

Artigos

Changes in the proteomic and polyamine profile induced by seed aging of *Cariniana legalis* (Martius) O. Kuntze

Alterações no perfil proteômico e de poliaminas induzido pelo envelhecimento de sementes de *Cariniana legalis* (Martius) O. Kuntze

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ABSTRACT

Studies on metabolic processes and seed germinability of native endangered species are essential for its conservation. The objective of this work was to evaluate the effect of accelerated aging on germination and on the proteomic and polyamine (PAs) profile in seeds of *Cariniana legalis*. The seeds were aged at 41, 43, 45 and 47°C for 24, 48, 72 and 96 h, under 100 % relative humidity using a completely randomized factorial design. The data were submitted to analysis of variance followed by Tukey's test. The temperature of 47°C was efficient in simulating seed aging, with no germination after 72 h of incubation. At 41°C, the germination did not change significantly until 96 h. The reduction in the concentration of total free PAs, mainly spermidine, and the down-accumulation of heat shock proteins in seeds aged at 47°C were associated with induction of seed aging. Furthermore, the down-accumulation of the aconitate hydratase protein and the up-accumulation of the alcohol dehydrogenase protein were associated with loss of seed germinability and rapid deterioration in seeds aged at 47°C. The aging of *C. legalis* seeds is related to alteration in the contents of PAs and accumulation of some proteins involved with the loss of germinability.

Keywords: Protein accumulation; Temperature; Seed deterioration; Endangered woody specie

RESUMO

Estudos sobre processos metabólicos e germinabilidade de sementes de espécies nativas ameaçadas de extinção são essenciais para sua conservação. O objetivo deste trabalho foi avaliar o efeito do envelhecimento acelerado na germinação e no perfil proteômico e de poliamina (PAs) em sementes de *Cariniana legalis*. As sementes foram envelhecidas a 41, 43, 45 e 47°C por 24, 48, 72 e 96 h, sob 100% de umidade relativa, usando um delineamento fatorial inteiramente casualizado. Os dados foram submetidos à análise de variância seguida de teste Tukey. A temperatura de 47°C foi eficiente em simular o envelhecimento das sementes, que não germinaram após 72 h de incubação. Em 41°C, a germinação não mudou significativamente até 96h. A redução na concentração de PAs livres totais, principalmente espermidina e a diminuição no acúmulo de proteínas de choque térmico em sementes envelhecidas à 47°C foram associados à indução do envelhecimento das sementes. Além disso, a diminuição no acúmulo da proteína aconitato hidratase e o aumento no acúmulo da proteína álcool desidrogenase foram associados à perda de germinabilidade das sementes e rápida deterioração em sementes envelhecidas à 47°C. O envelhecimento de sementes de *C. legalis* está relacionado à alteração no conteúdo de PAs e a alteração de acúmulo de algumas proteínas envolvidas na perda da germinabilidade.

Palavras-chave: Acúmulo de proteínas; Temperatura; Deterioração de sementes; Espécies arbóreas ameaçadas de extinção

1 INTRODUCTION

Cariniana legalis (Martius) O. Kuntze (Lecythidaceae), popularly known as jequitibá-rosa, is an endangered native species from Brazilian Atlantic Rain Forest (CARVALHO, 2005), classified in the vulnerable category by the International Union for Conservation of Nature (IUCN, 2021). Its propagation is commonly made by seeds (RÊGO, 2002). After dispersal, the *C. legalis* seeds can lose viability rapidly, even under controlled storage conditions at 4 - 6°C (SOUSA *et al.*, 2016; ARAGÃO *et al.*, 2019). Due to the ecological relevance of this species, physiological and biochemical studies are essential for seed conservation.

The loss of seed viability involves the deterioration process, undergoing physiological and biochemical metabolic changes, which can occur slowly or accelerated, depending on storage conditions and characteristics of each species (JYOTI; MALIK, 2013). In order to assess the seed germinability, the accelerated aging test can be useful to understand the changes that lead to the deterioration of seeds

(KRZYŻANOWSKI *et al.*, 1999). Originally, the purpose of the test was to estimate the longevity of stored seeds. Currently, it has been widely used as an approach to study once the deterioration processes occurring in this test is similar to what occurs in the natural aging of seeds, however, at an accelerated speed and in less time of experimentation (MARCOS FILHO, 2015).

