



Physiological and biochemical changes during the loss of desiccation tolerance in germinating *Adenantha pavonina* L. seeds

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ABSTRACT

We investigated the loss of desiccation tolerance (DT) in *Adenantha pavonina* seeds during germination. Seeds were subjected to imbibition for 0, 24, 36, 48, 60 and 81 h, then dried to their initial moisture content (13%), rehydrated and evaluated for survival (resumption of growth and development of normal seedlings) and membrane system integrity (electrolyte leakage). Embryonic axes of seeds subjected only to imbibition during the same early time periods were used to investigate the electrophoretic patterns of heat-stable proteins and the relative nuclear DNA content. In *A. pavonina* seeds, DT remained unchanged until 36 h of imbibition (resulting in germination and 82% normal seedlings), after which it was progressively lost, and seeds with a protruded radicle length of 1 mm did not withstand dehydration. The loss of desiccation tolerance could not be related to either membrane damage caused by drying or the resumption of the cell cycle during germination. However, the decrease in heat-stable protein contents observed throughout germination may be related to the loss of DT in *A. pavonina* seeds.

Key words: cell cycle, desiccation sensitivity, DNA content, drying, heat-stable proteins.

INTRODUCTION

Desiccation tolerance can be defined as the capacity of seeds to survive and maintain their physiological activities when subjected to a rigorous drying process. Orthodox seeds acquire the ability to tolerate desiccation during the late maturation phase (Bewley 1979). During the deposition of reserves, the moisture content of seeds is gradually reduced, causing a decrease in cell metabolism (Alpert and

Oliver 2002). During this time, molecular and metabolic changes that enable seeds to be dispersive and render them capable of tolerating long periods of drought are observed (Buitink et al. 2006, Angelovici et al. 2010, Huang and Song 2013).

To prevent or minimize the damage caused by desiccation in orthodox seeds, a series of repair mechanisms are activated, the regulation of which is highly complex (Farrant and Moore 2011, Maia et al. 2011, Gechev et al. 2012, Dinakar and Bartels 2013). Among these mechanisms, the synthesis of protective molecules, such as heat shock proteins

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and late embryogenesis abundant (LEA) proteins, is important (Pammenter and Berjak 1999, Hoekstra et al. 2001). LEA proteins are small hydrophilic, heat-stable molecules synthesized during late maturation in orthodox seeds (Delahaie et al. 2013). They are thought to act as antioxidants and as membrane and protein stabilizers during desiccation of seeds (Tunnacliffe and Wise 2007, Amara et al. 2012),

When orthodox seeds are hydrated and the germination process begins, desiccation tolerance remains unchanged. However, as germination advances, seeds reach a critical stage of imbibition, after which their desiccation tolerance is gradually lost (Hegarty 1988). DNA replication during germination is seen as an important marker of the transition of seeds, from being tolerant to sensitive to desiccation (Osborne and Boubriak 1994, Boubriak et al. 2000).

Several species of economic interest produce recalcitrant seeds, e.g., mango, rubber, cocoa and Paraná pine. Thus, attempts to elucidate the mechanisms related to the sensitivity of seeds to desiccation are essential for defining strategies for the conservation of these species. Studies in recalcitrant seeds are difficult to conduct due to the rapid loss of viability during seed storage. During germination, orthodox seeds develop a recalcitrant behavior (Sun 1999), and they can therefore be used to analyze molecular, metabolic and biochemical events related to sensitivity to desiccation.

In this study, changes in desiccation tolerance were examined in the seeds of *Adenanthera pavonina* L. during germination. This species belongs to the family Fabaceae-Mimosidae and is arboreal, native to Asia and well-adapted in Brazil (Corrêa 1978). Seeds are produced in large quantities and present orthodox behavior (Rocas 2002). The objective of this study was to investigate whether the loss of desiccation tolerance is associated with the membrane damage caused by drying, with the activation of the cell cycle, or with the levels of heat-stable proteins during germination.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *A. pavonina* L. were extracted from mature fruits collected in June 2011 in Muriaé, Minas Gerais, Brazil (21° 08' S; 42° 22' O). After opening the pods, the seeds were collected, processed and stored in a cold room at 5 °C and 60% relative humidity (RH) until the beginning of the tests. Immediately following seed processing, the initial moisture content of the seeds was determined.

