



PÓS GRADUAÇÃO
**BIOTECNOLOGIA
VEGETAL**

**MOBILIZAÇÃO DE RESERVAS EM EIXO EMBRIONÁRIO
DE SOJA (*Glycine max L. Merr.*) DURANTE A
GERMINAÇÃO**

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DARCY RIBEIRO – UENF

Campos dos Goytacazes – RJ

Julho de 2021

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Dissertação apresentada ao Centro de Biociências e
Biotecnologia, da Universidade Estadual do Norte
Fluminense, como parte das exigências para
obtenção do título de Mestre em Biotecnologia
Vegetal.

Orientadora: Dra. CLÍCIA GRATIVOL GASPAR DE MATOS

UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO - UENF
Campos Dos Goytacazes Julho/2021

FICHA CATALOGRÁFICA

UENF - Bibliotecas

Elaborada com os dados fornecidos pela autora.

M827 Moraes, Juliana Lopes.

Mobilização de reservas em eixo embrionário de soja (*Glycine max L. Merr.*) durante a germinação / Juliana Lopes Moraes. - Campos dos Goytacazes, RJ, 2021.

65 f. : il.

Inclui bibliografia.

Dissertação (Mestrado em Biotecnologia Vegetal) - Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Biociências e Biotecnologia, 2021. Orientadora: Clicia Grativol Gaspar.

1. Soybean. 2. Embryonic axis. 3. Germination. 4. Proteomics. 5. Biochemical quantification. I. Universidade Estadual do Norte Fluminense Darcy Ribeiro. II. Título.

CDD - 660.6

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Aprovada em 30 de julho de 2021

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Este trabalho foi realizado no Laboratório de Química e Função de Proteínas e Peptídeos (LQFPP) da Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), sob orientação da Drª Clícia Grativol Gaspar de Matos e financiado pela FAPERJ e UENF.

Agradecimentos

Agradeço primeiramente a Deus pela proteção, saúde, força e discernimento para conduzir esse trabalho que ocorreu em sua maior parte em meio a pandemia da COVID-19.

A toda minha família, que mesmo distantes, me apoiaram e entenderam minhas ausências.

A minha orientadora Clícia, por me conduzir nesta trajetória que não foi nada fácil em meio a pandemia, sendo compreensível e atenciosa. Minha imensa

gratidão pelas oportunidades, parceria, ensinamentos, paciência e puxão de orelha que foram necessários para meu crescimento pessoal e profissional na ciência. Você é uma profissional inspiradora!

Ao meu marido Wagner, o maior incentivador de toda essa experiência, agradeço por todo carinho, compreensão, e por estar sempre ao meu lado, me dando todo suporte.

Agradeço a todos os meus colegas pesquisadores do laboratório pelas trocas e experiências vividas, pela oportunidade de novos aprendizados e pela colaboração direta ou indireta no desenvolvimento deste trabalho. Em especial a Fernanda Coelho e Sara Sangi por todo suporte para a realização dos trabalhos de bancada, por sempre se prontificarem a me ajudar, a elas minha eterna gratidão.

A Fernanda, Walaci e Jacymara que são meus amigos para a vida, agradeço imensamente pela amizade, por todas as gargalhadas e pelo acolhimento diante dos obstáculos que surgiram nessa trajetória, sem vocês com certeza teria sido muito mais difícil.

Aos laboratórios LQFPP, LFBM, LBT e LBCT pela disponibilização dos equipamentos utilizados durante esse trabalho.

À UENF pela estrutura, suporte e oportunidade de completar o Mestrado gratuito e de qualidade em Biotecnologia Vegetal.

Aos professores por todo conhecimento compartilhado.

Dedicatória

Dedico esse trabalho a toda minha família que não tiveram a oportunidade de chegar onde cheguei e a mim, menina do interior, que mesmo diante do desconhecido nunca desisti, todos os desafios enfrentados e conhecimento adquirido me tornaram uma pessoa melhor me fazendo chegar até aqui.

LISTA DE ABREVIATURAS E SIGLAS

HAE	HORAS APÓS A EMBEBIÇÃO
SAM	S-ADENOSYLMETHIONINE SYNTHETASE
LEA	LATE EMBRYOGENESIS ABUNDANT PROTEIN
RMLC-LIKE	CUPINS
GS	GLUTAMINE SYNTHASE
WAXY	GRANULE-BOUND STARCH SYNTHASE
BMY	β -AMILASE
Susy	SUCROSE SYNTHASE
PFK	PHOSPHOFRUCTOKINASE

PK	PIRUVATE KINASE
PEP	phosphoenolpyruvate
UGDH	UDP-GLUCOSE PYROPHOSPHORYLASE-UDP-GLUCOSE DEHYDROGENASE
AXS	UDP-D-XYLOSE SYNTHASE
GLU	β -1,3 GLUCANASE
XTH	XYLOGLUCAN ENDOTRANSGLUCOSYLASE HYDROLASE
RS	RAFFINOSESYNTHASE
IMP	INOSITOL MONOPHOSPHATASE
ALDH	ALDEHYDE DEHYDROGENASE
ME	NADP-MALIC ENZYME
MDH	MALATE DEHYDROGENASE
OAA	Oxaloacetate
PEPCK	PHOSPHOENOLPYRUVATE CARBOXYKINASE
AST	ASPARTATE AMINOTRANSFERASE
APX	ASCORBATE PEROXIDASE
RPS	RIBOSOMAL PROTEINS
RPL	RIBOSOMAL PROTEINL
Trx	THIOREDOXIN
GSTF	GLUTATHIONE-S-TRANSFERASE
PDI	PROTEIN DISULFIDE ISOMERASE

Resumo

A produção de sementes é um dos principais segmentos do setor agrícola, não só no Brasil, mas em nível mundial. A soja (*Glycine max (L.) Merr.*) é uma cultura de grande importância para o agronegócio mundial. As sementes de soja possuem um alto valor nutricional, pois são ricas em proteínas, carboidratos complexos, fibras alimentares e óleos. Eventos bioquímicos e moleculares para mobilização de nutrientes dos tecidos de reservas durante o processo germinativo são bastante estudados. Entretanto, tais eventos são pouco elucidados no eixo embrionário. O objetivo deste trabalho foi analisar a mobilização de reservas em embriões de sementes de soja durante a

germinação. Através de análises histoquímicas foi observada uma reação positiva para proteínas em 3 horas após a imersão (HAE) em relação a 24 HAE. A análise histoquímica para amido evidenciou uma maior reação positiva em 24 HAE, mostrando que esse carboidrato é sintetizado ao final da germinação. Estudo proteômico comparativo entre 3 e 24 HAE indicaram a acúmulo de diversas proteínas no eixo embrionário da semente de soja. Na análise da via metabólica, proteínas parecem ser mobilizadas nas primeiras horas de germinação, juntamente com a sacarose, que por meio da glicólise pode alimentar a via *Perl* com piruvato para fornecimento de energia ao eixo embrionário. Embora a 13-LOX tenha sido encontrada em estádios iniciais de germinação, o consumo de lipídios de armazenamento ocorre posteriormente na germinação como forma de controle dos níveis de açúcares. Análises bioquímicas, por meio de quantificação, mostraram que o conteúdo de proteína, carboidratos e lipídios ao longo da germinação é coerente com o que foi reportado na proteômica. A análise de dois cultivares de soja BRS 284 e *Williams* 82, que diferiram no tempo de germinação, mostrou padrões distintos de mobilização de reservas ao longo da germinação. Os resultados apresentados neste trabalho forneceram informações sobre a mobilização de reservas em eixo embrionário de soja que propiciam a germinação e o estabelecimento de plântulas.

Palavras-chave: soja, eixo embrionário, carboidratos, proteômica comparativa, lipídeos.

Abstract

Seed production is one of the main segments of the agricultural sector, not only in Brazil, but throughout the world. Soybeans (*Glycine max* (L.) Merr.) is a crop of great importance for the agribusiness. Soybean seeds have a high nutritional value, being rich in proteins, complex carbohydrates, dietary fiber and oils. Biochemical and molecular events for nutrient mobilization from reserve tissues during the germination process are extensively studied. However, such events are poorly elucidated in the embryonic axis. The objective of this work was to analyze the mobilization of reserves in soybean seed embryos during

germination. The analysis of two soybean cultivars BRS 284 and Williams 82, which differed in germination time, showed distinct patterns of reserve mobilization throughout germination. Through histochemical analysis, a greater deposition of protein content was observed in 3 hours after imbibition (HAI) compared to 24 HAI. Histochemical analysis of starch showed a greater deposition in 24 HAI, showing that this carbohydrate is synthesized at the end of germination. Comparative proteomic study between 3 and 24 HAI indicated the differentiated regulation of several proteins in the embryonic axis of the soybean seed. In the metabolic pathway analysis, proteins seem to be mobilized in the first hours of germination, together with sucrose, which through glycolysis can feed the Perl pathway with pyruvate to supply energy to the embryonic axis. Although 13-LOX has been found in early stages of germination, consumption of storage lipids occurs later in germination to control sugar levels. Biochemical analysis, through quantification, showed that the protein, carbohydrate, and lipid content throughout germination is consistent with the proteomics data. The analysis of two soybean cultivars BRS 284 and Williams 82, which differed in germination time, showed distinct patterns of reserve mobilization throughout germination. The results presented in this work provided information on the mobilization of reserves in the embryonic axis of soybean that promote germination and seedling establishment.

Key words: soybean, embryonic axis, germination, proteomics, biochemical quantification.

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ou órgãos de reserva alimentar (cotilédones ou endosperma) e uma estrutura protetora, o tegumento da semente (testa). Essas estruturas irão formar a plântula durante a germinação, armazenar reservas para o crescimento da plântula e proteger a semente.
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1. Introdução

1.1 Soja: classificação e importância econômica

As sementes são a base econômica da agricultura, principalmente a soja *Glycine max* (L.) Merr.), cultura oleaginosa mais amplamente cultivada em todo o mundo. A soja pertence à família Fabaceae originada na China há mais de 3.000 anos. A Fabaceae, é terceiro maior grupo das angiospermas, compreende cerca de 770 gêneros e 19.500 espécies e apresenta grande variação morfológica e ecológica (AZANI et al., 2017). O gênero *Glycine* consiste em dois subgêneros, *Glycine* e Soja. A soja cultivada, *Glycine max*, pertence ao subgênero Soja (ANDERSON et al., 2019). É uma das principais commodities de exportação, fornecendo óleo e proteína para alimentação humana e animal (WIJEWARDANA et al., 2019) e representando 59% da produção mundial de oleaginosas em 2020 (<http://soystats.com/>).

Em escala mundial, os principais países produtores de soja são o Brasil com uma produção de 136 milhões de toneladas, seguido dos Estados Unidos com 112,5 milhões de toneladas, Argentina com 47 milhões de toneladas, China 19,6 milhões de toneladas e Paraguai com 9,8 milhões de toneladas. E dentre esses principais países produtores o destaque fica para o Brasil que, além de ser o maior produtor também é o maior exportador mundial do grão (Figura 1.A) (CONAB, 2021). No Brasil o maior produtor de soja é o estado do Mato Grosso, com produção de 35,947 milhões de toneladas, seguido do Rio Grande do Sul com 20,164 milhões de toneladas, Paraná com 19,872 milhões de toneladas e Goiás com 13,720 milhões de toneladas da oleaginosa, portanto, a soja possui uma importância para a economia brasileira (Figura 1.B) (Embrapa, 2021).