The process of deterioration in seeds involves many metabolic changes, including the reduction of metabolic and stress response proteins (ARAGÃO *et al.*, 2019; LERIN *et al.*, 2021). In this context, the use of comparative proteomics research appears as an important tool for identification of specific proteins that can be related with the loss of seed viability. During accelerated aging of seeds in *Vigna mungo*, the up-regulation of TU elongation factor (EF-TU) protein was correlated to an increase in germination, while, the down-regulation of actin protein was related to damage of cell membrane in aged seeds (SATHISH *et al.*, 2015). Seeds of *C. legalis* stored during 12 months at 6°C showed a reduction in the accumulation of ferritin and superoxide dismutase proteins, related to protection against oxidative stress and damage of cellular components, being associated to the loss of seed viability (ARAGÃO *et al.*, 2019). In addition, it has been showed that the storage of *C. legalis* seeds at 25°C, instead of 6°C, induced down-accumulation of proteins related to metabolic processes, such as aconitate hydratase, and cellular homeostasis, such as heat shock proteins (HSPs) and the down-accumulation of both proteins was related to decreased germination of stored seeds (LERIN *et al.*, 2021). Thus, the proteomic analysis coupled with accelerated aging approach may contribute to the knowledge of proteins involved in seed vigor and viability during storage.

Polyamines (PAs) are small aliphatic amines that have important regulatory roles in plant growth and development (KAUR-SAWHNEY *et al.*, 2003; KUSANO *et al.*, 2008), including seed development (SANTA-CATARINA *et al.*, 2006) and germination (PIERUZZI *et al.*, 2011). The most common PAs found in higher plants are spermidine (Spd), spermine (Spm) and putrescine (Put) (KUSANO *et al.*, 2008). During seed storage

at 4°C, a higher content of free Put in *C. legalis* seeds was associated with greater reduction in vigor and seedling emergence compared with to *C. fissilis* seeds (SOUSA *et al.*, 2016). However, little is known about PAs during seeds accelerated aging.

In this sense, the objective of this work was to evaluate the effect of accelerated aging on germination and on the proteomic and PAs profile in seeds of *C. legalis*.

2 MATERIAL AND METHODS

2.1 Plant material

Mature seeds were provided by the Caiçara Comércio de Sementes LTDA. Seed were collected soon after their dispersion, in September 2016, from trees in a natural area located in Brejo Alegre, SP, Brazil (21°10'S and 50°10'W). The analyses to determine the germinability were performed with non-stored dry seeds.

2.2 Accelerated aging of seeds

Seeds were placed on wire mesh screens and suspended over 40 ml of water inside plastic boxes (11 x 11 x 35 cm). The plastic boxes were then incubated in a biochemical oxygen demand (BOD)-type germination chamber (Eletrolab, São Paulo, Brazil) at 41, 43, 45 and 47°C for 0, 24, 48, 72, and 96 h in 100% relative humidity. The temperatures of 41 and 47 °C were used for the physiological and biochemical analysis. Non-aged seeds, i.e., seeds before start the accelerated aging experiment, were used as time zero. At each temperature (41 and 47°C) and time of incubation (0, 24, 48, 72 and 96 h) the analysis of seed moisture content, germination (%), germination speed index (GSI) and PAs content were performed. Proteomic analysis was performed using non-aged (time 0) and seeds aged by 48 h at 41 and 47°C, being the time of 48 h selected due to the significant differences in germination capacity of seeds comparing the two temperatures tested.

2.3 Germination analysis

This analysis was performed according to Brasil (2013), using four replicates (with 50 seeds each) from each treatment. Seeds were distributed upon sheets of germitest® paper (J Prolab, Paraná, Brazil) moistened with sterile distilled water at a ratio of 2.5 times the dry paper mass, and incubated in a BOD-type germination chamber at 25°C, with photoperiod of 8 h light/16 h dark, at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For germination speed index (GSI) analysis, the germination was evaluated daily until 28th day according to Maguire (1962), considering the protrusion of radicle from the coat-seed. The total germination (%) was obtained at 28th day considering the normal seedlings obtained, i.e., those with well-developed aerial parts and root systems, with the ability to continue further development (BRASIL, 2013).

2.4 Seed moisture content

This analysis was performed according to Brasil (2009) with modifications, using four samples (2 g fresh matter [FM] each) of seeds at each time of storage and temperature tested. First, the samples were weighed to obtain 2 g FM each sample, dried at 105°C for 24 h in a chamber with forced air circulation (Ethik technology, São Paulo, Brazil) and then, weighed again to obtain the dry matter (DM).