DORMANCY RELEASE AND SEED GERMINATION

The tip of the tegument was cut off with pliers in the lateral region opposite the hilum, according to the pre-germinating treatment chosen to break tegument dormancy (Silva et al. 2009). Next, the seeds were immersed in a 5% Captan® solution for five min, and four replicates of 25 seeds each, were subsequently placed on rolls of paper moistened with distilled water (2.5 times the weight of the dry paper) and incubated in a germinator at 25 °C (16 h dark/8 h light) (Silva et al. 2009). Germination was assessed daily up to the fifteenth day after sowing. Percentage germination was expressed as the number of normal seedlings developed.

Imbibition curve

To characterize the pattern of water imbibition by the seeds, two replicates of 12 seeds each were subjected to the pre-germination treatment and maintained under the same germination conditions described above. Then, each seed was individually weighed at regular periods over four days.

MOISTURE CONTENT DETERMINATION

The moisture content was determined using the oven-drying method at 105 °C for 24 h, with four replicates of 20 seeds each. The calculation was performed on a fresh weight basis, and the moisture content was expressed as a percentage.

ASSESSMENT OF THE LOSS OF DESICCATION TOLERANCE DURING GERMINATION

To evaluate the loss of desiccation tolerance during germination, different imbibition times were chosen based on the results obtained from the imbibition curve: 0 (control), 24, 36, 48, 60 and 81 h (when seeds were germinated with a 1 mm radicle length).

The seeds were subjected to pre-germination treatment and then sown. After each imbibition period, the seeds were transferred to an elevated screen placed into gerbox-type boxes containing activated silica gel at 20 °C until returning to their initial moisture content (with the time varying according to each period of the prior imbibition). The drying process was monitored through successive seed weighing, stopping at the point at which the desired seed weight was obtained, indicating the desired moisture content, as determined using the expression proposed by Cromarty et al. (1985). After observing the desired weight, the moisture content was measured to confirm whether the desired moisture content had been reached.

To ensure maintenance of the dry state following drying, after attaining the initial moisture content, the dry seeds were stored in a cold room for 72 h. Then, the seeds were pre-moistened in a humid atmosphere (100% RH) at 25 °C for 24 h and returned to the early germination conditions. The seeds that reinitiated development and became normal seedlings were considered tolerant to desiccation. The control consisted of fresh seeds, i.e., seeds subjected to pre-germination treatment, but not to the previously mentioned imbibition/drying process. Four replicates of 25 seeds each per treatment (imbibition times + control), were performed in this study.

ELECTROLYTE LEAKAGE ASSESSMENT

To analyze the integrity of the membrane system, an electrical conductivity test was performed. The seeds were imbibed for 24, 36, 48, 60 and 81 h, then dried to their initial moisture content and

pre-moistened as described above. The control consisted of pre-moistened fresh seeds. The test was performed with four replicates of 25 seeds each per treatment. The samples were weighed and stored in plastic containers with 150 mL of deionized water for 24 h at 25 °C (Hampton and TeKrony 1995). The electrolyte leakage for each solution was determined with an electrical conductivity meter, and the results were expressed as $\mu\text{S}\cdot\text{cm}^{-1}\cdot\text{g}^{-1}$.

DNA CONTENT ASSESSMENT

Quantification of the nuclear DNA content was conducted via flow cytometry. To this end, the embryonic axes of fresh seeds (control) after imbibition times of 24, 36, 48, 60 and 81 h were used to prepare nuclear suspensions. Root tips were subjected to nuclear isolation according to the methodology proposed by Carvalho et al. (2008) for subsequent analysis in a Partec PAS II / III flow cytometer (Partec® GmbH, Munster, Germany). To analyze nuclei stained with DAPI (4',6-diamidino-2-phenylindole), a high pressure mercury lamp (HBO-100 W) with KG 1, BG 38 and GG 435 filters was used. Each nuclear suspension was processed in the cytometer with at least 5,000 nuclei, and each treatment consisted of three biological replicates (one embryonic axis per replicate).