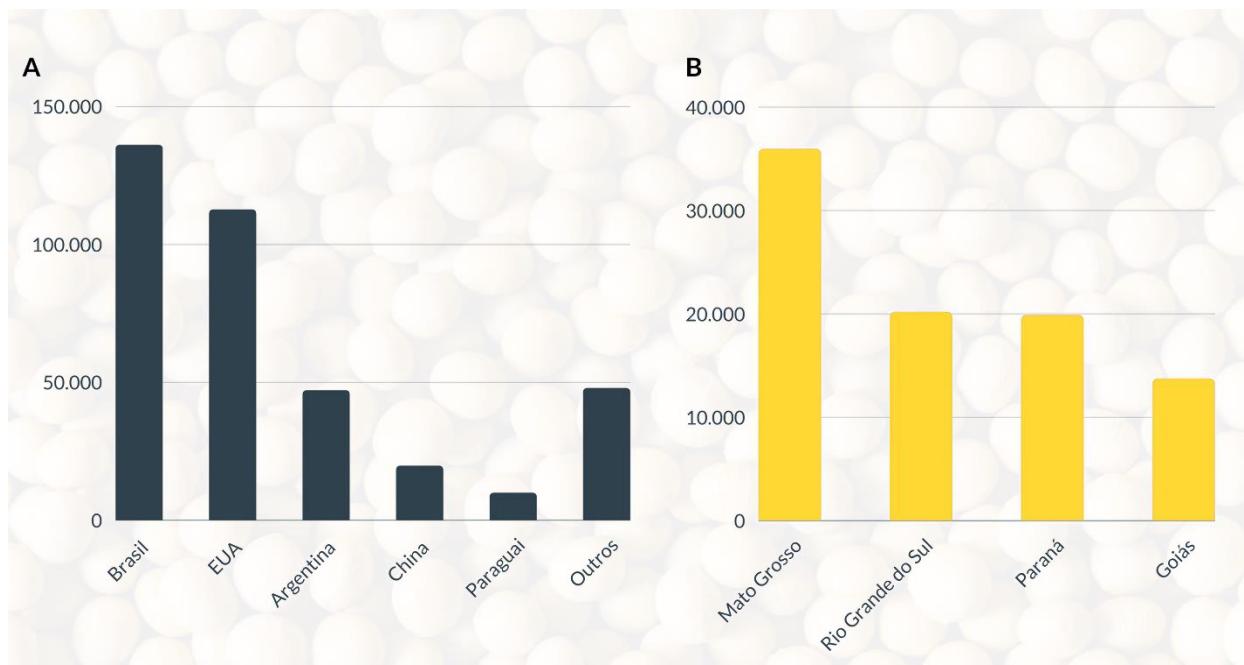


Figura 1. Dados econômicos da produção nacional e mundial da cultura da soja em milhões de toneladas. A) produção mundial de soja. B) produção nacional de soja (Embrapa Soja, 2021).

As sementes de soja são abundantes em proteínas e óleo vegetal (WANG; KOMATSU, 2017). A semente de soja, em média, é composta de aproximadamente 34% de proteína, 19% de óleo, 15% de carboidratos solúveis, 15% de carboidratos insolúveis (ANDERSON et al., 2019), que contribuem para sua importância no valor econômico e nutricional. Durante a germinação essas substâncias são mobilizadas e os produtos do catabolismo são utilizados para geração de energia e produção de matéria-prima para a construção de novas células e tecidos no decorrer do crescimento inicial das plântulas (MAYER POLJAKOFF-MAYBER, 1975).

A qualidade fisiológica das sementes depende da organização celular e da capacidade de mobilização de reserva para a formação de plantas mais vigorosas (DELGADO; COELHO; BUBA, 2015). Sementes de soja mais vigorosas detém maiores teores de proteína solúvel, amido e açúcares solúveis, além de maior capacidade de mobilização de reservas durante a germinação, originando plantas com melhor desempenho inicial (HENNING et al., 2010; PEREIRA et al., 2015).

As sementes de soja são constituídas por tecidos, incluindo o embrião, cotilédone e tegumento (Figura 2), que são os principais compartimentos da semente (ZHANG et al., 2021), apresentando origens e funções genéticas distintas (LIN et al., 2017).

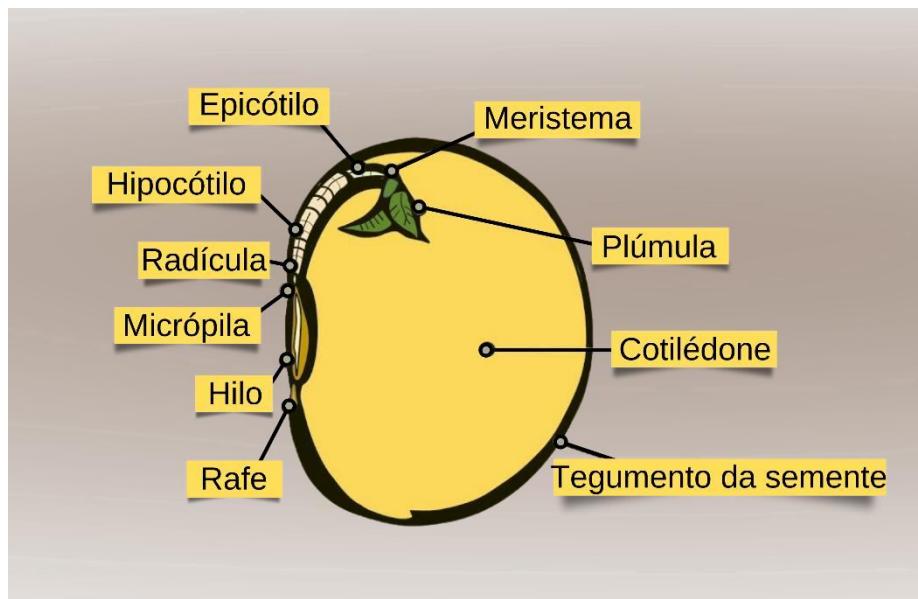


Figura 2. Imagem ilustrando a semente de soja (*Glycine max*) e suas estruturas. As sementes são as unidades de dispersão e propagação das espermatófitas. A semente madura é composta de três partes: uma raiz embrionária (radícula), tecidos ou órgãos de reserva alimentar (cotilédones ou endosperma) e uma estrutura protetora, o tegumento da semente (testa). Essas estruturas irão formar a plântula durante a germinação, armazenar reservas para o crescimento da plântula e proteger a semente. Imagem adaptada de Pioneer Seeds, 2021.

O embrião oriundo da dupla fecundação é subsidiado pelos cotilédones que atuam como órgãos de armazenamento de reservas fornecendo nutrientes que um embrião necessita para germinar. A fertilização dupla do óvulo pelo grão de pólen dá origem a semente que abriga o embrião zigótico que formará a nova

planta, bem como um tecido de armazenamento para fornecer nutrientes que vão subsidiar o crescimento das mudas após a germinação (RAJJOU et al., 2012). O tegumento, tecido materno que envolve o embrião e o cotilédone, fornece proteção e atua na distribuição de nutrientes entre os tecidos maternos e o embrião em desenvolvimento (LE et al., 2007; WEBER et al., 2005). Os compostos de reservas como proteínas, carboidratos e lipídios são sintetizados e se acumulam principalmente durante os estádios iniciais de maturação (LIN et al., 2017). O desenvolvimento da semente compreende sequências de processos metabólicos, enzimáticos, morfológicos e estruturais complexos que ocorrem em diferentes compartimentos da semente (SALGÓ; GERGELY; JUHÁSZ, 2005; LE et al., 2007; ZHANG et al., 2021).

1.2 Germinação de sementes de soja

A germinação da semente é uma etapa crucial na reprodução das espécies vegetais (FONSECA, 2015), é a primeira etapa essencial do ciclo de vida da planta. A germinação é o processo de desenvolvimento de sementes em uma nova planta, iniciando-se com a imbibição pela semente seca inativa onde há a hidratação dos tecidos do embrião, finalizando após a emergência da radícula (BEWLEY, BRADFORD, BLACK, 1994). Durante a germinação, as sementes utilizam os compostos de reserva para fornecer os nutrientes necessários na transição da fase heterotrófica para autotrófica.

Segundo HAN et al. (2013) há três fases que marcam os eventos da germinação. A fase I é marcada por uma rápida absorção de água pela semente, junto com o reparo de DNA danificado, além da retomada da via glicolítica e da via oxidativa das pentoses fosfato. A fase II é marcada pela síntese de mitocôndrias e a tradução de RNAm armazenado. Esta fase também é considerada uma fase ativa do metabolismo, pois é onde se inicia a mobilização de reservas. A fase III se caracteriza pela protrusão da radícula (Figura 3). Em sementes de soja, a fase I da germinação compreende o período de 0 a 12 (HAE). A fase II está entre 12 e 24 HAE. E a fase III ocorre depois das 24 HAE, dando início ao período pós-germinativo (HAN et al. 2013).

Os hormônios vegetais são moléculas necessários para propiciar a dormência, quebra da dormência e germinação de sementes. O ácido abscísico (ABA) e giberelinas (GAs) possuem atividade antagônica e são os principais reguladores desses processos. O ABA induz a manutenção da dormência, enquanto as GAs promovem a liberação da dormência e germinação das sementes (Figura 3) (CARRERA-CASTAÑO et al., 2020). A germinação de sementes termina quando há a protrusão da radícula (BEWLEY, 1997; SHU et al., 2016).

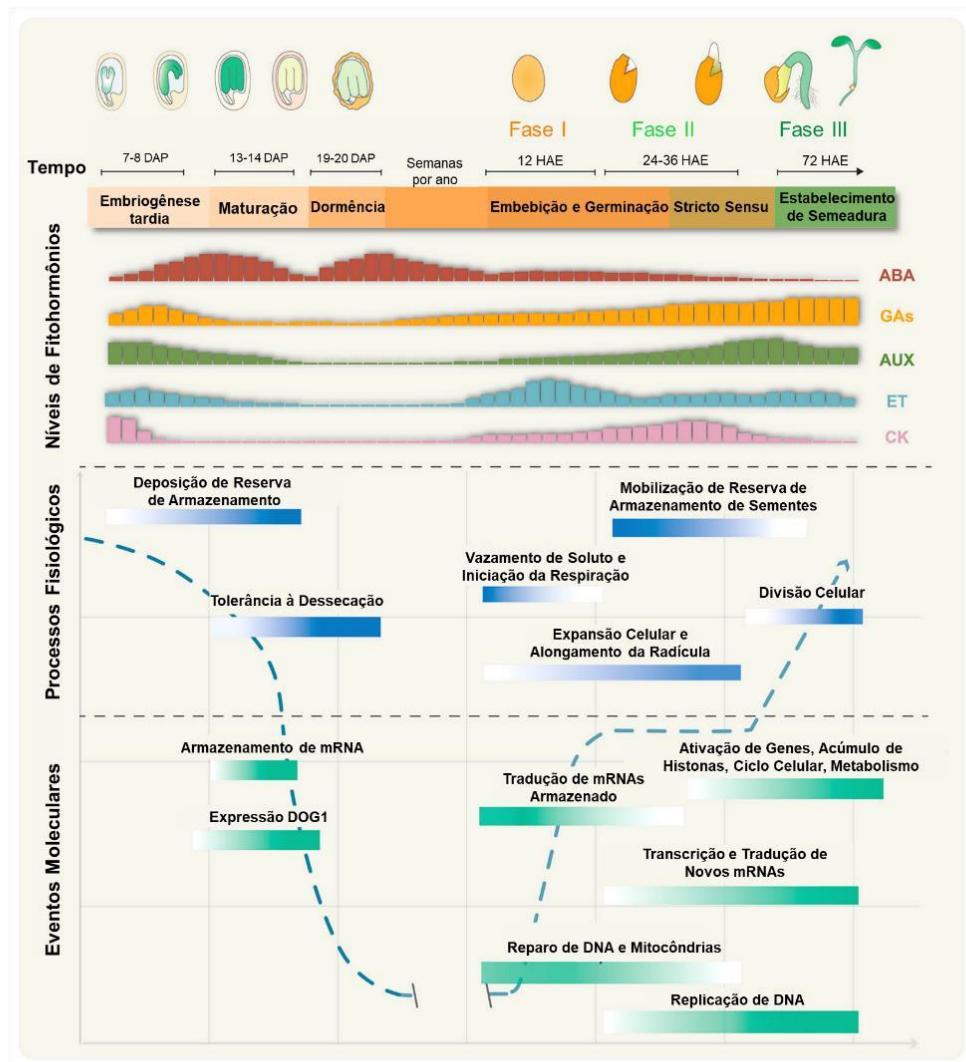


Figura 3. Processos moleculares, bioquímicos e fisiológicos na germinação das sementes de uma eudicotiledônea. À esquerda é representado os estádios finais de desenvolvimento da semente e a direita os estádios que marcam os eventos da germinação e estabelecimento da muda. Na lateral esquerda é mostrado mudanças nos níveis de fitohormônios específicos como ácido abscísico (ABA), giberelina (GA), auxina (AUX), etileno (ET) e citocinina (CK). Os processos fisiológicos são marcados pela deposição de reservas durante a maturação, tolerância a dessecação, vazamento de soluto, início da respiração celular, mobilização de reservas, divisão e expansão celular e alongamento da radícula regulados. Os eventos moleculares são marcados pelo armazenamento de mRNAs durante a maturação, tradução e transcrição de mRNAs, reparo de DNAs e mitocôndria, replicação do DNA e ativação de genes, acúmulo de histonas, ciclo celular e metabolismo. As barras em diferentes alturas indicam diferenças nos níveis de fitohormônios, sendo as barras mais altas referem-se a níveis mais elevados. A linha azul tracejada representa o nível de água em diferentes estádios de desenvolvimento do embrião e fases da germinação. DAP significa dias após a polinização e HAE significa horas após a imersão. Fonte: Adaptado de (LUJÁN-SOTO; DINKOVA, 2021).