2.5 Free-PAs determination

This analysis was performed according to Santa-Catarina *et al.* (2006), using three biological samples (200 mg FM each) of seeds from each treatment. Samples were ground with 5% (v/v) perchloric acid (Merck, Darmstadt, Germany) and free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck). Free PAs were identified by high performance liquid chromatography using a 5- μm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS) at 1 mL min⁻¹, at 40°C. The PA concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission), by comparison with the PAs standard Put, Spd and Spm (Sigma-Aldrich).

2.6 Comparative proteomic analysis

The proteomic profile analysis was performed using three biological samples (100 mg FM each) per treatment non-aged seeds (time zero) and seeds aged during 48h at 41 and 47°C.

Protein were extracted according to Lerin *et al.* (2021), using extraction buffer comprising 20 mM Tris-HCl (GE Healthcare, Piscataway, USA) pH 6.8, 1% dithiothreitol (GE Healthcare), 0.1% sodium dodecyl sulfate (GE Healthcare) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, USA). Protein concentration was measured using the 2-D Quant Kit (GE Healthcare). Before the trypsin digestion step, protein samples (100 µg from each biological replicate) were precipitated using the methanol/chloroform, and the samples were resuspended in a solution buffer (urea 7 M/thiourea 2 M). Protein digestion was performed by filter-aided sample preparation (FASP) methodology, according to Reis *et al.* (2021). Then, the peptides and proteins were quantified through a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), at a wavelength of 205 nm.

Mass spectrometry was performed using a nanoAcquity UPLC connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK) according to Passamani *et al.* (2020). Runs consisted of three biological replicates of 1 µg of digested peptides. During separation, samples were loaded onto the nanoAcquity UPLC M-Class Symmetry C18 5 µm trap column (180 µm × 20 mm) at 5 µL min⁻¹ for 3 min and then onto the nanoAcquity M-Class HSS T3 1.8 µm analytical reversed-phase column (75 µm × 150 mm) at 400 nL min⁻¹, with a column temperature of 45°C. Mass spectra were acquired by MassLynx v 4.0 software.

Spectra processing and comparative analysis were performed according to Passamani *et al.* (2020). Spectra processing and database searching were performed using ProteinLynx Global Server (PLGS) software v. 3.0.2 (Waters) and comparative

label-free quantification was performed using ISOQuant software v. 1.7 (DISTLER *et al.*, 2016). Briefly, for ISOQuant the following parameters were used to identify proteins: a 1% false discovery rate, a peptide score greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein were required for label-free quantitation using the TOP3 approach, followed by the multidimensional normalized process within ISOQuant. The proteomics data were processed against the *Helianthus annuus* protein databank (ID: UP000215914), which was selected based on the PhyloT phylogenetic tree proximity (LETUNIC; BORK, 2016) generated from all plant species (<http://itol.embl.de>), being the database available at UniProtKB (www.uniprot.org).

For comparative proteomic analysis, only proteins present in all three biological replicates, or absent (for unique proteins), were considered. Data were analyzed using Student's t-test ($P \leq 0.05$). Also, the criterion of Log_2 fold change (FC) was considered for the calculation of the differential accumulated protein (DAP), being considered up-accumulated when $\text{Log}_2 \text{FC} > 0.6$ and down-accumulated when $\text{Log}_2 \text{FC} < -0.6$. Description and functional annotation were performed for the differentially abundant proteins using OmicsBox software (<https://www.biobam.com/omicsbox>).