ELECTROPHORETIC PATTERNS OF HEAT-STABLE PROTEINS

The analysis of heat-stable proteins was performed using the embryonic axes of fresh seeds (control) and seeds imbibed for 24, 36, 48, 60 and 81 h. Sample preparation and protein extraction were performed according to the methodology proposed by José et al. (2005). A total of 25 μL of each sample was subjected to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) using 12.5% (separating) and at 6% (concentrating) gels. Electrophoresis was performed at 120 V, and the gels were stained with a 0.05% Coomassie Brilliant Blue solution (Alfenas 2006) for 12 h, then destained in a solution of 10% acetic acid.

DATA ANALYSIS

An entirely randomized experimental design was used in all experiments. The data were subjected to analysis of variance (ANOVA) and Tukey's test at a 5% probability using Sisvar software, version 5.3.

RESULTS

SEED GERMINATION AND IMBIBITION

The *A. pavonina* seeds had an initial moisture content of approximately 13%, and after breaking tegument dormancy, the mean percentage of germination was 94%.

The imbibition curve (Fig. 1) obtained in this study, showed a slow rate of absorption during the first hours of Phase I, which presented a mean duration of 42 h. The moisture content of the seeds was approximately 55% at the end of this phase. Then, Phase II began, which lasted for approximately 30 h and was characterized by the reduced rate of hydration observed in the plateauing of the curve. Visible germination (radicle protrusion) was observed starting at 72 h of imbibition, after which Phase III began.

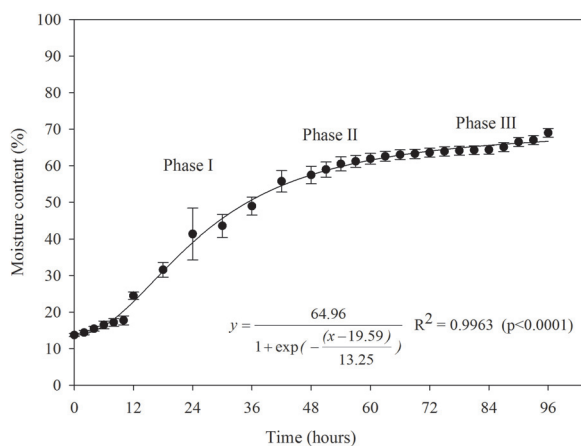


Figure 1 - Changes in moisture content during imbibition of *Adenanthera pavonina* seeds subjected to pre-germination treatment at 25 °C (16 h dark/ 8 h light). The bars represent the standard deviation. (Equation adjustment was significant when $p < 0.05$).

Based on these results, different imbibition times were selected to evaluate the loss of DT during germination: 0, 24, 36, 48, 60 and 81 h of imbibition. The imbibition time of 81 h was chosen to represent the seeds with 1 mm primary root because at this time, among the seeds that presented radicle protrusion, 47% exhibited a 1 mm primary root length.

LOSS OF DESICCATION TOLERANCE DURING GERMINATION

To determine the point at which DT is lost during germination, seeds were subjected to imbibition for different durations, then dried to their initial moisture content (13%), rehydrated and evaluated for survival (development of normal seedlings). The germination values related to the loss of DT are presented in Figure 2. In the early stages of imbibition (24 and 36 h), DT remained unchanged. Sensitivity to desiccation became evident after 48 h of imbibition, as seed survival declined from 82% to 54.71% after drying. There was no resumption of growth observed when seeds with a 1 mm radicle length were dried.

