU na regulação da germinação das sementes de *T. catharinensis?*

2) Estudar o papel do ácido giberélico (GA_3) e ácido abscisíco (ABA) e seus inibidores paclobutrazol (PAC) e fluridone (FLU) no controle da germinação de sementes de *T. catharinensis.*

Questões norteadora
- Como ABA e GA_3 e seus inibidores modificam a anatomia e a ultraestrutura celular na fase II da germinação das sementes de *T. catharinensis*?

3) Identificar as alterações celulares, por meio de microscopia óptica e eletrônica de transmissão durante as fases da germinação de sementes de *T. catharinensis.*

Questões norteadora
- Como GA_3 e ABA e seus inibidores, relacionam-se com o conteúdo endógeno de poliaminas livres e conjugadas, durante as três fases da germinação de sementes de *T. catharinensis?*

4) Identificar e caracterizar o conteúdo endógeno de Poliaminas livres e conjugadas, em resposta ao GA_3 e ABA e seus inibidores PAC e FLU durante as três fases da germinação de sementes de *T. catharinensis.*

Questão norteadora
- Antes da protrusão radicular, é observado divisão celular no eixo hipocótilo radicular? A aplicação de GA_3, ABA, PAC e FLU influenciam na atividade do ciclo celular?

5) Analisar atividade do ciclo celular no eixo hipocótilo radicular através da citometria de fluxo.

Questão norteadora
- Quais as proteínas envolvidas e as alterações na abundância em resposta ao GA_3 e ABA e seus inibidores na fase II do processo de germinação?

6) Analisar a abundância proteica diferencial em resposta ao GA_3 e ABA e seus inibidores PAC e FLU na fase II do processo de germinação sementes de *T. catharinensis.*

Questão norteadora
- Qual a relação entre a atividade das enzimas antioxidantes com aplicação de GA_3 e ABA e seus inibidores na germinação?

7) Determinar a atividade das enzimas antioxidantes (superóxido dismutase, catalase, ascorbato e glutationa redutase), em resposta ao GA_3 e ABA e seus inibidores PAC e FLU durante as três fases da germinação de sementes de *T. catharinensis.*

Questão norteadora
- Quais os efeitos da aplicação de PUT, SPD e SPM na regulação da germinação das sementes de *T. catharinensis?*

8) Verificar a influência de PUT, SPD e SPM no controle da germinação de sementes de *T. catharinensis.*

Questão norteadora

- Qual a relação entre a atividade das enzimas antioxidantes com aplicação de PUT, SPD e SPM na germinação?

9) Determinar a atividade das enzimas antioxidantes (superóxido dismutase, catalase, ascorbato e glutationa redutase) em resposta PUT, SPD e SPM durante as três fases da germinação de sementes de *T. catharinensis.*

Questão norteadora

- Como GA_3, ABA, PAC e FLU relacionam-se com a metilação global do DNA, durante as três fases da germinação de sementes de *T. catharinensis?*

10) Investigar a dinâmica da variação global da metilação do DNA, considerando três fases da germinação, em resposta às soluções exógenas de GA_3, ABA e seus inibidores PAC e FLU.

1.2 ESTRUTURA DA TESE

A tese está organizada em três capítulos, em formato de manuscrito. Anterior a estes, uma abordagem teórica sucinta para conhecimento do que o trabalho está abordando, juntamente com justificativa a fim de contextualizar o trabalho, bem como seus objetivos e questões da pesquisa.

O capítulo 1 intitulado - The physiological relationship between abscisic acid and gibberellin during seed germination of *Trichocline catharinensis* (Asteraceae) is mediated by polyamines and antioxidant enzymes, foi aceito para publicação no periódico "Journal of Plant Growth Regulation"

O capitulo 2 intitulado- The seed germination of *Trichocline catharinensis*, a Brazilian endemic species, includes cell division, ultrastructural changes and variations in the global DNA methylation

O capítulo 3 intitulado- Cellular alteration and differential protein abundance associated to the decision of radicle protrusion in *Trichocline catharinensis* (Asteraceae) seeds, a Brazilian wild species. Este capitulo está sendo formatado para submissão antes da defesa da tese.

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Capítulo 1 CAPÍTULO 1

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The physiological relationship between abscisic acid and gibberellin during seed germination of *Trichocline catharinensis* (Asteraceae) is mediated by polyamines and antioxidant enzymes

Abstract

The improvement of physiological knowledge about quality and seed germination control is an effective way to contribute to the use and conservation of neglected native species with ecological and economic value, such as *Trichocline catharinensis*, an endemic Asteraceae species from southern Brazil. We investigated the effects of solutions of gibberellin (GA_3), abscisic acid (ABA) and their inhibitors, paclobutrazol (PAC) and fluridone (FLU) on *T. catharinensis* seed germination as well their relationship with polyamines (PAs) and antioxidant enzymes. FLU and GA_3 increased seed germination speed compared to treatment with H_2O-only. ABA inhibited both speed and germination rate, while PAC severely inhibited seed germination. The stimulatory effect of GA_3 and FLU favored a balance of putrescine (PUT) and spermidine (SPD) over spermine (SPM). The balance of PAs (high ratio (PUT + SPD / SPM)) together with SOD, CAT and APX activities enzymes mediate seed germination of *T. catharinensis*, once these biomolecules showed an opposite accumulation pattern in germination delay (PAC and ABA) and (FLU, GA_3 and H_2O) stimulation. Applications of all PAs at 200µM had stimulatory effect on seed germination speed and in the increase in the oxidative activity. Our results suggest a direct relationship between PAs and antioxidant system with physiological mechanism of seed germination. These results improve the physiological knowledge of seed germination control in Asteraceae and give raise to a biological groundwork for future studies looking for use and conservation of biodiversity.

Keywords: Antioxidant enzymes; biodiversity; fluridone; paclobutrazol; polyamines; seed germination.

1. Introduction

Asteraceae is considered one of the largest flowering plant families, representing 8% to 12% of the angiosperm species in the world (Funk *et al.*, 2009).*Trichocline catharinensis* is a native and endemic species from the high-altitude fields (750-1500 m) of southern Brazil (Cabrera e Klein, 1973; Pasini e Ritter, 2012). Its rusticity and ornamental features have attracted attention and, recently, it was included on the "List of Future Plants for Southern Brazil"(Coradin *et al.*, 2011). However, anthropic intervention in the natural occurrence ecosystem has brought this species to vulnerable status (Bull e Maron, 2016). In addition, only a few studies have investigated seed germination of Brazilian Asteraceae species (Ferreira *et al.*, 2001; Gomes e Fernandes, 2002; Cury *et al.*, 2010; Bombo *et al.*, 2015), highlighting the importance of more information on the context of biodiversity conservation, developmental biology and agronomic features of these species.

Seed germination is the first developmental phase in the life cycle of a higher plant. It starts with water uptake by the (non-dormant) dry seed and ends with elongation of the embryonic axis (Bewley *et al.*, 2013). It is a process that involves the integration of environmental signals, endogenous hormones and a complex network of communication between cells and seed compartments (Holdsworth *et al.*, 2008; Leubner-Metzger *et al.*, 2010). The onset of seed germination is closely regulated by the endogenous balance of plant hormones, such as gibberellin (GA), abscisic acid (ABA) and ethylene, as well as other signaling molecules, that include reactive oxygen species (ROS) and polyamines (PAs) (Kucera *et al.*, 2005; Holdsworth *et al.*, 2008). GA and ABA contents tend to be negatively correlated and they alter the balance of germination repressors, which must be inactivated for germination to occur (Piskurewicz *et al.*, 2008). GA stimulate seed germination by degrading these repressors, while ABA induce them (Piskurewicz e Lopez-Molina, 2009). The manipulation of these repressors can be achieved by the application of inhibitors in the synthesis of GA and ABA (Piskurewicz *et al.*, 2008), such as paclobutrazol (PAC) and fluridone (FLU), respectively (Kusumoto *et al.*, 2006). Paclobutrazol (α-tert-Butyl-β-(4-chlorobenzyl)-1H-1,2,4-triazole-1-ethanol) inhibits the activity of *ent*-kaurene oxidase, which is an enzyme in the GA biosynthesis pathway that catalyses the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Hedden e Graebe, 1985). Fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl) phenyl]-4-(IH)-

pyridinone) inhibits the activity of phytoene desaturase I, which catalyses the conversion of phytoene to phytofluene, a key step in ABA biosynthetic route (Huarte e Benech-Arnold, 2010; Chen *et al.*, 2016). Moreover, the use of GA and ABA synthesis inhibitors provides information on how the relative content of these two hormones can elicit changes in other biomolecules, as, for example, polyamines (Piskurewicz *et al.*, 2008). It has been proposed that PAs may act as second messengers in mediating intracellular hormonal effects (Palavan e Galston, 1982) closely associated with seed germination.

PAs are low molecular weight polycationic aliphatic nitrogen-containing compounds of positive charge, allowing their binding to several cellular macromolecules (Kusano e Suzuki, 2015). In plants, putrescine (PUT) is a major diamine and a direct substrate for triamine-spermidine (SPD) and tetraamine-spermine (SPM) formation. PUT is synthesized from arginine (Arg) in a reaction catalyzed by Arg decarboxylase (ADC) or from ornithine by Orn decarboxylase (ODC) (Tassoni *et al.*, 2000; Mattoo e Handa, 2008). PAs are considered as a group of important growth regulators taking part in regulation of seed dormancy and germination, in both undisturbed and stressed conditions (for example, salinity) (Krasuska *et al.*, 2014). During germination *sensu stricto*, the enzymatic activity of the polyamines biosynthetic pathway, as well as their content, are modified mainly in the embryonic axes, although the content of individual PAs and seed sensitivity to them may vary, depending on the plant species (Krasuska e Gniazdowska, 2012).The action mechanism of polyamines during seed germination is not well understood. However, it could be closely related to the metabolism of hormones in the promotion of events of cellular division and elongation as well reserves mobilization (Krasuska *et al.*, 2014). In association with GA, PAs may play a role in cell division once PAs content increase by the application of GA (Shiozaki *et al.*, 1998). Also, a clear correlation was reported between PA concentration in embryonic axes of chick-pea and seed germination inhibition in presence of ABA (Bueno e Matilla, 1992). Moreover, it was suggested that the metabolism of GA and ABA might play a crucial role in the regulation of PAs upon seed germination (Huang *et al.*, 2017) together with ROS which are currently viewed as being essential within an oxidative window (Bailly *et al.*, 2008). Cellular antioxidant systems maintain intracellular redox homeostasis during germination, preventing accumulation of toxic amounts of ROS while allowing ROS-mediated signaling (Foyer e Noctor, 2005; El-Maarouf-Bouteau *et al.*, 2015). Enzymatic ROS scavenging mechanisms include superoxide dismutase (SOD), ascorbate

peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Apel e Hirt, 2004).

To the best of our knowledge, no studies have examined potential links of endogenous PAs and antioxidant enzymes with plant hormones effects in non-dormant seed of Asteraceae species. Considering that Asteraceae is an important plant family, both in terms of species number and economic value, we propose this study. We investigated the influence of GA_3, ABA and their synthetic inhibitors on seed germination over the three phases of seed germination, relating it to the PAs content and antioxidant enzymes (SOD, CAT, APX, CAT) activity. The relationship between the PGRs as well as their inhibitor and endogenous content of PAs and enzymes were made to disclose this crosstalk during the seed germination. Exogenous PAs effects on seed germination and antioxidant enzymes were also demonstrated and helped to clarify this relationship.

2. Material and methods

2.1 Plant material

Seeds of *T. catharinensis* were collected in two natural populations located in Curitibanos, Santa Catarina, Brazil (latitude 27° 18′ S, longitude 50° 38′ W, altitude 990 m; latitude 27° 36′ S, longitude 48° 27′ W, altitude 930 m). Damaged seeds were removed by hand-sorting. Selected seeds were immersed in ethanol 70% (v/v) for 1 min and disinfested in sodium hypochlorite (1% v/v) for 5 minutes. After that, the seeds were submitted to three washes in sterile distilled water.

2.2 Seed imbibition

Eight replicates of 25 seeds were placed in gerbox boxes with germitest paper moistened with 2.5 g of water per gram of paper. Boxes with seeds were placed in a BOD chamber at 20 ± 2ºC and photoperiod of 10h (142µmol m^{-2} s^{-1}). The seeds were weighed before imbibition, at 30-minute intervals for 6h, 1h intervals up to 12h, every 12h up to 6 days, and finally 24h intervals until 50% of the seeds showed radicle protrusion. The percentage of mass increment (I) over time, as a function of initial seed mass (Justo *et al.*, 2007), was calculated as (I %) = [(Mt-Mi) / Mi] x100, where Mi = initial fresh mass of the sample, and Mt = mass sample at time of collection (t). A 3rd degree equation adjusted to the

standard three-phase germination cycle was established to delineate the beginning, end and duration of the germination process. Afterwards, we determined the square root of derived 2nd degree equation and points of inflexion curves expressed as a percentage of mass increment per hours (%.h^{-1}), considering the midpoint of each interval for each value of x.

2.3 Seed germination

Applications of GA_3 (200µM) and ABA (200µM) (Sigma-Aldrich, St. Louis, MO, USA) and their respective biosynthetic inhibitors, FLU (200µM) and PAC (200µM) (Sigma-Aldrich) were evaluated. The concentrations applied were based on previous experiments with *T. catharinensis* and in the reports of Hu *et al.* (2012) and Kim *et al.* (2008). In all treatments, 10 ml of H_2O (as a control) or each of the chemicals cited above were added to the germitest paper containing the seeds. The gerboxes were incubated in the same conditions used in the seed imbibition experiment. PAC and FLU solutions were prepared by dissolving the compounds in acetone (0.1% v/v), followed by dilution with water (Kim *et al.*, 2008; Hu *et al.*, 2012). GA_3 was dissolved in ethanol (0.01% v/v) and ABA in NaOH (0.01% v/v), according to manufacturer's instructions. The concentrations of solvents were tested in previous experiments and shown to have no effect on germination. In all treatments, samples were collected in Phase I (18-22 h of imbibition), phase II (72-75 h of imbibition) and phase III (radicle protrusion, 1 mm length). For treatments with no germination, samples from phase III were collected at 30 days (720 h), along with the control treatment (H_2O). Radicle protrusion was considered the moment of seed germination. The experiment was monitored for 30 days. Samples were collected based on previous experiments performed at least three times. Four replicates with 20 seeds each were evaluated in all treatments. For endogenous polyamine quantification and analysis of antioxidant activity, samples were stored at –20°C until analysis.

2.4 Polyamine application

Applications of putrescine (PUT), spermidine (SPD) and spermine (SPM) (Acros Organics- Fisher Scientific) at the concentrations of 0, 200, 500, 1000 and 5000 µM were evaluated. Previous studies indicated that PAs at 0.20 to 1,4 mM seems to have an effect on plant metabolism (Li *et al.*, 2016). For each treatment, seeds were placed in gerboxes with germitest paper and 10 ml of each solution. The incubation was in a growth chamber as described for seed imbibition. Germination and germination speed index (GSI) were evaluated

from four replicates with 20 seeds each in all treatments. After germination, only the embryos submitted to a concentration of 200 µM were collected in Phase I (18-22 h of imbibition), phase II (72-75 h of imbibition) and phase III (radicle protrusion, 1 mm length). For analysis of antioxidant activity, samples were stored at –20°C until analysis.

2.5 Germination speed index (GSI)

Germination speed index was calculated as the total number of seeds germinated per day between sowing and germination divided by the number of days of the test (Maguire, 1962).

2.6 PAs determination

For PAs determination, three samples (200mg FM) of embryos from each treatment were ground in 1.6 ml of 5% (v/v) perchloric acid. Free and conjugated PAs were extracted, dansylated and quantified, according to Steiner *et al.* (2007) with modifications. Free PAs were directly determined from the supernatant. For conjugated PAs, HCl was added in the supernatant, heated 18h at 110ºC, and then dried and resuspended in perchloric acid (5%). Free and conjugated PAs were derivatized with dansyl chloride and quantified by HPLC using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient of absolute acetonitrile was programmed to 65% over the first 10 min, from 65 to 100% for 10 to 13 min, and 100% for 13 to 21 min, using 1 mL min^{-1} flow rate at 40ºC. PAs concentration was determined using a fluorescence detector with a wavelength of 340nm (excitation) and 510nm (emission). Peak areas and retention times were measured by comparison with standard PAs: putrescine, spermidine and spermine. The 1,7-diaminoheptane (DAH) was used as internal standard.

2.7 Antioxidant Enzyme Extraction and Assays

For the antioxidant enzyme assays, frozen embryos (300mg) were homogenized on ice with 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetra acetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP) using an Ultra-Turrax Homogenizer, according to Bailly e Kranner (2011) with modifications. The homogenate was centrifuged at 15 000×*g* for 20 min at 4°C and the supernatant was used for the determination of enzyme activities. All steps in extracting the enzymes were carried out at 4°C.

Catalase (CAT; EC 1.11.1.6) activity was estimated by the decrease in absorbance of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 7 min (Peixoto *et al.,* 1999). The 300 µl reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 12.5mM H_2O_2, and 10µl of enzyme extract. CAT activity was expressed as (µmol $min^{-1}mg^{-1}$protein). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was estimated following the decrease in absorbance at 290 nm for 10 min (extinction coefficient 2.8 mM^{-1} cm^{-1}) (Koshiba, 1993). The 300µl reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 50mM ascorbic acid, 4.75mM H_2O_2, 5mM EDTA and 10µl of the enzyme extract. APX activity was expressed (µmol $min^{-1}mg^{-1}$protein). Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by monitoring the 50% inhibition of photochemical reduction of NBT, at 560 nm, according to the method of Giannopolitis e Ries (1977). The 300uL reaction mixture contained 50mM potassium phosphate buffer (pH 7.8), 13mM methionine, 75µM NBT, 2mM riboflavin, 100nM EDTA, and 10µl of enzyme extract. The reaction mixtures were illuminated for 15 min. Glutathione reductase (GR; EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.22 mM^{-1} cm^{-1}) for 1 min in an assay mixture containing 50mM potassium phosphate buffer (pH 7.8), 1mM EDTA, 0.5mM oxidized glutathione, and 10µl of enzyme extract. The reaction was initiated by adding 0.5mM NADPH (Bailly e Kranner, 2011). GR activity was expressed (µmol $min^{-1}mg^{-1}$protein). Protein contents in the enzyme extracts were determined according to the method of Bradford (1976) at 595 nm, with bovine serum albumin (BSA) as standard. Activity of enzymes and protein content were performed by the use of a spectrophotometer Spectra-Max® 190 Microplate Reader.

2.8 Statistical analyses

Data normality was evaluated using the Shapiro-Wilk test. Analysis of variance was performed on all data set using variance analysis with two crossed fixed factors (phases of germination × plant growth regulator – PGRs or polyamines (PAs)) followed by the SNK test ($p < 0.05$) (Sokal e Rohlf, 1995) using the "R" statistical program (Team, 2014). Germination data were represented by the mean germination rate ($n = 4$) and evaluated by the confidence interval ($\alpha = 0.05$) for every evaluation day.

3. Results

Seed germination consists of three physiologically distinct phases

Water uptake of *T. catharinensis* seeds was well adjusted to the triphasic model (Fig. 1A), as defined by inflection points in the imbibition curve (Fig. 1B-D). In phase I, or imbibition *per se*, water entered in the *T. catharinensis* seeds rapidly and occurred within 9 hours (Fig. 1B). At phase II (Fig. 1C), water uptake was lower, increasing around 40% in comparison to a 105% mass increment of quiescent seeds in phase I. Radicle protrusion occurred at the end of this phase and marked complete germination (51% of seeds) when phase III started around 240 hours of imbibition (Fig. 1D). In this phase, further water uptake occurs as the young seedling becomes established.

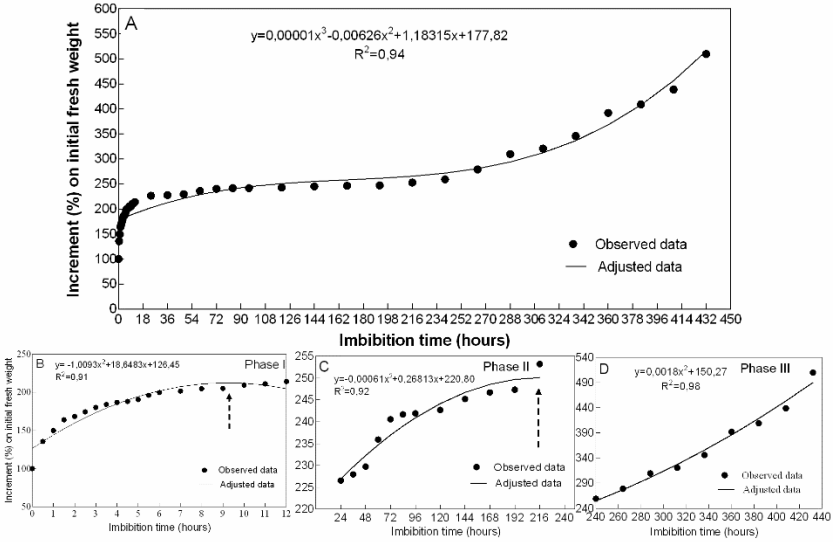

Fig 1: Imbibition curve of *T. catharinensis* seeds: Dynamics of increment on initial fresh mass in hours (%.h^{-1}) (A), phase I – arrow indicates the end of phase I and beginning of phase II (B), phase II- arrow indicates the end of phase II and beginning of phase III - Initial radicle emergence (C), Phase III - further water uptake with established seedling (D). Equations adjustment was significant when $p < 0.05$. (n=8)

Germination of T. catharinensis seed is stimulated by GA and fluridone and inhibited by ABA and paclobutrazol.