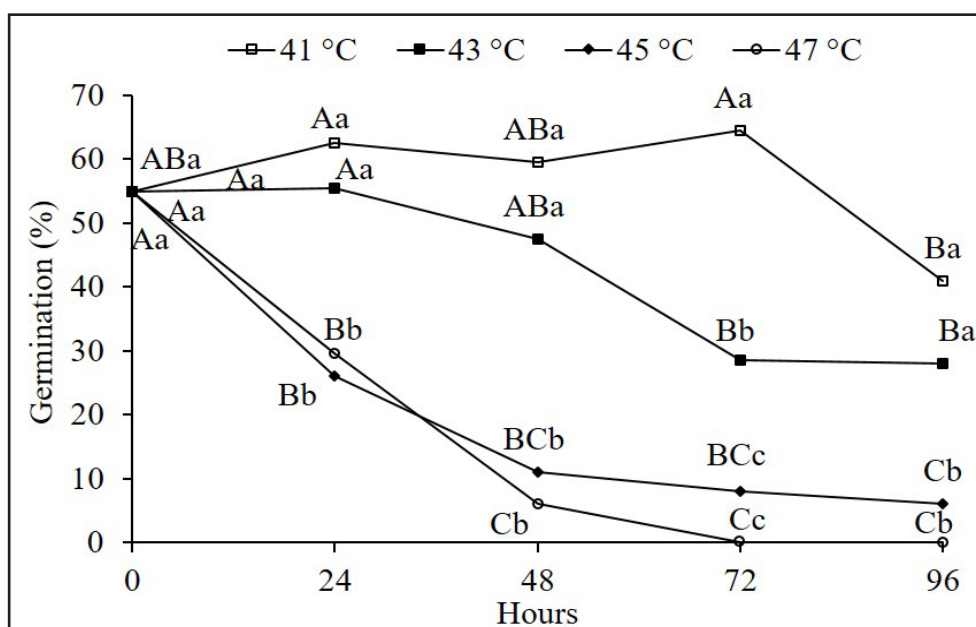
2.7 Statistical analysis

The first experiment with 4 temperatures (41, 43, 45 and 47°C) and 5 incubation times (0, 24, 48, 72, and 96 h), and then another experiment with 2 temperatures (41 and 47°C) and 5 incubation times (0, 24, 48, 72, and 96 h) were performed using a completely randomized factorial design. The germination, seed moisture content and GSI data were analyzed by Shapiro-Wilk and Bartlett tests for normal distribution and homogeneity, and were submitted to analysis of variance (ANOVA) ($P \leq 0.05$) followed by Tukey's test using the program R CORE TEAM (2018) (R Foundation for Statistical Computing, version 3.4.4, 2018, Vienna, Austria).

3 RESULTS AND DISCUSSION

Studies have shown a variation between the ideal temperatures used for the accelerated aging of seeds according to the species (CORTE *et al.*, 2010; GUEDES *et al.*, 2011). In this work, from the temperatures tested (Figure 1), it was observed that 41°C affected seed germination (Figure 2a) and GSI (Figure 2b) after 96 h of incubation, while at 47 °C, the germination (Figure 2a) and GSI (Figure 2b) decreased significantly with the increase of incubation time. These results demonstrate that the temperature of 41 °C was not efficient to simulate aging in *C. legalis* seeds until 72 h of incubation, on the contrary, this temperature and the high moisture degree stimulated the germination of the most vigorous seeds. However, the temperature of 47 °C stimulate aging of seeds in *C. legalis* compared to 41°C (Figure 2).

Figure 1 – Germination of *Cariniana legalis* seeds aged at 41, 43, 45 and 47°C before (non-aged, time zero) and after 24, 48, 72 and 96 h of incubation



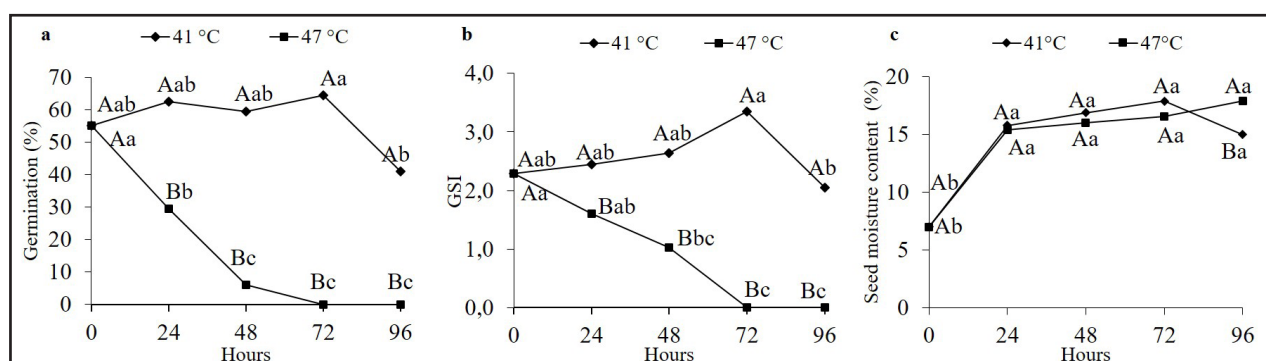
Source: Authors (2021)

In where: *Uppercase letters indicate significant differences between temperatures of aging. Lowercase letters indicate significant differences among the hours of aging. Means followed by the same letter do not differ statistically between them according to Tukey's test ($P \leq 0.05$). CV = Coefficient of variation ($n = 4$, CV germination = 28.86%).

For *Melanoxylon brauna*, 40°C did not affect seed germination until 72h of incubation, while 45°C affected the germination in the first 24 h of exposure (CORTE *et al.*, 2010). On the other hand, for *Dalbergia nigra* the 41 and 45°C only affected seed viability after 72 h, and the seed moisture content increased constantly during the 96 h of incubation at 45°C, resulting in more accelerated deterioration of seeds (GUEDES *et al.*, 2011).