MEMBRANE DAMAGE ASSESSMENT

The extent of damage caused by drying after different imbibition times was estimated according to the rate of electrolyte leakage from seeds that had been imbibed, dried and pre-moistened (Fig. 2). No significant difference in electrical conductivity was observed between solutions containing fresh seeds and solutions with dry seeds that had been imbibed for 48 h. However, there was a significant increase in electrolyte leakage from seeds imbibed for 60 h and then dried. The maximum electrical conductivity value was obtained in the solution of seeds with a 1 mm radicle length that were subsequently dried.

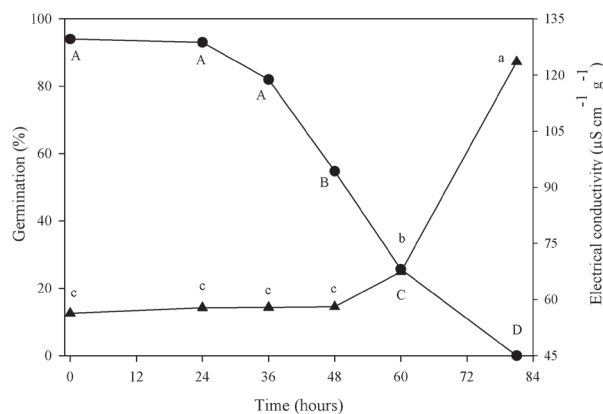


Figure 2 - Electrical conductivity (▲) and loss of desiccation tolerance (●) in seeds of *Adenanthera pavonina* subjected to different imbibition times, followed by drying to the initial moisture content. Germination results are expressed as the percentage of normal seedlings obtained after imbibition and drying. Capital letters compare the germination results, and lowercase letters compare the rates of electrical conductivity. Means followed by the same letter do not differ by Tukey's test at a 5% probability.

NUCLEAR DNA CONTENT

The flow cytometry analysis of *A. pavonina* radicles revealed that the majority of fresh seed nuclei exhibited a 2C DNA content (77%), while a small quantity of 4C DNA was present (5.8%) (Fig. 3). The DNA contents remained unchanged during the first 60 h of germination. However, as germination proceeded, a significant increase in the 4C DNA content was observed, while a significant decrease in the 2C DNA content occurred when the roots were 1 mm in length (approximately 19%).

ANALYSIS OF HEAT-STABLE PROTEINS

The relationship of desiccation tolerance to the SDS-gel electrophoretic profile of heat-stable proteins was examined in *A. pavonina* embryonic axes. The analysis was performed with fresh seeds undergoing germination. A set of four heat-stable maturation proteins (Fig. 4, arrows) were consistently present in fresh seeds. This set contained proteins with molecular masses of approximately 98, 96, 66 and 35 kDa. Levels of

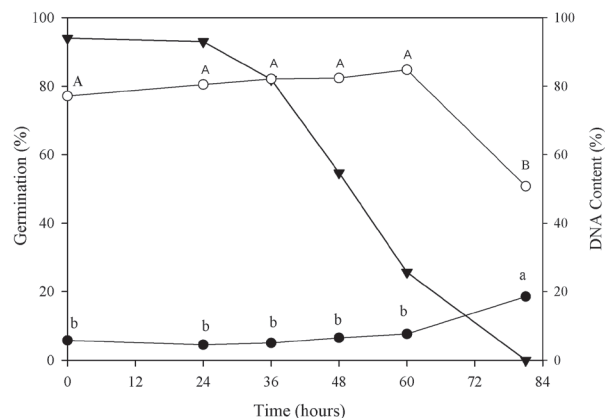


Figure 3 - Contents of 2C (○) and 4C (●) DNA and loss of desiccation tolerance (▼) in seeds of *Adenanthera pavonina* during and after germination. Germination results are expressed as the percentage of normal seedlings obtained after the treatment of seeds with different imbibition times, followed by drying to the initial moisture content. Capital letters compare the contents of 2C DNA, and lowercase letters compare the contents of 4C DNA. Means followed by the same letter do not differ by Tukey's test at a 5% probability.

these heat-stable proteins declined after 24 h of imbibition, which remained stable until 36 h of imbibition. After 48 h, the contents of the 98, 96 and 66 kDa fractions decreased, and after 60 h of imbibition, these fractions decreased sharply, as did the 35 kDa fraction. Only traces of the 66 and 35 kDa fractions were detected in seeds with a 1 mm radicle length.

