PHYSIOLOGICAL AND MOLECULAR RESPONSES OF *Talauma ovata* SEEDS TO DRYING AND IMBIBITION

ANDERSON CLEITON JOSÉ

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Orientador

Prof. Dr. Antonio Claudio Davide

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SUMMARY

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RESUMO

JOSÉ, Anderson Cleiton. **Respostas fisiológicas e moleculares em sementes de *Talauma ovata* à secagem e embebição.** LAVRAS: UFLA, 2007. 83p. (Tese - Doutorado em Engenharia Florestal)[*]

Estudou-se o efeito de taxas de secagem, expressão diferencial de proteínas pela técnica de eletroforese 2D e mudanças na expressão de genes relacionados com desenvolvimento (*ABI3*), ciclo celular (*CDC2-like*), cito-esqueleto (*ACT2*) e tolerância à dessecação (*PKABA1*, *2-Cys-PRX* e *sHSP17.5*), utilizando RT-PCR em sementes de *T. ovata*, uma espécie nativa de matas ciliares da Mata Atlântica brasileira. A taxa de secagem afetou a viabilidade das sementes, sendo que a secagem lenta resultou em maior sobrevivência comparando-se com a secagem rápida aos mesmos níveis de conteúdo de água. Extratos de proteína total de sementes frescas (0.28 g H_2O · g peso seco^{-1}), levemente secas (0.25 g H_2O · g ps^{-1} ps) e secas (0.10 g H_2O · g ps^{-1}), provenientes de secagem rápida, antes e após a embebição por 10 dias foram separadas em duas dimensões e após a revelação apresentaram em média 588 pontos de proteína, onde 21 apresentaram expressão diferencial, relacionada com dessecação e germinação, pelo aumento ou diminuição na expressão. Após sequenciamento (MS/MS), 3 proteínas produziram *spectra* com similaridade a um precursor de legumina de *Magnolia salicifolia*. Comparando-se a expressão destes pontos de proteínas identificados, com os dados de germinação foi possível sugerir o envolvimento deste precursor de legumina em eventos ocorrendo durante a secagem e subseqüente embebição de sementes secas. A análise da expressão gênica (após a secagem e embebição) revelou que a abundância de *ABI3* e *ACT2* não mudaram após a secagem, mas sim após a embebição das sementes. A expressão relativa de *CDC2-like* não alterou após uma secagem suave (0.25 g H_2O · g ps^{-1}), mas foi reduzida em sementes secas a 0.10 g H_2O · g ps^{-1}. Após a embebição, os níveis relativos de *CDC2-like* aumentaram. Transcritos de *PKABA1* e *sHSP17.5* não alteraram em abundância após a secagem a diferentes conteúdos de água, entretanto, seus níveis relativos aumentaram após a embebição. A quantidade relativa de *2-Cys-PRX* foi reduzida após a secagem a 0.10 g H_2O · g ps^{-1}. Não houve diferenças nos níveis de mRNA de *2-Cys-PRX* antes e após a embebição de sementes frescas e levemente secas, os quais foram reduzidos após 10 dias de embebição. Após a embebição de sementes secas (0.10 g H_2O · g ps^{-1}), a quantidade relativa de mRNA de *2-Cys-*

[*] Comitê Orientador: Antonio Claudio Davide (Orientador), Edvaldo A. Amaral da Silva - UFLA.

PRX aumentou para os mesmos níveis encontrados em sementes frescas. A expressão de *ABI3*, *ACT2* e *CDC2-like* sozinha não explica o comportamento da germinação de sementes de *T. ovata*. Aparentemente, as sementes comportam-se da mesma maneira nos primeiros dias de embebição, independentemente do conteúdo de água inicial e os efeitos deletérios da dessecação acontecem posteriormente. Os genes *PKABA1*, *sHSP17.5* e *2-Cys-PRX* não mostraram relação com a secagem, entretanto, há uma possível participação de *PKABA1* e *sHSP17.5* em mecanismos de proteção durante a embebição de sementes de *T. ovata*.

ABSTRACT

JOSÉ, Anderson Cleiton. **Physiological and molecular responses of *Talauma ovata* seeds to drying and imbibition.** LAVRAS: UFLA, 2007. 83p. (Thesis- Forest Engineering)*

The effect of drying rates and drying with subsequently imbibition was studied in seeds of *Talauma ovata*, a native species of riparian forests from Brazilian Atlantic Forest, regarding to differential protein expression using 2D-gel electrophoresis and changes in expression of some genes related to seed development (*ABI3*), cell cycle (*CDC2-like*) cytoskeleton (*ACT2*), and desiccation tolerance (*PKABA1*, *2-Cys-PRX* and *sHSP17.5*) by RT-PCR. Drying rate affected final viability of *T. ovata* seeds. Slowly dried seeds to 0.10 g $H_2O \cdot g^{-1}$ dw showed a higher survival when compared to seeds fast dried to the same water content level. Total protein was extracted and separated by 2D electrophoresis from fresh seeds (0.28 g $H_2O \cdot g^{-1}$ dw), mild dried seeds (0.25 g $H_2O \cdot g^{-1}$ dw) and seeds at low water content (0.10 g $H_2O \cdot g^{-1}$ dw), both from fast drying treatments, before and after imbibition for 10 days. The proteome profile revealed the presence of 588 spots on each silver stained gel. Analyzing the gels from different conditions enabled the identification of up to 21 protein spots that correlated with desiccation and germination, by increased or decreased expression. After MS/MS sequencing, 3 protein spots produced spectra that matched to a *Magnolia salicifolia* legumin precursor. By comparing the expression of these identified protein spots in the 2D-gels with the germination data, it is possible to suggest an involvement of this protein in events taking place during drying and subsequent imbibition of dried seeds. Analysis of gene expression (after drying and subsequently imbibition) reveled that the abundance of *ABI3* and *ACT2* did not changed after drying but increased after imbibition. Relative levels of *CDC2-like* did not changed after a mild drying 0.25 g $H_2O \cdot g^{-1}$ dw, but was down regulated when seeds were dried to 0.10 g $H_2O \cdot g^{-1}$ dw. After imbibition, the relative levels of *CDC2-like* increased. *PKABA1* and *sHSP17.5* transcripts did not changed in abundance after drying to different water contents, however, their relative levels increased after imbibition. The relative amounts of *2-Cys-PRX* were reduced after drying to 0.10 g $H_2O \cdot g^{-1}$ dw. There was no difference in *2-Cys-PRX* mRNA levels before and after imbibition of fresh and mild dried seeds, but it was reduced after 10 day of imbibition. After 10 days of imbibition of dried seeds (0.10 g $H_2O \cdot g^{-1}$ dw), the relative

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levels of 2-Cys-Prx mRNA increased to the same level of fresh seeds. The expression of *ABI3*, *ACT2* and *CDC2-like* alone do not explain the germination behaviour of *T. ovata* seeds. It seems that the seeds, irrespective to the initial water content, perform in the same way during the initial period of germination and the deleterious effects of desiccation will take place latter. *PKABA1*, *SHSP17.5* and *2-Cys-PRX* did not shown relation with desiccation. However, the expression pattern of *PKABA1* and *sHSP17.5* suggest the participation of these genes in protective mechanisms during the imbibition of *T. ovata* seeds.

CHAPTER 1

Running heading: Desiccation of *T. ovata* seeds.

Morphological aspects of fruits and seeds and effects of drying rates in the desiccation sensitivity of *Talauma ovata* A. St Hil seeds.

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Abstract

Talauma ovata seeds have been reported as desiccation sensitive. In order to study the effect of different drying rates on the final viability, seeds were dried over activated silica gel (fast drying) or salt solutions for different periods (slow drying). Fruits of *T. ovata* are dry capsules containing large number of seeds covered by a bright red aril. Seeds contain a small embryo, approximately, 1mm long and a thick seed coat. Mild drying transiently increased the final germination and the germination speed index, but further drying resulted in reduction in these parameters. Drying rate affected the final germination and vigor. Seeds that were slowly desiccated to 0.10 g $H_2O \cdot g^{-1}$ dw retain high viability when compared to seeds desiccated to the same water content level by fast drying method, although their vigor was reduced.

Introduction

As a result of deforestation, significant forest genetic resources are being threatened in various Brazilian ecosystems. The development of strategies for conservation and restoration of such degraded forests depends on the knowledge of plant propagation, e.g. seed germination and seedling growing conditions. Many species produce seeds that can be stored for long term and are available for use throughout the year, however, some plant species, especially those deriving from the tropics produce short-lived seeds.

Seeds can be sorted in three groups according to desiccation tolerance and storage conditions. Seeds that are tolerant to desiccation to low water content (lower than 0.10 g $H_2O \cdot g^{-1}$ dw) and storage at low temperature (usually -20^{o}C) are said to have an orthodox storage behaviour. On the other hand, recalcitrant seeds can not be dried to low water contents and may also be chilling sensitive. A group, showing an intermediate behaviour, are relatively more desiccation tolerant than recalcitrant species but less tolerant than orthodox. They can also be chilling sensitive especially if they are of tropical origin (Ellis et al., 1990; Hong & Ellis, 1996).

The degree of development and the drying rate are among the factors that can influence the desiccation sensitivity in recalcitrant seeds (Kermode & Finch-Savage, 2002; Wesley-Smith et al., 2001). Drying rate has been reported to affect the response of orthodox (Bewley & Black, 1994) and recalcitrant seeds regarding to desiccation tolerance. Fast dried embryonic axis of *Hevea brasiliensis* (Normah et al., 1986), *Araucaria husteinii* (Pritchard & Prendergast, 1986), *Mangifera indica* (Fu et al., 1990) *Quercus rubra* (Pritchard, 1991), *Quercus robur* (Finch-Savage, 1992), *Landolphia kirkii* (Berjak et al., 1990; Pammenter et al., 1991), *Avicena marina* (Farrant et al., 1993), *Euphoria longan* and *Litchi chinensis* (Fu et al., 1990; Fu et al., 1993), *Ekebergia capensis* (Pammenter et al., 1998), *Artocapus heterophyllus* (Fu et al., 1993; Wesley-

Smith et al., 2001) survived desiccation to lower water contents when compared with similar embryos dried more slowly. However, the effect of drying rate in whole seeds is much less pronounced when compared to embryonic axis (Farrant et al., 1985; Liang & Sun, 2002; Pritchard, 1991).

The reason why embryonic axis of recalcitrant species respond to differential drying rates is not well understood yet. The increase in the limit of water removal tolerated by embryos and seeds from desiccation sensitive species is attributed mainly to the reduction of the time of exposition to intermediate water contents, where most of the degenerative processes are thought to occur (Pammenter & Berjak, 1999; Wesley-Smith et al., 2001).

Information about seed and fruits characteristics can be useful to predict the seed storage behaviour. In an attempt to associate plant ecology, taxonomic classification, plant, fruit and seed characters to the seed storage behaviour, Hong et al. (1996) proposed the classification of some plant families based on the type of fruit. Besides the type of fruit, the size of the seeds and the water content at the time of the dispersion can also be an indicative of seed desiccation tolerance or sensitivity (Davide et al., 2001).

Seeds of *T. ovata* have been reported as desiccation sensitive (Carvalho et al., 2006; Lobo & Joly, 1996 and Chapter 2, in this thesis). Thus, this work aimed to study the morphological aspects of fruits and seeds and evaluate the effect of different drying rates in the viability of *T. ovata* seeds.