In the present results, the seed moisture content significantly increased after 24h of exposure at both temperatures (41 and 47°C), being significantly higher in seeds aged at 47°C only after 96h of incubation (Figure 2c). The high moisture content can increase the respiratory rate, restarting the metabolic activities of the seeds, and consequently, the depletion of reserve substances may occur (SHABAN, 2013). Besides seed moisture content contribute for seed deterioration, our results indicate that it was not the main factor to induce seed aging in *C. legalis*, since it increased in seeds at both temperatures (41 and 47°C) evaluated.

Figure 2 – Germination (a), germination speed index (b) and moisture content (c) of *Cariniana legalis* seeds aged at 41 and 47°C, before (non-aged, time zero) and after 24, 48, 72 and 96 hours of incubation

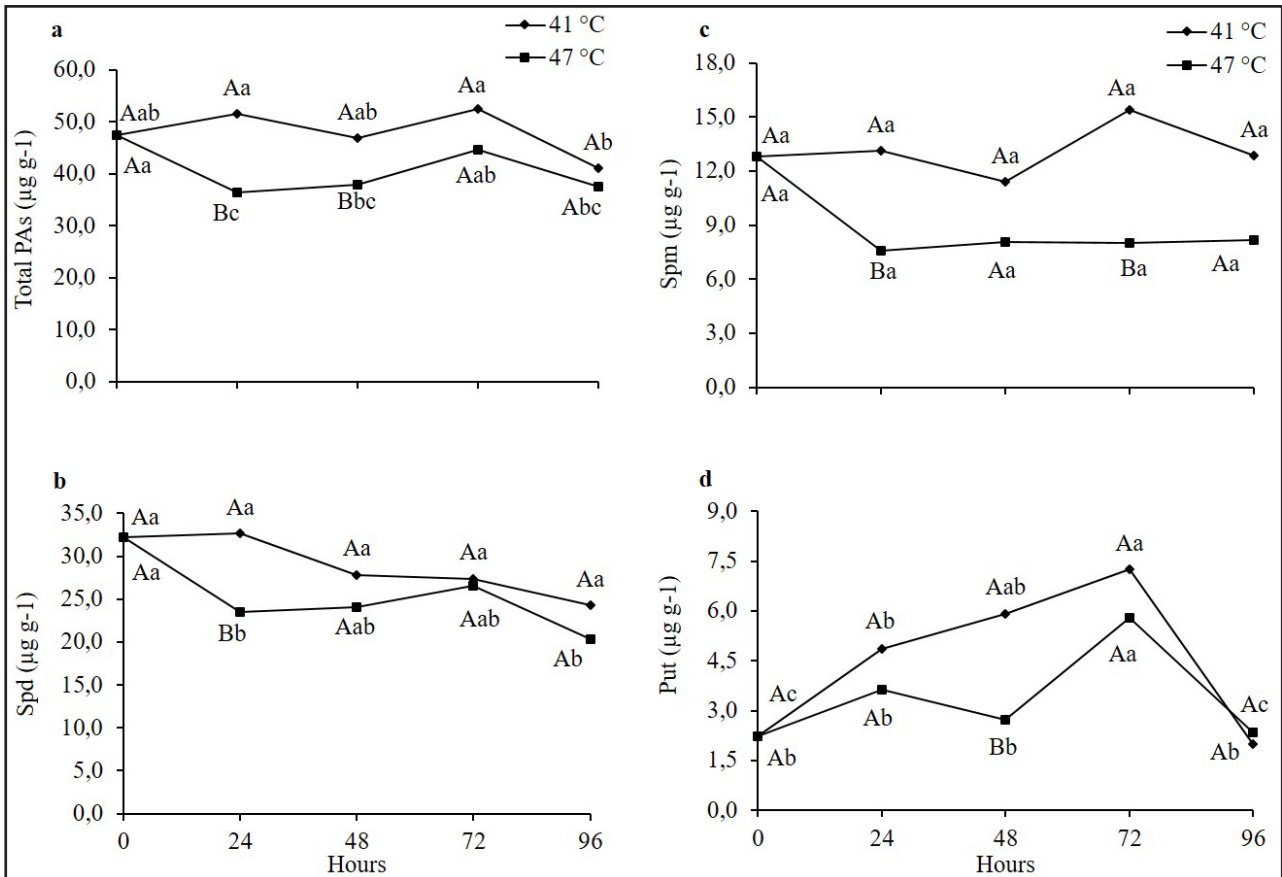


Source: Authors (2021)

In where: *Uppercase letters indicate significant differences between temperatures of aging. Lowercase letters indicate significant differences among the hours of aging. Means followed by the same letter do not differ statistically between them, according to Tukey's test ($P \leq 0.05$). CV = Coefficient of variation ($n = 4$, CV germination = 29.85%; CV GSI = 29.22%; CV seed moisture content = 10.24%).