A germinação de semente pode ser influenciada por diversos fatores internos. Por exemplo, concentrações de ácido abscísico (ABA) podem inibir a germinação. O ABA é um fitormônio que inibe a germinação impedindo os eventos de mobilização de reservas dos nutrientes acumulados durante a maturação da semente. Consequentemente, não há disponibilidade de energia para permitir o crescimento e desenvolvimento do eixo embrionário, impedindo, então, a germinação e o estabelecimento da plântula (KAZACHKOVA et al., 2016; TONINI et al., 2010). Outros fitohormônios como etileno, citocinina (CK), brassinosteroides (BRs), auxinas (AUX) e ácido jasmônico (JA) podem atuar em aspectos da dormência das sementes ou regulação da germinação (LUJÁNSOTO; DINKOVA, 2021).

Estudos com transcriptoma de eixos embrionários de semente de soja mostraram os aspectos regulatórios e metabólicos durante a germinação. Foi mostrado a ativação precoce de processos críticos para a germinação, como glicólise, ciclo de Krebs e remodelação da parede celular. Além disso, foi observado que em apenas 3 HAE, há uma regulação positiva preferencial de proteínas quinases e fatores de transcrição, sugerindo que regulação transcricional e pós-transcricional desempenha papéis importantes logo após a imersão da semente. Foi observado que as vias de mobilização de lipídeos e glioxilato também são transcricionalmente ativas nos eixos embrionários, indicando que o catabolismo local das reservas de lipídeos nos eixos embrionários contribui para a produção de energia durante a germinação (BELLIENY-RABELO et al., 2016).

1.32 Acúmulo e mobilização de nutrientes de reservas na semente

As sementes de espécies agrícolas, são consideradas alimento básico para a população mundial por acumularem reservas abundantes como carboidratos, óleos e proteínas durante a maturação. Essas reservas influenciam a germinação e estabelecimento de mudas, sendo cruciais nesse processo (HAN et al., 2013). A germinação da semente é composta por fenômenos metabólicos críticos, como a mobilização de reservas, que fornece precursores e energia para atividades biossintéticas (PINFIELD-WELLS et al.,

2005). As substâncias de reserva são utilizadas durante o processo germinativo e estabelecimento de mudas, onde são metabolizadas através de um sistema sincronizado que envolve a comunicação entre tecidos de reserva e o embrião (LIRA; 2010). A transição da fase heterotrófica para a autotrófica que culmina no estabelecimento das plantas depende da mobilização das reservas armazenadas (de cotilédones e endosperma) e da translocação de metabólitos solúveis para o eixo embrionário (Bewley et al., 2013, Silva et al., 2019). Essas macromoléculas acumuladas durante a maturação da semente são usadas como fonte de energia e carbono para a germinação e desenvolvimento de plântulas (Erbaş et al., 2016). Os principais grupos de reserva nutritiva que compõem as sementes são os carboidratos, as proteínas e os lipídios (BEWLEY et al., 2013). O catabolismo das reservas de proteína, lipídeos ou amido, acumuladas durante a maturação das sementes, apoiam a expansão celular, o desenvolvimento de cloroplastos e de estruturas celulares na plântula durante a germinação (PENFIELD, 2017).

A mobilização de diferentes reservas de nutrientes durante a germinação pode ser mediada por diferentes vias metabólicas em diferentes culturas (HAN et al., 2013). Por exemplo, diferentes vias de degradação podem atuar sobre a mesma reserva em distintas culturas. Estudos mostraram que em sementes de colza e milho, os óleos são degradados pela via independente da lipoxygenase clássica (LOX), mas em pepino o processo ocorre pela via dependente da LOX (FEUSSNER et al., 1995; FEUSSNER et al., 2001).

1.3.1 Proteínas

No Brasil, a soja é predominantemente utilizada para o processamento do grão em óleo e proteína (SMANIOTTO, 2013). Durante o desenvolvimento e maturação das sementes, as proteínas de armazenamento são alocadas em vacúolos de armazenamento de proteínas (PSVs) de maneira estável. Na germinação estas proteínas são degradadas para fornecer nutrientes para o embrião (JIANG et al., 2001). As proteínas são degradadas em aminoácidos durante o processo germinativo e podem seguir diferentes destinos, como permanecer no tecido de armazenamento ou serem transportados para tecidos

do eixo embrionário em desenvolvimento, para a síntese de diversas enzimas e proteínas estruturais (TAN et al., 2013).

Em relação à degradação do conteúdo de proteínas, existem vários tipos de enzimas proteolíticas envolvidas nesse processo, como as endopeptidases, que atuam no interior das cadeias e de acordo com suas especificidades podem apresentar diferentes classificações. Também já foram descritas as proteases cisteínicas e aspárticas, além das metaloproteases (KARMOUS et al., 2014) e serínicas (PRAXEDES-GARCIA et al., 2012).

As proteínas e proteases são direcionadas para esses compartimentos por diversas vias que se originam do retículo endoplasmático ou do Golgi, baseado na agregação e / ou na presença de determinantes de classificação específicos (VITALE; HINZ, 2005). As proteínas de armazenamento da semente de soja compreendem cerca de 35% do seu peso seco e são essenciais na germinação como fonte de nitrogênio. Estudos mostraram que 70% das reservas proteicas da semente de soja é composta de globulinas - glicinas (11S) e βconglicininas (7S). Além dessas, outras proteínas também já foram descritas em soja, as albuminas 2S e as prolaminas (TAN-WILSON; WILSON, 2012). Em semente de soja, foi relatado que as subunidades α e α' das β-conglicinina são as primeiras estruturas proteicas a serem degradadas.

Ao longo da germinação, essas proteínas são hidrolisadas em aminoácidos, e permanecem no tecido de armazenamento ou são translocadas para tecidos do eixo embrionário em desenvolvimento, para a síntese de enzimas e proteínas estruturais. Alguns aminoácidos podem ainda sofrer desaminação e serem utilizados na síntese de compostos que não contêm nitrogênio ou podem ser metabolizados para a produção de energia (TAN et al. 2013).

Em sementes de tremoço (*Lupinus luteus L.*), os aminoácidos oriundos da mobilização das reservas proteicas acumuladas são empregados na síntese de novos corpos proteicos e também utilizados como substrato para o processo respiratório (BOREK; RATAJCZAK, 2010). Foi mostrado em sementes de tomate que há um aumento no conteúdo proteico na fase de pós-germinação, sendo esse aumento, em alguns casos, antecede a quebra de triacilglicerol até mesmo por dois dias (LEWANDOWSKA 2016). Em sementes de girassol (*Helianthus annuus*), por exemplo, foi observado uma redução do conteúdo de proteínas

armazenadas no início da germinação, sendo essa redução coerente com o consumo da energia liberada pela degradação dessas reservas (ERBAŞ et al. 2016). Apesar disso, o padrão específico de consumo de proteínas varia de acordo com o cultivar avaliado (ERBAŞ et al. 2016). Em um estudo com dois cultivares de semente de girassol onde foi avaliado o padrão de consumo de reservas acumuladas, observou-se que os níveis de aminoácidos livres tendem a aumentar em ambos à medida que a germinação avança (ERBAŞ et al. 2016).

1.3.2 Carboidratos

Os carboidratos são a principal fonte de reserva de sementes de plantas cultivadas para o consumo humano (BEWLEY & BLACK, 1994). A oxidação de carboidratos constitui a principal via metabólica fornecedora de ATP que é a principal fonte de energia em todas as células (ROSCAMP; SANTOS, 2015). Durante o processo de desenvolvimento e maturação, as sementes de soja armazemam carboidratos solúveis, os quais podem desempenhar importantes funções na germinação, como tolerância à dessecação e tolerância ao estresse pelo frio (OBENDORF; SUZANNE, 2011).

Com base em suas propriedades físico-químicas nas plantas, os carboidratos podem ser divididos em dois grupos: carboidratos não estruturais e carboidratos estruturais. O primeiro grupo inclui açúcares de baixo peso molecular, oligossacarídeos e polissacarídeos de armazenamento (KARRLILIENTHAL et al., 2005). O segundo grupo abrange os polissacarídeos estruturais e inclui elementos de fibra alimentar (BACH KNUDSEN; EGGUM; JACOBSEN, 1987). Na semente de soja aproximadamente metade dos carboidratos é de origem não estrutural, incluindo açúcares de baixo peso molecular, oligossacarídeos e pequenas quantidades de amido, enquanto a outra metade são polissacarídeos de origem estrutural, incluindo uma grande quantidade de polissacarídeos pécticos (KARR-LILIENTHAL et al., 2005).

Em sementes de Lupino amarelo a sacarose atua no início da mobilização de amido nos cotilédones e no eixo embrionário de sementes durante a germinação. Além disso, foi observado um aumento no tamanho e número dos grânulos de amido em órgãos que são subsidiados pela sacarose (BOREK e

GALOR 2013). Os açúcares de baixo peso molecular são representados por pequenas quantidades de galactose, glicose, frutose e sacarose livres (KARRLILIENTHAL et al., 2005). O açúcar é utilizado para diversas funções importantes como no desenvolvimento de embrião, processo da germinação, regular os sinais que afetam a expressão de genes e, consequentemente, o desenvolvimento da planta (BEWLEY & BLACK, 1994). Em um estudo com sementes de girassol, foi observado que a glicose é a fonte primária de carboidratos utilizada durante a germinação. Além disso, foi registrado um aumento nos níveis totais de açúcares solúveis e na taxa de açúcar redutor após o período de 24 horas do início da germinação da semente. Isso pode sugerir uma grande conversão de reservas para açúcares, junto com a síntese do pigmento clorofila, a fim de iniciar o metabolismo fotossintético (ERBAŞ et al. 2016).

1.3.3 Lipídeos

Na soja, bem como em outras culturas de grãos oleaginosos, a produção e o acúmulo de óleo ocorrem durante o desenvolvimento e maturação das sementes (FEHR et al., 1971). Em sementes de soja, os lipídios são armazenados principalmente nos cotilédones, compreendendo 19% de seu peso seco. Eles são importantes como matéria-prima para consumo alimentar e inúmeras aplicações industriais, como combustível, biodiesel, óleos de motor, lubrificantes, poliésteres, pesticidas e tintas (THIEN NGUYEN et al., 2016). Os lipídios acumulados nas sementes de plantas são geralmente armazenados como triacilgliceróis (TAGs). A síntese de TAG é iniciada no citosol a partir da glicólise, os produtos resultantes desse processo são transportados para o plastídio para a síntese de ácidos graxos (ZHANG et al., 2019). Diversas plantas acumulam ácidos graxos na forma de TAGS como principais elementos de armazenamento nas sementes, fazendo das plantas uma fonte renovável expressiva dessa substância (THELEN; OHLROGGE, 2002). Os TAGs armazenados nas células das sementes das plantas em organelas designadas corpos oleosos (OBs) que atuam como fonte de energia para a germinação e crescimento pós-germinativo (HUANG, 1996; MURPHY, 2001).

Esses corpos lipídicos são compostos por TAG envolto por proteínas chamadas oleosinas, caleosinas e esteroleosinas, cobertos por uma monocamada de fosfolipídios. A oleosina é a proteína mais abundante dessa organela citoplasmática (HUANG, 1996). Acredita-se que a oleosina forme uma barreira esférica para impossibilitar a junção dos corpos oleosos durante o estado de dissecação da semente (LEPRINCE et al., 1997; MURPHY; VANCE, 1999). As caleosinas, além de englobar um número maior de espécies proteicas, podem substituir as oleosinas em seu papel de manter a estabilidade dos corpos lipídicos (JOLIVET et al., 2013).

Com relação ao catabolismo de lipídios armazenados pela semente existem muitas vias conhecidas. A via clássica se baseia na conversão de lipídeos em açúcares através da β -oxidação, ciclo do glicoxilato, ciclo da ácido cítrico e gliconeogênese (METTLER; BEEVERS, 1980). Em sementes de lupino, a quebra do conteúdo lipídico armazenado ocorre em função da diminuição da disponibilidade de açúcar (BOREK; KUBALA; KUBALA, 2012).

Em termos fisiológicos os lipídios apresentam diversas funções como compor a membrana, atuar na reserva de energia e servir como meio solvente para muitas substâncias lipossolúveis (GERDE; WHITE, 2008). Durante a maturação das sementes o TAG é acumulado e armazenado até a germinação, após esse processo é usado para nutrir o crescimento das plântulas (GRAHAM; EASTMOND, 2002). Nas sementes em germinação a mobilização lipídica começa com a quebra de TAGs em ácidos graxos livres e glicerol pelas lipases nos oleossomos. A energia (ATP) armazenada e os produtos oriundos da hidrólise de lipídios de armazenamento são cruciais para sustentar o crescimento embrionário do estádio de repouso para a fase ativa (AWATIF S. ALI ND ALAAELDIN A. ELOZEIRI, 2017). Para o desenvolvimento da semente de tomate, por exemplo, as reservas de lipídios acumulados podem desempenhar um papel primário e crucial na obtenção do aporte energético, por exemplo, onde o conteúdo lipídico armazenado é um tipo de nutriente predominante (ECKSTEIN et al., 2016).