Material and methods

Fruit collection and seed processing

T. ovata fruits were collected from 5 trees along the Rio Grande River near Lavras (MG, Brazil) in September 2006. After collection, fruits were left at room temperature to allow the completion of dehiscence, which occurred between 3 to 5 days. The red aril that covers the seed was removed by gentle rubbing on a mesh and rinsing with tap water. After cleaning, seeds were blotted dry with a paper towel to remove excess water, and stored at 5ºC in closed plastic bags. It was used 4 replications of 10 fruits and seeds for the morphological characterizations. Photographs were taken with the use of a digital camera. Images of fruits and seeds were taken by using a stereomicroscope when necessary.

Dehydration conditions

Desiccation was carried out at 20ºC in closed plastic containers (11.5 x 11.5 x 3.5 cm) containing either silica gel or salt solutions as described below.

Fast drying

Seeds were placed in plastic containers (460 cm^3) over 110g of activated silica gel, separated by a plastic mesh at 20ºC. After drying to target water content, seeds were stored hermetically at 20ºC for up to 24 hours before germination experiments and water content determination.

Slow drying

Seeds were placed in plastic containers (460 cm^3), at 20ºC over salt solutions providing 95% RH (5g LiCl/100mL H_2O), 89%RH (saturated solution of $MgSO_4 \cdot 7H_2O$) and 76%RH (saturated solution of NaCl) (Sun, 2002). To obtain samples with different water contents, seeds were kept for: 2 days at

95%RH; 4 days at 95%RH; 4 days at 95%RH + 4 days at 89%RH; 4 days at 95%RH + 4 days at 89%RH + 4 days at 76%RH.

Water content and water potential assessment

Water content of the whole seed was determined gravimetrically on four replications of five seeds by oven drying at 103ºC/17hours (ISTA, 2005). Water contents were expressed on a dry weight basis (g H_2O · g dry weight^{-1} or g H_2O · g^{-1} dw). For water potential measurements, a desorption isotherm using a range of lithium chloride solutions providing different relative humidity was established. Equilibrium relative humidity (eRH) of whole seeds was measured with a Rotronic Hygrometer (Probert et al., 2002) in four replicates of 25 seeds over 60 minutes. Water potential (Ψ_w) was calculated from eRH using the equation (Ψ_w) = (RT/V).ln (a_w), where *R* is the gas constant (8.314 J mol^{-1} K^{-1}), *T* is the temperature (Kelvin), *V* is the partial molal volume of water (18.048 ml mole^{-1}) and a_w is the water activity (eRH/100) (Sun, 2002).

Seed germination

After desiccation seeds were pre-imbibed on moist germination paper in gerbox for 48 hours. Subsequently, seeds had the surface sterilized with 1% sodium hypochlorite for 10 minutes and rinsed with distilled water. Germination assays were carried out with four replicates of 25 seeds. Seeds were incubated at 20ºC, with constant light. A seed was regarded as germinated when the radicle protruded 2mm through the seed coat. Germination tests were carried out for up to 35 days. The germination speed index - SGI (Maguirre, 1962) was used to quantify seed vigor. Data were compared by Scott and Knott test ($p \leq 0.05$) (Scott & Knott, 1974).

Results

General characteristics of fruits and seeds of T. ovata

The cone-like fruit of *T. ovata* is a capsule containing many seeds. Fruits were greenish, ovoid in shape, dehiscent, and contained around 65(±10) seeds each. Fruits collected before dehiscence (fig. 1-A) started opening after 2 days (fig. 1-B), continuing for up to 5 days.

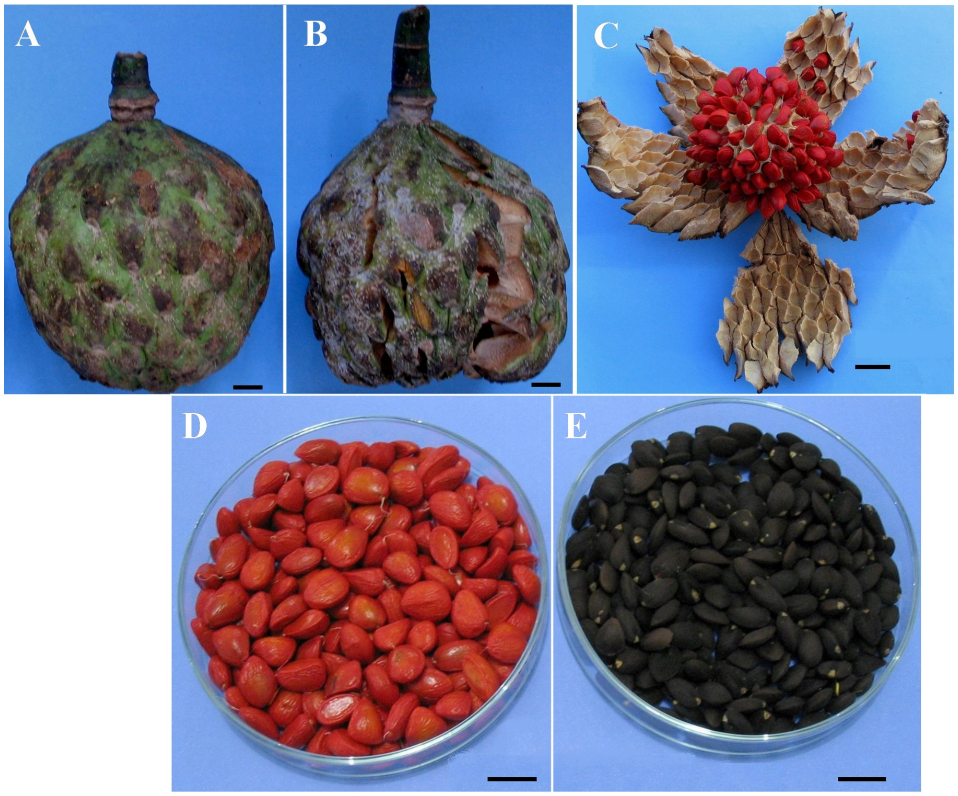

Figure 1. Fruits of *T. ovata* at the collection point (A), 2 days after collecting (B) and after opening, 3-5 days at room temperature (C). Seeds before processing, showing the bright red aril that covers the seed (D) and after removal of the aril (E). Horizontal black bars represent 1 cm (A, B, D and E) and 2 cm (C).

Seeds were black, covered by a bright red aril (fig. 2 A and B) and remained attached to the fruit by a lignin-fibril after fruit opening. A woody testa covered the soft oily endosperm with 32.7% oil content (Chapter 2, in this thesis). Seeds contain a small differentiated embryo (approx. 1 mm long) with embryonic axis and two cotyledons (fig. 2 A and B). Some morphological and physiological characteristics of fruits and seeds of *T. ovata* are presented in table 1.

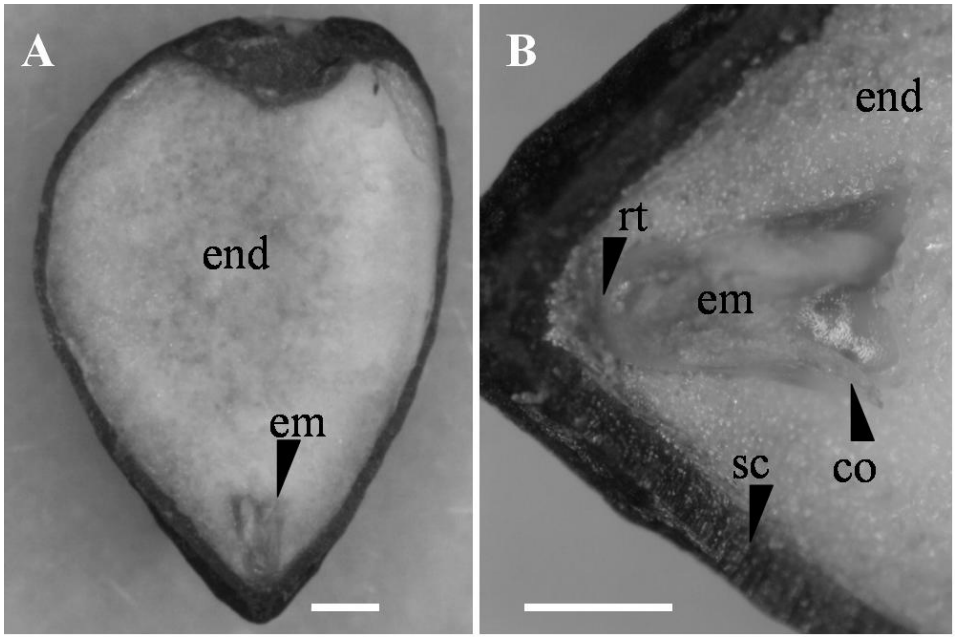

Figure 2. Transversal section of *T. ovata* seeds (A) and details of the embryonic axis region (B). end: endosperm, sc: seed coat, em: embrionic axis, rt: racicle tip, co: cotyledons. Horizontal white bars represents 1mm.

Table 1. Morphological and physiological characteristics of fruits and seeds of *T. ovata*.

Fruit size (cm)	10.90 (±1.93) x 5.99 (±0.48)
Seed weight (number of seeds/kg)	7437 ± 215
Seed size (cm)	0.97 (±1.1) x 0.62 (±0.9) x 0.42 (±1.3)
Seed water content (aril removed manually) (g $H_2O.g^{-1}$ dw)	0.33 ± 0.021
Seed water content (after processing) (g $H_2O.g^{-1}$ dw)	0.33 ± 0.023
Embryo size (mm)	0.92 ± 0.084

Effect of drying rates on germination of T. ovata seeds

Seeds of *T. ovata* exposed to silica gel dried very fast reaching low water content (0.11 g $H_2O \cdot g^{-1}$ dw) after 5.5 hours of drying. On the other hand, seeds equilibrated to different RHs for up to 4 days on each condition allowed the slow drying of the seeds (fig. 3).

Radicle protrusion was observed after 15 days of imbibition, being faster when compared to a seed lot collected in 2004 (Chapter 2, in this thesis). Germination is preceded by the seed coat rupture, what happened after 10 days of imbibition, and was an indicative of seed viability, since it was observed in germinating seeds but not in dead seeds.

A transient increase in germination was observed when the water content of the seeds was reduced to 0.22 g $H_2O \cdot g^{-1}$ dw in both fast and slow drying treatments. It was followed by a reduction in seed germination as the seeds dry, with marked differences between fast and slow drying methods (tab 2).

The vast majority of the fast dried seeds to 0.11 g $H_2O \cdot g^{-1}$ dw lost viability, while seeds that were slowly dried to the same water content level presented 74% of germination. After this point, water molecules are apparently

more firmly bind to macromolecules, since it is more difficult to remove from the seeds what can be seen in the desorption isotherm (Fig 4).