The content of total free PAs was significantly higher in seeds at 41 °C until 72 h of incubation compared to those at 47 °C. A decrease on contents of total free PAs was observed until 48 h of incubation at 47 °C, while at 41°C there was no significant differences during time of incubation (Figure 3a). The high content of total free PAs and the higher germination observed in seed aged at 41 °C indicate a positive correlation of PAs contents to seed viability. Among the free PAs, the Spd (Figure 3b) showed higher content compared to Spm (Figure 3c) and Put (Figure 3d). At 41 °C there was no significant differences for free Spd over time, while at 47 °C, the free Spd content decreased significantly after 24 and 96 h (Figure 3b). Free Spm content did not change over incubation time for the two temperatures (Figure 3c). The content of Put was the lowest among the evaluated PAs at both temperatures, being the highest content found after 72 h of incubation in both temperature, with no significant differences (Figure 3d). The highest content of free Spd and Spm compared to free Put content was observed in *C. legalis* seeds storage at 4 °C during 12 months (SOUSA *et al.*, 2016). This suggests a similar response in changing the PAs concentration induced both in seeds stored at 4 °C as in seeds under accelerated aging. Our results indicate the relevance of higher free Spd content (Figure 3) for germination process in seeds aged at 41 °C, while the content was negatively affected in seeds aged at 47 °C, affecting also seed germination (Figure 2). In addition, Sousa *et al.* (2016) showed a significant reduction on contents of total free PAs, Spd and Spm during storage at 4 °C, being related to a reduction on seedling emergence of *C. legalis*. Thus, a reduction/lower content on this PA could be related to a reduction on seed germination potential in *C. legalis* during accelerated aging.

Figure 3 - Endogenous contents ($\mu\text{g g}^{-1}$ fresh matter) of total free polyamines (a), and free spermidine (b), spermine (c) and putrescine (d) in *Cariniana legalis* seeds aged at 41 and 47 °C, before (non-aged, time zero) and after 24, 48, 72 and 96 h of incubation



Source: Authors (2021)

In where: *Uppercase letters indicate significant differences between temperatures of aging. Lowercase letters indicate significant differences among the hours of aging. Means followed by the same letter do not differ statistically between them according to Tukey's test ($P \leq 0.05$). CV = Coefficient of variation ($n = 4$, CV total free PAs = 6.63%; CV free spermidine = 12.77%; CV free spermine = 28.56%; CV free putrescine = 22.04%).

Proteomic analysis has made it possible to identify changes in the accumulation of proteins and, consequently, allow their association with the different development processes that are taking place (CHEN; HARMON, 2006). For seed conservation studies, proteomic analysis can be an important tool contributing to a better understanding

of the patterns of alteration of individual proteins and their association with the maintenance of seed viability during storage, as observed for *C. legalis* (LERIN *et al.*, 2021). In the present work a total of 202 proteins were identified (Supplementary Table 1), being highlighted the differentially accumulated proteins (DAPs) (Table 1). Comparing seeds aged at 41 °C during 48 h with non-aged seeds (time zero h), a total of 29 proteins were DAPs, being three up- and 20 down-accumulated, two proteins were unique in seeds aged at 41 °C during 48 h, while four were unique in non-aged seeds (time zero h) and 173 proteins were unchanged (Supplementary table 1; Table 1). In the comparison of seeds aged at 47 °C during 48 h with non-aged seeds (time zero h), 34 were DAPs, being eight up- and 21 down-accumulated and two proteins were unique in seeds aged at 47 °C during 48 h, while three were unique in non-aged seeds and 168 were unchanged (Supplementary Table 1; Table 1). Comparing seeds aged at 47 °C with seeds aged at 41 °C, both at 48 h, a total of 12 were DAPs, being five up- and four down-accumulated, two proteins were unique in seeds aged at 47 °C, one unique in seeds aged at 41 °C and 190 proteins were unchanged (Supplementary Table 1; Table 1). A higher number of DAPs was observed in seeds before aging (non-aged; time zero h) comparing to seeds aged at 41°C or 47°C during 48 h, being most of them down-accumulated in non-aged seeds. The down-accumulation can mean a decrease in protein accumulation with significant relevance for seed germinability, as showed by Lerin *et al.* (2021). The authors compared the proteomic profile of *C. legalis* seeds stored at 25 and 6°C with non-stored seeds, observing a higher number of DAPs, mainly down-accumulated, in non-stored seeds compared to seeds stored at 25 and 6°C (LERIN *et al.*, 2021).