Análise proteômica de corpos lipídicos em sementes maduras de *Jatropha curcas* observou-se que há uma interação significativa entre proteínas

de vacúolos de armazenamento, glioissomos e membrana plasmática (LIU et al., 2015).

2. Objetivo geral

Analisar o processo de mobilização de reservas em eixos embrionários de sementes de soja durante a germinação.

2.1 Objetivos específicos

- A** – Analisar a marcação histoquímica de proteínas de reserva e amido em tecidos de eixos embrionários durante a germinação
- B** - Analisar o perfil proteômico de eixo embrionário de sementes de soja horas após a embebição.
- C** – Quantificar proteínas, carboidratos e lipídios de eixo embrionário de sementes de soja horas após a embebição.
- D** – Analisar o conteúdo de proteínas e lipídios de eixo embrionário de sementes de duas cultivares de soja com diferença no tempo de germinação.

3. Resultados

O presente trabalho de dissertação foi realizado na forma de um artigo científico a ser submetido a um periódico internacional da área. Esse artigo compõe a seção de metodologia e resultados.

3.1 Artigo Científico

Mobilization of reserves in soybean embryonic axis during germination

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Abstract

Soybeans are a source of nutrients as they accumulate abundant reserves of proteins, carbohydrates and lipids during the maturation process. These storage macromolecules are a source of energy and carbon for seed germination and crop establishment, as their efficient mobilization paves the way for greater productivity. Here, we analyze the mobilization of nutrient reserves in soybean seed embryonic axes during germination in two cultivars, the BRS 284 and Williams 82. The ability to mobilize nutrients of two soybean cultivars - BRS 284 and Williams 82 – was analyzed. Through histochemical analysis, a higher protein deposition was observed at 3 HAI compared to 24 HAI. On the other hand, there was a higher starch accumulation at 24 HAI. Quantitative proteomic analysis revealed 141 proteins accumulated in the embryonic axis at 3 and 24 HAI. In metabolic pathway analysis we found that proteins are mobilized in the first hours of germination, together with sucrose, which through glycolysis fed the Perl's pathway with pyruvate for energy supply to embryonic axis. Although the 13-LOX was found at initial stages of germination, the consumption of storage lipids occurs later in the germination to supply the synthesis of sugars. Biochemical analyses showed that the content of proteins and lipids decreases during germination, while the amount of glucose varies in the hours of imbibition. The cultivars differed in the pattern of reserve mobilization throughout germination, which seems to impact their germination rate. Together, our results shed light on the regulation of reserve mobilization in the soybean embryonic axis throughout germination, which influences seedling performance.

Keywords: Soybean, seeds, embryonic axis, mobilization of reserves, germination.

Introduction

Soybean (*Glycine max* [L.] Merr.) is a versatile crop grown globally with diverse food and industrial uses and applications. In the 2020/2021 harvest, soybean was the main type of oilseed cultivated in the world, reaching a production of about 362 million tons (Shahbandeh, 2021). Soybean seed is composed of approximately 34% proteins, 19% oil, 15% soluble carbohydrates, and 15% insoluble carbohydrates (Wijewardana *et al.*, 2019). These storage macromolecules are a source of energy and carbon for seed germination and seedling development (Erbaş *et al.*, 2016; Wei *et al.*, 2020).

The propagation of higher plants depends on the formation and germination of seeds that are crucial for plant development, human food supply, conservation of genetic resources, and breeding programs (Bareke, 2018). Germination begins with imbibition - the absorption of water by the quiescent seed - and ends up with the elongation of the embryonic axis (Bewley, 1997), passing through a series of ordered physiological and morphogenetic processes (Zhang *et al.*, 2020). Seed germination is a

crucial growth process that ensures the successful establishment and productivity of soybean (Liu *et al.*, 2020). This process is composed of critical events, such as the mobilization of reserves, which provide precursors and energy for biosynthetic activities (Pinfield-Wells *et al.*, 2005). The catabolism of stored reserves, such as protein, oil, or starch, accumulated during the seed maturation process, supports cell expansion, the development of chloroplasts, and cell structures in the embryo during germination (Penfield, 2017).

Storage proteins represent most of the total protein content of the soybean seeds, largely determining their nutritional quality (Mouzo *et al.*, 2018). In legume seeds, 7S and 11S globulins are the main storage proteins (Kimura *et al.*, 2008). Globulins are proteins more widely distributed in eudicots and in some cereals (*Avena sativa*, and *Oryza sativa*), forming the main storage protein fraction (Kawakatsu and Takaiwa, 2017). The 7S globulins are known as β -conglycinin and 11S globulins are called glycinins. Glycinins and β -conglycinins represent about 70% of the total protein content of soybean seed (Thanh and Shibasaki, 1976). The synthesis of these storage proteins occurs during seed development at the maturation stage (Asakura *et al.*, 2012; Mouzo *et al.*, 2018). These proteins have nearly identical three-dimensional structures and belong to a superfamily of plant proteins called Cupins (Kawakatsu and Takaiwa, 2017), that can be mobilized during germination to support seedling growth and development (Kim *et al.*, 2011).

Triglycerides (TAGs) are also an important storage reserve in seeds of many plant species, called oilseeds, such as sunflower, canola, castor bean, corn, and soybean (Graham, 2008). TAGs are stored in plant seed cells in organelles called oil bodies (OBs) that act as a source of energy for germination and post-germination growth (Huang, 1996; Murphy, 2001). These OBs are surrounded by proteins called oleosins, kaleosins and sterolosins, covered by a monolayer of phospholipids (Huang, 1996). The mobilization of storage lipids starts with the beginning of seed germination (Kelly *et al.*, 2011). In germinating seeds, lipid mobilization initiates with the breakdown of TAGs into free fatty acids and glycerol by lipases in OBs. Stored energy (ATP) and products from the hydrolysis of storage lipids are crucial to sustain embryonic growth from the resting stage to the active phase (Ali and Elozeiri, 2017).

Carbohydrate is also an important constituent of soybean seeds (Dhungana *et al.*, 2017). A large proportion of soybean seed is comprised by insoluble polysaccharides, including pectin, cellulose, hemicellulose, and starch (Liu, 1997). In tomato, castor bean, and sunflower, an increase in the starch content was observed in seed germination and post-germination stages (Eckstein *et al.*, 2016). This transient accumulation of starch

during post-germination was also observed in soybean seeds, where a peak is reached after four or six days after sowing (Brown and Huber, 1988). On the other hand, the level of soluble carbohydrates slightly decreased in tomato seeds in the first days of germination, suggesting they are used as energy sources (Eckstein *et al.*, 2016). Their following increase may be the result of a transformation of fat into carbohydrates (Eckstein *et al.*, 2016; Erbaş *et al.*, 2016).

The formation of more vigorous seedlings is directly influenced by the physiological quality of the seed, which depends on cell organization and the efficient mobilization of reserves (Delgado *et al.*, 2015). The mobilization of stored reserves in cotyledons and endosperm and the translocation of soluble metabolites to the embryonic axis is crucial for the seedling establishment (Silva *et al.*, 2019). Although, the embryonic axis is responsible for the origin of the new seedling, few studies investigate the reserve mobilization in this particular tissue. Therefore, many questions still remain about biochemical and molecular events concerning the mobilization of nutrients on the embryonic axis. Here, we investigated reserve mobilization in soybean seed embryonic axes during germination. By histochemical analysis, we found differences in the accumulation of storage reserve proteins and starch between embryonic axis at 3 and 24 hours after imbibition (HAI). Comparative proteomic analysis identified 141 differentially accumulated proteins (DAPs), or unique proteins in the embryonic axis at 3 and 24 HAI. In functional annotation analysis, we found that proteins involved in carbohydrate and lipid metabolism are more accumulated at 24 than at 3 HAI, which could be corroborated with biochemical analysis. Considering that the ability to mobilize nutrients impacts seedlings performance, we compared two important soybean cultivars - BRS 284 and Williams 82. We found that BRS284 was able to mobilize reserves until 24 HAI, which could impact the rate of germination. Our data contribute to the understanding of how the soybean embryonic axis mobilizes seed reserves during germination by analyzing metabolic and molecular processes of germination, which has been considered crucial for the development of more vigorous seedlings.

Materials and Methods

Plant material and seed germination

Soybean seeds (cv. BRS 284) were disinfected with 2% sodium hypochlorite and germinated in Petri dishes containing 12 mL of water autoclaved on filter paper. The plates were placed in a biochemical oxygen demand incubator (B.O.D.) at 28 °C with a 12/12 h photoperiod, at light intensity of $28 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$. Three biological replicas (15 seeds each) were used for each time point. Dry seeds (no imbibed seeds) and seeds

with 3, 6, 12, and 24 hours after imbibition (HAI) had their embryonic axes removed manually. After removal, the embryonic axes were used for subsequent analysis.

For the germination rate, the cultivars BRS 284 and Williams 82 were germinated as described above. The number of germinated seeds was observed at the points 15, 18, 21, 24, 27, 30, 36, 42, and 48 HAI. The equation to calculate the germination percentage was $GP = \text{germinated seeds} / \text{total seeds} \times 100$. Two biological replicas (15 seeds each) were used for each time point. The embryonic axes at 3 and 24 HAI were manually removed and weighed to estimate fresh mass.

Histochemical analysis

Soybean seeds were germinated as described above and embryonic axes at 3 and 24 HAI were collected. Each embryonic axis was placed in a microtube containing Karnovsky solution (Karnovsky 1964). After fixation, the material was washed in 0.05 M sodium cacodylate buffer at pH 7.2 and dehydrated in an alcoholic series from 10 to 100%. Subsequently, the material was infiltrated with historesin (Historesin Leica, prepared according to the manufacturer's instructions) and incorporated into pure resin. The blocks were cut into 5 μm sections using an autotuning rotating microtome (RM 2155, Leica). The sections were adhered to histological slides containing distilled water and dried. Brightfield optical microscope (Axioplan ZEISS) coupled to the Canon Powershot A640 camera was used for the analyses. Images were captured using Axiovision 4.8 software (Carl Zeiss). Coomassie Blue dye was used for protein staining. A 0.25% solution of Coomassie Brilliant Blue (CBB) R250 in 7% Acetic Acid was used for 10 min at room temperature. Then the sections were washed in 7% acetic acid, 3 times for 5 min, followed by rapid washing in water (FISHER, 1968). The detection occurs through the binding of the dye to amino acid groups staining the proteins as blue. Lugol dye was used for starch detection. Lugol's solution (0.5% iodine solution added with 1% potassium Iodide) was used for 5-10 min followed by washing with water. In this procedure, the starch acquires a purple or brown coloration (Jensen, 1962).

Comparative proteomic analysis

Soybean seed embryonic axis at 3 and 24 HAI were powdered in liquid nitrogen. Three biological replicates (20 seeds each) were used for each time point. Samples (300 mg of fresh mass from each replicate) were mix in microtubes with 1 mL of extraction buffer consisted of 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% dithiothreitol (DTT, GE Healthcare), 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The mixture was vortexed and

centrifuged. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA). The methanol/chloroform methodology was used to precipitate protein samples (Nanjo *et al.*, 2012). The samples were then resuspended in a solution consisting of 7 M urea and 2 M thiourea, and protein digestion was performed using Microcon-30 kDa filter Units (Millipore) with the filter-aided sample preparation (FASP) methodology described by (Wiśniewski *et al.*, 2009), with some modifications. After digestion, the peptides from samples were quantified with NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and 1 µg of digested proteins were injected into a nanoAcuity UPLC mass spectrometer connected to an SYNAPT G2-Si Q-TOF instrument (Waters, Manchester, UK). Injections were performed randomly to avoid bias. Spectra processing and database searching were performed using ProteinLynx Global SERVER (PLGS) v.3.02 software (Waters), and label-free quantification analyses were performed using ISOQuant software v.1.7 (Distler *et al.*, 2014). For differential abundance analysis, a two-tailed Student's *t*-test was performed. Proteins with p-values ≤ 0.05 and log₂ of fold change (24HAI/3HAI comparison) ≤ -0.5 or ≥ 0.5 were considered as down- and up-accumulated, respectively. Functional annotation analysis of differentially accumulated proteins was performed based on the KO database (KEGG Orthology) using KOALA tools (available at <https://www.kegg.jp/blastkoala/>). KO numbers were used to determine the main metabolic pathways in the embryonic axis in the KEGG PATHWAY mapping database (<https://www.genome.jp/kegg/pathway.html>).