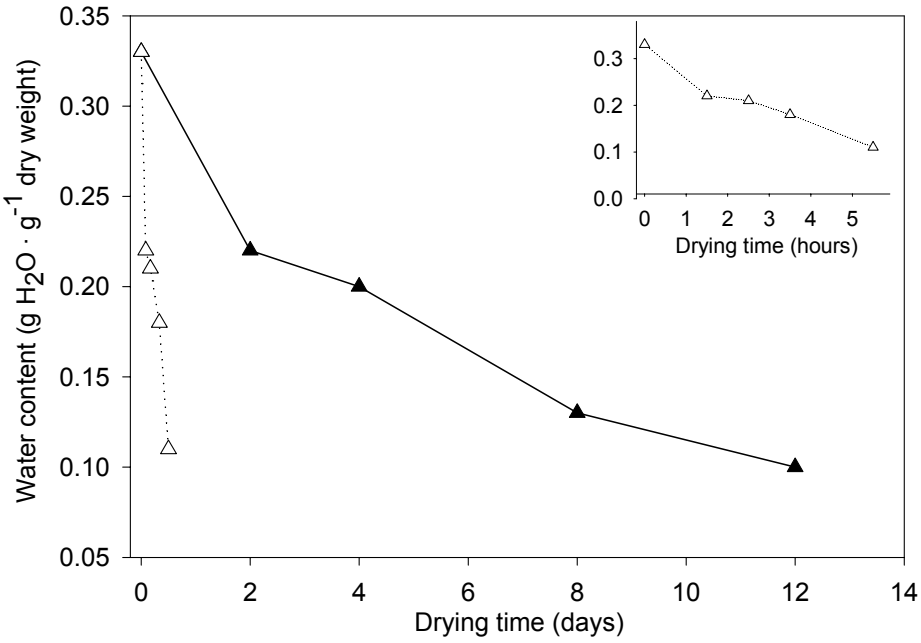

Figure 3. Drying rates of *T. ovata* seeds obtained by the different desiccation treatments. Fast (dotted line, open triangles) and slow drying (solid line, closed triangles). A detail of fast drying time courses is presented upper right.

Drying rate also affected the seed vigor, measured by the SGI (tab 2). In fast dried seeds it was reduced as seed dried. Slowly dried seeds kept the same SGI until 8 days of drying (0.13 g $H_2O \cdot g^{-1}$ dw). Drying further resulted in the reduction in the speed of germination index. Vigor also transiently increased after a mild drying in both fast and slow drying treatments (fig 5).

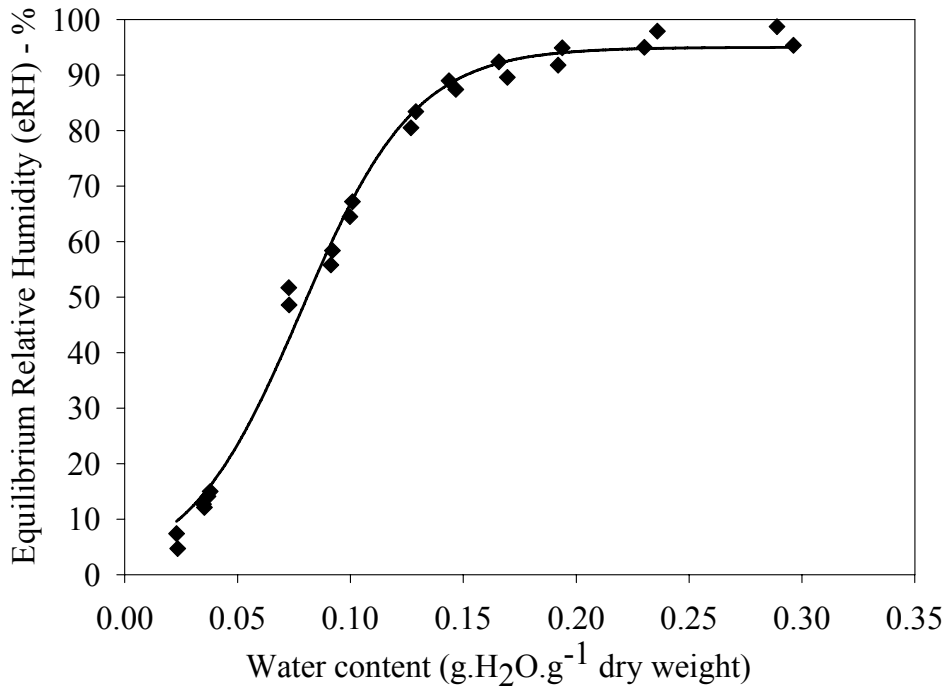

Figure 4. Desorption isotherm of *T. ovata* seeds dried at 20°C. Each data point represents the average of two measurements.

Table 2. Drying time courses, water content, survival (percentage of germination) and vigor (speed of germination index - SGI) of *T. ovata* seeds following fast or slow drying.

Treatment	Drying time	Water content (g $H_2O \cdot g^{-1}$ dw)	Percentage of Germination	SGI
Fresh	0	0.33	84 c	10.6 c
Fast drying	1.5 hours	0.22	94 d	16.5 f
	2.5 hours	0.21	82 b	12.2 d
	3.5 hours	0.18	79 b	11.1 d
	5.5 hours	0.11	19 a	1.3 a
Slow drying	2 days	0.22	92 d	16.4 f
	4 days	0.20	87 c	13.7 e
	8 days	0.13	85 c	11.7 d
	12 days	0.10	74 b	8.6 b

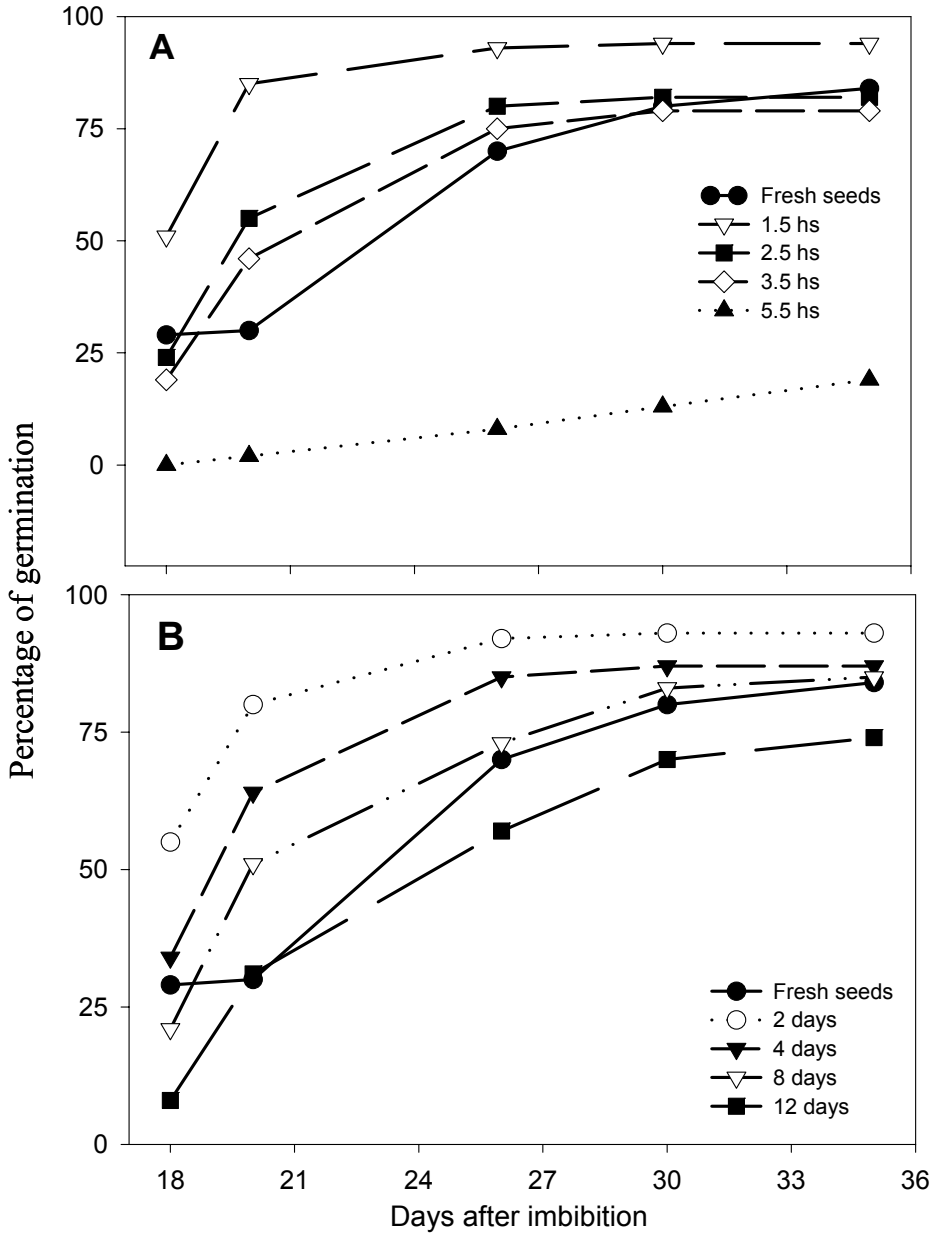

Figure 5. Effect of time of desiccation on germination course of *T. ovata* seeds, following fast (A) and slow drying (B).

Discussion

Drying rate affects survival of T. ovata seeds

Seeds of *T. ovata* are shed with relatively low water content. They are formed inside a dry fruit and are exposed to desiccation after fruit opening, where the aril seems to have an important role, preventing seed to fast desiccation. According to Hong et al. (1996), in general, species of the Magnoliaceae family have desiccation tolerant seeds.

The speed in which water is removed from seed tissues can affect its survival. Even in orthodox seeds, depending on the developmental status, it is a critical factor. Seeds of *Ricinus communis* do not withstand fast drying at early stages of development, but are able to germinate if slowly dried (Kermode & Bewley, 1985). According to Kermode & Finch-Savage (2002), an explanation for the survival of immature seeds after the slow drying is the operation of the protective mechanism during drying, what would not happen when seeds are fast dried, resulting in critical cellular damages. On the other hand, fast drying of recalcitrant seeds have been reported to improve the survival of seeds and embryos to lower water contents, because of the reduced period for damages accumulation during drying (Pammenter & Berjak, 1999).

Seeds of *T. ovata* showed significant differences in viability after drying to the lowest water content by using two drying rates (tab. 2). Seeds could be dried to lower water content using the slow drying procedure, what contrast with findings for recalcitrant seeds (Berjak et al., 1993; Fu et al., 1990). As an example, fast drying (60 min) of the desiccation sensitive seeds of *Artocarpus heterophyllus* resulted in 100% survival of embryonic axes, whereas no axes survived slow drying to the same water content (Wesley-Smith et al., 2001).

The drying rate used to redry osmoprimed seeds of *Brassica oleracea* has an effect on the behaviour of the seeds in physiological tests. Analyzing gene expression after two drying rates, Soeda et al. (2005) found differential

expression for genes correlated to *B. oleracea* seed stress tolerance. The LEA genes Em6 and RAB18 presented higher expression after a slow drying. The authors hypothesize that, during slow drying, the primed seeds are able to perceive the gradual increase in drought stress and respond by re-inducing RAB18 and Em6 gene expression, whilst during the fast drying the reduction in water content is too fast to allow seeds to respond by gene induction.

Although seeds could be dried to low water contents (0.10 g $H_2O \cdot g^{-1}$ dw), it is not possible to conclude that seeds of *T. ovata* acquired tolerance to desiccation, since viability after storage of these seeds were not evaluated. Instead, it is possible to infer that during slow drying some protection mechanism acts in order to avoid or reduce the damages caused by seed desiccation.