DISCUSSION

As imbibition progressed, drying of *A. pavonina* seeds to the initial moisture content (13%) caused a decrease in the survival rate of the seeds (Fig. 2). This indicates that the mechanisms related to the maintenance of tolerance to desiccation were inactivated with the progress of the germination process, contributing to an increase in the sensitivity of the seeds to desiccation.

According to Bewley and Black (1994), desiccation tolerance in seeds decreases during Phases I and II of imbibition and is lost during or after Phase III. This observation agrees with the

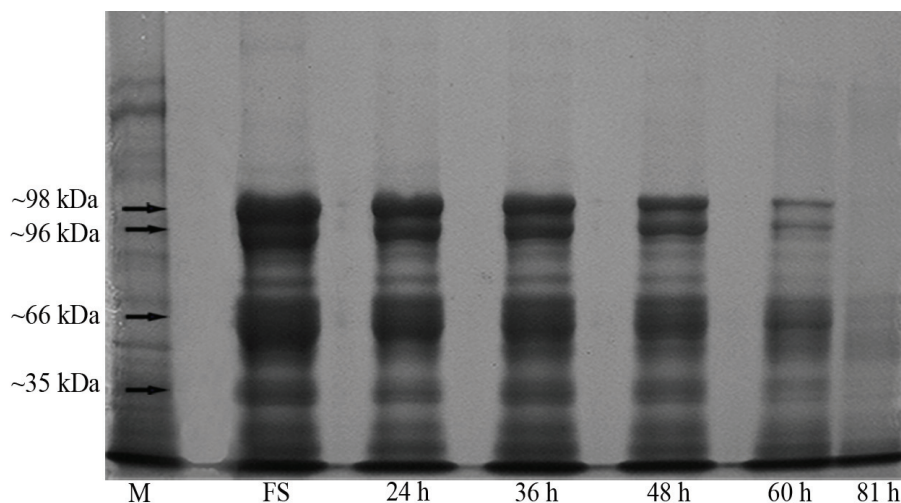


Figure 4 - Electrophoretic pattern of heat-stable proteins in the embryonic axes of *Adenanthera pavonina* seeds. M - molecular marker. FS - fresh seeds. 24-60 h - seeds im-bibed for 24, 36, 48 and 60 h. 81 h - seeds with a radicle 1 mm in length.

results obtained for the seeds of *A. pavonina*, as loss of tolerance to desiccation was observed 48 h after imbibition, i.e., during the transition between Phases I and II. In some species, sensitivity to desiccation is evident when the seeds are in a more advanced metabolic stage, i.e., the transition between Phases II and III, as observed in the seeds of *Glycine max* and *Zea mays* (Koster and Leopold 1988), *Pisum sativum* (Reisdorph and Koster 1999), *Triticum aestivum* (Miazek et al. 2001) and *Peltophorum dubium* (Guimarães et al. 2011).

The ability to tolerate desiccation is known to vary among seed tissues (Kermode and Finch-Savage 2002). According to Buitink et al. (2003), the radicle is the first structure to become sensitive to desiccation. In some species, however, this structure remains tolerant, only becoming sensitive with an increase in length. In the seeds of tomato (Lin et al. 1998) and *Medicago truncatula* (Faria et al. 2005), for example, loss of tolerance to desiccation occurs at the point at which the primary roots reach 2 mm in length. In the present study, no seed with 1 mm radicle survived after the drying process was performed. However, it was not possible to determine whether tolerance

to desiccation was lost in seeds with primary roots smaller than 1 mm because they were not evaluated in this study.