Quantification of total proteins

Protein concentration was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985). Embryonic axes at 0 (dry seeds) and 3, 6, 12 and 24 HAI were homogenized in monobasic sodium phosphate buffer 100 mM pH 7.6, shaken for 1 h, and centrifuged at 10.000 g for 10 min at 4°C. From the homogenate, 5 µl of supernatant was removed and diluted in 200 µL of BCA, before incubation at 37 °C for 30 min. Absorbance was read at 540 nm and protein concentration was determined using a bovine serum albumin (BSA) standard curve. The experiments were carried out with two biological replicates (15 seeds each) for each time point.

Glucose quantification

Glucose content was quantified using the Glucose Monoreagent K082 Enzyme Reaction Kit. Embryonic axes of 0 (dry seeds) and 3, 6, 12 and 24 HAI were macerated and homogenized in 50 Mm potassium phosphate buffer pH 6.8 at a ratio of 5 mg of

sample to 250 µL of buffer. The samples were shaken for 1h and centrifuged at 300 g for 5 min at 4°C. Samples were heated in a 37°C water bath for 10 min and read at 505 nm using Thermo Plate microplate reader (TP Reader), type B. The glucose quantification was performed with two biological replicas (15 seeds each) for each time point.

Triglyceride quantification

A TAG quantification assay was performed using the K117 Triglycerides Monoreagent Enzyme Reaction Kit. Embryonic axes of 0 (dry seeds) and 3, 6, 12 and 24 HAI were macerated and homogenized in Tween 20 in a proportion of 2 mg of sample to 100 µL of buffer and shaken for 1h. The homogenate was centrifuged at 6000 rpm for 10 min. The assay was performed with 10 µL of each sample with 1mL of reagent 1. The samples were heated in a water bath at 37°C for 10 min. Absorbance was read at 500 nm using Thermo Plate microplate reader (TP Reader), type B. Two biological replicates (15 seeds each) were used for each time point.

Western Blotting

To perform Western Blotting, a 12% polyacrylamide gel was prepared. The gels were polymerized according to defined concentrations of H₂O (3.3 mL), 30% acrylamide mix (4 mL), 1.5 M Tris pH 8.8 (2.5 mL), 10% SDS (0.1 mL), 10% APS (0.1 mL), and TEMED (0.004 mL). The STACK band of the gels was polymerized with H₂O (5.5 mL), 30% acrylamide mix (1.3 mL), 1.5 M Tris pH 6.8 (1 mL), 10% SDS (0.08 mL), 10% APS (0.08 mL), and TEMED (0.008 mL). We loaded 3 µL of the Molecular Weight Standard and the remaining wells were loaded with 15 µL of sample and 5 µL of sample buffer (20 µL per well). The transfer of the bands from the gel to the nitrocellulose membrane were performed in a period of 3 hours. The 10x transfer buffer (pH 8.3) made with 0.25 M tris, 1.92 M glycine and 20% methanol (v/v) was used. Blocking was performed overnight with PBS buffer and 2% skimmed milk powder. The buffer was composed of monobasic sodium phosphate (100 mM) and sodium chloride (500 mM). After that, the membrane was washed in distilled water. The primary antibody (anti-vicilin EPACE 10 produced in rabbit) was added at a ratio of 1:500, acting for 2 hours on the membrane. After this period, the membrane was washed with a PBS buffer (pH 7.6).

The membrane was immersed in a solution containing the secondary antibody (peroxidase-conjugated) goat anti-rabbit IgG (Sigma-Aldrich Corporation, St. Louis, MO, USA) diluted 1:1000 in blocking buffer for 1 h at room temperature; washing steps were as described previously. Immune reactions were developed by incubating the membranes in developer solution composed of 10 mg of 3,3-diaminobenzidine

tetrahydrochloride dissolved in 100 ml of 2M Tris-HCl buffer (pH 7.5) supplemented with 40ul of hydrogen peroxide (H₂O₂) and 300 μ l of 0.1 M imidazole for 10 min.

Statistical analysis

For fresh mass and quantification of proteins, TAGs and glucose, the mean and standard deviation were calculated. Statistical tests of one-way analysis of variance (ANOVA) followed by Tukey's test ($P \leq 0.05$) were performed using GraphPad Prism 7.0.

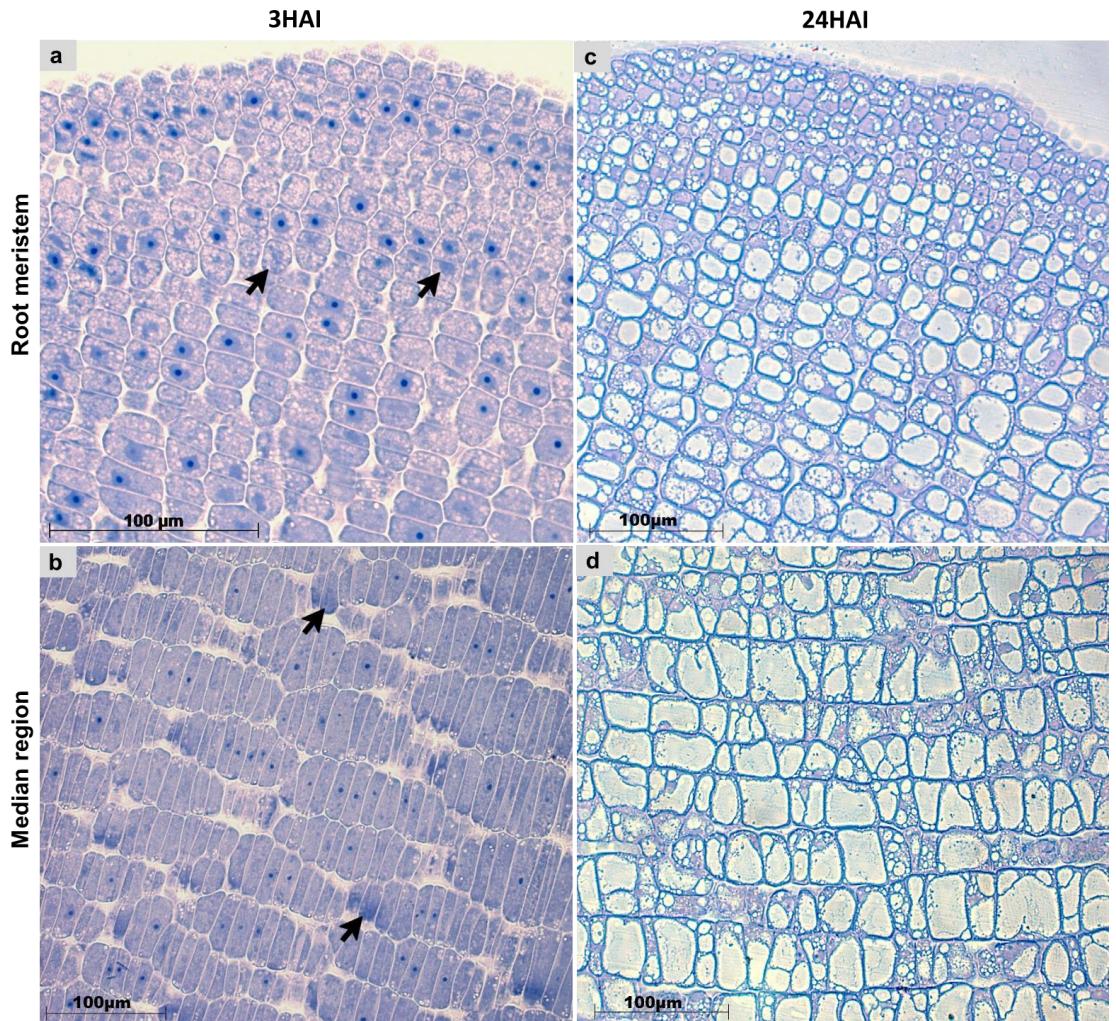
Results and discussion

Proteins and starch are inversely mobilized in the embryonic axis during germination

The mobilization of embryonic reserves is necessary for germination and starts during the initial stages of seed imbibition (Bewley, 1997; Nonogaki *et al.* 2010). Mobilization of seed reserves is generally considered a post-germination process (Eastmond and Graham, 2001; Pritchard *et al.*, 2002). However, studies have found that mobilization of seed reserves such as starch degradation (Galland *et al.*, 2017), protein (Yang *et al.*, 2007; Rosental *et al.* 2014) and lipid (Sreenivasulu *et al.*, 2008) occur during germination. To verify the presence of protein bodies in the embryonic axis during germination, we used CBB dye (Figure 1.a). The protein bodies were greater stained in the embryonic axis at 3 HAI than 24 HAI, suggesting a massive protein mobilization in this tissue during germination to supply the energy needs of the growing embryo. Storage proteins accumulate in abundance during seed development and maturation. Proteins synthesized and stored in abundance in the seed are degraded by proteases during germination and early seedling growth into free amino acids for biosynthesis and energy generation (Kim *et al.*, 2011; Tan-Wilson and Wilson, 2012). In sunflower seeds (*Helianthus annuus*), for example, a reduction in the content of stored proteins was observed as soon as germination began (Erbaş *et al.*, 2016). In the same study, the mobilization pattern of accumulated reserves in two cultivars of sunflower seeds showed that the levels of free amino acids increased during germination (Erbaş *et al.*, 2016).

Previous study with germinated *Yucca schidigera* seeds showed increase in starch while lipid reserves were consumed, attributing the increase in starch levels to the degradation of TAGs into glycerol and fatty acids, and then converted to carbohydrate (Horner and Arnott, 1966). In a study with *Lupinus luteus* L. seed, the mobilization of storage lipids during germination is controlled by changes in the sugar level, being strongly evidenced by the fact that the concentration of soluble sugars in tissues are kept at low levels (Borek *et al.*, 2006). It has also been reported that soluble sugars are

converted to starches as a strategy to control the level of soluble sugars in cells (Borek *et al.*, 2006). To verify the presence of starch granules in soybean embryonic axes during germination, we used Lugol staining. We found a greater accumulation of starch granules in the embryonic axes sections at 24 HAI when compared to 3 HAI. Soybean seeds mainly synthesize proteins and lipids during seed maturation; therefore, few starch biosynthesis enzymes could be detected at this stage (Han *et al.*, 2013). These enzymes possibly are synthesized in the late stages of seed germination, which is supported by the accumulation of starch granules at 24 HAI. Our results suggest the accumulation of starch granules at the embryonic axis tissues in the end of germination could be related to lipid mobilization controlled by the concentration of soluble sugars.