Another possible explanation for the differential response to drying rates in the present study is the fact that *T. ovata* seeds are dispersed with a lower water content compared to typical recalcitrant seeds, that are shed with water contents ranging from 0.54 to 9.0 g $H_2O \cdot g^{-1}$ dw (Hong et al., 1996 and references therein). It is thought that deleterious processes consequent of metabolic dysfunction and failure of antioxidant system at intermediate water contents (0.5-0.8 g $H_2O \cdot g^{-1}$ dw), leads seeds to loss of viability and a fast drying reduces the time that seeds are exposed to such processes (Pammenter et al., 1999; Pammenter & Berjak, 1999). Since *T. ovata* seeds are shed with water contents below these limits, the damages caused by metabolic dysfunction may be reduced and slow drying can provide time to seeds to respond to desiccation stress.

Desiccation to 0.10 g $H_2O \cdot g^{-1}$ dw, what is equivalent to a water potential of -53.7MPa, is close to critical water potential of *Acer platanoides*, *Azadirachta indica*, *Carica papaya* and *Coffea arabica* ((Sun & Liang, 2001), seeds with intermediate storage behaviour. At this water potential, most of the

non-freezable water seems to be removed from *T. ovata* seeds (Fig 4), what can make possible the storage at low temperatures. Further experiments should be conducted to assess the viability of slow dried seeds to lower water contents and viability after storage at low temperatures to determine the lowest water content tolerated by this species and the possibility of long term conservation.

This study developed a methodology to safely dry seeds of *T. ovata* to low water contents, a species previously described as desiccation sensitive and opened new perspectives for long-term conservation.

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CHAPTER 2

Changes in protein expression upon desiccation and imbibition of recalcitrant seeds of *Talauma ovata* A. St.-Hil.

Running title: Desiccation of *Talauma ovata* seeds

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Abstract

Seeds of *Talauma ovata*, a tree widely distributed in swampy soils of riparian forests from Brazilian Atlantic Forest, have been reported to have limited desiccation tolerance. The development of proper strategies for conservation of seeds relies on the understanding of mechanisms involved in desiccation tolerance and sensitivity. The effect of seed drying and imbibition was studied by differential protein expression using two dimensional gel electrophoresis. After drying to a range of water contents, seeds were germinated to assess viability. Seeds of *Talauma ovata* did not withstand desiccation down to 0.10 g $H_2O \cdot g^{-1}$ dw. The critical water content below which desiccation sensitivity became apparent was around 0.18 g $H_2O \cdot g^{-1}$ dw (-26.5 MPa). Total protein was extracted and separated by 2D electrophoresis from fresh seeds (0.28 g $H_2O \cdot g^{-1}$ dw), mild dried seeds (0.25 g $H_2O \cdot g^{-1}$ dw) and seeds at low water content (0.10 g $H_2O \cdot g^{-1}$ dw) before and after imbibition for 10 days. The proteome profile revealed the presence of 588 spots on each silver stained gel. Analyzing silver stained gels from different conditions (different water content, before and after imbibition) enabled the identification of up to 21 protein spots that correlated with desiccation and germination, by increased or decreased expression. After MS/MS sequencing, 3 protein spots produced spectra that matched to a *Magnolia salicifolia* legumin precursor. By comparing the expression of these identified protein spots in the 2D-gels with the germination data it is possible to suggest an involvement of this protein in events taking place during drying and subsequent imbibition of dried seeds.

Key Words: desiccation tolerance, legumin, protein expression, proteomics, *Talauma ovata*.

Introduction

Talauma ovata A. St.-Hil is a tree widely distributed in the Brazilian Atlantic Forest, ranging from north (Para State) to south (Rio Grande do Sul State). It is widespread in different vegetation types like rainforest, semideciduous forest and Cerrado (Brazilian savannah). However, its occurrence is particularly related to wet soils of riparian forests, a susceptible environment in various Brazilian ecosystems.

This species is used for restoration of riparian forests due to its adaptation to swampy soils. *T. ovata* has also been used in folk medicine as a febrifuge (Pio-Corrêa, 1984) and anti-diabetes (Morato et al., 1988) in some parts of Brazil. It is known that members of the Magnoliaceae family produce secondary metabolites such as phenolic compounds, sesquiterpene lactones, monoterpenes, alkaloids and volatile oils. Ethnomedicinal data for this plant family are reported from Eastern Asia, North America and Mexico (Schühly et al., 2001). From *T. ovata* some lignans, a large group of phenylpropanoid metabolites found in plants, have been isolated (Hoffmann et al., 1977; Stefanello et al., 2002). These compounds showed potential to inhibit *in vitro* growth of human tumor (Nascimento et al., 2004).

The tree is 20-30m high with 30-40 cm stem diameter, producing large amounts of seeds every year. Dispersion occurs between July and October mainly by birds that are attracted by the red aril covering the seed (Cazetta et al., 2002). The seeds are characterized by a woody testa, an oily endosperm and a small embryo, like many other Magnoliaceae species (Corner, 1976). Seeds from most species desiccate at the end of maturation, but not all seeds survive desiccation. Based on tolerance to seed desiccation, species can be divided into three groups. Orthodox seeds are tolerant to extreme desiccation and survive in the dehydrated state for long periods, which enables long-term storage at low temperature. In contrast, recalcitrant seeds do not tolerate desiccation or storage

at low water contents (Roberts, 1973). A third group, known as intermediate, has been proposed to share characteristics of the previous two groups (Ellis et al., 1990). Because of its low tolerance to desiccation, *T. ovata* has been classified as recalcitrant (Lobo & Joly, 1996).

Desiccation tolerance is a complex multigenic trait (Leprince et al., 1993; Vertucci & Farrant, 1995). It is the result of a complex cascade of molecular events, which can be divided into signal perception, signal transduction, gene activation and biochemical changes, leading to acquisition of desiccation tolerance (Bartels & Salamini, 2001; Phillips et al., 2002; Ramanjulu & Bartels, 2002). Desiccation tolerance is characterized by physical and chemical adjustments in order for the cells to withstand dehydration and resume biological activity after rehydration. Some of these mechanisms include a reduction in the degree of vacuolation, changes in the amount and nature of accumulated insoluble reserves, conformation of the DNA, chromatin and nuclear architecture, intracellular de-diferentiation, "switching off" of metabolism, activation of antioxidant systems, accumulation of putatively protective molecules like proteins (LEAs) and sugars, positioning of amphipathic molecules, protection of oil bodies by oleosins and the presence of repair mechanisms during rehydration (for review, see Pammenter & Berjak, 1999). At the moment, the majority of studies have compared desiccation-sensitive with desiccation-tolerant organisms in an attempt to understand the mechanisms that lead to death after drying of recalcitrant seeds. Despite the great number of studies with desiccation tolerant systems (e.g. Buitink et al., 2003), the immediate cause of the sensitivity to desiccation in recalcitrant seeds is still far from being understood.

Combined studies of seed physiology and molecular genetics have provided good insights into regulatory networks involved in development and germination of seeds (Koornneef et al., 2002). Proteomics is one of the most

important developments used for seed physiology studies in recent years (Bertone & Snyder, 2005; Bove et al., 2001; van der Geest, 2002). The study of the whole transcriptome and proteome has been suggested as useful in the identification of genes with differential expression (Koornneef et al., 2002) and proteomics has been extensively used in studies of seed development (Finnie et al., 2002; Gallardo et al., 2003; Hajduch et al., 2005; Schiltz et al., 2004) and germination (Gallardo et al., 2001, 2002, 2003; Rajjou et al., 2004; Wong & Abubakar, 2005). This paper describes the identification of proteins that are differentially expressed during desiccation and germination of *T. ovata* seeds

Material and Methods

Plant Material

Talauma ovata fruits were collected from 12 trees along the Rio Grande river near Lavras (MG, Brazil) in September 2004. After collection, fruits were left at room temperature to allow the completion of dehiscence, which occurred between 3 to 5 days. The red aril that covers the seed was removed by gentle rubbing on a mesh and rinsing with tap water. After cleaning, seeds were blotted dry with a paper towel to remove excess water, and seeds were stored at 5ºC in closed plastic bags. Seeds were deployed for experimental work within 2 months of harvest.

Desiccation conditions

Desiccation was carried out over a saturated 90% (w/v) lithium chloride solution, providing a relative humidity of 11% at 20ºC (Sun, 2002). Estimated moisture content was monitored each hour by the difference between initial weight of fresh and dried seeds. When seeds reached the estimated target moisture content, three samples were taken: one sample of 20 seeds for moisture content determination, one sample of 100 seeds for germination and one sample of 25 seeds for total protein extraction. The latter seeds were frozen in liquid nitrogen and stored at -70ºC until processing.

Water content, water potential and oil content assessment

Water content of the whole seed was determined gravimetrically on four replications of five seeds by oven drying at 103ºC/17hours (ISTA, 2005). Water contents were expressed on a dry weight basis (g H_2O · g dry weight^{-1}, or g H_2O · g^{-1} dw).

For water potential measurements, a desorption isotherm using a range of lithium chloride solutions providing different relative humidity was

established. Equilibrium relative humidity (eRH) of whole seeds was measured with a Rotronic Hygrometer (Probert et al., 2002) in four replicates of 25 seeds over 60 minutes. Water potential (Ψ_w) was calculated from eRH using the equation (Ψ_w) = (RT/V).ln (a_w), where *R* is the gas constant (8.314 J mol^{-1} K^{-1}), *T* is the temperature (Kelvin), *V* is the partial molal volume of water (18.048 ml $mole^{-1}$) and a_w is the water activity (eRH/100) (Sun, 2002).

Seed oil content was estimated using the supercritical fluid extraction method (Eller & King, 2000). An SFX 3560 (ISCO, USA) was used with the following extraction conditions: pressure 7500 psi; temperature 100°C; time 30 min; flow rate 2 $ml.min^{-1}$.

Seed germination and viability assessment

After desiccation, seeds were soaked on moist filter paper (filter paper 595, Ø 85 mm, Schleicher & Schuell, Dassel, Germany) in 9 cm Petri dishes for 24 hours with 5 ml distilled water. Subsequently, seeds were cleaned with 1% sodium hypochlorite for 10 minutes and rinsed with distilled water. Germination assays were carried out with four replicates of 25 seeds. Seeds were incubated at alternating temperature 20ºC/10ºC (day/night), with 8 hours light daily on two sheets of filter paper Schleicher & Schuell 595 wetted with 5ml of distilled water in disposable 9 cm Petri dishes. A seed was regarded as germinated when the radicle protruded 2mm through the seed coat. After 10 days of imbibition a sample of 25 seeds was taken for protein extraction. These seeds were frozen in liquid nitrogen and stored at -70ºC.

Germination tests were carried out for 80 days. Non germinated seeds after this period were evaluated by a cut test in order to assess viability of seeds. Seeds were regarded as dead if mushy, liquid or rotten endosperm was present. On the other hand, if a firm and white endosperm and embryo were present, seeds were scored as viable seeds (Gosling, 2002; ISTA, 2005) and reported as

"dormant" seeds. To confirm the applicability of the cut test, a tetrazolium test was performed using the same conditions, which showed the same results (data not shown), similar to results reported by Ooi & Whelan (2004).