The application of PGRs and their respective inhibitors had a significant effect on *T. catharinensis* seed germination. Seeds imbibed in water (H_2O) started germination at 4 days after imbibition, extending up to 30 DAI, with 96.25% of germination (Fig. 2). Seeds imbibed in FLU showed an increased germination speed, obtaining 50% of germinated seeds at 8 DAI and ending with 96.25% of germination at 25 DAI (Fig. 2). Seeds imbibed in GA_3 exhibited an initial behavior similar to that with H_2O treatment; however, at 10 DAI, a significant increase in germination percentage was observed (31.25%), compared to H_20 treatment (17.5%) (Fig. 2). Despite this, at 25 DAI, 98.75% of germination was observed, and similar values were detected in FLU (at 20 DAI) and H_2O (at 21 DAI) treatments. Also, in *T. catharinensis*, the final germination speed index (GSI) of GA_3-imbibed seed (14.8) showed no difference in relation to H_2O-imbibed seed (14.4), but both were significantly lower than that of FLU-imbibed seeds (22.8) (Table 1). The data also showed that ABA delayed germination time, starting only at 15 DAI and reduced the total percentage of seed germination (58.75%) (Fig. 2). Interestingly, when seeds were imbibed in the PAC solution, which is a chemical inhibitor of GA_3 biosynthesis, both a delay and a decrease in seed germination (7.5%) were observed (Fig. 2). Moreover, the germination speed index in *T. catharinensis* indicates an inhibitory effect of ABA (5.23) and PAC treatments (1.16) (Table 1).

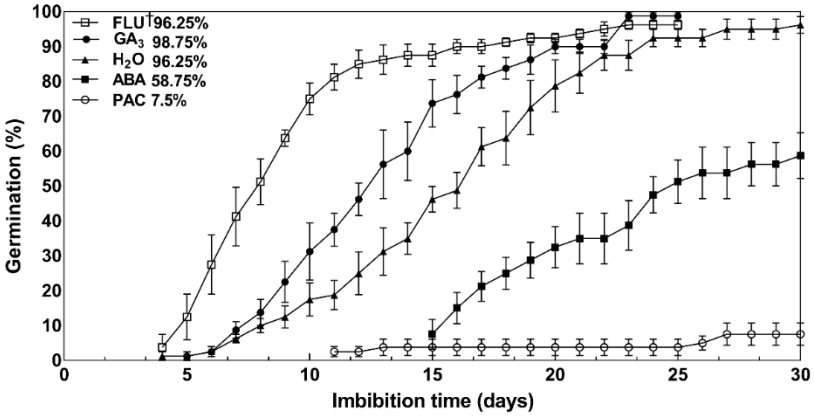

Fig 2: Dynamics of *T. catharinensis* seed germination (%) in water (H_2O), solutions of gibberellin (GA_3), abscisic acid (ABA), paclobutrazol (PAC) and fluridone (FLU) (200µM each). Data: means ± confidence interval (α= 0.05) (n = 4). † Absolute values in the graphic legend indicate the percentage of seed germination in the end of the experiment

Table 1. Germination speed index (GSI) of *T. catharinensis* seeds in water (H_2O), solutions of gibberellin (GA_3), abscisic acid (ABA), paclobutrazol (PAC) and fluridone (FLU) (200µM each).

Treatment	FLU	GA_3	H_2O	ABA	PAC
Germination speed index (GSI)	22.88 ± 1.54 a	14.80 ± 1.99 b	14.43 ± 2.14 b	5.23 ± 1.63 c	1.16 ± 0.89 d

Data mean ±SD. (n= 4). Different letters indicate significant differences at $p<0.05$, according to SNK test

Exogenous PGRs and their respective inhibitors affect the endogenous content of polyamines during *T. catharinensis* seed germination

For free PAs concentration (Fig. 3), the most pronounced differences were observed in the treatment with GA_3 and its inhibitor, PAC. Free putrescine (PUT) content in PAC-imbibed seeds, when compared to mature seed, increased

in phase I, but progressively decreased in phase II, reaching its lowest levels in phase III. On the other hand, during phase III, GA_3-imbibed seed showed higher free PUT content (Fig. 3A). H_2O- FLU- and ABA-imbibed seed, in phase I, showed a slight increase in PUT content, but with no differences among them, remaining similar until phase II. Notably, in phase III, seeds with lower germination percentages, from ABA and PAC treatments, had a significant reduction in PUT content when compared to H_2O, GA_3 and FLU treatments, indicating that higher PUT levels during phase III favors germination (Fig. 3A). Except for GA_3 and FLU treatments, the contents of free spermidine SPD decreased throughout germination (Fig. 3B). During phase III, a tendency towards higher contents of SPD was observed in FLU-, GA_3- and H_2O-imbibed seeds, when compared to the treatments that show few or no seed germinated (PAC and ABA – Fig. 3B). Considering this, free SPD content in phase III may also be associated with stimulation of *T. catharinensis* seed germination. Similarly, from mature seed, free spermine SPM content (Fig. 3C) progressively decreased, mainly in treatments FLU, GA_3 and H_2O that showed higher germination rates. Although not statistically significant, the highest contents of free SPM were observed in ABA-and PAC-imbibed seeds during phase III. These results could suggest an inverse relationship between free SPM content and *T. catharinensis* seed germination occurs at phase III.

The PAs ratio [(PUT+SPD)/SPM] increased in mature seed in phases I and II for all treatments, especially those that were associated with higher germination (Fig. 3D). At phase III, these values were significantly lower in ABA- and PAC-imbibed seeds (Fig. 3D), indicating that these values may be related do the delay and/or inhibition of seed germination.

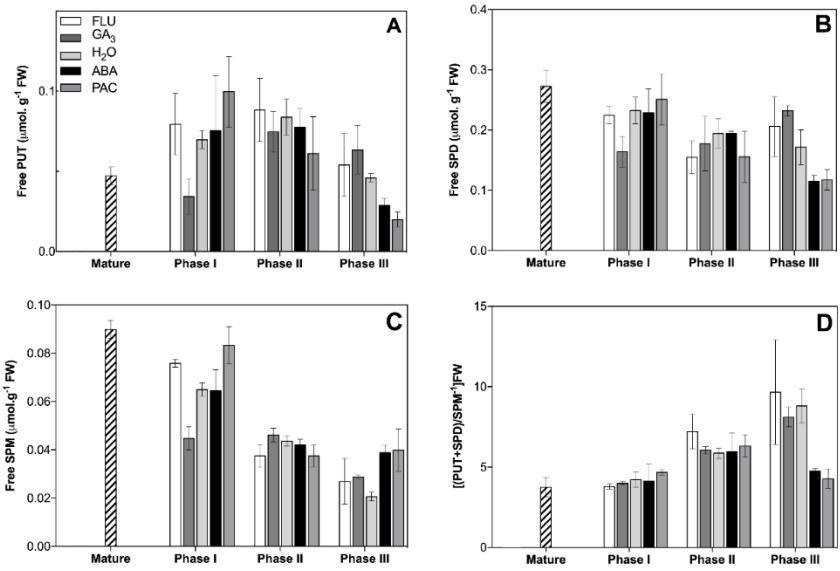

Fig 3: Endogenous contents (µmol g^{-1} FW) of free polyamines: PUT(A), SPD (B), SPM (C) and PAs ratio [(PUT+SPD)/SPM] (D) in mature and during seed germination (phases I, II e III) of *T. catharinensis*. Data mean ±SD (*n*=3). For statistical analysis see Supporting Information – Figure S2. PUT: putrescine; SPD: spermidine; SPM: spermine; FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol

Compared to free PAs, a different pattern of conjugated PAs content was observed during *T. catharinensis* seed germination (Fig. 4A-D). Conjugated PUT was observed in all treatments during seed germination; however, no detectable levels were observed in mature seeds (Fig. 4A). Although not statistically significant, an increase in conjugated PUT was observed in FLU-imbibed seed, showing high values at phase III (Fig. 4A). Conjugated SPD content (Fig. 4B) showed no significant differences among treatments and phases, but the seeds imbibed in GA_3, ABA and PAC showed undetectable levels of conjugated SPD throughout most of the germination process.

Conjugated SPM content (Fig. 4C) tended to decrease in all treatments, compared to mature seed. Total free PAs (Fig. 4D) showed an overall decrease as germination progressed for all treatments. Total PAs (free+conjugated) (Fig. 4E) did not show significant differences but appeared to decrease as germination progressed in most treatments, with the exception of the FLU treatment.

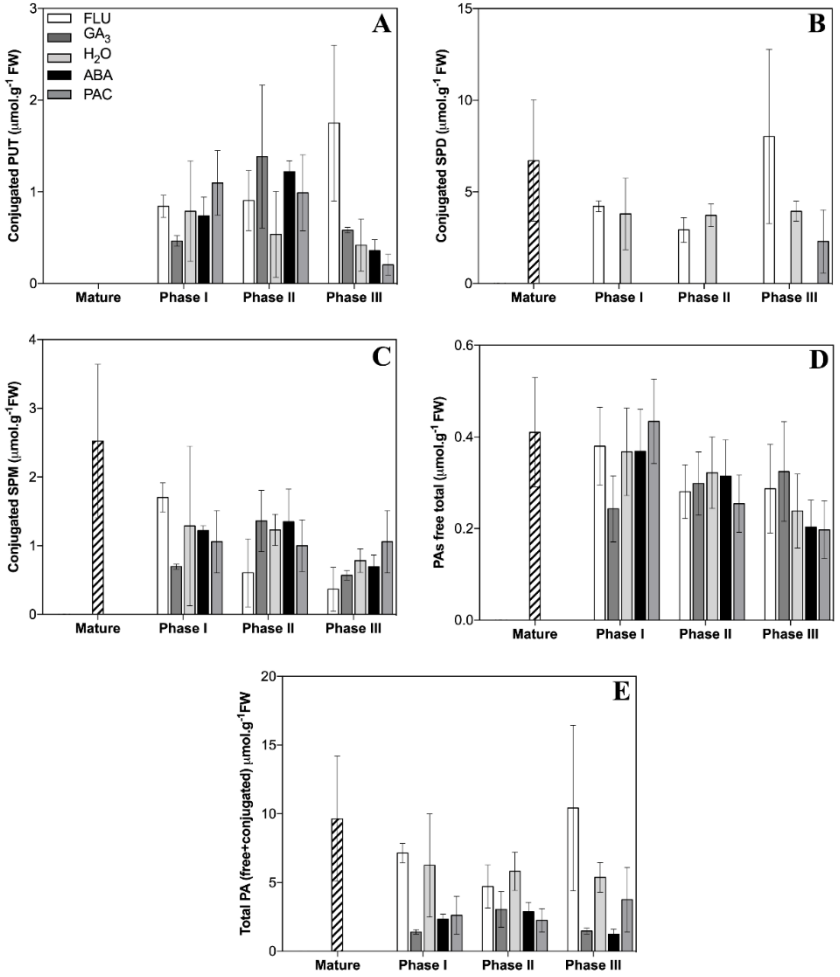

Fig 4: Endogenous contents (µmol g^{-1} FM) of conjugated PUT (A), conjugated SPD (B), conjugated SPM (C) Total free PAs (D) and total (free+conjugated) PAs (E) in mature

and during seeds germination (Phase I, II, III) of *T. catharinensis*. Data mean ±SD (*n*=3). For statistical analysis see Supporting Information – Figure S3. PUT: putrescine; SPD: spermidine; SPM: spermine; FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol

Exogenous polyamines stimulate seed germination

Exogenous polyamines PUT (Fig. 5A), SPD (Fig. 5B) and SPM (Fig. 5C) showed a stimulatory effect in seed germination speed and uniformity in comparison to water (H_2O). When applied in a 200 µM concentration, all PAs showed a stimulatory effect on seed germination speed (Fig. 5D). However, higher concentrations of SPD and SPM (5000 µM) delayed germination time (Fig. 5D) and inhibited significantly the final value of seed germination with 32.5% and 27.5% respectively (Fig. 5B-C). Interestingly, this effect was not observed with high doses of PUT (5000 µM), maintaining 86.25% of seed germination (Fig.5 A).

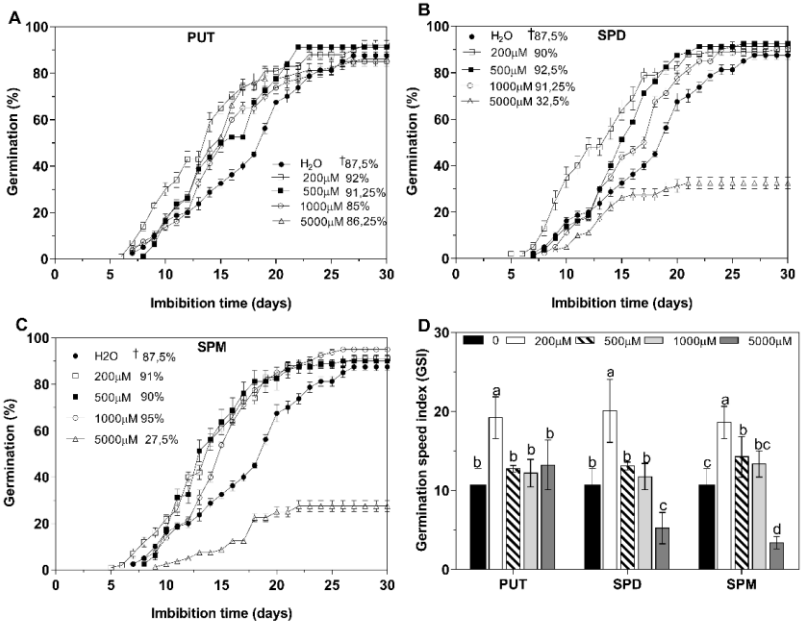

Fig 5: Dynamics of *T. catharinensis* seed germination (%) in water (H_2O), solutions of PUT (A), SPD (B), SPM (C) at the concentrations of 200, 500, 1000 and 5000 µM and germination speed index (GSI) (D). † Absolute values in the graphic legends of (A), (B) and (C) indicate the percentage of seed germination in the end of the experiment. Data: means ± confidence interval (α= 0.05) (n = 4). Letters in (D) compare different concentrations of each PA according to SNK test ($P \leq 0.05$). PUT: putrescine; SPD: spermidine; SPM: spermine

The enzymatic activity was affected by exogenous GA and ABA and this effect was mediated by polyamines.

During *T. catharinensis* seed germination, the antioxidant system was also affected by exogenous PGRs, as shown in (Fig. 6 A-D). SOD activity was high in the mature seed and remained high up to phase II in all treatments, while in phase III there was a significant reduction in the treatments FLU, GA_3, and H_2O, which showed higher germination (Fig. 6A). We observed no significant interaction to CAT activity during seed germination (Fig. 6B). APX activity significantly decreased from mature seed to Phase I, only in ABA treatment and, at phase II only in GA_3 treatment, however at phase III, all the treatments showed a significant APX activity reduction (Fig. 6C). Compared to mature seeds, at phase I no change in GR activity was observed in the treatments that presented highest percentages of germination (FLU, GA_3, H_2O), while a significantly increase appears in the treatments ABA and PAC, which showed low germination (Fig. 6D). At phase II, the ABA-imbibed seeds showed the highest GR activity and at phase III the activity decreased drastically. These results indicated that during the germination of *Trichocline* seeds there was an overall reduction in antioxidant activity between phase II and III, when there was root protrusion.

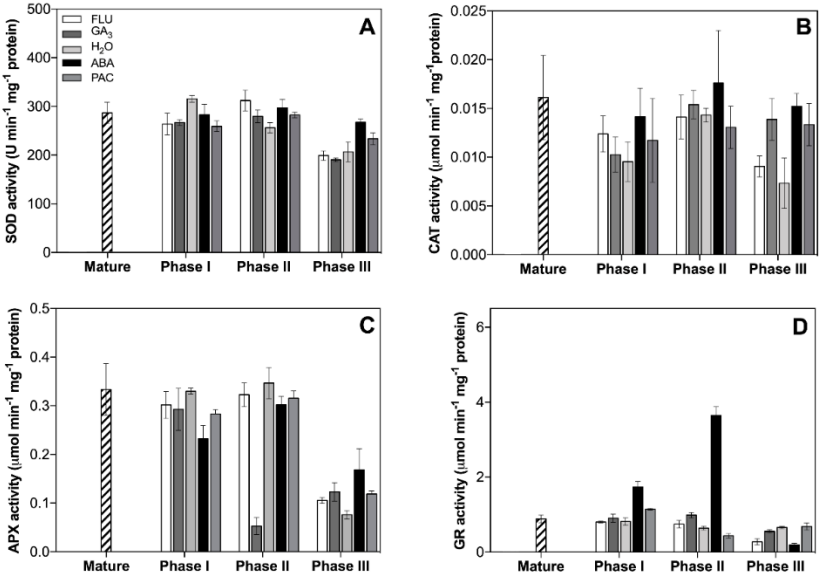

Fig 6: Antioxidant enzymes in *T. catharinensis* mature seed with application of FLU, GA_3, H_2O, ABA and PAC over the phase I, II, III of germination. (A) SOD activity, (B) CAT activity, (C) APX activity and (D) GR activity. Data mean ±SD (n = 4). For statistical analysis see Supporting Information – Figure S4. FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol

The enzymatic activity was affected by exogenous GA and ABA as we explained before and this effect seems to be mediated by polyamines. To clarify this effect, in the germination of *Trichocline* seeds, we evaluated the antioxidant system after application of PUT, SPD and SPM (200µM) (Fig.7A-D). We verified that from the mature seed the SOD activity had a decrease in SPM treatment at phase II, however at phase III, there was a significative reduction for all treatments (Fig. 7A). CAT activity increased significantly from mature seed to phase I in all treatments, with significant higher SPM within this phase (Fig.7B). In phase II a significant decrease activity occurred only for SPM treatment, while in Phase III this was also observed in SPM and H_2O treatment.

At phase III, SPD treatment showed highest activity, however not different from phase I and II (Fig7. B). Compared to mature seeds, APX activity showed a significant reduction at phase III in all treatment (Fig.7C). GR activity in comparison to the mature seed, at phase I, increased in all the PAs treatments, however it decreased again in all at phase III (Fig.7D). In the H_2O treatment, GR enzyme showed a significant increase at phase II (Fig. 7D). Our results indicated that by the application of PAs a general pattern of decreased enzyme activity between phase II and III was maintained (Fig.7).

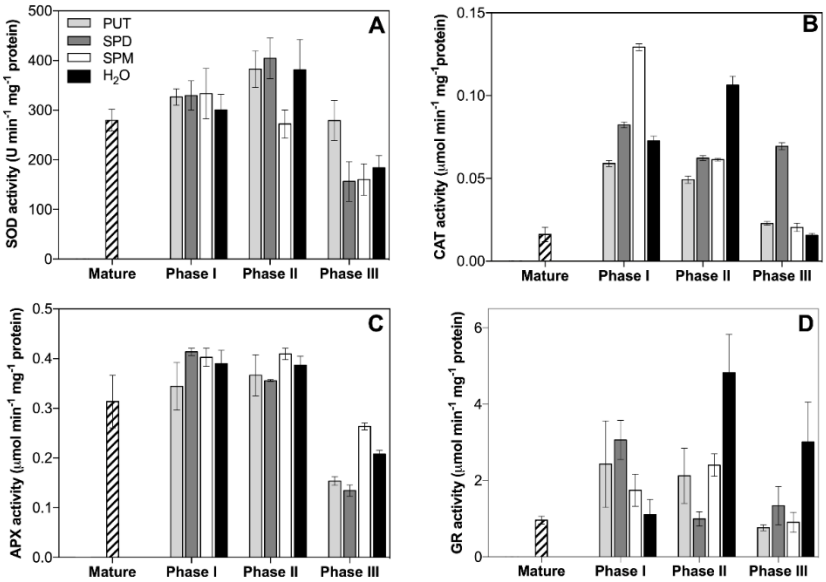

Fig 7: Antioxidant enzymes in *T. catharinensis* mature seed with application of PUT, SPD, SPM and H_2O over the phase I, II, III of germination. (A) SOD activity, (B) CAT activity, (C) APX activity and (D) GR activity. Data mean ±SD (n = 4). For statistical analysis see Supporting Information – Figure S5. PUT: putrescine; SPD: spermidine; SPM: spermine

4. Discussion

T. catharinensis seeds showed three distinct phases during germination similar to the other Asteraceae species, such as *Eremanthus incanus* (Davide *et al.*, 2008) and *Guizotia abyssinica* (Gordin *et al.*, 2012). We observed that seed germination exhibits three stages, which, according to (Bewley *et al.*, 2013), consists of: water imbibition (phase I); activation or germination *stricto sensu* (phase II); and completion of germination associated with radicle protrusion (phase III) (Fig. 1). In addition, we found a high rate of seed germination (96.25%) in H_2O, suggesting high physiological quality and high seed viability when compared to other species from the Asteraceae family, which may range from 2.40 to 93.0% (Ferreira *et al.*, 2001). In this family, plants produce a high number of seeds without embryos, thus leading to low germination rates (Gomes e Fernandes, 2002). However, sorting seeds to eliminate dead and malformed seeds, as well as those without embryos, resulting in a 100% germination rate in the Asteraceae *Lessingianthus bardanoides* (Cury *et al.*, 2010). We found a similar result; improved germination rates in *T. catharinensis* accompanied sorting seeds.

Endogenous GA and ABA levels may be altered by PAC and FLU supplementation, respectively (Kusumoto *et al.*, 2006), so we chose this approach to verify the role of GA and ABA on *T. catharinensis* seed germination. Compared to H_2O-only treatment, our results showed that GA_3 and FLU application stimulated *T. catharinensis* seed germination, while the opposite was observed for ABA and PAC treatments (Fig. 2). GA is thought to increase embryo growth during seed germination by stimulating cell division and elongation (Nieuwland *et al.*, 2016). GA also promotes the expression of genes encoding enzymes that mobilize reserves, including starches, proteins and lipids stored in the endosperm (Peng e Harberd, 2002). Moreover, GA is necessary to overcome the mechanical restraint conferred by the seed-covering layers by weakening the tissues surrounding the radicle through increased activity of cell wall-loosening enzymes (reviewed by Finch-Savage e Leubner-Metzger (2006) and Bewley *et al.* (2013). ABA, however, often works in opposition against GA_3 by restricting seed germination; several studies indicate that ABA probably acts to prevent cell division by blocking or retarding the cell cycle and/or inactivating DNA replication (Miransari e Smith, 2014). In many

species, ABA also prevents endosperm rupture (Kucera *et al.*, 2005; Finch-Savage e Leubner-Metzger, 2006; Leubner-Metzger *et al.*, 2010). A crucial point to understand the control of germination relies on the interactions between these two hormones. Through several intermediates, GA is capable of downregulating ABA biosynthesis, signaling pathways and responses, while ABA also counteracts GA action through the same means (Skubacz e Daszkowska-Golec, 2017; Liu e Hou, 2018). Therefore, we suggest that the application of GA or FLU on *T. catharinensis* seeds would shift the hormonal balance towards GA and, consequently, germination, while ABA and PAC would have an opposite effect. Grappin *et al.* (2000) observed that when FLU or GA were added to *N. plumbaginifolia* seeds, either prevented ABA biosynthesis and accelerated the germination rate. The inhibitory effect of ABA on seed germination was also reported in different species, i.e., *Coffea arabica*, *Prunus campanulata*, *Arabidopsis thaliana*, *Vellozia sp*, *Lolium rigidum*, *Leymus chinensis*, and *Myrica rubra* (Goggin *et al.*, 2009; Hu *et al.*, 2012; Vieira *et al.*, 2017), with a similar effect when compared to PAC application (Rademacher, 2000; Huarte e Benech-Arnold, 2010).