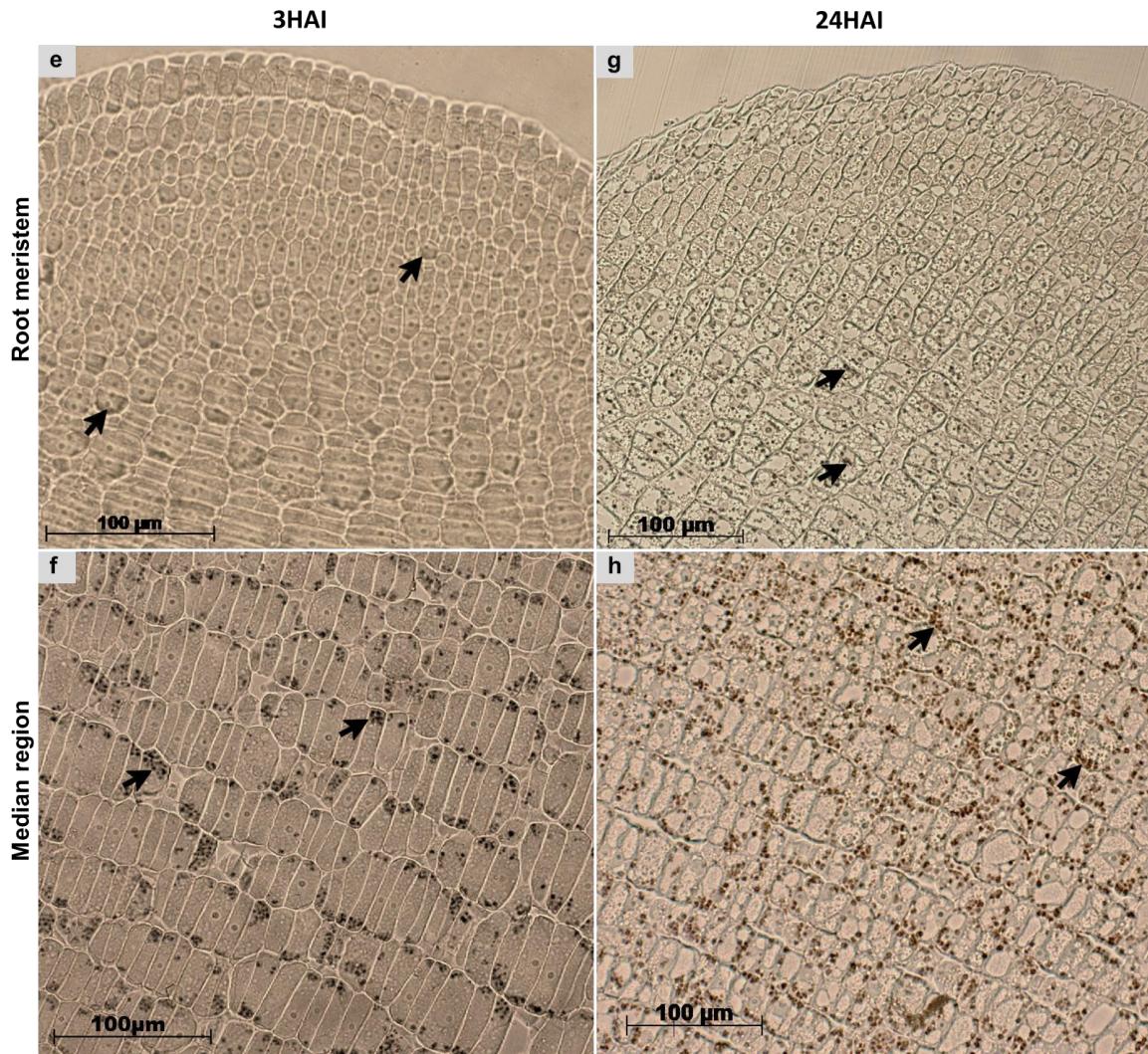


Figure 1. Histochemical analysis of the soybean embryonic axis at 3 and 24 hours after imbibition (HAI). Coomassie Brilliant Blue staining and Lugol. a-b Embryonic axes at 3 HAI. c-d Embryonic axes at 24 HAI. Protein bodies (arrow) can be observed in the cells of embryonic axes at 3 HAI but not in the embryonic axes at 24 HAI. Starch grains (arrow) are greater present in the cells of embryonic axes at 24 HAI. e-f Embryonic axes at 3 HAI. g-h Protein Embryonic axes at 24 HAI.

Comparative proteomic analysis of soybean embryonic axis during germination

In the present study, comparative proteomic analysis was used to uncover molecular mechanisms of reserves mobilization that take place on embryonic axes at 24 HAI compared to 3 HAI. The total of 141 proteins were differentially accumulated, or unique, in the embryonic axis at 24 HAI compared to 3 HAI (Figure 2.a). Of these, 70 proteins were differentially accumulated comparing seeds at 24 HAI with seeds at 3HAI

(24HAI/3HAI comparison), 23 proteins unique at 3 HAI, and 48 proteins unique at 24 HAI. In the volcano graphic, we showed the amount of differentially accumulated proteins by their significance (p-value). The orange dots represent the 39 differentially accumulated proteins at 3 HAI, while the 31 differentially accumulated proteins at 24 HAI were represented as blue dots (Figure 2.b).

For the functional annotation, we used the KOALA tools with the sequences of differentially accumulated proteins in the comparison 24 HAI/3 HAI. 62 DAP are related to 3 HAI (Figure 2.c). The largest number (24 proteins) of accumulated proteins in 3 HAI was from the genetic information processing category. The second largest functional category of proteins identified were carbohydrate metabolism with 7 proteins, aminoacids metabolism (with 1 protein), and lipid metabolism (with 1 protein). Others KEGG categories were also found (Figure 2.c).

79 DAP are related to 24 HAI (Figure 2.d). The largest number of differential accumulated proteins was related to the category of carbohydrate metabolism, with 23 proteins. The second-largest functional category of proteins identified was genetic information processing with 10 proteins, followed by lipid metabolism and amino acid metabolism with 6 proteins in each category. In these analyses, we found that proteins involved in carbohydrate and lipid metabolism are more accumulated in 24 HAI than in 3 HAI. Lipids are consumed through their conversion to carbohydrates. During germination, the degradation of lipids into fatty acids and glycerol is followed by the pathways of Acetyl-CoA metabolism and gluconeogenesis, mainly leading to the production of glucose and sucrose later, which are used for the growth of seedlings (Raineri *et al.*, 2016).

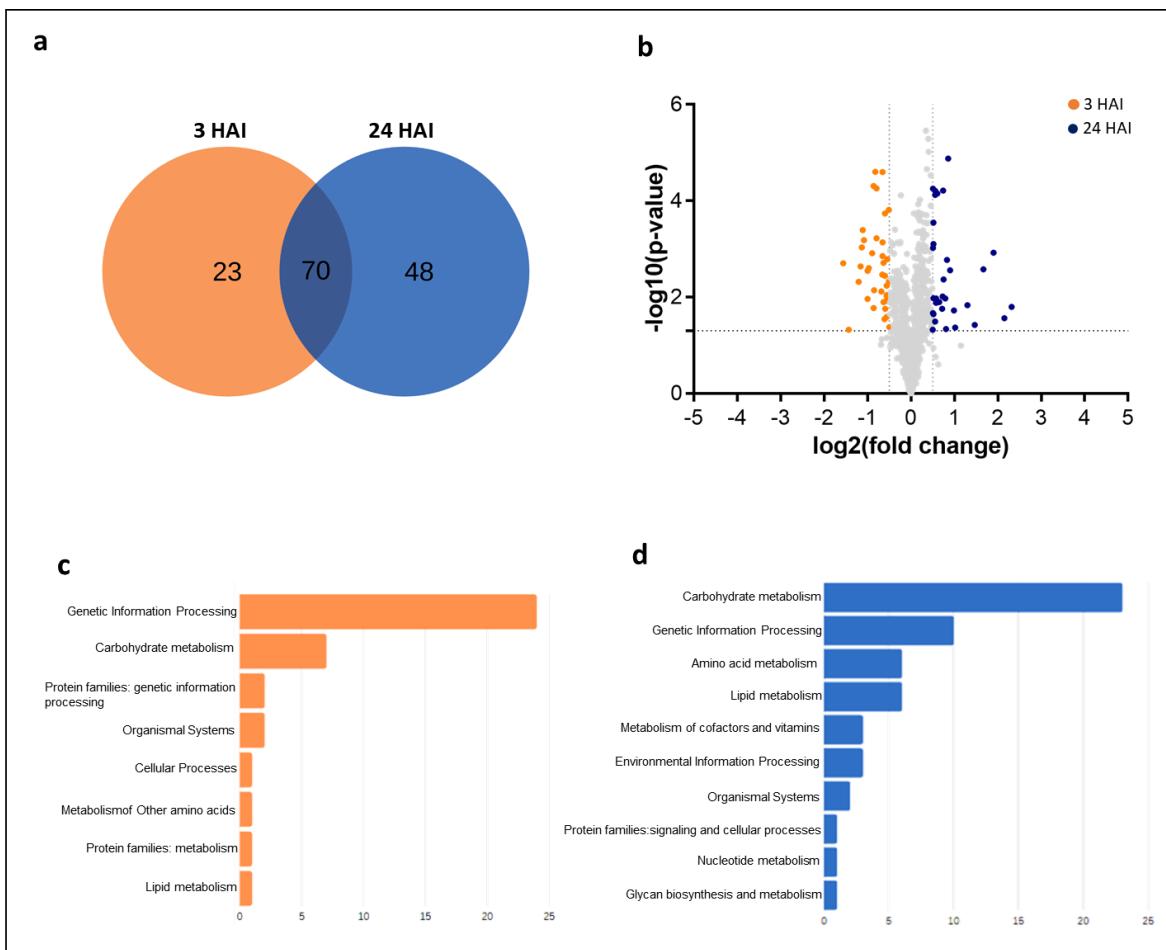


Figure 2. Quantitative analysis of proteomic data on the soybean embryonic axis at comparison of 24 HAI/3 HAI. A) Venn Diagram with differentially accumulated proteins. B) Volcano graph of different accumulated proteins in the comparison of seeds at 24HAI/3HAI. Orange dots: differentially accumulated proteins related to 3 HAI ($\log_2 \text{FC} \leq -0.5$ $p \leq 0.05$). Blue dots: differentially accumulated proteins related to 24 HAI ($\log_2 \text{FC} \geq 0.5$ $p \leq 0.05$). C e D) Horizontal bar graph showing the functional grouping of proteins differentially accumulated related to 3 (orange) and to 24 HAI (blue), using BlastKOALA tools.

Metabolic pathways involved with reserve mobilization in the embryonic axis

The mapping of the differentially accumulated proteins identified by proteomic analysis on metabolic pathways revealed important roles in reserve mobilization that occurs in the embryonic axis during seed germination (Figure 3). The differentially accumulated proteins with higher fold changes were S-ADENOSYLMETHIONINE SYNTHETASE (2.32) and LATE EMBRYOGENESIS ABUNDANT PROTEIN (-0.59) (Supplementary Table S1). Due to their importance as a source of amino acids and precursors of N compounds, the storage proteins LEAs and the RMLC-LIKE CUPINS were highlighted in our analyses (Figure 3). We found that LEA proteins and Cupins were down-accumulated in the comparison 24HAI/3HAI, showing a reduction in the accumulation during imbibition. LEAs can be classified into 8 subgroups (LEA1,

LEA2, LEA3, LEA4, LEA5, LEA6, Dehydrin and Seed Maturation Proteins), taking into account characteristics such as repeated motifs, amino acid composition and phylogenetic relationships (Hunault *et al.*, 2010; Wang *et al.*, 2017). These proteins accumulate in seeds during the later stages of embryogenesis (Hong-Bo *et al.*, 2005). We suggest that the strong presence of these proteins in the first hours of imbibition (Table 1) is explained by the two different types of water stress that the seed undergoes at the end of the maturation process followed by the quiescent stage and at the beginning of germination with tissue rehydration. In addition, LEAs can also be digested as storage proteins, to ensure the energy input required by the embryonic axis. As noted, at 24 HAI there is no presence of LEAs proteins. Likewise, cupins were higher accumulated at 3 HAI compared to 24 HAI (Figure 3). As part of cupins family, glycinins and β -conglycinin are the main storage proteins of soybean seeds (Tan *et al.*, 2013). The N-terminal region of the α and α' subunits of β -conglycinin are rich in glutamate, glutamine, aspartate, and asparagine, which, in addition to being used in protein synthesis, can be converted into citric acid cycle intermediates providing an energy supply or supporting the mobilization of lipid reserves by lipolysis, β -oxidation and the glyoxylate cycle (Lin *et al.*, 1982). Interestingly, we found early production of glutamine through the action of GLUTAMINE SYNTHASE (GS), which could be also involved with N-assimilation (Figure 3). These results suggest that the mobilization of storage proteins in the embryonic axis begins early in germination to meet energy demands and proteins biosynthesis.

Mature legume seeds, in addition to containing proteins and oils, contain important carbohydrate components, including starches and sugars (Tayade *et al.*, 2019). Concerning starch, enzymes GRANULE-BOUND STARCH SYNTHASE (WAXY) and β -AMILASE (BMY) were accumulated at 24 HAI. Carbohydrates are an essential component of legume seeds, they are mostly composed of starch, comprising 65-72% and 10-20% dietary fiber (Pehrsson, 2013). However, starch levels in legume seeds show a wide variation (Tayade *et al.*, 2019). In soybean embryonic axis, starch biosynthesis (WAXY protein) was positively regulated at 24 HAI, which corroborated with the increasing of starch granules in this time point (Figure 1). This starch accumulation was also supported by the identification of BMY uniquely at 24 HAI (Supplementary Table S1), suggesting that the starch is not a primary source of reserve mobilized during germination.

During maturation, soybean seeds accumulate soluble carbohydrates mostly as sucrose and galactosyl derivatives of sucrose - raffinose and stachyose - (Obendorf *et al.*, 2009). In the first day of germination, the amount of sucrose and sucrose-derived monosaccharides increased in the embryonic axis compared to 6 hours imbibed seeds

(Kuo *et al.*, 1990). Our proteomic data reveal that SUCROSE SYNTHASE (Susy) was significantly accumulated at 24 HAI (Figure 3). Susy can cleave sucrose in a reversible reaction to produce glucose and fructose. Sangi *et al.*, 2019 showed that glucose concentrations are more than ten times higher in the embryonic axis at 24 HAI compared to 3 HAI, which is corroborate with the higher accumulation of Susy in the end of germination (Figure 1). Also, the cell wall invertase genes were more expressed at the end of germination (24 HAI) (Bellieny-Rabelo *et al.*, 2016). Although Susy (Glyma.17G045800) was up-accumulated at 24 HAI on proteomic analysis, the expression pattern showed an increase at 6 HAI (Bellieny-Rabelo *et al.*, 2016). Accordingly, we found that the major glycolysis regulator in plants - PHOSPHOFRUCTOKINASE (PFK) and PIRUVATE KINASE (PK) - were regulated in the embryonic axis; PFK was up-accumulated at 3 HAI; PK protein (Glyma.02G165300.1) was up-accumulated at 3 HAI and Glyma.05G098000.1 uniquely identified at 24 HAI (Supplementary Table S1). These results suggested that sucrose is mobilized early in the germination to the triose phosphates and via ENOLASE (ENO) is converted to phosphoenolpyruvate (PEP), which PK convert to pyruvate. The upstream regulation of glycolysis via inhibition of PFK by PEP seems to have an important role in the balance of sucrose pools on embryonic axis during germination.