Total Protein Extraction

Total protein extracts were prepared from seeds dried at different water contents and from seeds subsequently imbibed for 10 days. Three samples of 5 seeds each were ground in liquid nitrogen using a mortar and pestle. Total protein was extracted on ice in 800µL of thiourea, urea lysis buffer containing 7M urea (Amersham Biosciences), 2M thiourea (Acros Organics), 18mM Trizma HCl (Sigma), 14mM Trizma base (Sigma), protease inhibitor cocktail Complete Mini (Roche Diagnostics), 12 units DNAse I (Roche Diagnostics), 20µL RNAse A (20mg/mL, Invitrogen), 0.2% (v/v) Triton X-100, 60mM CHAPS (Fisher Scientific) and 17.5mM DTT (Amersham Biosciences). After 20 minutes, tubes were centrifuged at 14,000 rpm for 10 minutes at 4ºC. Supernatant containing the total protein extract was centrifuged again and supernatant was stored at -20ºC in aliquots of 100µL.

Protein concentration in the various extracts was measured according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

Two-Dimensional Electrophoresis

Proteins were separated by isoelectric focusing (IEF), using 24 cm Immobiline DryStrips with a nonlinear pH gradient from 3 to 10 (Amersham Biosciences). Gel strips were rehydrated in the IPGphor system (Amersham Biosciences) for 14 hours at 20ºC in the thiourea/urea buffer containing 2M thiourea, 7M urea, 1.2% (v/v) DeStreak Reagent (Amersham Biosciences), 2% CHAPS (w/v) and 0.5% IPG buffer pH 3 to 10. A final volume of 450 µL of solution was applied to each strip holder. After positioning the gel strip in the

strip holder, 1.9 ml of cover solution (Amersham Biosciences) was applied and the strip holder lid replaced. Isoelectric focusing was then performed at 20ºC at 500 V for 1 hour, 1000V for 1 hour, 8000V for 8 hours and 20 minutes and 50V for up to 1 hour. Gel strips were stored at -70ºC in equilibration tubes (Amersham Biosciences) until second dimension electrophoresis. Before second dimension electrophoresis, the gel strips were equilibrated in 15 ml equilibration solution containing 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 75mM Trizma base pH 8.8, with 65 mM DTT (1^{st} step), without addition (2^{nd} step) and with 0.2 M iodoacetamide (3^{rd} step), 40 min per equilibration step. Equilibrated gel strips were placed on top of 1 mm vertical polyacrylamide gels containing 9.4% (v/v) acrylamide, 70mM Bis(acryloyl) piperazine, 0.3M Trizma base pH 8.8, 0.08% (w/v) ammonium persulphate and 0.04% (v/v) TEMED. Gel strips were sealed with 1% (w/v) low-melting point agarose (Fisher Bioreagents) containing 0.2% (w/v) SDS, 50mM Trizma base, 0.4 M glycine and trace bromophenol blue. Gels were left for 5 min to allow agarose solidification. Electrophoresis was performed at 25ºC in a buffer containing 0.1% (w/v) SDS, 95mM glycine and 12.5mM Trizma base, for 1 hour at 2.5V/gel and 4.5 hours at 17V/gel, in an Ettan DALTtwelve system (Amersham Biosciences). For each condition triplicate gels were electrophoresed using independent protein extracts.

Protein Staining and Analysis of 2D Gels

Gels were stained with the PlusOne Silver Staining Kit (Amersham Biosciences), or Colloidal Commassie Brilliant Blue G-250 (Sigma). Stained gels were scanned with the ImageScanner (Amersham Biosciences), equipped with Ulmax MagiScan 4.6 in the transmissive mode with 300dpi. Image analysis was carried out with ImageMaster 2D Platinum 5.0 (Amersham Biosciences). After spot detection, gels were aligned and matched, and then the quantitative determination of the spot volumes was performed. When necessary, gels were

normalized using a scatter plot fitting report method. Analysis of groups, classes and statistical tests (student's t-test) were then performed. Spots with a 2-fold difference and a significant result in the t-test ($P<0.05$) were regarded as differentially expressed.

Protein Identification by MS/MS

Protein spots with differential expression were manually excised from Coomassie Blue-Stained 2D gels using a sterile scalpel and digested by sequencing grade, modified porcine trypsin (Promega, Madison, WI). Samples were applied directly to the MALDI target plate and positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800-4000. Final mass spectra were internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data. The twenty strongest peaks, with a signal to noise greater than 50, were selected for CID-MS/MS analysis.

For CID-MS/MS, source 1 collision energy of 1 kV was used, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and polynomial order 4); peak detection used a minimum S/N of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Filters of S/N 20 and 30 were used for generating peak lists from MS and MS/MS spectra, respectively.

Mass spectral data obtained in batch mode were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 1.9). Batch-acquired MS and MS/MS spectral data were submitted

to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.5) to Mascot. Search criteria included: Maximum missed cleavages, 1; Variable modifications, Oxidation (M), Carbamidomethyl; Peptide tolerance, 100 ppm; MS/MS tolerance, 0.1 Da.

Results

General characteristics of fruits, seeds and effect of drying on germination of *T. ovata* seeds

Fruits were greenish, ovoid in shape, dehiscent, and contained around 65(±10) seeds each. Seeds were black, covered by a red aril and remained attached to the fruit by a lignin-fibril after fruit opening. A woody testa covered the oily endosperm (32.7% oil content). Seeds contained a small embryo (approx. 1 mm long) with embryonic axis and differentiated cotyledons.

Fresh seeds were exposed to a RH of 11% at 20ºC for different periods of time. Seed water content decreased with progressing time. Fig.1 shows the effect of the drying on germination and viability of *T. ovata* seeds. Desiccation damage was observed when seeds were dried to water content below 0.18g $H_2O \cdot g^{-1}$ dw, which is equivalent to a water potential of -26.5MPa. Further drying resulted in an increase in the number of dead seeds and, conversely, a reduction in germination could be observed.

Germination was typically very slow, with radicle protrusion initiating 40 days after imbibition (results not shown). This was preceeded by the start of seed coat rupture after 10 days of imbibition, which was indicative of seed viability since it was observed in germinating and dormant seeds but not in dead seeds. After 80 days the majority of seeds had either germinated or died. Seed germination was higher in seeds dried to intermediate water contents (0.25 to 0.18 g $H_2O \cdot g^{-1}$ dw) than fresh seeds (0.28 g $H_2O \cdot g^{-1}$ dw). The number of dead seeds was relatively constant down to 0.18 g $H_2O \cdot g^{-1}$ dw of water content. Below this point, dehydration caused an increase in the number of dead seeds. It shows that the critical water potential (below which desiccation sensitivity became apparent) was around -26.5 MPa (0.18 g $H_2O \cdot g^{-1}$ dw).). However, at low water potential, some seeds were still viable. It is important to mention that although the vast majority of the seeds for all the different water contents had

either germinated or died as above-mentioned, a reasonable number of dormant seeds (27%) was found after 80 days of imbibition of fresh seeds, this number was reduced to about 7% when seeds were dried to 0.25 g $H_2O \cdot g^{-1}$ dw.

Seeds were also dried at 11% of relative humidity at 5ºC to test if the causing of dying was due to the temperature or water content. However, the viability after drying at 5ºC was always lower when compared to seeds dried at 20ºC (data not shown).

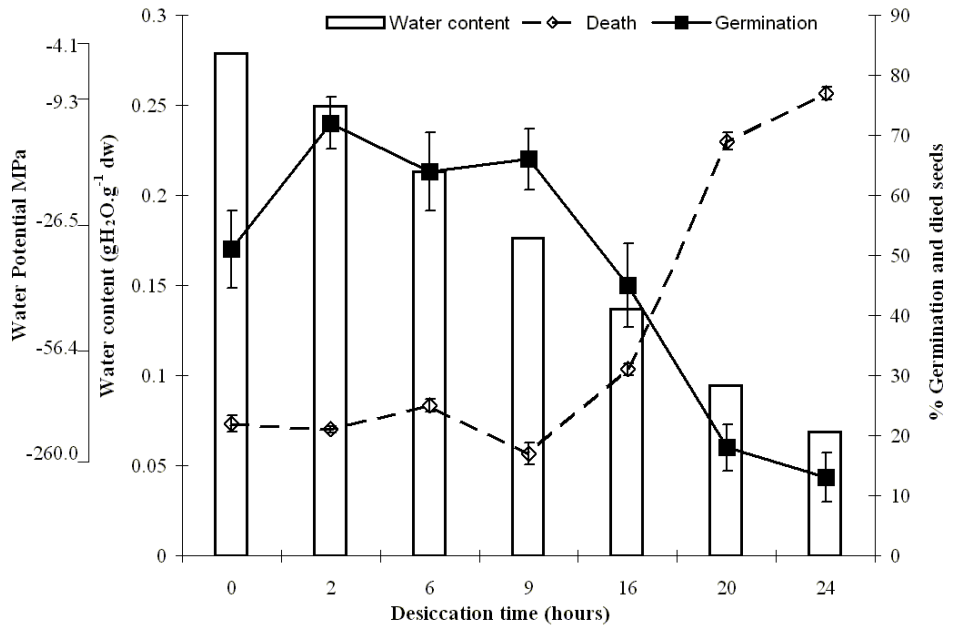

Fig.1. Effect of drying (11% RH at 20ºC) on germination and viability of *T. ovata*. Final germination was scored after 80 days of incubation at 20/10ºC. An axis for water potential is presented for reference to water content. Error bars represent the standard error of the mean (when bigger than symbols).

Proteome analysis

Based on desiccation sensitivity and number of dormant seeds after 80 days of imbibition, three samples were selected for proteomics studies. Fresh seeds (0.28 g $H_2O \cdot g^{-1}$ dw) with high viability; mild dried seeds (0.25 g $H_2O \cdot g^{-1}$ dw) with reduced dormancy and high viability; and seeds at low moisture content (0.10 g $H_2O \cdot g^{-1}$ dw) with reduced viability. The proteome profile of *T. ovata* seeds revealed the presence of 588±69 spots on each silver stained gel. After alignment, 61% of the spots were matched. A high experimental reproducibility was achieved. A characteristic result for *T. ovata* seeds was the presence of highly abundant proteins, which are most probably storage proteins. A large majority of the proteins were focused between pI 5-8. Differential expression was found only for proteins with a molecular mass between 15 and 50 KDa.