Table 1 – Differentially accumulated proteins (DAPs) in seeds of *Cariniana legalis* during accelerated aging at 41 and 47°C by 48 h comparing to non-aged (time zero h) seeds

Accession	Peptide report	Score	Description	Comparisons*		
				Seeds aged at 41°C / non-aged seeds	Seeds aged at 47°C / non-aged seeds	Seeds aged at 47°C / seeds aged at 41°C
A0A251UTE2	15	7403	Alcohol dehydrogenase	-	-	Up
A0A251UJ14	14	10280	Enolase	Down	Down	-
A0A251V8J6	2	2350	Proteasome subunit	Down	Down	-
A0A251UU89	3	1246	Proteasome subunit	Down	Down	-
A0A251RVM7	9	4797	Fructose-bisphosphate aldolase	Down	Down	-
A0A251VS40	9	3589	Fructose-bisphosphate aldolase	-	Down	-
A0A251TFL1	2	701	Fructose-1,6-bisphosphatase	Down	-	-
A0A251V7G1	18	4751	Aconitate hydratase	-	Down	Down
A0A251VA91	3	3608	Aconitate hydratase	-	Down	-
A0A251RZ67	4	726	Cysteine synthase	-	Down	-
A0A251SCF6	3	1692	14-3-3-like protein	-	Up	Up
A0A251VH41	4	1343	Malate dehydrogenase	Down	Down	-
A0A251TZ13	4	3503	Malate dehydrogenase	-	Down	-
I6LNU0	7	5836	Phosphoglycerate kinase	Down	Down	-
A0A251VGX1	5	1874	14-3-3 protein	Down	-	-
A0A251SIN0	5	2015	14-3-3 protein	Down	-	-
A0A251STB4	4	1950	Translation elongation factor	Unique in unaged seeds	Unique in unaged seeds	-
A0A251TST3	13	7394	Translation elongation factor	Down	-	-
A0A251RY06	3	3690	Ferritin	unique at 41 °C	unique at 47 °C	-
A0A251SUY5	8	1366	Heat shock protein	-	Down	Down
A0A251UM28	26	14226	Heat shock protein	-	-	Down

Source: Authors (2021)

In where: * = Proteins were deemed up-accumulated if the \log_2 value of the fold change (FC) was greater than 0.60 and deemed down-accumulated if the \log_2 value of the FC was less than -0.60, as determined by Student's T-Test (two-tailed; $P \leq 0.05$).

Among the DAPs (Table 1), some proteins were highlighted according to their involvement in the accelerated seed aging, as the HSPs. The HSPs proteins were down-accumulated in the comparisons of seeds aged at 47 °C with non-aged seeds (A0A251SUY5) and in seed aged at 47 °C with seed aged at 41 °C (A0A251SUY5 and A0A251UM28) (Table 1). The HSPs, together with the late embryogenesis abundant (LEA) proteins, are considered a signature for desiccation tolerance, being accumulated in the late development of seed to survive the quiescent state (RAJJOU; DEBEAUJON, 2008). A reduction in the accumulation of HSPs protein was also found in *C. legalis* seeds stored at 6 °C for 12 (ARAGÃO *et al.*, 2019) and 24 months (LERIN *et al.*, 2021) and was related to the reduction on seed germination potential due to the aging of seeds. In this work, the decrease in germination of *C. legalis* seeds aged 47 °C can be associated to a decreased in contents of free PAs, mainly Spd (Figure 2b). In view of that Spd are more accumulated in the maturation phase, at the end of seeds development (SANTA-CATARINA *et al.*, 2006) as well the HSPs (Table 1), these molecules can have an important role to understanding the seed aging and loss of viability in *C. legalis*.

Moreover, the aconitate hydratase proteins (A0A251V7G1 and A0A251VA91) were also down-accumulated in seeds aged at 47 °C compared to both, non-aged seeds and seeds aged at 41 °C (Table 1). This protein have been associated to reserve substances mobilization and degradation used during seed germination (HE *et al.*, 2011). Proteomic analysis in *C. legalis* indicated that this protein was only accumulated in viable mature non-stored seeds in comparison with seeds stored at 6 and 25 °C (LERIN *et al.*, 2021). In this sense, the down-accumulation of these proteins may be associated with the aging of seeds, consequently impairing the degradation and metabolization of reserve substances necessary for germination in *C. legalis*.