UDP-GLUCOSE PYROPHORYLASE (UGP) is a key enzyme in UDP-glucose production, which is involved in several metabolic pathways such as cell wall synthesis and raffinose and stachyose oligosaccharides (Ciereszko *et al.*, 2001; Kleczkowski, 1994; Amor *et al.*, 1995). In soybean embryonic axis, UGP was upaccumulated at 24 HAI (Figure 3). The UDP-glucose made by the action of UGP can be directed to cell wall metabolism, which is linked with the up-accumulation at 24 HAI of cell wall synthesis and remodeling enzymes -UDP-GLUCOSE DEHYDROGENASE (UGDH), UDP-D-XYLOSE SYNTHASE (AXS and UXS), β -1,3 GLUCANASE (GLU), XYLOGLUCAN ENDOTRANSGLUCOSYLASE HYDROLASE (XTH) (Figure 3).

Together with the increase of UGP in the end of germination, we found RAFFINOSE SYNTHASE (RS) and INOSITOL MONOPHOSPHATASE (IMP) with higher accumulation at 24 HAI (Figure 3). The IMP converts inositol to *myo*-inositol, which are essential to raffinose biosynthesis. RFOs play an important role in desiccation tolerance and declines during soybean germination (Koster and Leopold, 1988). This suggests that after breakdown, the biosynthesis of RFOs is induced in the embryonic axes at the end of germination.

Considering the oxygen sup-optimal levels in seeds during the first hours of imbibition, alternatives pathways such as fermentation take place to provide the necessary ATP for germination, when pyruvate usage by respiratory pathways is limited. In support to this, we found two ALDEHYDE DEHYDROGENASE (ALDH) proteins uniquely accumulated in soybean embryonic axis at 3 HAI (Glyma.17G091000.1) and 24 HAI (Glyma.01G031500.1) (Figure 3). ALDH enzymes use NAD⁺ or NADP⁺ as a cofactor to convert acetaldehyde, produced by ethanolic fermentation, in acetate, which is subsequently used for Acetyl-CoA synthesis (MIN et al., 2019). Other alternative pathway that contributes with the production ATP at initial stages of germination is the Perl's pathway (Rosental et al., 2014). In this pathway, pyruvate is converted in malate by NADP-MALIC ENZYME (ME); the MALATE DEHYDROGENASE (MDH) produces oxaloacetate (OAA), which can be converted to PEP by PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) (Weitbrecht et al., 2011). The action of PK converts PEP to pyruvate, which can enter in TCA cycle via Acetyl-CoA or reinitiate de Perl's pathway. The ASPARTATE AMINOTRANSFERASE (AST) can feed the Perl's pathway producing OAA from aspartate (Weitbrecht et al., 2011). The NADH recycling in the pathway can be performed by ALDH. Our proteomic data showed that two ME proteins were uniquely accumulated at 3 (Glyma.08G201200.1) and 24 HAI (Glyma.04G086300.4) (Figure 3). The MDH and AST were found only at 24 HAI (Figure 3). Together with the PK, ME produces malate in the beginning of germination, which can start the Perl's pathway. Once activated, MDH and AST will feed the pathway with OAA. Although we did not find PEPCK in our proteomic data, its higher expression in the embryonic axis at 24 HAI (Bellieny-Rabelo et al., 2016), corroborates this hypothesis. With the action of ALDH, the NADH can be recycling in the pathway.

The triacylglycerol (TAG) breakdown is the initial step in oil utilization, which is catalyzed by TAG lipase in the lipid bodies to produce free fatty acids and glycerol (Eastmond, 2006). Besides TAG lipases, lipid bodies membranes are composed of oleosin, caleosin, and 13-LOX (Feussner et al., 2001). During germination, the 13-LOX are involved with the mobilization of storage TAGs for β-oxidation through oxygenation of free linoleic acids (Feussner et al., 2001). A special 13-LOX isoform in cucumber oxygenates TAGs without the action by a lipase and is synthesized at early stages of germination (Feussner et al., 1997). Our proteomic data reveal that different lipoxygenase 13 I (13-LOX I) proteins were accumulated at 3 and 24 HAI, while 9-LOX was only accumulated at 24 HAI (Figure 3). These soybean LOX proteins were classified based on the study of Song et al., (2016). Besides, we found that the oleosin (OLE) was uniquely accumulated at 3 HAI (Figure 3). These results suggest that mobilization of TAGs starts with the 13-LOX action at initial stages of

germination, which will decrease the amount of OLE proteins. In the next steps, the free fatty acids are then transferred to the glyoxysome and subsequently catabolized by β -oxidation to generate acetyl-CoA which is converted to sugars by the glyoxylate cycle and gluconeogenesis (Bunkelmann *et al.*, 1995; Graham *et al.*, 2002). The unique accumulation of ASCORBATE PEROXIDASE (APX) at 24 HAI further supported β -oxidation.

A considerable number of proteins involved in mitochondrial repair were found to be positively regulated at 3 HAI, such as RIBOSOMAL PROTEIN S (RPS) and RIBOSOMAL PROTEIN L (RPL). In one study, early ribosomal RNA synthesis was shown in germinating embryos (Spiegel *et al.*, 1975). Ribosomal proteins (RPs) act in the formation and stabilization of the ribosomal complex and in mediating protein synthesis (Ban *et al.*, 2000; Barakat *et al.*, 2001; Hanson *et al.*, 2004). Previous studies indicated that components required for the resumption of protein synthesis in germination and ribosomes were already present in maize quiescent embryo axis (QEAs) (Beltránpeña *et al.*, 1995; Bewley, 1997).

Seeds contain THIOREDOXIN (Trx) systems for reduction of the storage proteins during germination (Alkhalfioui *et al.*, 2007). Lozano *et al.*, (1996) also suggested the potential role of Trx in storage redox metabolism and proteolysis during germination. The resumption of germinative metabolism promotes a high production of reactive oxygen species; TRX acts conferring protection against oxidative damage generated in this process (Aalen, 1999), corroborating with our proteomic analysis where Trx were uniquely accumulated at 3 HAI (Figure 3). The GLUTATHIONE-S-TRANSFERASE (GSTF) have a role on metabolic detoxification and can also be oxidized during imbibition (Alkhalfioui *et al.*, 2007), which agrees with its up-accumulation at 3 HAI (Figure 3). PROTEIN DISULFIDE ISOMERASE (PDI) was also found to be oxidized during imbibition (Alkhalfioui *et al.*, 2007) and was uniquely accumulated at 24 HAI (Figure 3). Together, the Trx and glutathione-mediated reduction starts early in the embryonic axis imbibition and seems to be important for metabolic detoxification during reserves mobilization.

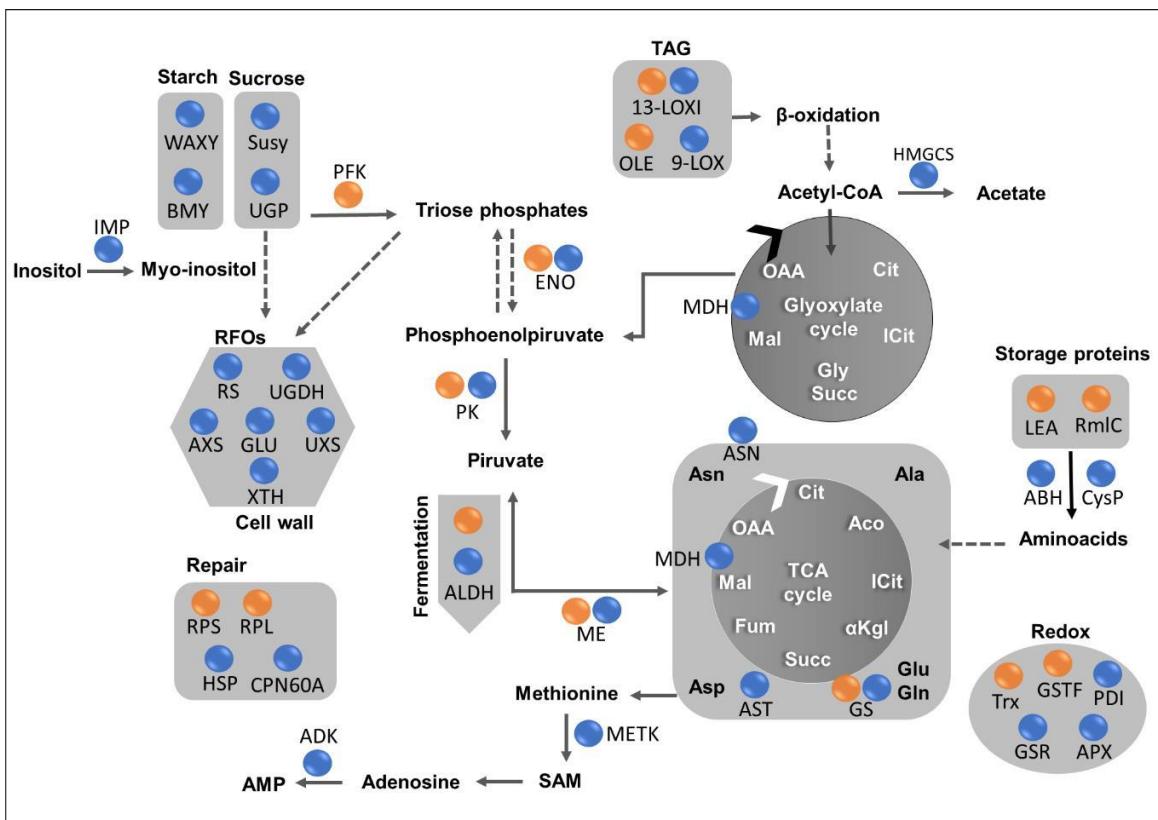


Figure 3. Metabolic pathways involved with reserve mobilization in soybean embryonic axis during germination as reflected by proteomic data. Orange circles indicate positively regulated proteins at 3 HAI; Blue circles indicate positively regulated proteins at 24 HAI. Uniquely accumulated proteins were also shown. Dashed arrows represent intermediate metabolic reactions. Genes highlighted in the metabolic pathway: s-adenosylmethionine synthetase (SAM), late embryogenesis abundant protein (LEA), cupins (RMLC-LIKE), glutamine synthase (GS), granule-bound starch synthase (WAXY), β-amilase (BMY), sucrose synthase (Susy), phosphofructokinase (PFK), piruvate kinase (PK), phosphoenolpyruvate (PEP), UDP-glucose pyrophosphorylase-UDP-glucose dehydrogenase (UGDH), UDP-D-xylose synthase (AXS), β-1,3 glucanase (GLU), xyloglucan endotransglucosylase hydrolase (XTH), raffinose synthase (RS), inositol monophosphatase (IMP), aldehyde dehydrogenase (ALDH), NADP-malic enzyme (ME), malate dehydrogenase (MDH), Oxaloacetate (OAA), phosphoenolpyruvate carboxykinase (PEPCK), aspartate aminotransferase (AST), ascorbate peroxidase (APX), ribosomal proteins (RPS), ribosomal protein (RPL), thioredoxin (Trx), glutathione-s-transferase (GSTF), protein disulfide isomerase (PDI).

Content of total protein, triglycerides, and glucose in the embryonic axis

Proteins, starch and lipids are hydrolyzed in seed storage tissues and then converted to sugar (sucrose) and amino acids (asparagine and glutamine), which are available for plant growth and development (Derek Bewley and Black, 2013). Considering our proteomic data, we evaluate the content of proteins, TAGs, and glucose in the embryonic axis throughout germination. The total protein dosage revealed a significant decrease at 3 HAI with a major reduction at 24 HAI (Figure 4.a). This data suggests that

the mobilization of proteins in the embryonic axis starts early at 3 HAI and decreases until 24 HAI, which corroborated with the accumulation of storage proteins like Cupins and LEAs at 3 HAI, but not at 24 HAI (Figure 3). To further confirmed the mobilization of storage proteins, we performed western blotting with anti- Vicilin, member of the Cupin family. The results of the western blot showed a reduction in the molecular weight of the Vicilins in the embryonic axis during germination (Figure 4.b). This reduction was more pronounced at 24 HAI, corroborating with the higher accumulation of cysteine proteases found on the proteomic analysis (Figure 3). These results indicate that storage proteins are mobilized in the embryonic axis since the beginning of germination through the action of proteases to release free-amino acids for the synthesis of new proteins.