Based on these findings and our results, we propose that the germination of *T. catharinensis* seeds is controlled by a balance between ABA and GA in a manner similar to that of other angiosperm seeds. Once this was confirmed, we investigated how these hormonal manipulations might be related to the endogenous content of PAs. The metabolism of PAs appears to be involved in a broad spectrum of biological activities, i.e., cell division and elongation, membrane stabilization, plant growth, plant adaptation toward abiotic stresses (Igarashi e Kashiwagi, 2000; Saha *et al.*, 2015). In seeds, PAs function as regulators during seed development. In the study of Sánchez-Rangel *et al.* (2016), with *Arabidopsis* transgenic lines, the silencing of ADC genes (amiR:*ADC*-L2 cell line) resulted in decreased endogenous PA concentrations, and this was thought to be responsible for the significant decrease in seed size and the arrest of embryo development at the torpedo stage in the transgenic lines. Moreover, changes in either PAs profile or ratio was correlated with specific stages of embryo development, suggesting that PAs are potential markers of seed development (Silveira *et al.*, 2004; Urano *et al.*, 2005). In our experiments, FLU, GA_3 and H_2O-only applications were not only associated with the highest germination rates (Fig. 2), but also comparatively higher concentrations of PUT and SPD concentrations that showed the highest germination rate (Fig.3A-B). GA in involved in regulating PA (PUT and SPD) content, as well as two enzymes related to PA biosynthesis, ADC (arginine decarboxylase) and ODC

(ornithine decarboxylase) (Galston e Sawhney, 1990). Further, PAs appear to play a positive role during germination, as reported in germination of dormant apple embryos, which were stimulated by PUT and SPD (Krasuska *et al.*, 2017). The stimulation of germination observed by PAs may also be related to its effects in hormones content. For example, SPD supplementation significantly increased endogenous indole-3-acetic acid (IAA) and GA content during wheat (*Triticum aestivum* L.) seed germination, and was also associated with reserve mobilization through increased α-amylase and β-amylase activities during the germination of *Trifolium repens* seeds (Yang *et al.*, 2016). PAs, in association with GA, may also play a role in cell division and cell expansion (Kaur-Sawhney *et al.*, 2003). SPD application in sweet corn (*Zea mays*) seeds significantly increased GA and ethylene contents, but reduced ABA concentration in embryos during seed imbibition (Huang *et al.*, 2017). So, the high contents of free PUT and SPD found in FLU and GA_3 treatments, as well the reduction of these PAs observed in ABA and PAC treatments (Fig. 3A, B) might indicate, at least in part, a relationship between germination-stimulating agents (FLU, GA_3) and germination-inhibiting agents (ABA, PAC), and their effects on endogenous PAs, as observed in the germination of *T. catharinensis* seeds. Based on these results, we observed that the (PUT+SPD)/SPM ratio value indicates a clear and significant relationship between exogenous hormones (ABA, GA_3), its inhibitors (FLU, PAC), and endogenous PAs (PUT, SPD, SPM) at phase III of *T. catharinensis* seed germination. High PAs ratio values were associated with seed germination stimulation while low ratio values were associated with seed germination inhibition in this species (Fig. 3D).

To investigate if PAs stimulate germination in *T. catharinensis*, we investigated whether the application of different concentrations of PUT, SPD and SPM would positively affect the percentage of germination and GSI (Fig. 5). Seeds were able to germinate when treated with all the PAs, but very high doses of SPM or SPD appeared to have a toxic effect on the seeds, as reflected by the reduced germination percentage (Fig. 5A –C). Curiously, high concentrations of PUT, unlike SPD or SPM, did not reduce the germinative capacity of the seeds. A 200 µM concentration of all PAs appeared to significantly speed up germination (Fig. 5D), indicating that the already-high germinative potential of these seeds could be improved even further. Since PAs can interconvert from PUT to SPM and back (Yang *et al.*, 2016), an input in any of these forms could be converted to a PA ratio that favors germination. Furthermore, the PAs catabolic process through diamine oxidases (DAO) and

polyamine oxidases (PAO) produce hydrogen peroxide (H_2O_2) and this could affects germination (Moschou *et al.*, 2008; Jiménez-Bremont *et al.*, 2014).

Our results showed that SPD and SPM gradually decrease during seed germination (Fig. 3B, C). These results are different than those of several species, where PAs, and especially SPM, accumulated during embryogenesis. In sweet corn seeds, SPM in free form had an apparent significant effect on seed quality in the mature (Cao *et al.*, 2010). SPM is involved in membrane stability and osmotic adjustment, both important processes during the desiccation phase of seeds (Li *et al.*, 2015). Furthermore, high SPM content in mature *Arabidopsis thaliana* seeds conveyed enhanced dormancy (Mirza e Rehman, 1998). Even if *T. catharinensis* seeds did not show a dormant state, we observed that high free SPM content in mature seeds may be associated with high viability and vigor (Fig. 2; Table 1).

Our results also demonstrate that total conjugated PAs (Fig.4) contents were affected by application of GA_3 and ABA, especially due to conjugated SPD contents (Fig. 4B). In these two treatments, no conjugated SPD was observed at any phase of seed germination, regardless whether the seed germinated or not (Fig. 4B). Conjugated PAs help regulate the concentration of free PAs (Kusano e Suzuki, 2015), and, thus, their presence may be advantageous for the long-term regulation of free PAs content in germinating seeds. FLU, PAC and H_2O-only treatments showed conjugated SPD during phase III, while GA_3 and ABA treatments did not (Fig. 4B). This leads us to suggest a relationship between the role of the molecule (PGRs or enzyme inhibitors) and conjugated SPD content (Fig. 4B). Therefore, variation in PAs contents (free + conjugated) seems to be intrinsic to seed germination, as corroborated by the maintenance of the content of conjugated PAs, especially PUT and SPM, in the treatments that showed high germination rate in all phases of germination (Fig.4 A, C). This could also be associated with antioxidant ability of PAs to improve antioxidant defense systems and to counteract radical oxidative species (ROS) created by abiotic (Kuznetsov *et al.*, 2006). These results indicate that there might be a crosstalk between free and conjugated PAs and antioxidant enzymes during seed germination, once we clearly observe the link between PAs, PGRs and oxidative system.

PAs may further aid in seed germination through neutralizing ROS generated through abiotic stress or cellular growth (Saha *et al.*, 2015) We observed that SOD, CAT and APX activities (Fig. 6A, B, C), in response to PGRs, were maintained at higher levels from the mature seed to Phase II which indicates, apart from PAs, an antioxidant system that may control ROS during

water loss and subsequent rehydration at the beginning of the germination (Bailly *et al.*, 2004; Niedzwiedz-Siegien *et al.*, 2004). In phase III, however, this system apparently becomes less active. Interestingly, when we applied PAs, we observed similar results (Fig. 7A-D), however, antioxidant enzyme activity increased from the mature seed stage until phase II. From these results, we suggest that the application of PAs increased oxidative activity at the beginning of germination (Fig.7A-D) and this may be related to the GSI and improvement to plantlet uniformity(Fig. 5 A-D). In many species, phase II, which involves the activation of the regulatory system of germination, ROS production increases (see the review by Bailly *et al.* (2008) and Gomes e Garcia (2013)). This was associated with reserve mobilization through oxidative modifications of stored proteins and as signals to mobilize reserves during growing seedling (Barba-Espin *et al.*, 2010). This could also be the case for *T. catharinensis*, since a steady decrease of antioxidant enzymes could allow the buildup of ROS. We must consider that ROS must undergo rigorous control to fulfill its role as cellular messengers; therefore, the seeds must have an efficient antioxidant system to regulate ROS concentration (El-Maarouf-Bouteau and Bailly 2008). This observation corresponds also with to the "oxidative window" hypothesis where ROS may play a role in cell signaling by interplaying with the hormone signaling pathways or by triggering the cellular events such as cell wall loosening and reserve mobilization (Job *et al.*, 2005; Barba-Espin *et al.*, 2010). Below this window, the amount of ROS during imbibition is too low to allow germination and above window is too high and was suggested that this can induce cellular oxidative damage that prevents or delay germination (Bailly *et al.*, 2008). Our work corroborates this hypothesis once PUT and SPD showed higher endogenous contents in FLU, GA_3, H_2O-only treatments, which showed higher germination, which is related to regulation of oxidative stress and seed germination (Tiburcio *et al.*, 1994). Moreover, from mature seed SOD, CAT, APX, GR activities increased significantly when seeds were treated with PUT and SPD (Fig. 7A-D) especially at the first phase of seed germination. These results may be able to be related to the observation that PA application strongly improve seed germination uniformity (Fig. 5A-C) and even high concentrations of PUT could not reduce seed germination (Fig. 5A). PAs inhibit oxidative stress in plants, acting as free radical scavengers or by binding to antioxidant enzyme molecules to eliminate ROS (Alcázar *et al.*, 2010) and significantly enhance the activity of both enzymatic and non-enzymatic antioxidants (Radhakrishnan e Lee, 2013). According to Li *et al.* (2016) SPD was able to stimulate the antioxidant system (SOD, POD, CAT, APX enzymes) that moderates the

oxidative stress in seed germination under water drought. SPD and SPM also have been enhanced seed desiccation tolerance associated by the control of transcript levels of antioxidant enzyme-encoding genes (Tanou *et al.*, 2009). These findings and our results suggest a possible balance between PAs and the antioxidant enzymatic system in the regulation of oxidative stress and the stimulation of seed germination. Thus, we suggest that both endogenous and exogenous PAs can participate in ROS-mediated signaling and, specifically in seeds, which has been reported an important role in cell wall loosening during germination (Gupta *et al.*, 2016; Wojtyla *et al.*, 2016).

On the contrary, ABA and PAC treatments, which showed low percentages of germination, the endogenous PUT and SPD contents decreased in phase III (Fig. 3 A-B), while the endogenous SPM content (Fig. 3C) and antioxidant enzyme—mainly SOD—activity tended to increase (Fig. 6A). SOD plays an essential role in scavenging ROS, and H_2O_2 produced via SOD action is further scavenged by peroxidases (POD) and CAT (Jaleel *et al.*, 2009). We observed maintenance of CAT activity in ABA treatment (Fig. 6B) and greater SOD and APX activity, at phase III, in ABA and PAC treatment comparatively than the other treatments. These results may be associated with ABA's role in promoting the expression of genes encoding SOD, CAT, APX enzymes, leading to an increased synthesis of these enzymes (Hu *et al.*, 2006). Because of this, ROS accumulation may have remained below a certain threshold, not triggering the necessary signals for germination (Jiang e Zhang, 2003; Hu *et al.*, 2006). According to Leymarie *et al.* (2011) there are variations in quantities and moments of ROS production, suggesting that ROS can assume distinct roles during germination. Also, for germination to be stimulated, it is essential the precise regulation of the accumulation of ROS by the cellular antioxidant system leading to achieve a balance between oxidative signaling and damage effects (Foyer e Noctor, 2005; Diaz-Vivancos *et al.*, 2013; Wojtyla *et al.*, 2016). In barley (*Hordeum vulgare*) and pea (*Pisum sativum*) seeds, the molecular mechanisms regulated by the interplay between ABA and GA seems to be influenced by ROS signaling; H_2O_2 stimulation of seed germination was paralleled by a reduction in endogenous ABA (Barba-Espin *et al.*, 2010; Wang *et al.*, 2011). The altered germination resulting from PAC and ABA treatment caused, especially at phase III, the different allocation pattern of endogenous PAs (low (PUT + SPD / SPM) ratio) and increased antioxidant enzymes activity, and this may lead to unbalanced ROS production, which may be above or below the oxidative window delaying or inhibiting germination.

The results from this study highlight the function of PAs in stimulating germination through improving the speed and uniformity (Fig. 5A-D), and this effect was mediated by antioxidant enzymes activity. The balance of PAs (high (PUT + SPD / SPM) ratio) (Fig. 3D) together with SOD, CAT and APX activities (Fig. 6A-C) mediated seed germination of *T. catharinensis*, and these, in turn, were affected by substances that either delayed (PAC and ABA) or stimulated (FLU, GA_3, and H_2O-only) seed germination.

5. Conclusions

Our results confirmed the direct and opposite effects of ABA, GA_3 and their inhibitors (FLU and PAC) on the control of *T. catharinensis* seed germination. FLU and GA_3 stimulate germination while ABA delayed and PAC inhibited seed germination. Moreover, the PGRs and their inhibitors stimulate a direct effect on PAs metabolism in all germination phases and this allow us to disclose the relationship between these molecules and how this affects *T. catharinensis* seed germination. An increase of free endogenous PUT and SPD content was identified at phases III associated with the stimulatory effect of GA_3 and FLU on seed germination while SPM increased content occurs when the effects were inhibitory (ABA and PAC). The low PAs ratios in mature seed in contrast with high value in phase III indicate the direct relationship of PAs with physiological mechanism of seed germination stimulation underpinned by exogenous PAs results. Application of PAs promotes an increase in the oxidative activity and this related to the germination speed index and uniformity improvement. FLU, GA_3, H_2O-only treatments, which showed higher germination have high PUT and SPD content endogenous and this is related to the regulation of oxidative stress through of the activity of the enzymes SOD, CAT and APX. These results improve the physiological knowledge of seed germination control in Asteraceae point out the early first description of relationship between hormones, polyamines and antioxidant enzymes in this process.

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Capítulo 2 CAPITULO 2

The seed germination of *Trichocline catharinensis,* a Brazilian endemic species, includes cell division, ultrastructural changes and variations in the global DNA methylation

Abstract

The completion of germination of seeds of *Trichocline catharinensis* (a Brazilian endemic species) is marked by the appearance of the radicle through the surrounding micropylar endosperm and exotesta. Ultrastructural analysis shows increased vacuolization in the cells of the hypocotyl-radicular axis, due to the transformation of protein storage vacuoles to typical vacuoles occurred prior to the initiation of cell elongation. Using flow cytometry observed cells with replicated 4C DNA content (G2 phase of cycle cellular) before germination is complete. This is related to the reduction of levels of global DNA methylation (GDM) observed after 72h of imbibition, when DNA replication is possibly initiated. When germination is inhibited by PAC, inhibitor of GA_3 synthesis, the levels of GDM decrease dramatically in phase III. We observed in the seed mature compacted chromatin in electron-dense regions (heterochromatin), becomes loosened during germination in regions of euchromatin. As we observed, there are few studies that correlate ultrastructural changes with molecular processes before the protrusion of the radicle. These observations suggest a contributing role of cell division to radicle growth during germination in *Trichocline* and provide information that may be useful in agronomically important practices, such as seed priming, to promote the conservation of germplasm of native species.

Keywords: cell elongation; epigenetics; protein storage vacuoles; cell cycle; chromatin; native species

1 Introduction

Trichocline catharinensis (Cabrera) is a Brazilian endemic Asteraceae (Cabrera e Klein, 1973). It is considered a species of current or potential economic value, which due to its rusticity and ornamental characteristics have encouraged the use of this species in plant breeding programs, ornamental crops and ecological programs for ecosystem recovery (Long *et al.*, 2015; Manning *et al.*, 2016). The knowledge about germinative of seeds behavior is fundamental to the use of native species in ornamental crops and ecological programs for ecosystem recovery (Normah *et al.*, 2013), once that the germination is an important stage in the plant life cycle because it is a prerequisite for seedling establishment (Bewley *et al.*, 2013). In addition, seed biology is a major research topic of importance for food security and climate change (Nonogaki, 2017; Penfield e Macgregor, 2017).

Seed germination is a complex process and we need to understand the underlying molecular, hormonal, and morphological aspects (Steinbrecher e Leubner-Metzger, 2018). At maturity, a typical angiosperm diaspore (seed/fruit) consists of an embryo and its 'coats', which might include living endosperm, exotesta (seed coat derived from the outer integument) and pericarp (fruit coat) (Steinbrecher e Leubner-Metzger, 2018) Quiescent seeds are characterized by an absence of hormone controlled dormancy and their germination can simply be triggered by imbibition (Weitbrecht *et al.*, 2011; Obroucheva *et al.*, 2017) Thus, the seed germination begins with uptake of water and ends with emergence of the embryonic axis through its surrounding structures (Bewley *et al.*, 2013). An essential event for the completion of germination *sensu stricto* is elongation of specific cells to result in radicle protrusion (Novikova *et al.*, 2014; Rewers e Sliwinska, 2014). Whereas cell elongation is a fundamental process during early seed germination, the initiation of cell division varies between species and can occur simultaneously with elongation or later on (Obroucheva *et al.*, 2012). The cell division is generally considered to occur only at the completion of germination (Bewley *et al.*, 2013). However, in germinating seeds of certain species such as *Nicotiana*, *Solanum*, and *Arabidopsis*, cell division activation has been found to precede radicle protrusion (Masubelele *et al.*, 2005). Flow cytometric analysis of the cell cycle in different parts of the embryo during imbibition has allowed verification of where, and at which phase of germination, DNA synthesis occurs (Sliwinska *et al.*, 2009). Studies using flow cytometry

have demonstrated that DNA replication is initiated in the radicle tip cells during hydro- or osmopriming of seeds of *Capsicum* (Saracco *et al.*, 2008) and *Beta vulgaris* (Śliwińska, 2000).

The DNA replication is related reduction of global DNA methylation (GDM) (Zluvova *et al.*, 2001). DNA methylation generally refers to an addition of a methyl group onto the C5 position of cytosine to form 5-methylcytosine (5mC) (Bird, 2002). DNA methylation was studied in wheat seeds during germination and a rapid reduction of global DNA methylation (GDM) levels was observed in connection with increasing metabolic activity (Drozhdeniuk *et al.*, 1976). Similar results were reported Zluvova *et al.* (2001) where a rapid decrease GDM levels was reported during the seed germination of *Silene latifolia,* and this event occurred before cell division had begun. Changes in the spatial distribution sequences DNA methylated are accompanied by changes in chromatin organization (Van Zanten *et al.*, 2011). Several authors reported that the highly compacted seed chromatin becomes loosened during germination (Bartels *et al.*, 2018). The chromatin compaction in *Arabidopsis* seeds quickly decreases upon imbibition (Van Zanten *et al.*, 2013). The integrating molecular, epigenetic, ultrastructural and morphological aspects are key to advance our understanding of the complex process of seed germination (Steinbrecher e Leubner-Metzger, 2018). In this sense, we analyzed the structural changes and activity of the cellular cycle associated with the protrusion of radicle in seeds of *T. catharinensis*. Also, in order to gain insight epigenetic associated with germination of seeds, the present study investigated the dynamics of global DNA methylation (GDM) variation, considering three phases of germination, in response to the exogenous solutions of GA_3, ABA and their inhibitors PAC and FLU. In addition, we observed the chromatin ultrastructure in seed mature and in the phase II of seed germination.

2 Material and methods

2.1 Plant material

Seeds of *T. catharinensis* were collected in two natural populations located in Curitibanos, Santa Catarina, Brazil (latitude 27° 18′ S, longitude 50° 38′ W, altitude 990 m; latitude 27° 36′ S, longitude 48° 27′ W, altitude 930 m). Damaged seeds were removed by hand-sorting. Selected seeds were immersed in ethanol 70% (v/v) for 1 min and disinfested in sodium hypochlorite (1% v/v)

for 5 minutes. After that, the seeds were submitted to three washes in sterile distilled water. One specimen was stored in Flor Herbarium (UFSC-Brazil) under registration number FLOWER 38056.

2.2 Seed imbibition and germination

Eight replicates of 25 seeds were placed in gerbox boxes with germitest paper moistened with 2.5 g of water per gram of paper. The seeds were weighed before imbibition, at 30-minute intervals for 6h, 1h intervals up to 12h, every 12h up to 6 days, and finally 24h intervals until 50% of the seeds showed radicle protrusion. The percentage of mass increment (I) over time, as a function of initial seed mass (Justo *et al.*, 2007), was calculated as (I %) = [(Mt-Mi) / Mi] x100, where Mi = initial fresh mass of the sample, and Mt = mass sample at time of collection (t). The seeds were inoculated in gerbox boxes, under germitest paper moistened with 10 ml of solutions of GA_3, ABA and their respective PAC and FLU inhibitors (200μM). In the treatment H_2O (control) was moistened with sterilized distilled water. All treatments were placed in a BOD chamber at 20 ± 2ºC and photoperiod of 10h.

2.3 Morphological analyses

Seed samples were collected during germination and analyzed in a Leica EZ4 HD stereo microscope equipped with the Leica EZ4 image capture and Leica LAS EZ software.

2.4 Light microscopy

Samples were collected in the three phases of germination and were fixed in phosphate buffer 0.1M (pH 7.2) containing 2.5 % formaldehyde at room temperature for 48h, according to Schmidt *et al.* (2009) with modifications. Subsequently, the samples were dehydrated. The samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany) and cut into semi-thin sections (4μm). Sections were stained with toluidine blue (TB-O) (O'brien *et al.*, 1964). LM sections were analyzed in a Leica DM2500 microscope equipped with the OPT 5.1 MP scientific camera and OPTHD software.