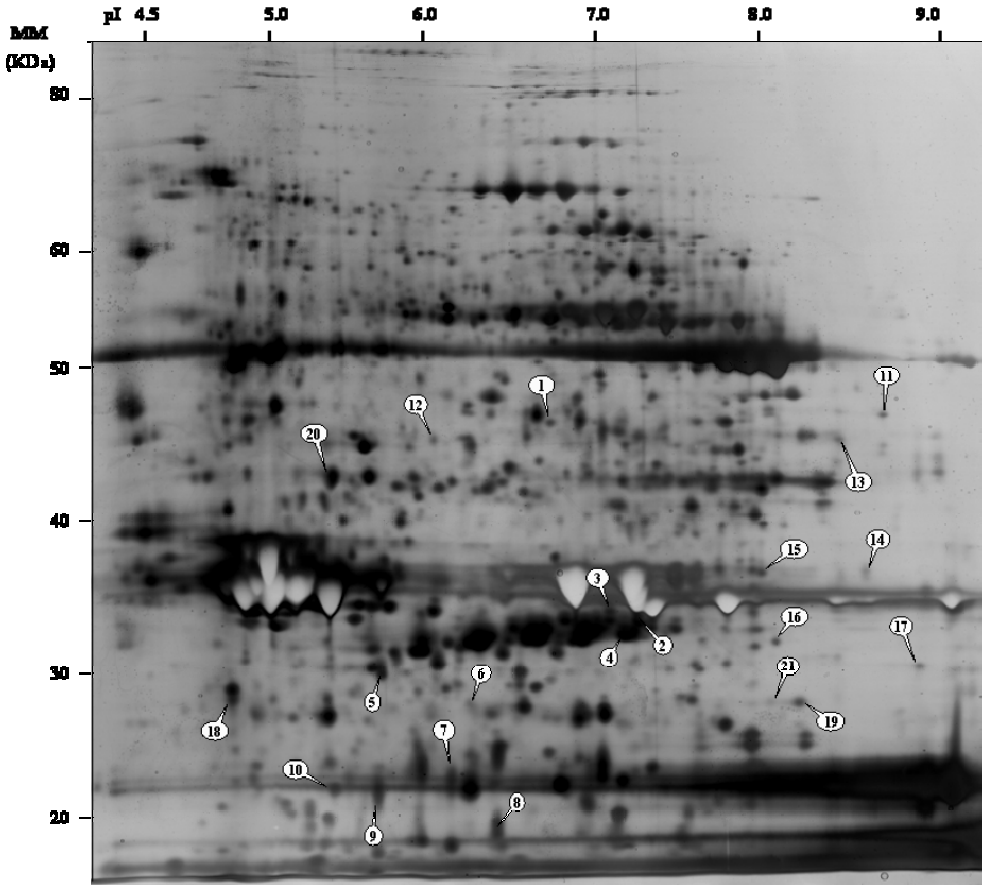

Fig.2. Silver stained two-dimensional gel of total protein from *T. ovata* seeds. Labelled protein spots presented differential expression during drying and after drying and imbibition.

Comparing silver stained gels from different conditions (different water content, before and after imbibition) enabled identification of up to 21 protein spots with differential expression (Fig.2). Of these, 13 could be identified in Coomassie-stained gels for manual spot picking, in-gel digestion and peptide mass spectrometry.

Protein spots were sorted in three groups, based on the profile of expression after drying or after imbibition, when there was no significative change in expression after drying. In the first group (Fig.3), protein abundance after desiccation was reduced. On the other hand, proteins from group two (Fig.4) showed higher expression after desiccation of fresh seeds. A third group (Fig.5) contains proteins showing lower expression after drying fresh seeds to 0.25 g $H_2O \cdot g^{-1}$ dw and subsequently, higher expression if seeds were dried to a low water content (0.10 g $H_2O \cdot g^{-1}$ dw). In general, for all the three groups the responses were similar for both dried seeds and during subsequent imbibition. However, this was not true for all spots as can be seen in Fig.4 (protein spot number 8) and Fig.5 (protein spots number 12, 15, 16, 18 19 and 21). In the case of spots 8, 18 and 21, after imbibition, a mirrored image of gene expression could be noticed compared to the un-imbibed state.

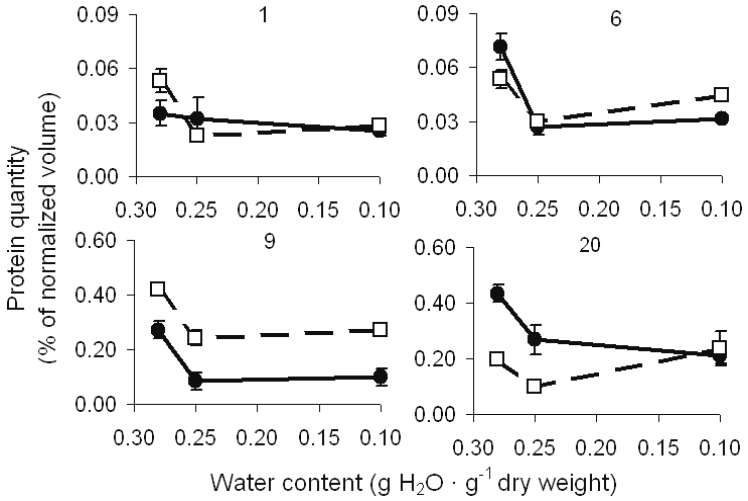

Fig.3. Expression profiles of proteins from 2D-gels of *T. ovata* seeds showing lower expression after desiccation (solid lines, closed circles). Dashed lines and open squares represent expression during subsequently imbibition.

Of the 13 protein spots submitted for identification by mass spectrometry, 10 did not match to any sequences in databases and 3 matched the same protein, a *Magnolia salicifolia* legumin precursor (accession n^{o} gi-793854)**.** The peptide sequence GLLLPSFDNAPR produced by MS spectra from protein spots number 2 (Fig.4) and 21 (Fig.5) matched with the N-terminal cupin domain, while the sequences ADVYNPQAGR and EEIAVFAPR from protein spot number 5 (Fig.4) matched with the C-terminal cupin domain and a down-stream region, respectively.

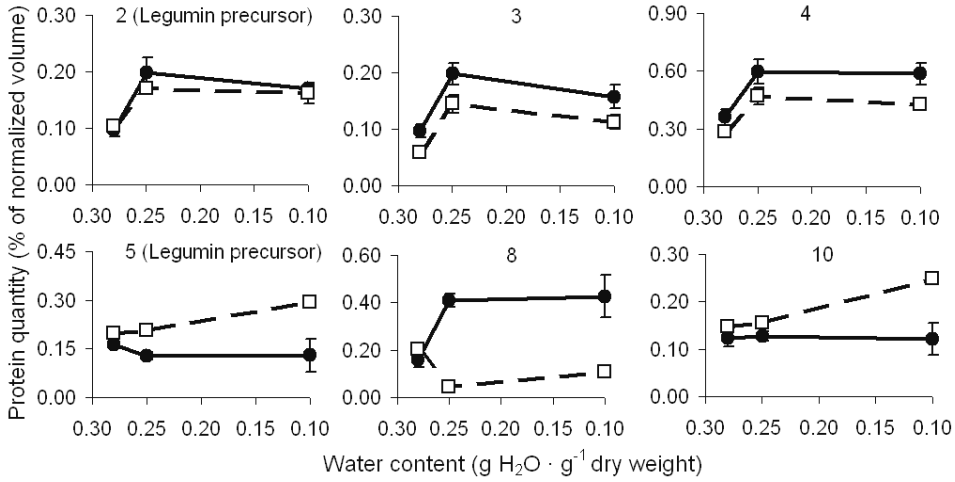

Fig.4. Expression profiles of proteins from 2D-gels of *T. ovata* seeds showing higher expression after desiccation (solid lines, closed circles). Dashed lines and open squares represent expression during subsequently imbibition.

For protein spot number 2 (pI 6.8, Mw 32.0), an increased abundance was observed both after drying fresh seeds to 0.25 g $H_2O \cdot g^{-1}$ dw and subsequent imbibition treatment ($P<0.05$). The expression remained at an elevated level after further drying to 0.10 g $H_2O \cdot g^{-1}$ dw and subsequent imbibition. Protein spot number 5 (pI 5.6, Mw 28.7) showed a decrease in abundance after drying

treatment, while an increase was noticed after imbibition of seeds dried to a water content of 0.10 g $H_2O \cdot g^{-1}$ dw. On the other hand, protein spot number 21 (pI 7.8, Mw 29.0) showed a transient decrease in abundance after drying to 0.25 g $H_2O \cdot g^{-1}$ dw and a contrasting expression behaviour after imbibition of the seeds. Abundance of spot 21 was much lower than that of spots 2 and 5.

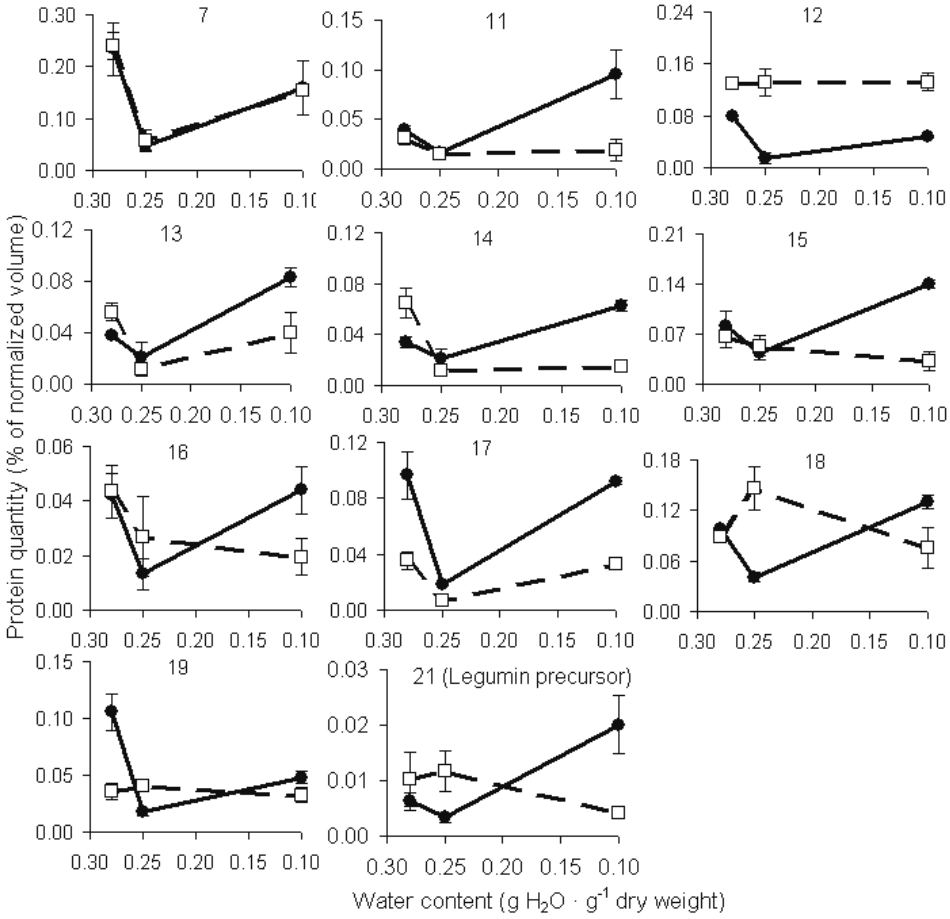

Fig.5. Expression profiles of proteins from 2D-gels of *T. ovata* seeds showing transiently lower expression during desiccation (solid lines, closed circles). Dashed lines and open squares represent expression during subsequently imbibition.

Discussion

Effect of drying on germination and viability of *T. ovata* seeds

Recalcitrant seeds are those that can not survive desiccation to low water contents, usually below 0.10 g $H_2O \cdot g^{-1}$ dw (Ellis et al., 1990; Hong et al., 1996; Roberts, 1973). The loss of viability in *T. ovata* seeds occurred after drying to water contents below 0.18 g $H_2O \cdot g^{-1}$ dw. This corresponds to a water potential of -26.5MPa, which is also close to the critical water potential of -23MPa found by Sun & Liang (2001) for *Artocarpus heterophyllus* and *Hevea brasiliensis* and -20MPa found by Pritchard, (1991) for *Quercus rubra*, all desiccation sensitive species.

After mild drying, before reaching the critical water content, a slight increase in germination was observed. This has been attributed to a continuation of the maturation process in recalcitrant seeds of *Inga vera* (Faria et al., 2004). This observation, inversely related to the fraction of dormant seeds, which was higher in fresh seeds (0.28 g $H_2O \cdot g^{-1}$ dw) compared to mild dried seeds (0.25 g $H_2O \cdot g^{-1}$ dw). Further drying to 0.10 g $H_2O \cdot g^{-1}$ dw concurred with a larger fraction of dormant seeds again (data not shown). Changes in dormancy in relation to desiccation have been reported in papaya seeds, where dormancy is induced when seeds are dried (Wood et al., 2000).