The proteasome subunit (A0A251UU89 and A0A251V8J6) proteins were down-accumulated in seeds aged in both temperature (41 and 47 °C) compared to non-aged seeds (Table 1). The proteasome activity is required for germination (CHIU *et al.*, 2016) and the accumulation of these proteins is involved with degradation of

stocking proteins in viable quiescent seeds (HE *et al.*, 2011). Down-accumulation of the proteasome subunit protein has already been found in *C. legalis* seeds during storage at 6 and 25 °C (LERIN *et al.*, 2021), indicating that down-accumulation is directly related to the loss of germinability of the seeds in this species.

The malate dehydrogenase proteins were down-accumulated in seeds aged in both temperature, at 41 °C (A0A251VH41) and at 47 °C (A0A251VH41 and A0A251TZ13) compared to non-aged seeds (Table 1). The lack of activity of this enzyme in aged seeds of *Arabidopsis* led to the reduction of reserve accumulation and rapid loss of seed viability (SEW *et al.*, 2016). In seeds of *Zea mays*, the elimination of malate dehydrogenase activity resulted in reduced ATP supply and impaired tricarboxylic acid cycle, altering the substrate availability for amino acid biosynthesis (CHEN *et al.*, 2020). In this way, the reduction on accumulation of malate dehydrogenase protein in *C. legalis* seeds aged indicate the negative effect caused by seed aging, which was more visible at 47 °C. Moreover, the fructose-bisphosphate aldolase (A0A251RVM7), phosphoglycerate kinase (I6LNU0) and enolase (A0A251UJ14) proteins were down-accumulated in seeds aged at both temperature (41 and 47 °C) compared to non-aged seeds (Table 1), while the fructose-bisphosphate aldolase (A0A251VS40) was down-accumulated only in seeds aged at 47 °C compared to those non-aged (Table 1). These proteins are involved in glycolysis, one of the primary sources of energy for germination (HE; YANG, 2013), the reduction on its accumulation show the negative effect of temperatures on metabolism of seeds.

One translation elongation factor (A0A251STB4) protein was unique in non-aged seeds compared to seeds aged at 41 and 47 °C and down-accumulated (A0A251TST3) in seeds aged at 41°C compared to non-aged seeds (Table 1). This protein play a central role in the elongation phase of protein synthesis (URSIN *et al.*, 1991), being critical for the mechanisms of translational control required during *Z. mays* seed germination (DINKOVA *et al.*, 2011). In our results, the presence (unique protein) of translation elongation factor protein in non-aged seeds may be important for proteins synthesis

needed during germination, while the lower accumulation in seeds aged at 41 and 47°C compared to non-aged seeds may affect the biosynthesis of new proteins, which may impair the development seedlings.

The alcohol dehydrogenase (A0A251UTE2) protein was up-accumulated in seeds aged at 47°C compared to those aged at 41 °C (Table 1). This protein is an enzyme in the fermentation (SCHIFFERDECKER *et al.*, 2016), which is an alternative pathway for glycolysis in cells under stress condition (STROMMER, 2011). Seed deterioration is due, at least in part, to the accumulation of acetaldehyde protein, and the alcohol dehydrogenase enzyme acts detoxifying toxic acetaldehyde produced from to pyruvate, leading to synthesis of ethanol (BUCHER *et al.*, 1995; ZHANG *et al.*, 1997). Thus, the up-accumulation of this protein in seeds aged at 47 °C compared to 41 °C may be related to the investment in detoxification in cells under stress condition, which can lead to the loss of germinability and rapid seed deterioration in *C. legalis*.

4 CONCLUSION

Our results showed that the accelerated aging at 47 °C was efficient in simulating the deterioration process in *C. legalis* seeds compared to 41 °C and accelerated aging can be used as a tool to study the ideal temperature for seed conservation in this species. The reduction on the total free PAs and Spd contents and down-accumulation of HSPs in seeds aged at 47°C was associated with the induction of seed aging. The down-accumulation of proteins, such as the aconitate hydratase and up-accumulation of the alcohol dehydrogenase protein, were associated to the loss of seed germinability and rapid deterioration in seeds aged under 47 °C temperature. These results contribute to the knowledge on the biochemical alterations involved in germinability during aging, contributing for further studies relate to storage and conservation of *C. legalis* seeds.

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