Cellular membranes are particularly vulnerable to damage caused by desiccation. During the drying of seeds, accumulation of reactive oxygen species and free radicals is observed in cells (Leprince et al. 1994, Ntuli et al. 2011), resulting from the rupture of plastids and from the electron transport system of the mitochondria (Pammenter and Berjak 1999). These compounds can damage proteins, lipids and nucleic acids, causing permanent damage to enzymes, chromosomes and membranes. Lipid peroxidation reduces the fluidity of cell membranes, interfering with their selective permeability during rehydration (Walters et al. 2002).

Thus, the integrity of membranes is crucial to maintain the viability of seeds (Kermode and Finch-Savage 2002). For this reason, orthodox seeds are dispersed with mechanisms that attempt to minimize the damage caused by desiccation. Among these mechanisms, it has been suggested that the accumulation of sucrose and oligosaccharides of the raffinose family (raffinose, stachyose and verbascose) can reduce the harmful

effects of desiccation on cell membranes (Hoekstra et al. 2001, Buitink et al. 2002). During desiccation, these sugars are inserted into the polar regions of the lipid bilayer, maintaining the proper spacing of the lipid groups and the structure of the membrane (Walters et al. 2002, Vicré et al. 2004).

Evaluation of electrolyte leakage from seeds of *A. pavonina* via electrical conductivity was performed to investigate whether the loss of desiccation tolerance is associated with possible damage to the membrane caused by drying, since previously, the relationship between desiccation tolerance and the integrity and stability of seed membranes has been reported (Corbineau et al. 2004, Koster and Leopold 1988, Spanò et al. 2011). In the present study, sensitivity to desiccation was evident after 48 h of imbibition. However, the increased rate of electrolyte leakage observed in the seeds indicated that drying caused damage to cell membranes only after 60 h of imbibition. Although an increase in electrical conductivity corresponds to a loss of tolerance to desiccation in the seeds of pea (Reisdorph and Koster 1999, Koster et al. 2003), canola (Bagniewska-Zadwoma 2008) and *Phaleria macrocarpa* (Ahmed Asrity et al. 2013), it is likely that in the present work, the electrical conductivity test tended to overestimate the ability of seeds to tolerate desiccation.

It has been suggested that the loss of tolerance to desiccation during germination coincides with the level of activity of the embryonic cells of seeds, mainly at the root tip (Osborne et al. 2002). Radicle growth is initially driven by the cell expansion process. Subsequently, usually after radicle protrusion, cell division begins (Bewley and Black 1994). Interphase, the stage that precedes cell division, is divided into three phases: G_1 (pre-synthesis), S (DNA synthesis) and G_2 (post-synthesis). Cells with a 2C nuclear DNA content are found in G_1 phase, while cells with a 4C DNA content are observed in G_2 phase. The C constant is the nuclear DNA content for the haploid condition.

The cytometric analysis revealed that the radicles of the fresh seeds of *A. pavonina* exhibited a high relative 2C DNA content (Fig. 3). This suggests that the majority of cells were retained in G_1 phase, while a small number were in G_2 phase of the cellular cycle. This is a prevalent characteristic in orthodox seeds (Bino et al. 1993, Castro et al. 2000, Vázquez-Ramos and Sánchez 2003, Gendreau et al. 2008), although there are reports of dispersed orthodox seeds with high 4C DNA contents (approximately 40%) (Faria et al. 2005, Guimarães et al. 2011). High 2C DNA contents have also been observed in the mature seeds of intermediate and recalcitrant species, such as *Castanea sativa* (Bino et al. 1993), *Inga vera* (Faria et al. 2004) and *Coffea arabica* (Silva et al. 2008), indicating that the relationship between tolerance to desiccation and events inherent to the cellular cycle, is weak.

The low 4C DNA contents found in fresh seeds may be explained by the effects of desiccation during the late maturation process in orthodox seeds. According to Deltour and Barys (1985), the moisture content of embryonic cells declines rapidly when seeds are dehydrated, and metabolism is consequently reduced, while cytoplasmic viscosity is increased, precluding the formation of the complex structures necessary for the activity of the cell cycle.