As suggested by our proteomic analysis, TAGs can be mobilized by β -oxidation and then converted to soluble carbohydrates by the glyoxylate cycle and gluconeogenesis in the embryonic axis tissues (Figure 3). Thus, we investigate the TAGs content in the embryonic axis during germination. The TAGs dosage showed a large decrease at 24 HAI compared to the dry seed and initial hours after imbibition (Figure 4.c). This result suggests that the consumption of storage lipids occurs later in the germination to supply the synthesis of sugars that will be destined to starch accumulation, cell wall biosynthesis, and respiratory pathway, which is supported by the accumulation of flavodoxin-like quinone reductase protein at 24 HAI (Supplemental Table S1). To further analyze the partition of carbohydrates in the embryonic axis, we performed a glucose dosage. The glucose content varies throughout the germination points with significant decrease at 3 HAI and 12 HAI (Figure 4.d). This profile suggests that glucose pool can be consumed early in the germination to meet the energy demands for embryonic axis growth. In sunflower seeds, the primary carbohydrate source used during germination is glucose. Furthermore, an increase in the total soluble sugar levels was reported in sunflower after the 24-hour period (Erbaş *et al.*, 2016). In soybean embryonic axis, the pool of soluble sugars only changed after radicle protrusion (Supplementary Figure S1), which agrees with the accumulation of RS and IMP at 24 HAI (Figure 3).

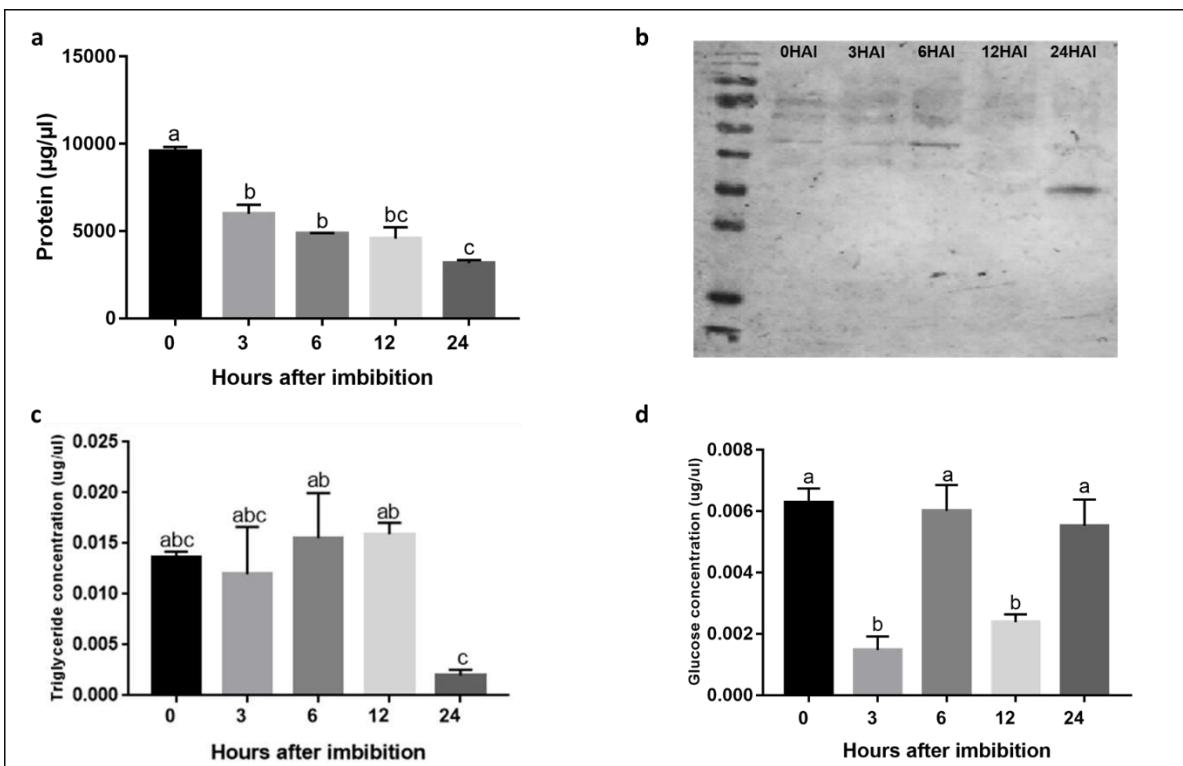


Figure 4. Protein, TAGs and glucose content of soybean embryonic axes on dry seeds and 3, 6, 12 and 24 HAI. a) Protein dosage using BCA method; b) Western blot with anti-vicilin; c) TAGs dosage; and d) Glucose dosage. Means followed by different letters showed significant differences ($P \leq 0.05$) according to Tukey's test.

Soybean cultivars show divergence in mobilization of reserves during germination

Sensu stricto germination is the complex metabolic process starting with the absorption of water from dry seeds (imbibition) to root protrusion (Weitbrecht *et al.*, 2011; Rajjou *et al.*, 2012). More vigorous seeds show higher capacity of reserves mobilization throughout the germination, which affect the radicle protrusion and later crop establishment (Henning *et al.*, 2010). To evaluate the capacity of nutrients mobilization, we compared two important soybean cultivar - BRS 284 and Williams 82. The BRS 284 conventional soybean cultivar reached the highest germination percentage at 24 HAI, with 90% of germinated seeds. In the same time point, Williams 82 germinated 40% of the seeds (Figure 6.a). The germination of BRS284 began at 15 HAI and remaining stable after 24 HAI, while Williams 82 was slower during the hours of imbibition, therefore, requiring more time to complete germination (Figure 6.a). We then compared the fresh mass of embryonic axes of these cultivars at 3 and 24 HAI (Figure 5.b). The fresh mass of BRS 284 was smaller than Williams 82 at 3 HAI, while at 24 HAI BRS284 showed the highest fresh mass. This result suggests that in the beginning of Williams 82 germination

there is greater water absorption, promoting the growth of the embryonic axis. However, during the imbibition hours the germination process becomes slower. To verify whether the prolonged germination period of Williams 82 could be related to mobilization of reserves, we quantify proteins and TAGs contents in the embryonic axis from dry seeds, 3, 6, 12 and 24 HAI. Protein and TAGs dosages showed no significant difference between the analyzed points (Figures 6.c and 6.d). We found in the analysis of total protein a slight decrease in protein content after seed imbibition, maintaining minimal changes as germination progresses (Figure 6.c). For TAGs, a slight decrease at 12 HAI was observed, although there is no significant difference (Figure 6.d). When compared to BRS284, proteins and TAGs are not mobilized during imbibition hours until 24 HAI, where almost all BRS284 seeds germinated.

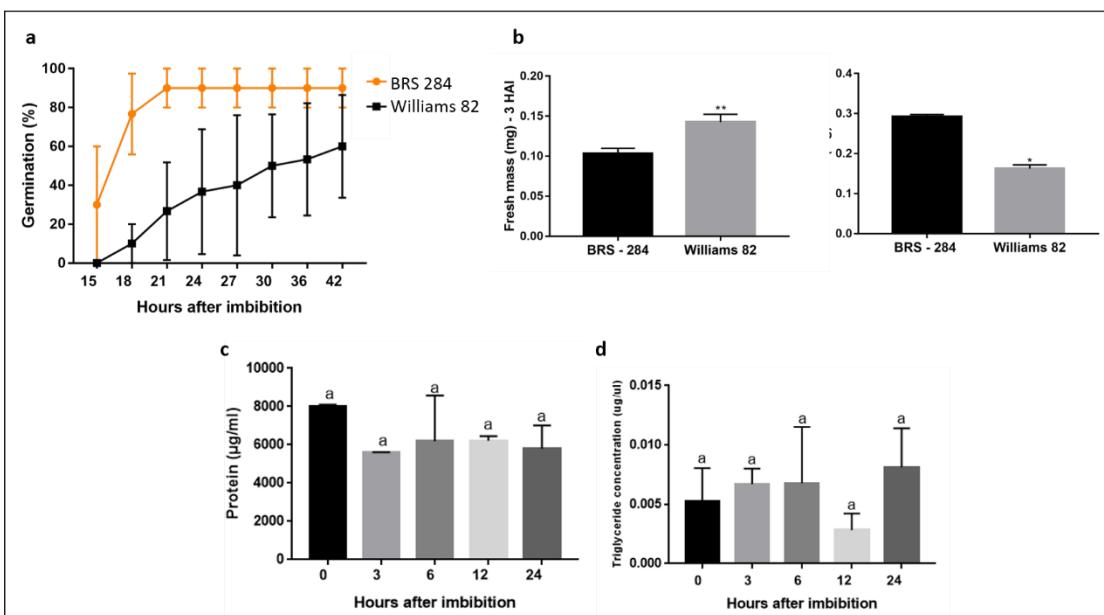


Figure 5. Germination analysis of soybean embryonic axes from cv. BRS284 and cv. Williams 82. a) Germination rate of seeds from cv. BRS 284 and Williams 82; b) Percentage of fresh mass of embryonic axes at 3 and 24 HAI from cv. BRS 284 and Williams 82; c) Protein dosage using BCA method from Williams 82; d) TAGs dosage Williams 82. Means followed by different letters showed significant differences ($P \leq 0.05$) according to Tukey's test. *Indicate significant difference $P \leq 0.05$; ** Indicate significant difference $P \leq 0.01$.

Conclusion

In this study, we focused on the identification of molecular events that are involved with reserve mobilization in the soybean embryonic axis during germination. Through histochemical analysis, a higher protein deposition was observed at 3 HAI compared to 24 HAI. We showed that this protein abundance decreases as germination progresses to supply the energetic need of the growing embryo. Starch was highest

accumulated at 24 HAI, showing that this carbohydrate is synthesized late in germination. The starch granules may be related to lipid mobilization controlled by the concentration of soluble sugars. By quantitative proteomics, we found 141 differentially accumulated proteins in the embryonic axis comparing 24 with 3HAI. Metabolic pathway analysis revealed important roles of several proteins differentially or uniquely accumulated in the comparison 24 HAI/3HAI in the reserve mobilization that occurs in the embryonic axis during seed germination. Proteins seem to be mobilized in the first hours of germination and, together with carbohydrates, they are the main reserve compounds that act to supply energy to the embryonic axis. Although proteins related to lipid metabolism were up-regulated in the first hours of imbibition, the breakdown of lipids only occurred in the last hours of germination. Through biochemical analyses we show that the content of proteins and lipids decreases during germination, while the amount of glucose varies in the hours of imbibition. We show that in the Williams 82 cultivar there is no mobilization of TAGs and proteins during germination as occurs in the BRS 284 soybean. Here, we showed the mobilization of storage macromolecules that are a source of energy and carbon for seed germination and crop establishment since efficient mobilization reflects in the formation of more vigorous seedlings.

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4. Considerações Finais

Neste estudo analisamos a mobilização de nutrientes reserva no eixo embrionário de semente de soja durante a germinação. Na análise histoquímica, a mobilização de proteínas foi majoritariamente em 3 HAE, enquanto em 24 HAE houve maior acumulação de amido. Os corpos proteicos diminuem à medida que a germinação progride, corroborando com a degradação de proteínas para fornecer aporte para o crescimento do embrião. Os grânulos de amido podem estar relacionados com a mobilização lipídica controlada pela concentração de açúcares solúveis. A proteômica quantitativa mostrou que 141 proteínas foram diferencialmente acumuladas no eixo embrionário na comparação 24

HAE/3HAE. Em 3 HAE as principais proteínas identificadas estão relacionadas ao processamento de informação genética, seguido das proteínas relacionadas com o metabolismo de carboidratos. Em 24 HAE, as principais proteínas identificadas estão relacionadas com metabolismo de carboidratos, seguido pelo processamento de informação genética, metabolismo de lipídios e metabolismo de aminoácidos. Nesta análise, mostramos ainda que as proteínas e carboidratos solúveis são a fonte primária de reserva para o crescimento do eixo embrionário. Por outro, lipídios são mobilizados mais tarde na germinação e parecem estar envolvidos com a homeostase de açúcares. A dosagem de conteúdo de proteínas, lipídios e glicose corroborou os resultados da proteômica. A comparação da mobilização de reservas em dois cultivares importantes de soja mostrou que não há mobilização de TAGs e proteínas durante a germinação da cv. Williams 82, como ocorre na cv. BRS284. Juntos, os nossos resultados revelaram proteínas, carboidratos e lipídeos de armazenamento mobilizados no eixo embrionário durante a germinação fornecendo informações importantes da atuação desses compostos para propiciar a germinação.

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