Although *T. ovata* seeds were sensitive to desiccation, some seeds were still viable after drying to 0.07 g $H_2O \cdot g^{-1}$ dw (-93 MPa). Many factors, like seed maturity, desiccation rate, imbibitional injury, chilling sensitivity, and dormancy, have been described to show an important role in storage behaviour. The manipulation of water content and germination of seeds that experience some of these factors might be rather complex. As an example, neem (*Azadirachta indica*) seeds are chilling sensitive at high water content and sensitive to imbibitional stress at low water contents (Sacandé, 2000).

Effect of drying and drying/imbibition treatments on the patterns of protein expression in *T. ovata* seeds

After analyzing the 2D gel, the proteins could be sorted into 3 groups, according to the pattern of expression (Figs.3, 4 and 5). Proteins from group 1 (Fig.3) show a decrease in abundance after drying fresh seeds. Down-regulated expression in a set of genes related to maturation was also observed when the drying maturation phase starts in *Medicago truncatula* seeds (Gallardo et al., 2003). Some genes with high expression during maturation of *Brassica oleracea* seeds are a sub-group of late-embryogenesis abundant and storage proteins (Soeda et al., 2005).

In spite of the exact function of several down-regulated genes being unknown, the logical presumption is that some genes are down-regulated because of the fact that their product might not be suited to the new physiological condition caused by dehydration stress (Ramanjulu & Bartels, 2002). Studies have revealed that transcripts encoding proteins relevant to photosynthesis in *Craterostigma plantagineum* are down-regulated and have been estimated to represent 36% of the total number of genes altered during the dehydration process (Bockel et al., 1998).

Group 2 proteins (Fig.4) showed an increase in abundance after drying of the seeds. This pattern of expression coincides with stress tolerance. After imbibition of fresh and dried seeds, spots number 2, 3 and 4 from group 2 showed the same expression pattern as in the dry state (Fig.4), albeit lower. It seems that these proteins that are induced during desiccation are reduced during subsequent imbibition. It is known that, with the initiation of drying, there is a shift in gene expression, compelling seeds to a different physiological status, where genes coding for protective molecules play an important role (Ramanjulu & Bartels, 2002). Proteins related to desiccation tolerance and involved in metabolic changes (Avelange-Macherel et al., 2006; Leprince et al., 2000;

Walters et al., 2002), protection against oxidation and other putative protective molecules show a particular expression pattern during desiccation (Pukacka & Ratajczak, 2005). Small heat-shock proteins (*sHSPs*) (Wehmeyer & Vierling, 2000), and late embryogenesis abundant (LEA) proteins (Boudet et al., 2006; Gallardo et al., 2001), are amongst the molecules with increased abundance during drying. It is important to notice, however, that the expression of a gene alone, during water stress, does not guarantee that the seed becomes able to survive desiccation (Bray, 1993; Collada *et al.*, 1997).

On the other hand, many other genes show a different pattern of expression during germination, with a decrease in abundance after imbibition. Dehydrins, proteins from the group of Lea proteins, are reported to have decreased expression at the start of germination (Boudet et al., 2006; Buitink et al., 2003; Close, 1997; Roberts et al., 1993). Some other proteins, with high expression in the dry state, but decreased abundance during germination are oleosin, RAB18, EM6, Histone H1and Napin (Soeda et al., 2005) and cytosolic GAPDH (Gallardo et al., 2001).

Protein spots number 5 and 10 showed a significant up regulation ($P<0.05$) after imbibition, but only for seeds that were dried to the lower water content. After analyzing the expression pattern of these two proteins, it is possible to suggest their participation in a protective mechanism during rehydration of the dried seeds. Some Lea proteins and HSPs may provide this protective function not only in the dry state, but also throughout germination. This pattern was observed by Gallardo et al. (2001) and DeRocher & Vierling (1995) in seeds of *Arabidopsis thaliana* and *Pisum sativum*, respectively.

Proteins from the group 3 (Fig.5) presented the most intriguing expression pattern that seems related to final germination of *T. ovata* seeds (Fig.1). These results suggest the regulation of a number of genes when fresh seeds are dried to 0.25 g $H_2O \cdot g^{-1}$ dw, since all the spots from this group were

down-regulated when seeds were mild dried, with the expression being up-regulated again with further drying to 0.10 g $H_2O \cdot g^{-1}$ dw. The expression pattern during progressing desiccation invertedly reflects the germination capacity, and indicates that low abundance of these proteins is somehow associated with onset of germination. The transient decrease seems to testify that high abundance is important if absence of germination is anticipated by the seed. After imbibition, the expression for some spots were similar compared to only dried seeds (protein spots number 7, 13 and 17), suggesting that these proteins, induced during drying, are still present after imbibition of dried seeds. On the other hand, protein spots number 21 showed an opposite pattern of expression comparing dried seeds with dried and imbibed seeds, with an increase upon imbibition of seeds with higher moisture content and a strong decrease of seeds with low moisture content. This is suggestive of a transient increase in expression that only occurs if seeds are imbibed after drying to low water content, weakly mimicked if not dried to such low water content.

Expression of a legumin precursor during drying and imbibition of *T. ovata* seeds

Legumin and vicilin are the two major seed storage proteins accumulated in large quantities during the development of *P.sativum* L. seeds (Chandler et al., 1983). They are encoded by multi-gene families of at least 40 genes for *P. sativum* (Casey et al., 2001). The transcripts encoding storage proteins are amongst the most abundant in the embryo. Its accumulation is temporally and spatially regulated during seed development (Abirached-Darmency et al., 2005; Kroj et al., 2003).

In this study three protein spots matched a legumin precursor from *Magnolia salicifolia.* This protein is the product of a multi-gene family (Casey et al., 2001), which may explain the presence of more than one spot for the same

protein. A multi-gene family consists of group of genes from the same organism that encode proteins with similar sequences either over their full length or limited to a specific domain. Sheoran et al. (2005) also identified 11 protein spots in 2-DE gels as legumin-like proteins and Gallardo et al. (2003) found 2 spots, identified as precursor forms of legumin, while studying processes related to reserve accumulation during seed development of *M. truncatula*. Post translational modifications (PTM) may also explain this, since two separate spots (2 and 21) match with the same oligopeptide sequence. The shift between spots number 2 and 21 (3kDa and about 1.0 pI unit) might be explained by ubiquitination, which is the modification of a protein by the covalent attachment of one or more ubiquitin monomers (a small conserved regulatory protein). It acts as a tag that signals the protein-transport machinery to carry the protein to the proteasome for degradation. This PTM is related to signal destruction (Mann & Jensen, 2003). In fact, legumin expression has been described as regulated by post-translational modifications (Jung et al., 1997). Its precursor is cleaved post-translationally and assembled into 11S hexamers and then deposited within specific regions of the inclusion bodies (Stoger et al., 2001).

During desiccation of fresh seeds, a transient up-regulation of protein expression was observed in spot number 2 (Fig.3). The opposite expression pattern was found for protein spot number 21, which could be explained by post-translational modification of this legumin precursor. These changes are related to differences in germination (Fig.1), where seeds dried to a water content of 0.18 g $H_2O \cdot g^{-1}$ dw have a higher germination compared to fresh seeds and seeds dried to a water content of 0.10 g $H_2O \cdot g^{-1}$ dw.

In the literature there are many reports of increased germination after a mild drying of recalcitrant seeds. This has been attributed to a continuation of the maturation process in recalcitrant seeds during the drying treatment (Faria et al., 2004; Pammenter et al., 1998 and references therein). Indeed, Abirached-

Darmency et al. (2005), studying the expression of legumin during the development of *M. truncatula* and *P. sativum* seeds, suggest the use of legumin A gene as a marker for embryo development, since this gene is highly expressed, specifically during the embryogenesis (embryo development), similar to the 2-D protein electrophoresis results of Gallardo et al. (2003). Boudet et al. (2006) identified a pea (*P. sativum*) legumin precursor homolog in *M. truncatula* radicles associated to desiccation tolerance, but it was attributed to the digestion of storage proteins during germination. These results suggest a function for legumin during desiccation and modify its role as a marker for seed development in *T. ovata*, indicating a range of other proteins that may serve as a marker.

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CHAPTER 3

Changes in gene expression during drying and imbibition of desiccation sensitive seeds of *Talauma ovata* A. St.-Hil.

Running title: Desiccation of *Talauma ovata* seeds

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Abstract

Seeds of *Talauma ovata*, a tree from Brazilian Atlantic Forest, were dried to different water contents to assess the viability and the expression at mRNA level of genes related to seed development (*ABI3*), cell cycle (*CDC2-like)*, cytoskeleton (*ACT2*) and desiccation tolerance *(PKABA1*, *2-Cys-PRX* and *sHSP17.5*). The viability of the seeds did not change after a mild drying, but a reduction in the number of dormant seeds was observed at the end of the germination period, when the initial water content was reduced about 10% (from 0.28 to 0.25g H_2O • g^{-1} dw). Drying seeds to 0.10g H_2O • g^{-1} dw led to loss of viability. Abundance of Abi3 and Act2 did not change after drying but increased after imbibition. Relative levels of *CDC2-like* did not change after a mild drying 0.25g H_2O • g^{-1} dw, but was down regulated when seeds were dried to 0.10g H_2O • g^{-1} dw. After imbibition, the relative levels of *CDC2-like* increased. *PKABA1* and *sHSP17.5* transcripts did not change in abundance after drying to different water contents, however, their relative levels increased after imbibition. The relative amounts of *2-Cys-PRX* were reduced after drying to 0.10g H_2O • g^{-1} dw. There was no difference in *2-Cys-PRX* mRNA levels before and after imbibition of fresh and mild dried seeds, but it was reduced after 10 days of imbibition. After 10 days of imbibition of dried seeds (0.10g H_2O • g^{-1} dw), the relative levels of *2-Cys-PRX* mRNA increased to the same level of fresh seeds. The expression of *ABI3*, *ACT2* and *CDC2-like* alone do not explain the germination behaviour of *T. ovata* seeds. It seems that the seeds, irrespective to the initial water content, perform in the same way during the initial period of germination and the deleterious effects of desiccation will take place latter. *PKABA1*, *sHSP17.5* and *2-Cys-PRX* did not show relation with desiccation. However, the expression pattern of *PKABA1* and *sHSP17.5* suggest the

participation of these genes in protective mechanisms during the imbibition of *Talauma ovata* seeds.

Key words: cell cycle, citosqueleton, gene expression, seed desiccation, desiccation tolerance, seed development, *Talauma ovata*.

Introduction

Desiccation tolerance in organs such as seeds and pollens is a widespread phenomenon among higher plants. Seeds that exhibit the ability to withstand removal of water from their tissues to permit storage at low temperatures can be kept in seed banks for periods of time varying from decades to thousand years, are called orthodox seeds regarding to the storage behaviour. However, not all seeds species tolerate drying to such low water contents. Recalcitrant seeds have high water content at shedding and do not withstand desiccation down to 0.10 g $H_2O \cdot g^{-1}$ dw (Hong et al., 1996).