2.5 Transmission Electron Microscopy (TEM)

Samples were collected in the three phases of germination and were fixed in 0.1M sodium cacodylate buffer (pH 7.2) containing 2.5%

glutaraldehyde for 48h, to according Schmidt *et al.* (2009) with modifications. The material was post-fixed with 0.1M sodium cacodylate buffer containing 1% osmium tetroxide for 5h, dehydrated in an increasing series of acetone aqueous solutions, and then embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections (60nm) were collected on grids and stained with aqueous uranyl acetate followed by lead citrate. Three grids for each treatment were then examined in the JEM 1011 TEM (JEOL Ltd., Tokyo, Japan) at 80 kV.

2.6 Global DNA methylation analysis

For the Global DNA methylation analysis, seeds were imbibed in the exogenous solutions (GA_3, FLU, ABA, PAC) and H_2O and were collected in Phase I (18-22 h of imbibition), phase II (72-75 h of imbibition) and phase III (radicle protrusion, 1mm length). For treatments with no germination, samples from phase III were collected at 30 days (720 h), along with the control treatment (H_2O). Radicle protrusion was considered the moment of seed germination. DNA extraction was performed in samples consisting of three different biological replicates, for each treatment. Nucleic acids digestion procedures were based on the method described by Johnston *et al.* (2005) and Fraga *et al.* (2012). HPLC analysis was performed according to Johnston et al. (2005). 5mdC quantification (%) was performed according to 5mdC concentration divided by 5mdC concentration plus dC concentration multiplied by 100. The obtained peak area was analyzed by LC Solution software (Shimadzu, Kyoto, Japan). Data were analyzed by "R" statistical program (Team, 2014) and submitted to ANOVA. Treatments were compared by Student–Newman–Keuls (SNK) post hoc test ($p<0.05$).

2.7 Flow cytometry

For FCM analyses, the embryos were dissected into the radicle after 150h of imbibition in the exogenous solutions (GA_3, FLU, ABA, PAC) and H_2O. Samples were prepared as described previously by Rewers *et al.* (2009) with modifications. Plant tissue was chopped with a sharp razor blade in a plastic Petri dish with 1 ml nucleus-isolation buffer (100 mM Tris, 2.5 mM $MgCl_2.6H_2O$, 85 mM NaCl, 0.1% Triton X-100; 2% PVP, pH 7.0). After chopping, the suspension was passed through a 50µm mesh nylon filter and added Propidium iodide and RNase (50µg ml^{-1}) for DNA staining. For each sample, the

fluorescence of 10000 nuclei was analysed using a FACSCanto II (BD Biosciences™, USA) flow cytometer. Analyses were performed on four replicates. Histograms were analysed using a Flowing Software 2.5.1, and the percentage of nuclei with different DNA contents were calculated.

3 Results and discussion

3.1 Exotesta rupture and endosperm rupture are sequential events during the seed germination of **Trichocline catharinensis** *and includes cell division, ultrastructural changes and variations in the global DNA methylation*

The seed of *Trichocline*, is inside ripe cypsela, that showed pericarp (pe), seed coat (exotesta-ex) and remaining endosperm surrounding the embryo (1a-I). Morphologically, the seeds of *T. catharinensis* were obovoid, with a papyraceous seed coat (exotesta), and an average length of 0.6 mm (Fig. 1a-II). Germination begins with water uptake by the seed (imbibition), which in the *Trichocline* seed, as well as in the *Glycine max* seed (Pietrzak *et al.*, 2002), have the hilum and the micropyle as the water entry sites. This is necessary to trigger the signaling cascade, and thus initiate the complex set of processes that ends with the emergence of the embryonic axis, usually the radicle, through the structures surrounding it (Obroucheva *et al.*, 2017) defined as "*Germination sensu stricto*". This does not include seedling growth; this start after germination is completed, defined as post-germination events (Bewley *et al.*, 2013). At the beginning of the *germination sensu stricto*, we observe hydration of the pericarp (Fig.1a-III). The formation of a protuberance with the elongation of the hypocotyl-radicular axis (HRA), which ends in the protrusion of the radicle through the surrounding structures (endosperm, exotesta and pericarp) (Fig. 1a-IV). The hypocotyl curved, raising the cotyledons above the soil, making them chlorophyllous (Fig. 1a-V e VI). We observed the formation of short and translucent root hairs (Fig. 1a-VI detail). Hypocotyl and primary root posterior growth; the persistent cotyledons expanded completely (Fig. 1a-VII). These stages evidenced that the germination of this species is of the epigeal type and shows phanerocotyledonar seedlings (Souza, 2003).

Observations in LM show the structural modifications in HRA after imbibition (Phase II) that promote germination. We observed evident intercellular spaces in the region of the fundamental meristem (later called the cortex) (Fig. 1b arrows). According to Evert (2006) intercellular spaces extend into meristematic tissues, where the dividing cells are respirating heavily. This is confirmed when we observe, in the outer layers of the pericycle (p), an endodermal layer (1) that divides periclinal (in a longitudinal orientation), giving rise to a new layer of the fundamental meristem (2) (Fig.1b-c). This is described at the *Arabidopsis* root apex (Dolan *et al.*, 1993). In the phase III, we observed in LM, three cell layers of the micropylar endosperm (me) vacuolated and collapsed (Fig. 1d). Also, we observe that the exotesta (ex), already showed evidence of rupture (Fig.1d-e). According to Müller *et al.* (2006) and Yan *et al.* (2014) upon imbibition, after testa rupture in *Nicotiana* and *Lactuca*, mannans are degraded in the micropylar endosperm (me) to promote endosperm weakening. In this region the accumulation of pectic substances was evidenced, proved by the differentiated staining with the use of TB-O (Fig. 1e detail). The endosperm cell walls have diverse structure and compositions of hemicellulose and pectin monomers (Lee *et al.*, 2012). Cell wall remodeling enzymes (CWREs) are important for synthesizing, loosening and reinforcing the cell walls (Müller *et al.*, 2006; Yan *et al.*, 2014). Both the composition and abundance of CWREs alter the tensile properties that allow tissue to rupture (Lee *et al.*, 2012), which could be the cause of the opening at the micropylar end (Fig. 1f arrow). The opening observed in *Trichocline*, are also presented in *Nicotiana tabacum* seeds, the radicle emerges through an orifice in the endosperm that has a smooth outline. This hole always forms at the micropylar end and results from the degradation of the endosperm (Leubner-Metzger, 2007). The rupture of the endosperm is a limiting process of germination, observed in sp. Asteraceae (*Lactuca*) and Solanaceae (*Solanum*, *Nicotiana tabacum*, *Capsicum* and *Datura* species), data reviewed by (Steinbrecher e Leubner-Metzger, 2017; Steinbrecher e Leubner-Metzger, 2018). These findings strongly suggest that exotesta rupture and from endosperm are sequential events during the germination of *T.catharinensis*. Also, the rupture of the endosperm is a limiting process in the germination of seeds *Trichocline.*

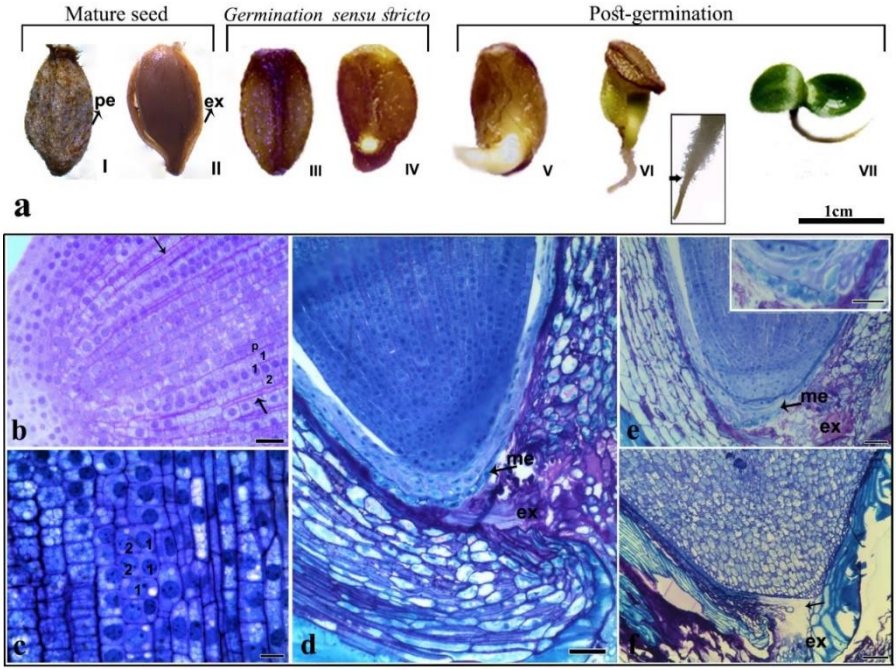

Fig. 1 Morpho histological observations of the seed of *Trichocline catharinensis.* a. Mature seed- I. ripe cypsela, that showed pericarp (pe). II. seed with a papyraceous exotesta. *Germination sensu stricto*- III. hydration of the pericarp. IV. elongation of hypocotyl-radicular axis, which ends in the protrusion of the radicle. Post-germination events – V and VI. The hypocotyl curved, raising the cotyledons above the soil, making them chlorophyllous. VI. formation of short and translucent root hairs (detail). VII- hypocotyl and primary root posterior growth; persistent cotyledons expanded completely. Phase II in b and c. longitudinal section of HRA in LM showed intercellular spaces evident in the region of the fundamental meristem (arrows). In the outer layers of the pericycle (p), an endodermal layer (1) divides periclinal (in a longitudinal orientation), giving rise to a new layer of the fundamental meristem (2). Phase III in d. longitudinal section showed three cell layers of the micropylar endosperm (me) vacuolated and collapsed. Also, we observe that the exotesta (ex), already showed evidence of rupture. e. In the endosperm cells, it showed accumulation of pectic substances (detail). f. The loosening of endosperm cells, allow tissue to rupture causing an opening at the micropylar end (arrow). Bars. b-e-detail 25µm. c. 10µm. d-f. 50µm.

Observations in TEM of the HRA showed cells with protein storage vacuoles (PSVs), consisting of a crystalloid matrix and small lytic vacuoles (LV) (Fig.2a). In phase I, PSVs initiate the proteolysis, by the fusion of the LV (Fig.2b-c). The cells showed cytoplasm with a large number of free ribosomes (Fig.2b arrowhead) or clusters (polyribosomes) (Fig 2d detail). In addition, rough endoplasmic reticulum (RER), mitochondria (M) and Golgi (G) (Fig. 2c-e detail). These characteristics are indicative of a possible onset of protein synthesis. The ribosomes are recruited to synthesis complexes of polyribosome proteins using mRNAs stored in the dried seed (Bewley *et al.*, 2013). According to Obroucheva *et al.* (2017) at the end of the first imbibition phase, all major metabolic systems are activated. We observed intercellular traffic via plasmodesmata (Fig. 2b arrows) indicating that the metabolism is being initiated and activated.

In phase II, there was an increase in LV in PSVs (Fig.2f). This mobilization, as noted, does not occur simultaneously in all cells (Fig.2g-h). The PSVs presented several stages of degradation, according to the LV fusion. The lipid bodies (LBs), remained around the PSVs. More intercellular spaces were observed in phase II compared to phase I (Fig. 2f asterisk). Nucleus showed nucleolus (NU) with heterochromatin (arrowhead) and euchromatin (arrow) distinct (Fig.2h). According to Obroucheva *et al.* (2012) an additional increase of the water content in phase III is required for the onset of growth in the embryonic axes. We also observed that the intercellular spaces were maintained in phase III (Fig. 2i-j asterisk). The PSVs undergo dedifferentiation of the crystalloid matrix and coalescence of the LV, causing the formation of a central vacuole, occupying a large part of the cytoplasm (Fig. 2i-j). The organelles M, Golgi, glyoxysomes (GL) and RER were reduced to one layer, close to the cell membrane (Fig.2k). In this phase, the vacuoles (v) show full of fibrillar material and small membrane fragments, due to the numerous fusions and intravacuolar digests (Fig. 2i-j) related to the mobilization of the reserves. According to Zheng e Staehelin (2011) in *Nicotiana tabacum* radicle cortex cells, lytic vacuoles are formed by a process involving PSV fusion, storage protein degradation and the gradual substitution of intrinsic α-tonoplast protein (TIP) of the PSV with the γ-TIP lytic vacuole (LV) marker protein. This aqueous lytic vacuolar compartment has to be formed to provide turgidity pressure, directing cell elongation to promote radicle protrusion (Maeshima *et al.*, 1994; Inoue *et al.*, 1995). Protein

storage vacuoles for lytic vacuoles occur clearly prior to the initiation of cell elongation at the elongation zone of the radicle (Novikova *et al.*, 2014). Plant cells adjust the extensibility of cell walls by remodeling major wall components, cellulose microfibrils and /or pectin/ hemicellulose matrix (Schopfer e Plachy, 1985). Cell wall acidification increases by membrane H+ ATPase and results in the activation of some cell wall enzymes, in particular xyloglucan endotransglicosylase (XET), methyl esterase and expansins. Expansins are proteins that interrupt the hydrogen bonds between cellulose and hemicelluloses. Thus, the mechanism of "acid growth" begins to operate and prepare the cells for the elongation at the end of the germination (Obroucheva e Antipova, 2000). We observed mobilization of the lipid reserves, evidenced by the lower relative volume of (LB) that were closer to the membrane (Fig 2k). Reserves are mobilized, and converted into easily transportable low molecular weight metabolites into growing regions, in support of the energy production (Bewley *et al.*, 2013).

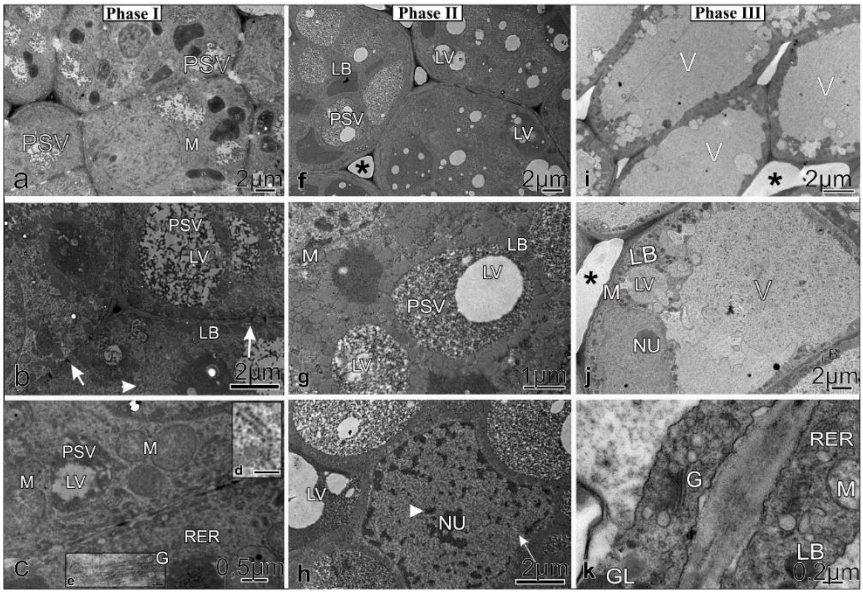

Fig.2 Observations on transmission electron microscopy (TEM) during germination of *T. catharinensis* seeds: **Phase I** a. cells with protein storage vacuoles (PSVs), consisting

of a crystalloid matrix and small lytic vacuoles (LV). b. PSVs initiate the proteolysis, by the fusion of the LV. Presence of lipid bodies (LB) near the cell membrane and around the PSVs. plasmodesmata in the cellular wall (arrows). Free ribosomes were observed in the cytoplasm (arrowhead). c. mitochondria (M) and rough endoplasmic reticulum (RER). d. detail polyribosomes. e. detail golgi (G). **Phase II** f. The PSVs presented several stages of degradation, according to the LV fusion. intercellular spaces (asterisk).g. LBs around PSVs. h. Nucleus showed nucleolus (NU) with heterochromatin (arrowhead) and euchromatin (arrow) distinct. **Phase III** i. formation of a central vacuole, occupying a large part of the cytoplasm. j. coalescence of the LV with central vacuole. k. Organelles close to cell membrane -Golgi (G), glioxysomes (GL), rough endoplasmic reticulum (RER), mitochondria (M). Also, close to cell membrane lipid bodies (LB).

In the seeds of *T. catharinensis*, in addition to the micropylar endosperm rupture and HRA elongation observed by cell changes, we also observed the onset of cell division. Flow cytometric histograms from embryonic nuclei after 150h imbibition in H_2O, showed one large peak, corresponding to the 2C DNA content (G1 phase of the cell cycle), and the second smaller peak, corresponding to nuclei with replicated 4C DNA content (G2 phase) (Fig. 3a). The most commonly accepted view is that the initiation of DNA synthesis leading to mitotic activity only contributes to post-germination root growth (Sliwinska *et al.*, 2009; Bewley *et al.*, 2013). However, in some species such as *Nicotiana tabacum*, *Solanum* and *Arabidopsis*, cell division was detected prior to radicle protrusion (De Castro *et al.*, 2000; Masubelele *et al.*, 2005). To better understand the activity of the cellular cycle and in order to gain insight epigenetic associated with germination of seeds, we apply GA_3, ABA and theirs PAC and FLU inhibitors. Since the relative abundance of these hormones constitutes a metabolic threshold mechanism that regulates the germination process (Weitbrecht *et al.*, 2011). Flow cytometric histograms in response of GA_3, ABA and their inhibitors PAC and FLU, after 150h of imbibition, also showed one large peak, corresponding to the 2C DNA content (G1 phase of the cell cycle), and the second smaller peak, corresponding to nuclei with replicated 4C DNA content (G2 phase) as observed in seeds imbibition in H_2O (Fig. 3a-e). We observed that the percentage of nuclei with 4C DNA is higher in seeds embedded in FLU, GA_3 and H_2O. In these treatments there is no delay in germination, as observed in the imbibition curve (Fig. 4a-b-c), at the end of phase II, there is an increase in uptake water and start of phase III. In these treatments, we observed decreased global DNA methylation (GDM) levels (75h after imbibition), the that

appear to occur prior to DNA replication (Zluvova *et al.*, 2001). In addition to this, flow cytometric studies show that there is an increase in the proportion of 4C nuclei in the axis which coincides with a considerable increase in length of the hypocotyl (Sliwinska *et al.*, 2009). We observed that more than 80% of the nuclei were in the G0 / G1 phase of the cell cycle (having 2C DNA), and there were no more than 12% of 4C (G2) nuclei. These results strongly suggest that there is no endopolyploidization in *Trichocline*, which is similar to the *Helianthus annuus* species, both Asteraceae, which probably completed germination due to cell division (Rewers e Sliwinska, 2014). These data confirm that together with elongation of the radicle cells, cell division occurs in seeds of *Trichocline.*

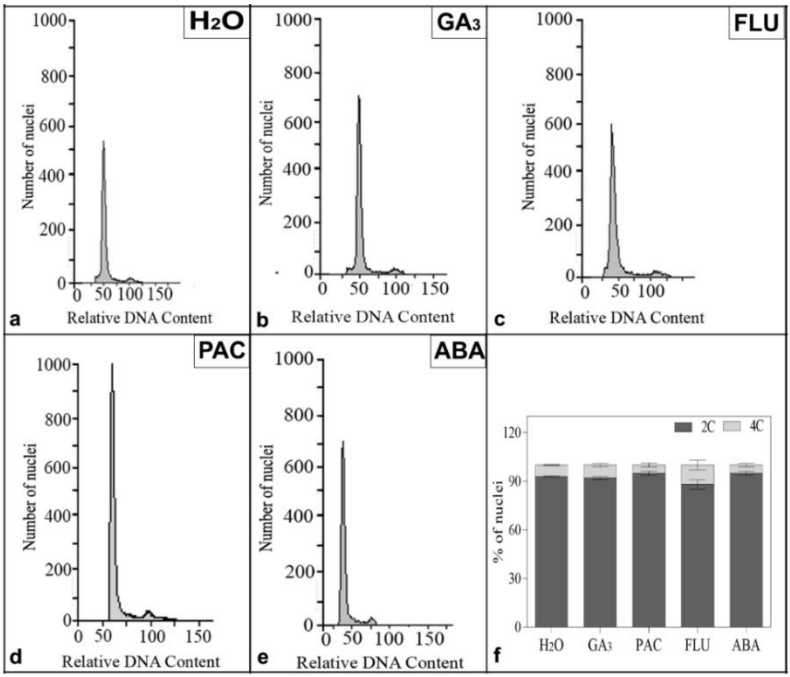

Fig. 3 DNA histograms of nuclear preparations from hypocotyl-radicular axis de *Trichocline catharinensis* after 150h of imbibition in the exogenous solutions of: a. H_2O. b. GA_3. c. FLU. d. PAC. e. ABA. f. Proportion of nuclei with different DNA contents in H_2O, GA_3, FLU, PAC, ABA.

The level of GDM in the mature dry seed was high (50%), associated with higher chromatin compaction (heterochromatin) (He) (Fig. 4; Fig. 5a). In the dry seeds, GDM is concentrated on chromocenters (more compacted regions), which appear electron-dense in TEM analysis (Van Zanten *et al.*, 2011). We observed these regions in *Trichocline* seeds closer to the periphery of the nuclear membrane (Fig.5a (Ed)). Similar results were also observed by Kawakatsu et al. (2017); Meng et al. (2012); van Zanten et al. (2011). After 72h of imbibition (Phase II) GDM levels decreased in all treatments, possibly related to metabolic activities and transcriptional activation, initiated by the germination process (Fig.4a-e). It is also related to the elongation and DNA replication, as we observed in our results, which prior to cell division decreases GDM levels. This is in line with previous reports of global DNA methylation declines during germination (Meng *et al.*, 2012; Bräutigam *et al.*, 2013; Van Zanten *et al.*, 2013). In addition, when we analyzed in TEM, the nucleus of the cells after 75h of imbibition, we observed the most decondensed chromatin (regions of euchromatin (Eu)), and without electron-dense regions in comparison to dry seed (Fig.5b-f). The changes in chromatin organization are accompanied by changes in the spatial distribution of methylated DNA sequences (Van Zanten *et al.*, 2011). During seed imbibition, when chromatin decondenses, GDM sequences become dispersed over the nucleus (Mathieu *et al.*, 2003; Van Zanten *et al.*, 2011) and lack visible chromocenters (Mathieu *et al.*, 2003). In the treatments FLU and GA_3, significantly showed greater reduction of GDM compared to H_2O in phase II (Fig.4). According to Manoharlal *et al.* (2018) the GA_3 application induced a similar declining trend in global DNA methylation. GA_3 may have indirect effect on DNA methylation by increasing the mobility and/or modification of linker histones (H1 and its variants) (Wierzbicki e Jerzmanowski, 2005).