Resumption of the cell cycle was demonstrated by the increased 4C DNA and reduced 2C DNA contents observed at 60 h after germination. However, in the seeds of *A. pavonina*, loss of tolerance to desiccation was initially observed after 48 h of imbibition; i.e., the development of sensitivity to desiccation preceded the activation of cellular division. Although it has been proposed that cells with a 4C DNA content are more sensitive to stress conditions because they are more vulnerable to factors inducing mutation (Deltour and Barys 1985, Sliwinska 2009), cells with a 2C DNA content have been shown to be sensitive to

desiccation in the seeds of *A. pavonina*, *Medicago truncatula* (Faria et al. 2005) and *Peltophorum dubium* (Guimarães et al. 2011).

Orthodox seeds also accumulate proteins as a mechanism for repairing the damage caused by desiccation, including LEA proteins. One feature of LEA proteins is their hydrophilicity and high proportion of charged amino acids, which contribute to their heat and acid stability (Amara et al. 2012). In general, they are considered to contribute to diverse protective mechanisms upon desiccation: replacement of water, stabilization of macromolecules and cellular structures, ion sequestration and membrane integrity maintenance, alone or in combination with sugars (Hoekstra et al. 2001, Tunnacliffe and Wise 2007, Battaglia et al. 2012).

Studies show that the synthesis of LEA proteins, coincides with the acquisition of desiccation tolerance during the development of seeds in *Ricinus communis* (Han et al. 1997), *Arabidopsis thaliana* (Bies et al. 1998), *Prunus amygdalus* (Campalans et al. 2000), *Glycine max* (Samarah et al. 2006) and *Nelumbo nucifera* (Shen-Miller et al. 2013). In addition, the disappearance of LEA proteins and heat-stable proteins, was related with loss of desiccation tolerance in radicles of *M. truncatula* (Boudet et al. 2006) and seeds of soybean (Blackman et al. 1991), respectively.

Here we tested the hypothesis that heat-stable maturation proteins are associated with desiccation tolerance in *Adenantha pavonina* seeds. The emergence and development of sensitivity to desiccation after 48 h of imbibition, coincided with the reduction of heat-stable protein contents in *A. pavonina* seeds (Fig. 4). Our data indicate that heat-stable maturation proteins somehow play an important role in seed-water interactions, protecting seeds against the damage caused by desiccation. It is known that the maintenance of the ability to tolerate desiccation, depends on the joint and integrated action of the various repair

mechanisms in seeds. The lack or inefficiency of one or more protection mechanisms determines the degree of sensitivity to desiccation in different species (Berjak and Pammenter 2013).

Considering all of the physiological events analyzed in this study, only the reduction of heat-stable protein contents can be related to the loss of tolerance to desiccation in the seeds of *Adenantha pavonina*.

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RESUMO

Nós investigamos a perda da tolerância à dessecação (TD) em sementes de *Adenantha pavonina* durante a germinação. Sementes submetidas à embebição por 0, 24, 36, 48, 60 e 81 h foram secas até à umidade inicial (13%), reidratadas e avaliadas quanto à sobrevivência (retomada do crescimento e formação de plântulas normais) e à integridade do sistema de membranas (extravasamento de eletrólitos). Eixos embrionários de sementes submetidas apenas à embebição, pelos mesmos tempos citados anteriormente, foram utilizados para avaliar os padrões eletroforéticos de proteínas resistentes ao calor e o conteúdo relativo de DNA nuclear. Em sementes de *A. pavonina*, a TD foi mantida por até 36 h de embebição (resultando em germinação e 82% de plântulas normais), após o que foi gradativamente perdida, até que sementes com 1 mm de raiz primária não sobreviveram após a secagem. A perda da TD não pôde ser correlacionada com danos causados por secagem ao sistema de membranas, nem com a retomada do ciclo celular durante a germinação. No entanto, a redução do conteúdo de proteínas resistentes ao calor, ao longo da germinação, pode estar relacionada com a perda da TD em sementes de *A. pavonina*.

Palavras-chave: ciclo celular, sensibilidade à dessecação, conteúdo de DNA, secagem, proteínas resistentes ao calor.

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