However, at phase III of seed germination , we observed significant differences. The exogenous PAC resulted in a gradual reduction in GDM levels from 29% in phase II to 7% in phase III (Fig. 4e). Moreover, the structure of the chromatin in TEM after 75 h of imbibition in PAC is more decondensed (Fig.5f). PAC treatment, by inhibiting germination, resulted in abiotic stress, which generally leads to decreased levels of methylation (Peng e Zhang, 2009). The germination of rice seeds in a salt solution, cause a decondensation of chromatin (Santos *et al.*, 2011). Several reports have demonstrated that global demethylation can affect genome integrity and gene regulation (Koji *et al.*, 2008; Anderson *et al.*, 2009; Shimoda *et al.*, 2014). The regulation of gene expression under stress is not limited to the promoter region but, can be achieved by

changing the methylation status of the coding region, leading to a differentiated expression of genes or deletion in the expression of genes related to germination (Peng e Zhang, 2009). In tobacco plants subjected to stress (e.g., aluminum, salt, cold, or oxidative stress) acquired a lower DNA methylation specifically at a gene coding for a protein, which is associated to its higher transcription (Choi e Sano, 2007). In addition, it has been suggested that oxidative damage as a result of stress (exogenous application of PAC) may cause variations in DNA methylation patterns (Cerda e Weitzman, 1997). The results implied that the epigenetic mechanisms dependent on the DNA methylation appear to influence gene expression during the germination in *Trichocline*, which was also confirmed by regions of euchromatin after imbibition.

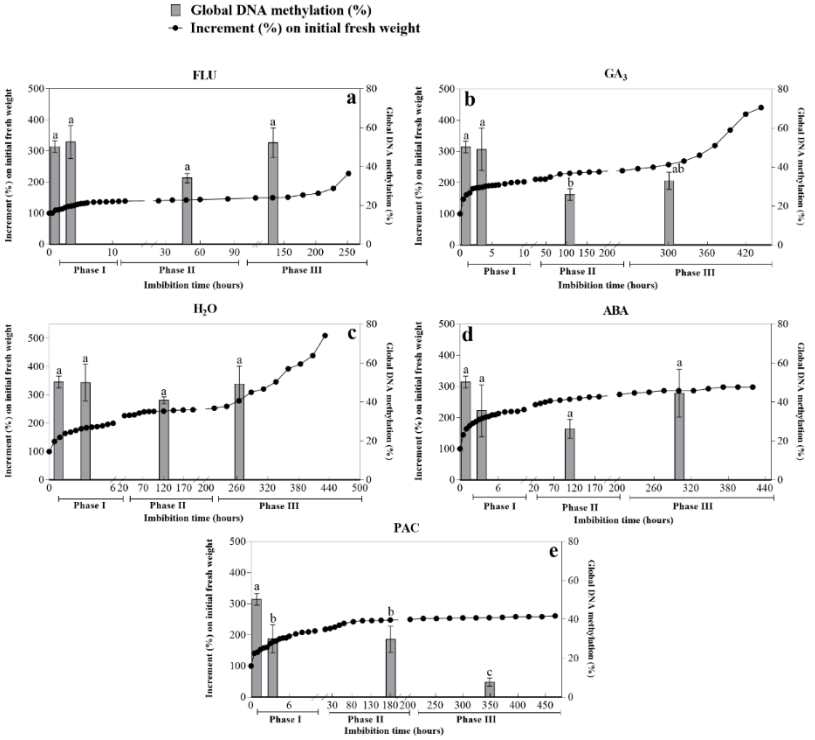

Fig.4 Dynamics of global DNA methylation of *T. catharinensis* in mature seed and the three phases of germination in exogenous solutions of fluridone (FLU) (a), gibberellin (GA_3) (b), water (H_2O) (c), abscisic acid (ABA) (d), paclobutrazol (PAC) (e). Data mean ±SD (*n*=4). Means followed by the same letter are not significantly different by the SNK test ($p < 0.05$)

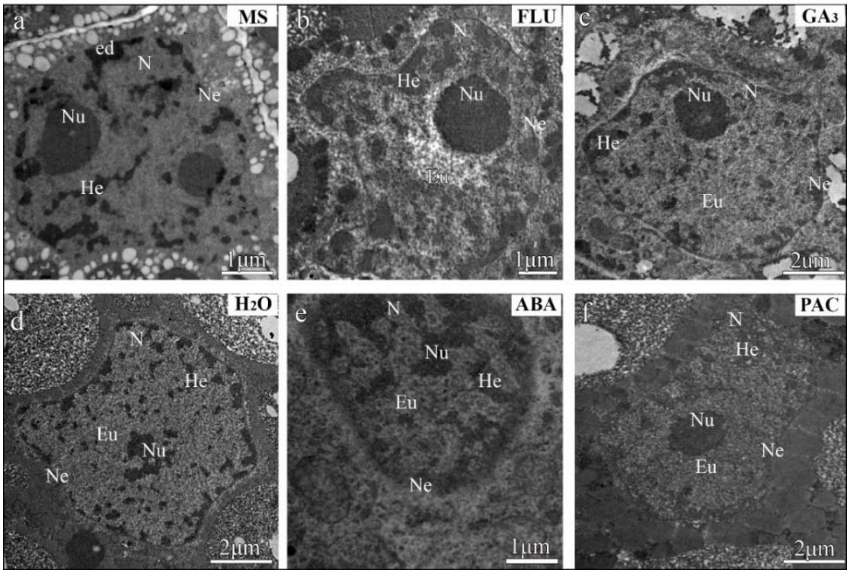

Fig.5 Ultrastructural characterization of cells from *T. catharinensis* embryo by TEM analysis, showed cells with nuclei (N), nucleolus (Nu) showing euchromatin (Eu) and heterochromatin (He) regions near the periphery of the nuclear envelope (Ne) more electron-dense (Ed) **a**. mature seed. **b**. cells imbibed-FLU. **c**. cells imbibed-GA_3. **d**. cells imbibed-H_2O. **e**.cells imbibed-ABA. **f**. cells imbibed-PAC. FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol.

In conclusion, our results confirm that for the protrusion of the radicle in the seed of *Trichocline* (Asteraceae) exotesta rupture and endosperm rupture are sequential events. The observed cell elongation during germination is accompanied by the enlargement of vacuoles due to the transformation of protein storage vacuoles to typical vacuoles occurred prior to the initiation of cell elongation. Together with elongation of the HRA cells, we observed in analysis of flow cytometry, cells with replicated 4C DNA content (G2 phase of cycle cellular) before germination is complete. This is related to the reduction of levels of GDM observed after 72h of imbibition, when DNA replication is possibly initiated. In the seeds imbibed FLU, GA_3 and H_2O, showed higher number of cells with nuclei 4C DNA. When we applied PAC inhibitor of GA_3 synthesis,

we observed the levels of GDM decrease dramatically in phase III. In addition, there is no fresh weight increased (FW) at this phase, that is the germination is inhibited. In the mature seed we observed compacted chromatin in electron-dense regions (heterochromatin), becomes loosened during germination in regions of euchromatin. As we observed, there are few studies that correlate ultrastructural changes with molecular processes before the protrusion of the radicle. These information has implications both in the research to determine fundamental processes involved in germination, and for seed producers at the beginning of ornamental crops. Furthermore, this information may be useful in agronomically important practices, such as seed priming, to promote the conservation of germplasm of native species.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Capítulo 3 Cellular alteration and differential protein abundance associated to the decision of radicle protrusion in *Trichocline catharinensis* (Asteraceae) seeds, a Brazilian wild species.

Abstract

This is the first study that to gather ultrastructural characteristics and comparative proteomic analysis in response to GA_3, ABA and its PAC and FLU inhibitors associated to the decision of radicle protrusion during seed germination. Analysis of PCA explain 84% of the variation in protein abundance and revealed distinct classes of proteins associated the effects of FLU, GA_3, PAC, ABA and H_2O. GA_3 stimulate germination, observed by intense cellular activity and mobilization of reserves. These responses relate to up-accumulation of proteins associated energy metabolism and redox regulation. GA_3 appeared to be involved in controlling the abundance of several proteins associated with radicle protrusion, since when we inhibited GA biosynthesis with PAC, the cells remain at phase II. PAC imbibed seeds down-accumulated proteins related to energy metabolism and redox regulation, associated to this, showed few organelles and plasmodesmata associated to the low metabolism activation, and no reserves mobilization. In addition, in the treatment with PAC, we observed few intercellular spaces in hypocotyl-radicular axis, which influences elongation and growth. Exogenous ABA up-accumulated proteins related to osmotic stress response and showed osmotolerance which was observed by the plasmalema retraction, as well the coalescence of vacuoles, surrounded protein reserves (PSVs). Also, ABA down-accumulated proteins related to cell structure and growth, and this may be associated with interruption the extension of the hypocotyl-radicular axis, which showed flattened cells. In response to this, ABA delays and decreases the percentage of germination (58.75%). Our results with applications of GA_3, ABA and their inhibitors, besides the biological relevance in germination, revealed cellular response mechanisms associated to differential protein abundance from an Asteraceae wild from southern Brazil. These differentially accumulated proteins associated to the cell changes are critical for to transition water absorption to the radicle protrusion.

Key words: cell biology, proteome, seed physiology, plant hormones, wild plants

1. Introduction

Seed germination is a complex and critical process in the life cycle of higher plants. By definition, seed germination start with the uptake of water and is completed with the emergence of the radicle from the seed coat (Bewley *et al.*, 2013). In general, the progress of seed germination can be divided into three phases. In the first phase, there is fast water uptake by the dry seed until all the seed contents are fully hydrated. The second phase is a period of limited water uptake, associated with several cellular and biochemical events, including signal transduction, regulation of gene expression, reactivation of metabolism and regulation of redox homeostasis (Bailly *et al.*, 2008; Bewley *et al.*, 2013). This is followed by phase III, with resumption of water uptake, micropylar endosperm rupture, testa rupture, and radicle protrusion, that is, the completion of germination *sensu stricto* (Weitbrecht *et al.*, 2011). Among the three phases, phase II is the most critical because all necessary metabolic pathways and physiological processes that control the next phase are reactivated (Bewley *et al.*, 2013; Galland *et al.*, 2014).

The onset of seed germination is closely regulated by the endogenous balance of plant hormones, such as gibberellin (GA), abscisic acid (ABA) (Holdsworth *et al.*, 2008). GA and ABA contents tend to be negatively correlated and they alter the balance of germination repressors, which must be inactivated for germination to occur (Piskurewicz *et al.*, 2008). GA promotes seed germination by degrading these repressors, while ABA induce them (Piskurewicz e Lopez-Molina, 2009). The manipulation of these repressors can be achieved by the exogenous application of inhibitors in the synthesis of GA and ABA (Piskurewicz *et al.*, 2008), such as paclobutrazol (PAC) and fluridone (FLU), respectively (Kusumoto *et al.*, 2006). Paclobutrazol (α-tert-Butyl-β-(4-chlorobenzyl)-1H-1,2,4-triazole-1-ethanol) inhibits the activity of *ent*-kaurene oxidase, which is an enzyme in the GA biosynthesis pathway (Hedden e Graebe, 1985). Fluridone (1-methyl-3-phenyl-5-[3-trifluoromethyl) phenyl]-4-(IH)-pyridinone) inhibits the activity of phytoene desaturase I, which catalyses the conversion of phytoene to phytofluene, a key step in ABA biosynthetic route (Huarte e Benech-Arnold, 2010; Chen *et al.*, 2016). The use of inhibitors of GA and ABA synthesis provides information on how the relative content of these two hormones affect biochemical profile and ultrastructural characteristics

during seed germination. Morphological and ultrastructural analysis provides intercellular and intracellular inherent characteristics of the events that promote biological development (Verdeil et al. 2007). Usually, in seed reports structural analyzes are related to identify the reserve storage and mobilization during development or germination, respectively(Pritchard *et al.*, 2002; Penfield *et al.*, 2004; Bolte *et al.*, 2011; Novikova *et al.*, 2014; Obroucheva *et al.*, 2017). The complexity of seed tissues and the difficulties in access deep tissues *in vivo* as well preparing good samples for microscopy can have difficult these studies in seeds (Fotouo-M *et al.*, 2015). Ultrastructural alterations at specific phases of seed germination, in response application exogenous hormones, has been poorly reported in the scientific literature and usually are not associated to biochemical profile.

On the other hand, proteomic studies have been shown to be a powerful tool for monitoring the physiological status of plant cells and tissues under specific developmental conditions (Rose et al., 2004). In addition, proteomics offers an opportunity to examine simultaneous changes in and to classify temporal patterns of protein abundance during seed germination (Rajjou *et al.*, 2011; Wang *et al.*, 2013). Considerable proteomics studies on seed germination in several species has been performed, such as on *Arabidopsis thaliana*, *Oryza sativa* (Kim *et al.*, 2008; Liu *et al.*, 2015; Liu *et al.*, 2016), *Zea mays* (Huang e Song, 2013), *Pisum sativum* (Wang *et al.*, 2012), *Lactuca sativa* (Wang *et al.*, 2015), *Triticum aestivum* (He *et al.*, 2015; Liu *et al.*, 2018). However, relatively few studies of differential protein abundance on seed germination in response to exogenous GA and ABA hormones and their respective inhibition PAC and FLU were performed. Moreover, we observe that the biochemical and cellular mechanism in the germination of wild species, it is not as explored as in Arabidopsis and cultivated crops. Further, cell biology methods remarkably extend the knowledge provided by proteomic studies and might be fundamental for the functional complementation of proteomic data (Takáč *et al.*, 2017). In this sense and considering that the reactions which occur in the germination, are catalyzed or mediated by different proteins it is valuable to identify protein profile associated to the cellular alterations in wild species, especially from a biodiverse plant family as Asteraceae. Asteraceae is considered one of the largest flowering plant families, representing 8% to 12% of the angiosperm species in the world (Funk *et al.*, 2009). In addition, only a few studies have investigated seed germination of Brazilian Asteraceae species (Ferreira *et al.*, 2001; Gomes e Fernandes, 2002; Cury *et al.*, 2010; Bombo *et al.*, 2015).

Based on this, we propose to study *Trichocline catharinensis* seeds, a wild and endemic species from the high-altitude fields (750-1500 m) of southern Brazil (Cabrera e Klein, 1973; Pasini e Ritter, 2012). In order to understand the transition of water uptake and radicle protrusion, the present work analyzed cellular alterations and differential protein abundance in response to GA_3 and ABA and their inhibitors PAC and FLU at phase II of *Trichocline catharinensis* seed germination.

2. Material and methods

2.1 Plant material

Seeds of *T. catharinensis* were collected in January (2017) in two natural populations located in Curitibanos, Santa Catarina, Brazil (latitude 27° 18′ S, longitude 50° 38′ W, altitude 990 m; latitude 27° 36′ S, longitude 48° 27′ W, altitude 930 m). Damaged seeds were removed by hand-sorting. Selected seeds were immersed in ethanol 70% (v/v) for 1 min and disinfested in sodium hypochlorite (1% v/v) for 5 minutes. After that, the seeds were submitted to three washes in sterile distilled water.

2.2 Seed germination and Germination speed index (GSI)

Exogenous applications of GA_3 (200µM) and ABA (200µM) (Sigma-Aldrich, St. Louis, MO, USA) and their respective biosynthetic inhibitors, FLU (200µM) and PAC (200µM) (Sigma-Aldrich) were evaluated. The concentrations applied were based on previous experiments with *T. catharinensis* and in the reports of Hu *et al.* (2012) and Kim *et al.* (2008). In all treatments, 10 ml of H_2O (as a control) or each of the chemicals cited above were added to the germitest paper containing the seeds. PAC and FLU solutions were prepared by dissolving the compounds in acetone (0.1% v/v), followed by dilution with water (Kim *et al.*, 2008; Hu *et al.*, 2012). GA_3 was dissolved in ethanol (0.01% v/v) and ABA in NaOH (0.01% v/v), according to manufacturer's instructions. We considered the germination percentage (%) and germination speed index (GSI). GSI was calculated to according the Maguire's index. It is considered as the total number of seeds germinated per day between sowing and germination divided by the number of days of the test (Maguire, 1962; Brown e Mayer, 1988). Germination percentage is calculated by the

cumulative number of daily germinated seeds with respect of the total seed evaluated (Ranal e Santana, 2006).

2.3 Light Microscopy (LM)

For light microscopy studies, embryos after 75h of imbibition in the exogenous solutions (GA_3, FLU, ABA, PAC) and H_2O were fixed in phosphate buffer 0.1M (pH 7.2) containing 2.5 % formaldehyde at room temperature for 48h, according to (Steiner *et al.*, 2015) with modifications. Subsequently, the samples were dehydrated. The samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany) and cut into semi-thin sections (4μm). Sections were stained with toluidine blue (TB-O) to according O'brien *et al.* (1964). LM sections were analyzed in an Olympus BX 41 microscope equipped with Image Q Capture Pro 5.1 Software (QImaging Corporation, Austin, TX, USA). The length the hypocotyl-radicular axis (HRA) and mean size cell (length and width) for each treatment were determined on longitudinal sections using the OPTHD software. For the HRA, were used five measurements for each treatment. For the mean size cell, were used twenty measurements for length and width for each treatment.

2.4 Transmission Electron Microscopy (TEM)

For electron microscopy studies, embryos after 75h of imbibition in the exogenous solutions (GA_3, FLU, ABA, PAC) and H_2O were fixed in 0.1M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 48h, to according Schmidt *et al.* (2009) with modifications. The material was post-fixed with 0.1M sodium cacodylate buffer containing 1% osmium tetroxide for 5h, dehydrated in an increasing series of acetone aqueous solutions, and then embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections (60nm) were collected on grids and stained with aqueous uranyl acetate followed by lead citrate. Three grids for each treatment were then examined in the JEM 1011 TEM (JEOL Ltd., Tokyo, Japan) at 80 kV.

2.5 Proteomic analyses

Total protein extraction

Protein extracts were prepared in biological triplicate (100 mg) for each treatment (H_2O, GA_3, FLU, ABA, FLU) in phase II. Samples collected were frozen in liquid nitrogen, pulverized in a bead mill and macerated with extraction buffer containing 20 mM Tris-HCl (GE Healthcare, Uppasala, Sweden) pH 6.8, 1% dithiothreitol (DTT- GE Healthcare), Sodium dodecyl sulfate 0,1% (SDS- GE Healthcare), 1 mM phenylmethanesulfonyl fluoride (PMSF- Sigma-Aldrich) and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were agitated during 30 min, followed by centrifugation at 10 min for 4ºC at 16,000 g. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

Protein digestion

Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology to remove any interferent from samples (Nanjo *et al.*, 2012). After protein precipitation, samples were resuspended in urea 7M/thiourea 2M solution for proper resuspension. Protein digestion was performed using the filter-aided sample preparation (FASP) methodology as described by Wisniewski *et al.* (2009) with modifications. Before starting the digestion procedure, an integrity test was made to check for damaged filter units (Hernandez-Valladares *et al.*, 2016), thus only the working units were used. After that, 100 µg of protein samples were added to the Microcon-30 kDa filter units (Millipore) (Lipecka *et al.*, 2016) washed with 200 µL 50 mM ammonium bicarbonate (solution A) and centrifuged at 10,000 g for 15 min at 25°C (otherwise stated, all centrifugation steps were performed at this condition). This step was repeated once for complete removal of urea before reduction of proteins. Next, 100 µL of 50 mM DTT (GE Healthcare) freshly made in solution A were added, gently vortexed and incubated for 20 min at 60 °C (1 min agitation and 4 min stopped, 650 rpm). Then, 200 µL of 8M Urea in 50 mM ammonium bicarbonate (solution B) were added and centrifuged for 15 min. For protein alkylation, 100 µL of 50 mM iodoacetamide (GE Healthcare) freshly prepared in solution B were added, gently vortexed and incubated for 20 min at 25 °C in the dark (1 min agitation and 19 min stopped, 650 rpm). Next, 200 µL of solution B were added and centrifuged for 15 min. This step was repeated once. Then, 200 µL of solution A were added and centrifuged for 15 min. This step was

repeated twice. In the last washing, it should remain approximately 50 µL of sample. For protein digestion, 25 µL of 0.2% (v/v) RapiGest (Waters, Milford, CT, USA) and 25 µL of trypsin solution (40 ng/µL, V5111, Promega, Madison, WI, USA) were added, gently vortexed and incubated for 16 h at 37 °C (1 min agitation and 4 min stopped, 650 rpm). For peptide elution, the filter units were transferred for new microtubes and centrifuged for 10 min. Then, 50 µL of solution A were added and centrifuged for 15 min. This step was repeated once. For RapiGest precipitation and trypsin inhibition, 5 µL of 15% trifluoroacetic acid (TFA, Sigma-Aldrich) were added, gently vortexed and incubated for 30 min at 37 °C. Then, samples were centrifuged for 15 min, the supernatants collected and vacuum dried. Peptides were resuspended in 100 µL solution of 95% 50 mM ammonium bicarbonate, 5% acetonitrile and 0,1% formic acid. The resulting peptides were quantified by the A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for ESI-LC-MS/MS analysis. During separation, the samples (1 µg of digested protein) were loaded onto the nanoAcquity UPLC 5-µm C18 trap column (180 µm x 20 mm) at 5 µL min-1 for 3 min and then onto the nanoAcquity HSS T3 1.8-µm analytical reverse-phase column (75 µm x 150 mm) at 400 nL min-1. The column temperature was 45°C. For peptide elution, a binary gradient was used: mobile phase A consisted of water (Tedia, Fairfield, USA) and 0.1 % formic acid (Sigma-Aldrich), and mobile phase B consisted of acetonitrile (Sigma-Aldrich) and 0.1 % formic acid. Gradient elution was performed as follows: 7 % B for 3 min, ramping from 7 to 40 % B until 90.09 min; ramping from 40 to 85% B until 94.09 min; holding constant at 85 % until 98.09 min; decreasing to 7% B until 100.09 min; and holding constant at 7 % B until the end of the run at 108.09 min. Mass spectrometry was performed in positive and resolution mode (V mode), with a resolution of 35000 FWHM with ion mobility and in the data independent acquisition mode. The ion mobility wave velocity was set to 600 m s-1; transfer collision energy was ramped from 19 to 45 V in high-energy mode; the cone and capillary voltages were 30 and 2,800 V, respectively; and the source temperature was 70 °C. The nano flow gas was set to 0.50 Bar, and the purge gas flow ranged from 145 to 150 L h-1. The TOF parameters included a scan time of 0.5 s in the continuum mode and a mass range of 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (Sigma Aldrich) at 100

fmol µL-1 was used as an external calibrant, and lock mass acquisition was performed every 30 s.

Proteomics data analysis

Spectra processing and database searching conditions were performed by Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis used the following parameters: Apex3D of 150 counts for low energy threshold, 50 counts for elevated energy threshold, and 750 counts for intensity threshold; one missed cleavage, minimum fragment ion per peptide equal to two, minimum fragment ion per protein equal to five, minimum peptide per protein equal to two, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY), and a default false discovery rate (FDR) value at 1% maximum, peptide score greater than four, and maximum mass errors of 10 ppm. Protein identification was performed against *Helianthus annuus* (Common sunflower) databank obtained from UniProt database (http://www.uniprot.org/taxonomy/4232). Label-free relative quantitative analyses were performed based on normalized total ion counts. After the data were processed, only the proteins present in all 3 runs were considered. Differential regulation was performed in terms of protein accumulation comparing H_2O, GA_3 and FLU treatments with PAC treatment, and ABA and PAC treatments with H_2O treatment. Data were analyzed using the Student's t test (two-tailed). Proteins with P values of $P < 0.05$ were considered upregulated if the $\log_2$ of the fold change (FC) was greater than 0.5 and down-regulated if the $\log_2$ of FC was less than -0.5. Principal components analysis (PCA) were generated using PAST software 3.04 (Hammer Ø *et al.*, 2001). Finally, proteins were blasted against the Nonredundant (nr) Plants/Viridiplantae_Protein_Sequences database using the Blast2GO software (www.blast2go.com) for protein description and cellular component term annotation (Conesa et al., 2005).

3. Results

*3.1 ABA and GA_3 and their inhibitors modify seed germination, and this can be identified by cellular alteration at phase II of **T. catharinensis** seed germination*

Exogenous PGRs solutions and their inhibitors have a significant effect on germination of *T. catharinensis* seeds (Fig.1). FLU imbibed seeds (96.25%), GA_3 (98.75%) and H_2O (96.25%) showed similar value of germination percentage (Fig. 1a-b-c). The imbibition curve of these treatments was also similar, only FLU showed an advance imbibition, reflecting increased germination speed index (GSI), comparatively to the GA_3 and H_2O. GA_3 and H_2O showed no difference in GSI (14.8 and 14.4) (Fig. 1 a-b-c). The lengths the hypocotyl-radicular axis (HRA) in longitudinal section, in the H_2O, GA_3 and FLU treatments are 400 to 500 µm (Fig. 2a-b). The cells in these treatments, have a mean size between 20 µm of length and 27 µm in width (Fig. 2c). LM analysis of these treatments in details showed HRA cells with high content of storage reserves, vacuolization, presence of intercellular spaces and turgescence as consequence of imbibition (Fig.2d-f-h arrows). The cells of the cotyledons showed more vacuolization in the seeds imbibed in GA_3 and FLU (Fig.2g-i). Otherwise, the ABA imbibed seeds showed a reduction in the percentage of germination (58.75%) and this was more expressive in PAC treatment when the seed germination reach only 7.5% (Fig. 1d-e). In these two treatments, a distinct standard was identified in the imbibition curve, which help us to explain the physiological effect on seed germination percentages (Fig.1 d-e). The long imbibition time and GSI decrease indicates an inhibitory effect of ABA (5.23) and PAC treatments (1.16) (Fig.1 d-e) on seed germination. At phase II cellular alterations were observed by LM analysis of HRA and cotyledons cells (Fig.2j-l). ABA and PAC imbibed seeds showed a lower cellular elongation of HRA region, especially in ABA (234 µm) (Fig.2b-c) which also showed cells with lower length (12.5µm) and higher width (28.30µm) (Fig. 2c) resulting in flattened cells (Fig. 2j, arrow). Cotyledons cells in ABA treatment showed lower vacuolization (v) and plasmalemma retraction (Fig. 2k arrow). Similarly, in PAC treatment, it was observed few intercellular spaces in HRA region compared to other treatments (Fig. 2l) and the cotyledons cells showed plasmalemma retraction (Fig.2m arrows). All these cellular characteristics observed by LM analysis at phase II of seed germination inspirited us to study ultrastructural changes associated to the seed physiological behavior in response to hormones and its inhibitors.

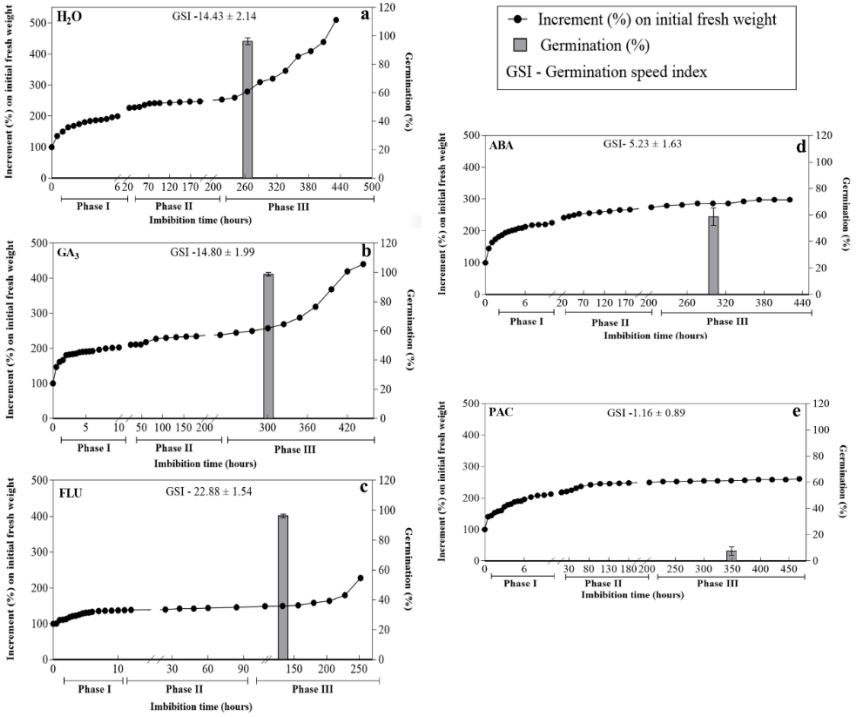

Fig.1 Imbibition curve (fresh mass in hours (%.h^{-1})), seed germination percentage and germination speed index (GSI) of *T. catharinensis* seeds in exogenous solutions of FLU (a), GA_3 (b), H_2O (c), ABA (d), and PAC (e). FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol. Data mean ±SD. (n= 4)

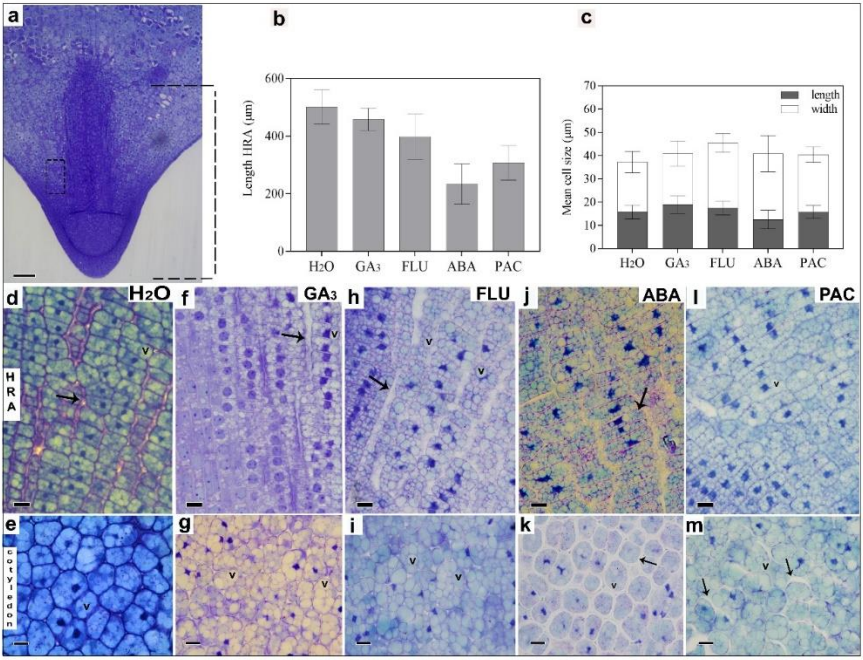

Fig. 2 Light microscopy analysis of hypocotyl radicular axis (HRA) and cotyledon of *Trichocline catharinensis* at phase II of seed gemination. **a.** longitudinal section indicating HRA region and cell enlargement region. **b.** Length of HRA region (µm) in seeds imbibed in the solutions GA_3, ABA, PAC, FLU and H_2O for 75h. **c.** Cell size average (µm) in length and width. Data mean ±SD. **H_2O** imbibed seed **(d-e) d.** detail of HRA cells showed intercellular spaces (arrow) and vacuolization (v). **e**. cotyledon cells showing vacuolization (v). **GA_3** imbibed seed **(f-g) f**. detail of HRA cells showed intercellular spaces (arrow) and vacuolization (v). **g**. cotyledon cells showed increased vacuolization (v). **FLU (h-i)** imbibed seed **h.** detail of HRA cells showed intercellular spaces (arrow) and vacuolization (v). **i**. cotyledon cells showed increased vacuolization (v). **ABA** imbibed seed **(j-k) j.** detail of HRA cells showed flattened cells (arrow). **k**. retraction of the plasmalemma in cotyledon cells (arrow) and low vacuolization (v). **PAC (l-m)** imbibed seed **l**. detail of HRA cells showed vacuolization (v) and few intercellular spaces **m.** retraction of the plasmalemma in cotyledon cells (arrow). Bars =100 µm and 10µm. FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol.

Cellular characteristics, at phase II, of seed germination gives us pivotal information about the differences associated to the decision of radicle protrusion from seed coat, once this phase is associated to the main metabolic alteration in this process. Cells from GA_3 imbibed seed, showed reserve mobilization, compared to treatment with H_2O (Fig.3a-d). In these cells protein storage vacuoles (PSVs) showed intense proteolysis on what the degradation of the crystalloid matrix was observed by fusion of the lytic vacuoles (LV) (Fig. 3d-e). GA_3 also showed lipid reserves mobilization, since we did not observe surrounding the PSVs, but only close to the plasmatic membrane (Fig.3d). Cells from GA_3 imbibed seeds also showed intense communication by the presence of plasmodesmata (Fig. 3d-f arrow) and activation of cellular metabolism with presence of mitochondria (M), glyoxysomes (GL), rough endoplasmic reticulum (RER), and ribosomes (R) in the cytoplasm (Fig.3e-f). Cellular organization in FLU imbibed seeds (Fig. 3g-h-i) were similar to H_2O treatment (Fig. 3a-b); cytoplasm with innumerous PSVs, surrounded by LBs (Fig. 3g-h). The cells from FLU and H_2O imbibed seeds also indicate the activation of metabolism once it was observed the presence of mitochondria (M), rough endoplasmic reticulum (RER), glyoxysomes (Fig. 3b-3g) and plasmodesmata (Fig.3c- 3h arrow). The ultrastructural characteristics observed in cells from GA_3, FLU and H_2O imbibed seeds could be associated to the seed germination once indicate reserve mobilization and presence of organelles associated to activation of metabolism. These facts it was even more evident once the cells from ABA and PAC imbibed seed showed contrasting characteristics. ABA promoted cellular reduction of cytoplasm by coalescence of vacuoles (Fig.3j-k) and the PSVs are retained at the periphery of them (Fig.3k-l arrows) while LBs remained at the periphery of the cell (Fig. 3k-l asterisk). It was also observed in seed cells submitted to ABA that the cell wall presented multiple layers (Fig.3l detail) and retraction of the plasmalemma (Fig.3k star). In addition, low number of plasmodesmata (p) was observed indicate poor communication between the cells (Fig.3l). In the treatment with PAC, cells presented cytoplasm filled by reserve compounds, both PSVs and LBs (Fig.3m-n). Moreover, low number of organelles associate to metabolism activation was observed as well poor connection between them by the plasmodesmata (Fig.3o arrow).

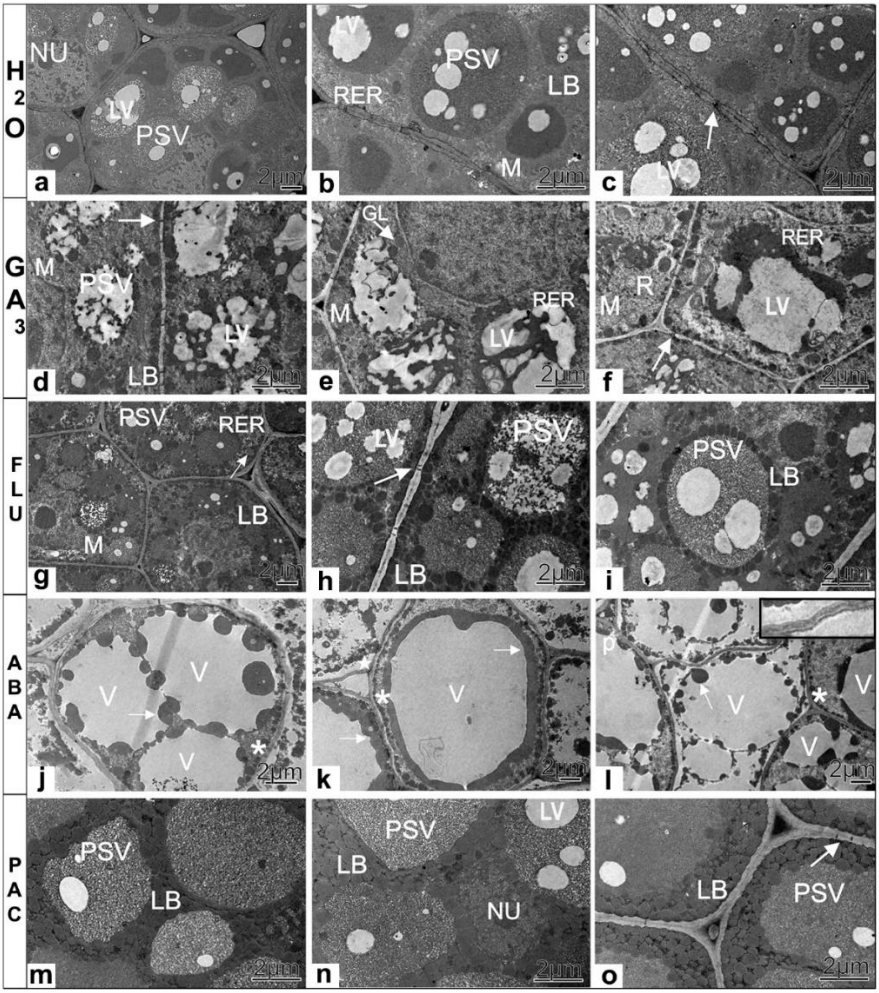

Fig.3 Transmission electron microscopy analysis of hypocotyl radicular axis (HRA) region in GA_3, ABA, PAC, FLU and H_2O imbibed seeds cells of *Trichocline catharinensis* after 75h of imbibition. **H_2O** imbibed seed **(a-b-c). a.** Cells with protein storage vacuoles (PSVs) and lytic vacuoles (LV). **b**. Lipid bodies (LB) surround PSV,

cytoplasm with mitochondria (M), rough endoplasmic reticulum (RER). **c**. presence of many plasmodesmata (arrow). **GA_3** imbibed seed **(d-e-f). d**. Proteolysis of PSVs. LBs close to membrane. Detail of the cell wall with many plasmodesmata (arrow) **e**. Cytoplasm with the presence of mitochondria (M), glyoxysomes (arrow), rough endoplasmic reticulum (RER). fragmentation of PSVs, with the fusion of LV. **f**. cells with GA_3 show intense cellular activity; mitochondria (m), ribosomes (r), plasmodesmata (arrow). **FLU** imbibed seed **(g-h-i)**. **g.** cellular organization similar to the H_2O. PSVs surrounded by LBs, mitochondria (m), rough endoplasmic reticulum (RER), glyoxysome (arrow). **h**. proteolysis in PSVs, fusion of LV. cell wall with plasmodesmata (arrow) **i.** PSVs surrounded by LBs. **ABA** imbibed seed **(j-k-l) j.** vacuoles (v) with protein reserves periphery the vacuolar membrane (arrows). LBs (asterisk) remain close to the cell membrane. **k**. formation of a central vacuole (v) by fusion with smaller vacuoles. junction of the protein reserves periphery of the vacuolar membrane (arrow). cytoplasm and LBs (asterisks) were reduced to periphery of the cell membrane. Some cells presented plasmalemma retraction (star). **l.** cell wall with multilayer (detail). reduced communication between cells, few plasmodesmata (p). reduction of the cytoplasm periphery of the membrane cell (asterisk). **PAC** imbibed seed **(m-n-o)**. **m**. LBs filled the whole cytoplasm. **n.** the structure of PSVs, with LV showed no change. nucleus with nucleolus (NU) **o.** There is little communication through plasmodesmata (arrow). FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol.

3.2 Functional classification and comparative protein accumulation between GA_3, ABA and their biosynthesis inhibitors PAC and FLU at phase II of the seed germination

In order to link cellular information and metabolic activation after seed imbibition and before radicle protrusion, we performed the proteomic analysis at phase II of seed germination. A total of 270 proteins (Table S1) were identified by LC-MS/MS which were classified by the Blast2GO software into 14 functional categories (Fig. 4a). The most abundant group were the proteins related to energy metabolism (20.81%) (glycolysis, TCA cycle, fermentation, gluconeogenesis and glyoxylate cycle). The second protein group (13.01%) involved biosynthesis, modification and folding of proteins. The third proteins group (13.01%) involved in redox regulation. Other functional groups included signaling (11.15%) stress response (9.66%), proteolysis (8.55%), metabolic processes (4.84%), amino acid metabolism (4.83%), RNA (3.34%), cell structure and growth (2.60%), uncharacterized proteins (1.85%), lipid metabolism (1.11%) and DNA (0.74%). These results show an overview of the functional processes that are related to phase II of *T. catharinensis* seed

germination. The differential accumulation analysis was performed comparing H_2O, GA_3 and FLU treatments against PAC treatment, and comparing ABA and PAC treatments against H_2O treatment. In H_2O treatment the proteins up-accumulated when compared with PAC treatment and these proteins were associated with stress response (2), protein biosynthesis (2) and signaling (2) (Fig.4b). The proteins up-accumulated by GA_3 treatment are most associated with functional groups as energy metabolism (3), redox regulation (3) signaling (2) and stress response (2) (Fig.4c). In FLU imbibed seeds, the up-accumulated proteins are mainly were associated to stress response (2) (Fig.4d). In ABA treatment, the proteins up-accumulated comparatively to H_2O treatment were associated with signaling (4), energy metabolism (3) and protein biosynthesis (3) (Fig.4e). On the other hand, in this treatment the proteins down-accumulated were related to protein biosynthesis (2) and stress response (2) (Fig.4e). In PAC imbibed seeds, the proteins related protein biosynthesis (4) and signaling (4) were up-accumulated while the proteins associated with protein biosynthesis (2), signaling (2) and stress response (2) were down-accumulated (Fig.4f).

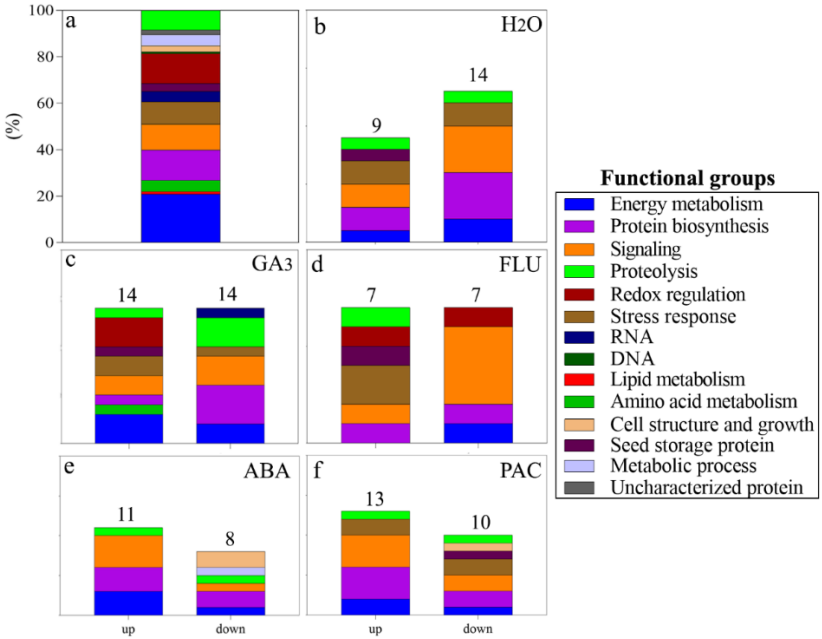

Fig.4 Functional classification of the identified proteins in all treatments (a) and differential protein accumulation in phase II of the *T.catharinensis* seed germination in H_2O (b), GA_3 (c), FLU (d), ABA (e) and PAC (f). Up, the number of proteins increases accumulation. Down, the number of proteins decreases accumulation. The comparative accumulation analysis was performed comparing H_2O, GA_3 and FLU treatments against PAC treatment, and ABA and PAC treatments against H_2O treatment. FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol

Additionally, we analyze the abundance proteins in response to GA_3 and ABA and their inhibitors in the decisive phase of seed germination by principal components analysis (PCA) (Fig.5). Component 1 showed the abundance proteins patterns in relation to the treatments applied. Component 2 showed the abundance protein arrangement among them. PCA analysis explained 84% of the variation in protein abundance and showed clearly the opposite effects of the treatments in proteins associated to promotion of seed germination (FLU, H_2O,

GA_3) and to delay or inhibit them (ABA and PAC). From this analyses, all these proteins (41) were classified into their respective biological processes (Blast2GO and UniProtKB) (Fig.5. Table1) looking for to understanding what of them are directly involved in radicle protrusion decision. Also, the proteomic profile indicates what this proteins are differently affected in GA_3, ABA, PAC, FLU and H_2O imbibed seed at phase II of *Trichocline* seed germination.

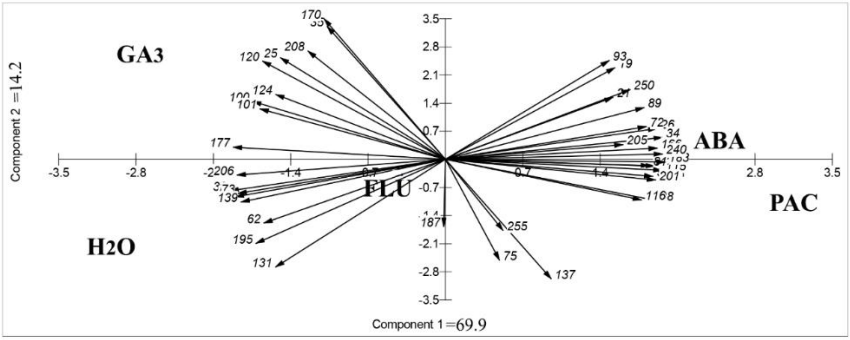

Fig.5 PCA analysis of proteome profile in phase II of the *T.catharinensis* seed germination, in H_2O, FLU, GA_3, ABA and PAC. FLU: fluridone; GA_3: gibberellin; H_2O: water; ABA: abscisic acid; PAC: paclobutrazol

Table 1 Differentially accumulation of proteins identified by comparative proteomic analysis in the embryo of *T. catharinensis* in phase II of the seed germination.

Prot Number	Protein description	Accession	Functional groups	Differential accumulation [a]				
				H_2O/PAC	GA_3/PAC	FLU/PAC	ABA/H_2O	PAC/H_2O
P19	ras-related protein RABA6a-like	A0A251U522	Signaling	Down	Unchanged	Down	Up	Up
P21	17.9 kDa class II heat shock protein-like	A0A251V797	Stress response	Down	Unchanged	Unchanged	Unchanged	Up
P25	superoxide dismutase [Mn], mitochondrial	A6XIG5	Redox regulation	Unchanged	Up	Unchanged	Unchanged	Unchanged
P26	nuclear transport factor 2	A0A251SJ77	Signaling	Down	Down	Down	Up	Up
P30	hydroxyproline-rich glycoprotein family protein	A0A251SYR0	Cell structure and growth	Unchanged	Unchanged	Unchanged	Down	Unchanged

P34	putative alpha/Beta hydrolase fold protein	A0A251V3Y5	Proteolysis	Down	Unchanged	Unchanged	Up	Up
P35	probable aldo-keto reductase 2	A0A251TBW8	Redox regulation	Unchanged	Up	Unchanged	Unchanged	Unchanged
P38	heat shock cognate 70 kDa protein 2-like	A0A251SZ04	Stress response	Unchanged	Down	Unchanged	Unchanged	Unchanged
P39	eukaryotic translation initiation factor 5A	A0A251RWG2	Protein biosynthesis	Up	Up	Up	Down	Down
P50	elongation factor 1-beta 2-like	A0A251UEP1	Protein biosynthesis	Up	Unchanged	Unchanged	Down	Down
P55	eukaryotic translation initiation factor 6-2	A0A251VDF0	Protein biosynthesis	Down	Down	Unchanged	Unchanged	Up
P62	serine/threonine-protein kinase RIPK-like	A0A251SDJ9	Signaling	Up	Unchanged	Unchanged	Unchanged	Down

P72	temperature-induced lipocalin-1-like	A0A251UK13	Stress response	Up	Up	Up	Unchanged	Down
P73	temperature-induced lipocalin-1	A0A251VPI2	Stress response	Up	Up	Up	Down	Down
P75	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	A0A251SC90	Energy metabolism	Unchanged	Down	Unchanged	Unchanged	Unchanged
P76	14-3-3-like protein	A0A251VGX1	Signaling	Down	Down	Down	Unchanged	Up
P81	eukaryotic translation initiation factor 5A-like	A0A251SAL4	Protein biosynthesis	Down	Down	Down	Up	Up
P89	eukaryotic translation initiation factor 5A	A0A251RNJ4	Protein biosynthesis	Down	Down	Unchanged	Up	Up
P93	pyruvate decarboxylase 1	A0A251SBK9	Energy metabolism	Unchanged	Unchanged	Unchanged	Up	Unchanged

P100	calmodulin-7-like isoform X2	P93171	Signaling	Unchanged	Up	Unchanged	Unchanged	Unchanged
P101	puromycin-sensitive aminopeptidase-like isoform X2	A0A251VA09	Proteolysis	Unchanged	Up	Unchanged	Unchanged	Unchanged
P115	14-3-3 protein 10	A0A251VGY2	Signaling	Unchanged	Unchanged	Unchanged	Up	Unchanged
P116	polyubiquitin 11	A0A251TC36	Proteolysis	Unchanged	Down	Unchanged	Unchanged	Unchanged
P120	luminal-binding protein 5	O04223	Stress response	Unchanged	Up	Unchanged	Unchanged	Unchanged
P124	glutathione S-transferase-like	A0A251VJT1	Redox regulation	Unchanged	Up	Up	Unchanged	Unchanged
P131	heat shock cognate 70 kDa protein-like	A0A251UWB5	Stress response	Up	Unchanged	Up	Down	Down
P137	proteasome subunit beta type-1	A0A251VCW8	Proteolysis	Unchanged	Down	Unchanged	Unchanged	Unchanged
P139	proteasome subunit beta type-1	A0A251SYL1	Proteolysis	Up	Up	Up	Down	Down

P166	actin-7	B8Y645	Cell structure and growth	Down	Down	Unchanged	Unchanged	Up
P170	ATP synthase subunit beta, mitochondrial-like	A0A251TYG9	Energy metabolism	Unchanged	Up	Unchanged	Unchanged	Unchanged
P177	oil body-associated protein 1A	A0A251TCF1	Seed storage protein	Up	Up	Up	Down	Down
P183	beta-glucosidase 24-like	A0A251SZC2	Energy metabolism	Down	Down	Unchanged	Up	Up
P187	selenium-binding protein 1-like	A0A251UA71	Redox regulation	Unchanged	Unchanged	Down	Unchanged	Unchanged
P195	guanine nucleotide-binding protein subunit beta-like protein	A0A251TJH9	Signaling	Up	Up	Up	Down	Down
P201	aspartic proteinase	A0A251SHZ3	Proteolysis	Unchanged	Down	Unchanged	Unchanged	Unchanged

P205	malate dehydrogenase, mitochondrial	A0A251TLI1	Energy metabolism	Down	Unchanged	Unchanged	Unchanged	Up
P206	glyceraldehyde 3-phosphate dehydrogenase	A0A251U0Y5	Energy metabolism	Up	Up	Unchanged	Down	Down
P208	Enolase	A0A142K3L6	Energy metabolism	Unchanged	Up	Unchanged	Unchanged	Unchanged
P240	guanine nucleotide-binding protein subunit beta-like protein	A0A251SEI5	signaling	Down	Down	Down	Up	Up
P255	aconitate hydratase, cytoplasmic-like	A0A251V3V8	Redox regulation	Unchanged	Unchanged	Down	Unchanged	Unchanged
P267	elongation factor 2	A0A251T9L1	Protein biosynthesis	Down	Down	Unchanged	Up	Up

[a] Differentially accumulated proteins were selected based on a max fold change of at least 1.5 and ($p < 0.05$) (t test). H_2O,GA_3 and FLU treatments were compared with PAC treatment. ABA and PAC treatments were compared with H_2O treatment.

4. Discussion

4.1 Cellular alteration and protein abundance are associated to the decision of radicle protrusion from seed coat

The germination of *T. catharinensis* seeds was promoted by GA_3, FLU and H_2O application, while the opposite was observed for ABA and PAC treatments (Fig. 1). In plants, it is well documented that GA and ABA levels can be altered by PAC and FLU supplementation, respectively (Kusumoto et al., 2006). These results were confirmed by structural observations in LM and TEM analysis (Fig.2-3) and in protein abundance (Fig.4-5). GA_3 imbibed seeds showed higher vacuolization with mobilization of protein and lipid reserves in cotyledons as well in HRA region cells (Fig.2f-3d). It is quite well know that GAs stimulate the mobilization of storage reserves, as PSVs by acidification of vacuoles which hydrolyzing the stored polymers in its lumen, often with the use of pre-existing enzymes (Cooley *et al.*, 1999; Lee *et al.*, 2015). In our work, GA_3 effect also result in cytoplasm activity, evident intercellular communication, through plasmodesmata and thin cell wall. Together with cellular alteration GA_3 up-accumulated proteins related energy metabolism, redox regulation and stress response which result in seed germination (Fig. 4 Table 1). The important role of the plant hormones gibberellins (GAs) in promoting seed germination is indicated by several observations. The first one is the induction of the expression of genes encoding enzymes hydrolyzing the endosperm, which confers part of the mechanical resistance to radicle protrusion, as demonstrated in tomato (Groot e Karssen, 1987), tobacco (Müller *et al.*, 2006) and barley (Schuurink *et al.*, 1992). The second mechanism consists of a direct stimulating effect on the growth potential of the embryo and seedling, as suggested for Arabidopsis (Karssen *et al.*, 1989). In *Acer platanoides,* the role of GA in promoting seed germination can be performed by up-regulated proteins: ATPase (associated energy metabolism) EF-2, (protein biosynthesis) and proteasome proteins (proteolysis) (Pawłowski, 2009). In FLU imbibed seeds, observed cellular alterations similar to H_2O and up-accumulated proteins to stress response and down-accumulated proteins related the signaling. Application of ABA affect the HRA elongation (Fig. 2b), by reducing the mean cell length (Fig. 2j). Physiological studies on the effect of ABA during germination have delineated that: ABA may or may not prevent (1) external rupture of the testa (2) extension of the HRA (3) and interruption of HRA protrusion by

the endosperm (Müller *et al.*, 2006; Steinbrecher e Leubner-Metzger, 2016). In sunflower seeds, application of exogenous ABA inhibits only the extension of the HRA, while its removal leads to the conclusion of germination (El-Maarouf-Bouteau *et al.*, 2015). These reports corroborate with the results observed in our work with *T. catharinensis* seeds, which also is an Asteraceae *sp*. However, we must consider that ABA can be involved in different responses in plants, and that its exogenous application can have multiple effects after imbibition. In our work, it was clearly showed by cellular analysis that application of ABA delays the degradation of protein and lipid reserves (Fig.2o; Fig.3n) as well reported by Müller *et al.* (2006) and this possible to limit the availability of energy and nutrients to the growth potential of the embryo. This was also observed in *Arabidopsis*, what exogenous ABA delays seed germination, in part, reducing lipid degradation (Penfield *et al.*, 2004). This can be explained also once the genes involved in the conversion of triacylglycerols (TAG) into soluble sugars are down-accumulated in response to ABA (Pritchard *et al.*, 2002). The plasmalema retraction observed as an ABA effect on the cells (Fig.2k; 3k star) as well the coalescence of vacuoles, surrounded protein reserves (PSVs) while lipids bodies are close to the cell membrane can be explain at least in part by the ABA function in osmotolerance. ABA and osmotic stress interact at a common point that controls the water relations of the embryo specifically during the stretching phase (Müller *et al.*, 2006; Finkelstein, 2013). This phase is characterized by the resumption of rapid water absorption, due to increased growth of the active embryo (Schopfer e Plachy, 1985). According to Schopfer e Plachy (1985) the inhibitory effect of ABA on water absorption has been shown to be rapidly eliminated after removal of the hormone. One possible explanations for this effect is that with exogenous application of ABA, there is an accumulation of transcription factor (FT) - ABA-Insensitive 5 (*ABI5*), and regulation at the level of transcription is important for the ABA response (Piskurewicz *et al.*, 2008). In addition, ABA added exogenously or produced in response to osmotic stress prevents germination and confers osmotolerance by stimulating de novo accumulation of FT-*ABI5* (Lopez-Molina *et al.*, 2002; Piskurewicz *et al.*, 2008). Another characteristic associated with ABA accumulation is the thickening observed in the cell wall (Fig.3l detail). Exogenously applied ABA induced enhanced accumulation of pectic arabinan in cells of root meristem of Arabidopsis (Talboys *et al.*, 2011). This may influence mechanical property of the cell wall, and explain at least in part, the effect observed in the HRA elongation in this work. Pectin-degrading enzymes can be down-accumulated by water stress with consequences on

cell expansion (Pandey *et al.*, 2010). During water stress, the amount of side chains it seems increased, with consequent effects on the viscosity state of the cell wall (Le Gall et al., 2015). ABA is a fundamental molecule that strongly regulates the result of the interaction between plants and a harmful environment (Kundu e Gantait, 2017). Moreover, the link of this cellular alteration with proteins accumulation was evident, once ABA imbibed seed showed down-accumulated proteins related to energy metabolism and cell structure and growth (Fig. 4, Table 1). PAC application showed cells with few organelles and plasmodesmata associated with low metabolism activation, and no reserves mobilization (Fig.3g-h). When mobilization of PSVs are inhibited, the vacuolar coalescence is concomitantly prevented (Hwang *et al.*, 2003). In *Arabidopsis* seeds, PAC application blocks lipid catabolism (Penfield et al., 2004). In soybean seeds, with inhibition of GA biosynthesis by PAC, hypocotyl elongation and mobilization of storage reserves were inhibited (Ren *et al.*, 2007). These finds confirm the results of our work and can associated to the PAC protein profile in comparison the GA_3 (Fig. 4, Table 1). PAC up-accumulated proteins associated signaling and biosynthesis protein e down-accumulated proteins related to energy metabolism and stress response as we forward discuss.

4.2 Overview of functional and differential protein accumulation in response to GA_3 and ABA and their inhibitors associated to promotion or inhibition of seed germination

During the germination of *T. catharinensis* seeds, the time interval between 72 and 75 h after imbibition, we determined as phase II (*sensu stricto*) (Fig.1). In non-dormant seeds the proteins synthesized during the first hours of imbibition are associated with the seed maturation program and the germination program being activated only later during the 8-24 h imbibition window (Galland *et al.*, 2014). Considering this, we performed the proteomic profile analysis in phase II of the germination process. Related to this, it is valuable to identify protein profile considering, a wild species from biodiverse plant family (Asteraceae). In addition, germination is a very interesting process, during which a series of physiological and biochemical reactions, including signal transduction, regulation of gene expression, reactivation of metabolism and regulation of redox homeostasis occur (Bewley *et al.*, 2013; Pawlowski e Staszak, 2016; Liu *et al.*, 2018). Our results showed that the main processes associated with up-accumulated proteins are related to energy metabolism, involving glycolysis, TCA cycle, fermentation and glyoxylate cycle (20.81%),

biosynthesis, protein modification and folding (13.01%) and redox regulation (13.01%). These results have also been identified by other proteomic studies, with different species: *Lepidium sativum* (Müller *et al.*, 2010) *Arabidopsis thaliana* (Galland *et al.*, 2014), *Oryza sativa* (Han, C. *et al.*, 2014), *Pisum sativum* (Wang, W.-Q. *et al.*, 2012), in which translation, detoxification, amino acid metabolism and energy metabolism are mainly activated during phase II germination. PCA analysis revealed considerable differences in patterns in the expression of the proteins associated to promotion or inhibition of seed germination in response of exogenous GA_3, ABA and their inhibitors (Fig.5). The analysis of PCA were responsible for explaining 84% of the variation in protein abundances. In subsequent section we discuss the function of these proteins in metabolic pathways relating to the role of GA_3, ABA and their inhibitors in the seed germination.

4.3 Proteins related to stress response

In the treatments with the highest percentage of germination, GA_3, FLU and H_2O up-accumulated heat shock 70 kDa proteins (HSPs) (P131). These proteins are involved in maintenance of cellular homeostasis and proper protein biogenesis (Lin *et al.*, 2001). Heat shock proteins help in the refolding of damaged proteins and stabilize proteins at intermediate stages of folding or degradation and the seed germination, with the return to full metabolism, is very stressful for the plant (Bailly, 2004; Bewley *et al.*, 2013). These results suggest that under stress conditions, folding and processing of proteins are improved in order to maintain the normal function of the proteins and thereby complete the germination of the seeds. Similar results with increased in HSPs abundance in *Oryza sativa* seeds germination were observed by Liu *et al.* (2015). Also, Yang *et al.* (2009) found that this protein showed high accumulation during germination of *J. curcas* seeds. Another chaperone protein up-accumulated by GA_3 (P120) was a luminal-binding protein 5 (BiP 5), which is required for protein synthesis, folding, and secretion in the ER (Zhu et al., 2014). In GA_3, H_2O and FLU treatment, the proteins (P72 and P73) temperature-induced lipocalin-1 (TIL) was up-accumulated, and down-accumulated in ABA and PAC, which may be associated to the elimination of lipophilic molecules that could be harmful. The scavenging of free radicals generated after abiotic stress conditions, preserving cell viability and helping to restore the membranes integrity (He, X. *et al.*, 2015; Porro *et al.*, 2017) together with TIL1 that is an essential component for thermotolerance (Chi *et al.*, 2009). The protein P21

(17.9 kDa class II heat shock protein-like) showed up-accumulation in PAC. Small heat shock proteins (sHSPs) are a large group of proteins, range in size from 12 to 42 kD. sHSP accumulation could also be triggered by other stress conditions, such as drought, salinity, and cold, and by developmental regulation, such as seed germination (Zhang *et al.*, 2018). Extensive studies on the function of sHSPs commonly depict them as ATP-independent molecular chaperones (Koo *et al.*, 2015). During germination, HSP17.9 disappear after the major storage proteins, which are degraded (Coca *et al.*, 1994; Wehmeyer *et al.*, 1996), this accumulation observed in PAC may be due to low reserve mobilization and the stress conditions generated from it.

4.4 Proteins related to redox regulation process

In this study, GA_3 imbibed seed showed up-accumulated proteins superoxide dismutase (SODs) (P25), probable aldo-keto reductase 2 (35), glutathione S-transferase (P124). All of them can reduce the target proteins disulfide and keep these functional proteins (Buchanan and Balmer, 2005). Also, reducing oxidized proteins is another critical way to cope with ROS (He, M. *et al.*, 2015). The protein P35 has been reported to detoxify a wide variety of lipid peroxidation or glycolysis derived cytotoxic compounds (Colrat *et al.*, 1999). Seed germination is usually accompanied by extensive changes in the redox state of seed proteins (Bailly *et al.*, 2008). Proteins present in an oxidized form in dried seeds were converted to the reduced state after imbibition (Alkhalfioui *et al.*, 2007). In the germination of *T. catharinensis* seeds, GA_3 and FLU treatments that result in higher germination showed intensive regulation of oxidative stress through the activity of SOD enzymes, CAT and APX (Lando *et al.*, (*In Press*) 2018).

4.5 Proteins related to energy metabolism

Only in GA_3 imbibed seed, the proteins P170 (ATP synthase subunit beta, mitochondrial-like) and P208 (enolase) were up-accumulated. The enolase (P208) is a metalloenzyme that, as part of the glycolytic pathway, catalyze the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate (PEP) (Canback *et al.*, 2002). This process is required for ATP production, and in addition serves as a precursor for the synthesis of aromatic compounds and secondary metabolites (Vermerris and Nicholson, 2006). The synthesis of ATP is mediated by the enzyme ATP synthase (P170), which plays a key role in

energy transduction in living cells (Walker *et al.*, 1982). The potential to produce ATP, largely reflect metabolic activity, and was correlates with germination percentage in *Beta vulgaris* and Brassica (Lunn e Madsen, 1981) similarly to the observed in our work in GA_3 imbibed seed (98,75%)(Fig.1). In addition, in our results, GA_3 stimulates cellular activity, showing associated organelles (mitochondria, RER and glyoxysomes) (Fig. 3d-e-f). This finds suggest that in the GA_3 imbibed seeds, the germination may be associated with increased respiration due to the gain of mitochondrial functions. In seed germination of *Pinus massoniana*, GA_3-treated seeds began to respire first and consumed oxygen at the fastest percentage (Guangwu e Xuwen, 2013). Similar results were found in tomato (*Lycopersicon esculintum*), oat (*Avena sativa*), and sugar beet (*Beta vulgaris*) (Gui *et al.*, 1991; Lecat *et al.*, 1992; Rochalska e Orzeszko-Rywka, 2008). Also, it was reported that GA_3 stimulates the activity of energy metabolism proteins (Pawłowski, 2009; Pawlowski e Staszak, 2016) which confirm our suggestion. The protein P206 (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, that is a central step in metabolism linking the energy consuming with the energy producing steps of glycolysis (Plaxton, 1996). The GAPDH is a critical enzyme for respiration, which is one of the most important metabolic processes for germination (Leon *et al.*, 2006). The P206 were up-accumulated in GA_3 imbibed seeds and H_2O and down-accumulated in ABA imbibed seeds and PAC which can be associated to the opposite treatments effect in in seed germination percentage (Fig.1) and cellular features (Fig.3). In support of this results, the protein P93 (pyruvate decarboxylase 1 (PDC) is up-accumulated in ABA imbibed seeds. The ethanolic fermentation pathway branches off the main glycolytic pathway at pyruvate, which PDC catalyzes the first step in this pathway (Kürsteiner *et al.*, 2003). This suggests that ABA imbibed seeds have a lower threshold of oxygen levels needed to promote germination and presence of immature or damaged mitochondria (Rosental *et al.*, 2014). Alternative pathways can provide energy for germination, however, the toxicity of fermentation products can be a limiting factor for seed germination (Benvenuti e Macchia, 1995). This finds can be associated to the delay and low percentage of germination observed in ABA treatment. The protein P183 (*β*-glucosidase 24-like) this is associated in key developmental processes, such as growth, pathogen defense and hormone hydrolysis (Hu *et al.*, 2012). The P183 were up-accumulated in response the ABA and PAC. Lee *et al.* (2006) provided direct evidence that ABA-GE (conjugation form) can be hydrolyzed in response to stress by a member of multigene family

of β-glucosidases, resulting in an increase in the concentration of active ABA. ABA added exogenously confers osmotolerance, overall, the ABA-dependent pathways are involved essentially in osmotic stress gene expression (Lee *et al.*, 2006; Tuteja, 2007). The cellular ABA level is constantly adjusted to respond to changing physiological and environmental conditions (Wang *et al.*, 2011). Possibly ABA levels increased with exogenous application and inhibition of GA synthesis by PAC, since up-accumulated the protein β-glucosidase compared to GA_3 and H_2O treatments which they were down-accumulated. The protein P205 (malate dehydrogenase, mitochondrial (mMDH) showed up-accumulated in PAC imbibed seeds comparatively to H_2O. mMDH has multiple roles; MDHs play a critical role in the tricarboxylic acid cycle in the mitochondria, where they generate NADH by oxidizing malate to oxaloacetate (Sew e Ströher, 2016). A redox equilibrium mediated by mMDH also is important, several reports have indicated that MDHs have a close relationship with stress resistance (Wang *et al.*, 2010). The accumulation of this protein in PAC imbibed seeds, may be associated with the stress generated, since the beginning of the low germination percentage, only occurred after a long time of imbibition.

4.6 Proteins related to protein biosynthesis

The protein eukaryotic translation initiation factor 5A (P39) were up-accumulated in GA_3, H_2O and FLU imbibed seeds and protein elongation factor 1-beta 2-like (P50) were up-accumulated in H_2O. The protein P39 (EIF-5A) are involved in multiple biological processes, including protein synthesis regulation, translation elongation, programmed cell death (Xu *et al.*, 2011; Wang, L. *et al.*, 2012) while eIF5A plays roles in regulating osmotic stress (Ma *et al.* (2010). However, it was reported that the activity of eIF5A is not absolutely essential for general protein synthesis but facilitate this by acting as a nucleocytoplasmic shuttle protein, selectively translocating specific subsets of mRNAs from the nucleus to the cytoplasm (Henderson e Hershey, 2011). Already, the elongation factor 1-beta (EF-1β) (P50) participates in the polypeptide elongation cycle during protein synthesis (Janssen e Möller, 1988). In our work, we also observed significant up-accumulation of these proteins in ABA and PAC imbibed seeds. The proteins P55 (eukaryotic translation initiation factor 6-2) and homologous protein P81, P89 and P267 (elongation factor 2) were up-accumulated in ABA and PAC imbibed seeds. It seems that ABA controls protein synthesis both negatively and positively in barley aleurone cells by regulating the level of

mRNA and possibly also by changing the efficiency of translation (Higgins *et al.,* 1982). In Arabidopsis, were observed that protein synthesis activity even substantially increased for the seeds imbibed in ABA (Chibani *et al.,* 2006). Thus, seeds can acquire the capacity to reprogram the pattern of protein biosynthesis during imbibition, allowing the conclusion or not of germination (Rajjou *et al.,* 2004). It can be suggested that in the seeds imbibed in ABA and PAC, for the long time of imbibition, there could be a reprogramming of these proteins related to protein biosynthesis (P55, P81, P89 and P267), which would have relation with the result (delay and inhibition of germination).

4.7 Proteins related to signaling

Seeds are able to activate a series of mechanisms to respond to many biotic and abiotic stresses during germination (Yu *et al.,* 2014). Cells recognize external stresses and induce many signal proteins, including signaling receptor kinases, calcium-dependent protein kinases, G-proteins and 14-3-3 proteins (Yu *et al.,* 2014). In the GA_3 imbibed seeds, the protein Ca^{2+} signaling-related, calmodulin (P100) were up-accumulated. Ca^{2+} is a prominent second messenger in signal transduction cascades in plants and is involved in a wide variety of growth and development processes, including plant responses to various environmental stimuli (Buchanan *et al.,* 2015). The involvement of calcium signaling in seed germination has been documented (Lu *et al.,* 2008; Han, Chao *et al.,* 2014) which explain this protein abundance observed in our work in this treatment. In H_2O imbibed seeds, the protein serine/threonine-protein kinase RIPK-like (P62) were up-accumulated. This protein P62, is a receptor-interacting protein (RIP) kinases are involved in protein phosphorylation, and this is one of the most important regulatory mechanisms known to control protein activity and cellular signaling (Chevalier e Walker, 2005). Other protein associated the signal transducing, is the heterotrimeric G-proteins (G-proteins) (P195) that are up-accumulated in H_2O, GA_3 and FLU imbibed seeds. A heterotrimeric G protein mediate many of the signals that seeds use to control germination (Assmann, 2002). They are associated in the signaling in GA-stimulated expression of several genes, including expression of the gene encoding the hydrolytic enzyme α-amylase, which breaks down carbohydrate reserves and thus mobilizes nutrients that nourish the young seedling (Assmann, 2005). Seed germination is the outcome of an integration of many signals, that determination of the hormone sensitivities, we observe in our results, that the

G protein, also up-accumulated in response to the ABA and PAC. Associated this, the protein P76 and P115 (14-3-3-like protein) were up-accumulated in seeds imbibed in PAC and ABA. This 14-3-3 proteins integrate multiple hormone pathways, thus controlling a specific physiological process (e.g., ABFs factors in GA and ABA signaling) (Camoni *et al.*, 2018). Alternatively, 14-3-3 protein can regulate hormone action at the post-translational level, by modulating the activity of proteins in signaling cascades (e.g., IAA, ABA) or in biosynthetic pathways (e.g., ethylene, GA) (Camoni *et al.*, 2018). Also, 14-3-3 isoforms are constituents of transcription factor complexes and interact with components of abscisic acid (ABA)-induced gene expression machinery (Fulgosi *et al.*, 2002). Based in this finds our results suggest that the accumulation observed in treatments (ABA and PAC) is associated to ABA response, since when we inhibit GA synthesis, the hypothesis is that ABA levels are maintained in the seed. It was reported that these 14-3-3 proteins control the activity of the plasma membrane H+ -ATPase, and this makes 14-3-3 a prime candidate for molecular master regulator of the cellular osmo-pump activity by ABA which was associated to the controlling the growth of the embryonic root during seed germination (Van Den Wijngaard *et al.*, 2005). Our results from LM and TEM analyses, showed lower cellular elongation of HRA, especially in ABA (Fig.2b-c) and changes associated to osmoregulation (Fig.3j,k,l). Together with these results, other proteins associated with signaling, ras-related protein RAB (P19) and nuclear transport factor 2 (NTF2) (P26), were up-accumulated in ABA and PAC imbibed seeds. Ras-related protein is a small GTPase superfamily endosomal protein involved in endocytosis (Ueda *et al.*, 2001). They are involved in the transport and coupling of specific vesicles trafficking which is important for the location of hormone receptors and other elements of the signaling machinery (Aoki *et al.*, 2006). Interference with these processes would be expected to have effects upon cell wall metabolism, and this is probably important in tip growing systems (Blatt e Campanoni, 2006). This may be associated with ABA and PAC treatments, which showed alterations in HRA elongation, plasmalema retraction and especially in ABA thickening in the cell wall (Fig.2b,k,m; Fig.3l). The protein NTF2 is involved in bi-directional translocation of macromolecules, ions and small molecules between cytoplasm and nucleus (Zhao e Meier, 2011). This protein is associated the GTPase in the transport of macromolecules into and out of the nucleus via the establishment of a protein gradient between the cytoplasm and nucleoplasm (Quimby *et al.*, 2000). We suggest that the nuclear import of

proteins selectively may in the response of the seeds to the reduction of GA levels, which later results in the delay or inhibition of germination.

4.8 Proteins related to proteolysis

During germination, proteasome activity increases and is up-accumulated in H_2O, GA_3 and FLU imbibed seeds (P139, proteasome subunit beta type-1). In agreement with this, recent proteomic studies have shown that some proteasome subunits are synthesized de novo during seed germination in Arabidopsis and Oryza (He *et al.*, 2011; Galland *et al.*, 2014; Chiu *et al.*, 2016). That suggest an active proteasome is required for germination, since in the treatments with inhibition or delay (PAC and ABA) in seed germination, the P139 were down-accumulated. Imbibition marks a significant change in protein metabolism, once the proteins that were synthesized and stored in abundance in the seed are broken down to free amino acids for biosynthesis and energy generation (Tan-Wilson e Wilson, 2012). The protein aspartic proteinase (ASPG) (P201) is down-accumulated by GA_3. ASPG is involved in storage protein degradation, it hydrolyses seed storage proteins to provide amino acids for seed germination (Mutlu e Gal, 1999). In *Lepidium sativum*, aspartic protease increased from 8 h to 18 h during germination (Müller *et al.*, 2010). Similar results were observed in *Magnolia* (Lu *et al.*, 2016) and *Vigna* (Kulkarni e Rao, 2009). Moreover, during germination, the accumulated of aspartic proteinase leads to enhanced ABA sensitivity (Yao *et al.*, 2012). Thus, aspartic proteinase initially up-accumulated and then down-accumulated. This explains our results, and we confirm in TEM analysis, that the mobilization of reserves is early with GA_3 (Fig. 3-d-e-f). Associated to this, the protein puromycin-sensitive aminopeptidase-like isoform X2 (P101) is up-accumulated by GA_3. The small peptides produced by the joint action of proteases are first transported and then hydrolyzed by aminopeptidases (APs) to amino acids (Mikola, 1983). APs are major N-terminal modifying enzymes which catalyze the hydrolysis of amino acids from the N-terminus of peptides and proteins (Matsui *et al.*, 2006). Confirming that GA_3 anticipates the mobilization of reserves in comparison to FLU and H_2O, and provides free amino acids required for the synthesis of new proteins. The protein degradation also occurs under conditions that induce oxidative stress (Palma *et al.*, 2002). The protein P34 (putative alpha/Beta hydrolase fold protein (ABH) are up-accumulated in the ABA and PAC imbibed seeds. BHs are commonly associated with housekeeping roles that participate in the breakdown and recycling of cellular metabolites, exercising play key

regulatory roles in metabolism (Mindrebo *et al.*, 2016). Phospholipases, many belonging to the plant ABH family, are key to generating chemical signals at the cell membrane to regulate cell function and respond to environmental cues (Canonne *et al.*, 2011). ABHs also participate in the breaking of carbon-carbon bonds, decarboxylation reactions (Auldridge *et al.*, 2012; Mindrebo *et al.*, 2016). The up-accumulation in these treatments may be due to the increase in signaling under stress conditions generated by the exogenous application.

4.9 Proteins related to seed storage protein

In cells, lipids required for energy supply are stored in lipid bodies (LB), also called lipid droplets or oil bodies (OBs) (Purkrtova *et al.*, 2008). The oil body-associated protein 1A (P177) were up-accumulated in GA_3, H_2O and FLU imbibed seeds. We observed in the ultrastructural analysis of cells, mobilization the LBs these treatments (Fig.3 a-d-g). Storage lipids are mobilized rapidly during seed germination and early seedling establishment (Bewley *et al.*, 2013). Germination percentages decrease when there is expansion of OBs, which may push the nuclei to the cell periphery and may prevent them from playing their roles, inducing cell degeneration and death (Shimada *et al.*, 2008). Moreover, the average size of the OBs reduces the accessibility of lipases to the TAGs during germination (Siloto *et al.*, 2006), which explains the results observed in seeds imbibed in PAC, that showed cells with cytoplasm filled by lipids bodies.

4.10 Proteins related to cell structure and growth

Proteins involved in cell wall modifications and growth were down-accumulated by ABA (P30, hydroxyproline-rich glycoprotein family protein). Hydroxyproline-rich glycoproteins (HRGPs) (P30), a major group of wall glycoproteins, play important roles in plant growth and development (Johnson *et al.*, 2017). Also, HRGPs are thought to exhibit broad functionality, involved in cell division and expansion, participates in cell wall formation by interacting with various cell wall components, among other functions (Johnson *et al.*, 2017; Li *et al.*, 2018). The negative regulation of this protein by ABA, it may explain in part, the results observed as the lower elongation of the HRA, showed flattening of the cells. Actin-7 (ACT-7) (P166) it is a fundamental part of the cytoskeleton, involved in several basic developmental processes including the establishment of cell polarity, cell division plane determination, cell wall deposition, and cell elongation (Meagher *et al.*, 2000). We observed that

ACT-7 down-accumulated in GA_3 and H_2O imbibed seeds and up-accumulated in PAC imbibed seeds. It seems that the patterns of *ACT7* expression are altered by treatment with exogenous hormones, indicating that *ACT7*, unlike most other actins, is responsive to the changing environment of the plant (Mcdowell *et al.*, 1996; Kandasamy *et al.*, 2001). Furthermore, expression of other actin may overlap ACT7 expression suggesting some functional redundancy between members of the actin family (Gilliland *et al.*, 2003), which may explain in part this accumulation observed in PAC imbibed seeds, which inhibited germination and showed lower HRA elongation.

5 Conclusions

The results of the present work showed by PCA analysis, differential protein abundance in response to GA_3, ABA and their inhibitors PAC and FLU in association, cell alterations at phase II, of *Trichocline catharinensis* seed germination. These changes are associated to the transition of water uptake to the radicle protrusion. GA_3 imbibed seeds, improve enhancing cellular activity and mobilization of reserves, by up-accumulated thirteen proteins associated energy metabolism and redox regulation, resulting in 98,75% of germination. With opposite response mechanism, PAC imbibed seeds, down-accumulated proteins associated energy metabolism and redox regulation. The cells remain in phase II, implying in cellular alterations as low metabolism activation, and no reserves mobilization, explaining the 7% of germination. These ultrastructural changes are associated with up-accumulation of proteins related to signaling in PAC. The application of exogenous ABA retards the transition from the water absorption phase II to III, resulting in osmotolerance, observed by the plasmalema retraction, as well the coalescence of vacuoles. ABA up-accumulated proteins related to osmotic stress response and down-accumulated proteins related to cell structure and growth, that interruption the extension of the HRA, showed flattened cells. FLU and H_2O imbibed seeds showed cell changes similar, and up-accumulated proteins energy metabolism and stress response, resulting in 96% of germination. Our findings providing new information of cellular response mechanisms associated with proteins, from water uptake to the decision of radicle protrusion from a wild Asteraceae *sp* from southern Brazil. Also, it is a starting point for establishing new studies on seed physiology, considering especially, wild plants families.

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os resultados apresentados no presente trabalho, aprimoram o conhecimento fisiológico sobre qualidade e controle da germinação de sementes, e dão origem a uma base biológica para futuros estudos que buscam o uso e a conservação de espécies nativas com valor ecológico e econômico. O sucesso de um cultivo, seja ele comercial ou não, se dá em grande parte, pela viabilidade e germinação das sementes. Portanto, a biologia das sementes deve estar sempre no tópico das pesquisas sobre uso e conservação da biodiversidade. Esse trabalho teve como proposta examinar o papel do GA e o ABA no controle da germinação a fim de compreender como os níveis relativos destes dois hormônios são coordenados na semente. Para isso foi usado os inibidores de biossíntese do GA- PAC e ABA- FLU. Isso permitiu entender a relação dos hormônios com outras biomoléculas associadas a germinação, como proteínas, poliaminas e enzimas antioxidantes. Os resultados mostraram que esses compostos exercem efeitos na germinação das sementes de *T. catharinensis.* FLU e GA_3 mostraram efeitos estimulantes na germinação. ABA mostrou atraso e baixa porcentagem de germinação 58,75% e PAC inibiu 7.5%. Os resultados confirmam que há uma coordenação dos níveis de GA e ABA na semente para que ocorra a germinação. Para estudos posteriores, a quantificação endógena de ABA e GA são necessários nas três fases da germinação, para comprovar, se quando há inibição da síntese de GA, aumenta os níveis de ABA endógeno ou quando a síntese de ABA é inibida, os níveis de GA aumentam e após quanto tempo de embebição, esse antagonismo acontece. Com os resultados obtidos, observamos que há uma relação direta entre PAs e principalmente GAs. Para futuros estudos, a quantificação endógena dos hormônios faz-se necessária e possivelmente torne claro o crosstalk entre essas moléculas. Uma vez que PAs exógenas (200 µM) promoveram a germinação das sementes. PAs também mostraram uma relação com o sistema antioxidante. O que pode ser base para novos estudos envolvendo tolerância a dessecação, “priming” de sementes visando a conservação das sementes.

A análise de citometria mostrou uma porcentagem pequena de células com conteúdo de DNA 4C (fase G2 do ciclo celular), o que juntamente com as observações estruturais confirmam o início da divisão celular antes da protrusão da radícula. As análises em citometria precisaram ser repetidas algumas vezes, para ter a maior padronização possível do material, que apresentou dificuldades

devido a grande variação apresentada nos histogramas, pela espécie não ser domesticada. Alguns cuidados são essenciais para a qualidade dos resultados, como a utilização de PVP, e o cuidado na separação das amostras, quando separar eixo radicular dos cotilédones, para que esteja o mais homogêneo possível. A metilação global do DNA, mostrou uma redução nos níveis, na fase II, relacionando-se às atividades metabólicas e à ativação transcricional, iniciada pelo processo de germinação. Interessante que quando inibimos a germinação, os níveis de metilação caem significativamente na fase III. Possivelmente há silenciamento de genes positivamente relacionados com a germinação. Para tornar mais claro essa repressão da germinação com os mecanismos epigenéticos, mais estudos aprofundados sobre quais as regiões metiladas e silenciadas são necessários. Novos estudos, relacionados a remodelação da cromatina por meio de ubiquitinação, metilação e acetilação de histonas, podem desempenhar um papel significativo na regulação da germinação de sementes.

As análises estruturais mostraram que a conclusão da germinação está baseada na ruptura do endosperma micropilar e ruptura da exotesta. O alongamento celular é precedido da formação de vacúolos através da degradação dos vacúolos de armazenamento de proteínas. As analises estruturais mostram as peculiaridades de cada semente e geram informações de como o processo de germinação é iniciado e concluído. Estudos que mostram o transporte de GA e ABA, em diferentes domínios no eixo embrionário – vascular, endoderme, córtex são fundamentais para uma melhor compreensão da interação entre os diferentes tipos celulares no embrião que geram o potencial de crescimento para que ocorra a protrusão radicular. Quando aplicamos GA, ABA, PAC e FLU observamos que os resultados apresentados na germinação, condizem com as observações ultraestruturais e análise proteômica. A análise dos componentes principais (PCA) explicou 84% da variação na expressão das proteínas e revelou classes distintas de proteínas associadas aos efeitos de FLU, GA_3, PAC, ABA e H_2O. Essas proteínas diferencialmente acumuladas associadas às alterações celulares são críticas para a transição da absorção de água para a protrusão da radícula. Os efeitos da aplicação GA_3, ABA, PAC e FLU durante a embebição são resumidos na Figura 1.

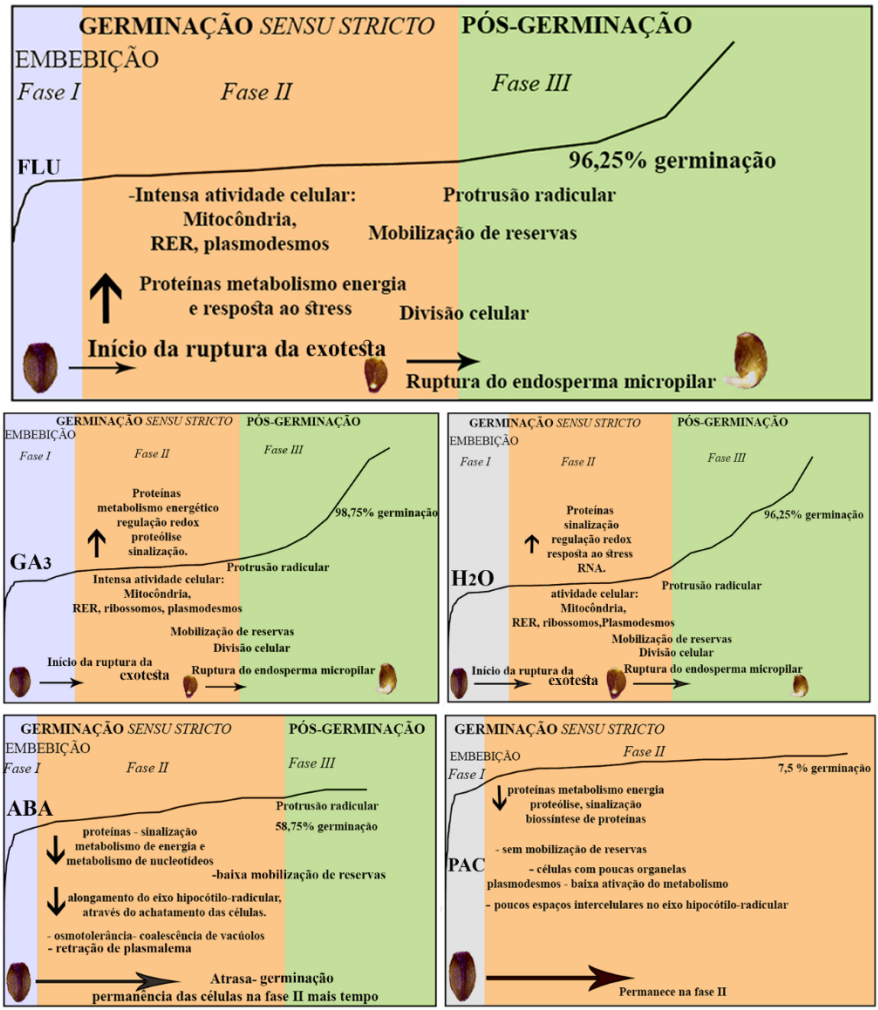

Fig.1 Esquema ilustrativo, mostrando a curva de embebição e as principais alterações associadas a cada solução aplicada FLU, GA_3, H_2O, ABA e PAC. Na curva são apresentadas as principais alterações celulares juntamente com aumento ou diminuição das proteínas em relação aos grupos funcionais em resposta as soluções aplicadas. Como resultado ao final da curva é apresentado a porcentagem de germinação obtida e as características morfológicas associadas a protrusão da radícula e início da fase III.

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