Although all seeds experience some reduction in water content after maturation, the limit tolerated by recalcitrant species is narrow, with considerable variation between species (Farrant et al., 1996). During the development, some recalcitrant seeds like *Camellia sinensis* (Berjak et al., 1993), *Quercus robur* (Finch-Savage, 1992), *Landolphia kirkii* (Vertucci et al., 1995) increase in desiccation tolerance. A few recalcitrant species can also exhibit some degree of dormancy at shedding (Liu et al., 2005).

Study of gene expression, associated with desiccation tolerance and sensitivity, has been used in order to understand mechanisms leading recalcitrant seeds to death after drying (eg. Faria et al., 2006). With the initiation of the drying there is a shift in the gene expression compelling seeds to a different physiological status, where genes coding for protective molecules have an important role in desiccation tolerant material (Ramanjulu & Bartels, 2002). Genes coding for products associated to tolerance, especially those involved in metabolic changes (Avelange-Macherel et al., 2006; Leprince et al., 2000; Walters et al., 2002), protection against oxidation (Pukacka & Ratajczak, 2005), and other putative protective molecules show a particular expression pattern during desiccation.

The phytohormone abscisic acid (ABA) regulates many complex plant physiological processes including seed development, embryo development, seed dormancy, transpiration, and adaptation to environmental stresses (Finkelstein et al., 2002; Finkelstein, 2006) and play an important role in seed desiccation tolerance. ABA-Insensitive3/Viviparous1 (*ABI3*/*VP1*) are transcription factors that mediate ABA responses in seeds from a range of species (Jones et al., 1997; Parcy et al., 1994; Zeng et al., 2003). ABA acts through *ABI3*/*VP1* proteins, during the latter stages of seed development, which promote seed maturation processes including storage reserve deposition, the acquisition of desiccation tolerance and dormancy (Li & Foley, 1997; McCarty, 1995). Seeds of severe *abi3* mutants in Arabidopsis accumulate less storage protein, are desiccation-intolerant and germinate precociously (Nambara et al., 2000). Studies with transgenic plants overexpressing *ABI3* indicated that this gene is able to direct the expression of seed-specific genes in response to ABA in tissues other than seeds (Parcy et al., 1994).

Actin (*ACT*) and tubulin are the major components of the plant cytoskeleton. Actin is thought to be required for correct cell-division, plate alignment and synthesis, cell shape determination, cell-polarity establishment, cytoplasmic streaming, organelle movement, and tip growth (for a review see Meagher et al., 2005).

The cell division cycle is controlled by the molecular machinery that ensures the fidelity of DNA replication and responds to signals from both the external environment and intrinsic developmental programs. A central role in the regulation of the cell cycle is played by the cyclin-dependent kinases (*CDKs*) (den Boer and Murray, 2000; De Veylder et al., 2001). *CDKs* govern, as in other eukaryotic organisms, the plant cell cycle (Inzé, 2005). Cell division is controlled by the activity of *CDK* complexes. In addition to their association with CYCs (Cyclins), the activity of *CDKs* is also regulated by other

mechanisms, including activation of *CDKs* through phosphorylation of Thr-161 by a *CDK*-activating kinase, inactivation of the CDK/CYC complex via phosphorylation of the Thr-14 and Tyr-15 residues by WEE1 kinase, and degradation of CYC subunits. *CDKs* are also involved in the regulation of transcription and mRNA processing (Loyer et al., 2005).

Proteins kinases have also a critical role in the perception and transduction of cellular responses to environmental stresses (Holappa et al., 2005). PKABA1 is a serine/threonine protein kinase belonging to SnRK2 group. Its transcription is induced by ABA and occurs in embryos and seedlings (Hrabak et al., 2003). *PKABA1* is transcriptionally up-regulated in response to environmental stress via changes in ABA concentration in vegetative tissues and developing embryos of wheat (Holappa & Walker-Simmons, 1995). *PKABA1* mRNA levels increase as ABA content increases in developing seed embryos and reach high levels at seed maturity. Levels remain high in isolated embryos treated with ABA but decline in germinating seeds. *PKABA1* is induced rapidly in seedlings when ABA levels increase in response to environmental stress like cold temperature and osmotic stress (Holappa & Walker-Simmons, 1995). *PKABA1-like* genes have been identified in many plant genomes including *Triticum aestivum* protein kinase 3 (*TaPK3*), soybean *SPK3*, Arabidopsis *SNRK2E* genes, and *Medicago truncatula* (Holappa & Walker-Simmons, 1997; Yoon et al., 1997; Yoshida et al., 2002) and are associated to acquisition of desiccation tolerance (Faria et al., 2006).

Reactive oxygen species (ROS) play a significant role in causing damage to living cells under severe stress conditions. To deal with oxidative stress, complex protective mechanisms have been evolved by plants to mitigate and repair the damage initiated by free radicals (Price et al., 1994). The primary constituents of these protective mechanisms include enzymes such as superoxide dismutase (SOD), catalases and peroxidases, and free-radical scavengers such as

carotenoids, ascorbate, tocopherols, glutathione S-transferases (GST) and glutathione peroxidases (GPX) that catalyze the scavenging of ROS (Mowla et al., 2002; Roxas et al., 2000). Peroxiredoxins (PRXs) are a family of thioredoxin-dependent peroxidases (Horling et al., 2002) characterized as peroxidases with broad substrate specificity, reducing diverse peroxides such as H_2O_2, alkyl hydrogen peroxides and peroxinitrite to water and the corresponding alcohol, and water and nitrite, respectively (Bryk et al., 2000). Usually, plant Prxs are classified as belonging to one of four subgroups, called 2-Cys Prx, 1-Cys Prx, type II Prx, and Prx Q (Rouhier et al., 2001; Rouhier & Jacquot, 2002). The expression of this gene is up-regulated in Arabidopsis plants in response to salt stress and in response to pathogen infection (Rouhier et al., 2004; Charlton et al., 2005). These antioxidants can also remove damaging reactive oxygen species produced by products of desiccation and respiration during late embryogenesis, imbibition of dormant seeds and germination (Stacy et al., 1996).

Another group of plant proteins with synthesis associated with stress is the heat shock proteins (HSP). HSPs are divided into low molecular mass proteins of approximately 15–28 kDa (sHSPs) and high molecular mass proteins of more than 30 kDa (HMM HSPs). sHSPs were first discovered as gene products whose expression is induced by heat and other forms of abiotic stresses (Feder & Holfmann, 1999). Nowadays, it is recognized their importance as molecular chaperones (Collada et al., 1997, Lee & Vierling, 2000). Some sHSPs are developmentally regulated, with accumulation starting at mid-maturation and increasing in abundance as the seeds dehydrate. During germination, the developmentally regulated sHSPs are relatively abundant for the first few days and then decline quickly (Coca et al., 1994; DeRocher & Vierling, 1994; Wehmeyer et al., 1996).

This work aimed to study the expression, at mRNA level, of five genes related to seed development, cell cycle, cytoskeleton and desiccation tolerance during drying and subsequently imbibition of desiccation sensitive seeds of *Talauma ovata* (Magnoliaceae), a tree from the Brazilian Atlantic Forest, in order to understand better the mechanism, leading recalcitrant seeds to death after desiccation and during imbibition.

Material and Methods

Plant Material

Talauma ovata fruits were collected from 12 trees along the Rio Grande river near Lavras (State of Minas Gerais, Brazil), in September 2004. After collection, fruits were left at room temperature to allow the completion of dehiscence, which occurred between 3 to 4 days. The red aril that covers the seed was removed by gentle rubbing on a mesh and rinsing with tap water. After cleaning, seeds were blotted dry with a paper towel to remove excess of water, and stored at 5ºC in closed plastic bags. Seeds were deployed for experimental work within 6 months of harvest.

Desiccation conditions

Desiccation was carried out over a saturated 90% (w/v) lithium chloride solution, providing a relative humidity of 11% at 20ºC. Estimated water content was monitored each hour by the difference between initial weight of fresh and dried seeds. When seeds reached the estimated target water content, three samples were taken: one sample of 20 seeds for water content determination, one sample of 100 seeds for germination and one sample of 50 seeds for RNA extraction. The latter seeds were frozen in liquid nitrogen and stored at -70ºC until processing.

Water content assessment

Water content of the whole seed was determined gravimetrically on four replications of five seeds by oven drying at 103ºC/17hours (ISTA, 2005). Water contents were expressed on a dry weight basis (g H2O • g dry weight^{-1}, or g H_2O • g^{-1} dw).

Seed germination and viability assessment

Before germination seeds were pre-humidified by incubation on moist filter paper (filter paper 595, Ø 85 mm, Schleicher & Schuell, Dassel, Germany) in 9 cm Petri dishes for 24 hours with 5 ml distilled water. Subsequently, seeds had the surface sterilised in 1% sodium hypochlorite for 10 minutes, rinsed with distilled water and imbibed in 5ml of distilled water. Germination was carried out with four replicates of 25 seeds. Seeds were placed in 9cm Petri dishes on two sheets of filter paper (Schleicher & Schuell 595). During imbibition, seeds were incubated at alternating temperature 20ºC/10ºC (day/night), with 8 hours light daily.

A seed was regarded as germinated when the radicle protruded 2mm through the seed coat. After 0, 2 and 10 days of imbibition, a sample of 50 seeds was taken for RNA extraction. These seeds were frozen in liquid nitrogen and stored at -70ºC. Germination experiments were carried out for 80 days. Non germinated seeds after this period were evaluated by a cut test in order to assess viability of seeds. Seeds were regarded as dead if mushy, liquid or rotten endosperm was present. On the other hand, if a firm and white endosperm and embryo were present, seeds were scored as viable seeds (Goslin, 2002; ISTA, 2005) and reported as "dormant" seeds.

Total RNA isolation

Total RNA was extracted from intact seeds (without the seed coat). For each treatment, 50 seeds were frozen in liquid nitrogen and stored at -70°C. Seeds were ground with a mortar and pestle in liquid nitrogen and added to a tube containing 1 ml of phenol:chloroform (5:1) plus TLE grinding buffer (0.18M Tris, 0.09M LiCl, 4.5mM EDTA, 1% SDS, adjusted to pH 8.2) and 5µL of β-mercaptoethanol that was added to the extraction buffer. After centrifugation, the upper phase was transferred to a new tube containing 1ml of

phenol-chloroform (1:1), centrifuged again and the supernatant was washed with chloroform to remove any residual phenol. RNA was precipitated overnight at -20°C in 0.1 volume 3 M sodium acetate and 2.5 volume of 100% ethanol, and then resuspended in 20 µL DEPC water. RNA quality was analyzed in 1.0% agarose gel electrophoresis, stained with ethidium bromide and quantified with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf, Hamburg, Germany).

Tab. 1. Treatments applied to *Talauma ovata* seeds in order to study changes in gene expression during desiccation and after imbibition.

Treatments	**Sample**
F0	Fresh seeds (control, 0.28 g $H_2O \cdot g^{-1}$ dw)
MD0	Mild dried seeds (0.25 g $H_2O \cdot g^{-1}$ dw)
D0	Dried seeds (0.10 g $H_2O \cdot g^{-1}$ dw)
F2	Fresh seeds after 2 days of imbibition
MD2	Mild dried seeds after 2 days of imbibition
D2	Dried seeds after 2 days of imbibition
F10	Fresh seeds after 10 days of imbibition
MD10	Mild dried seeds after 10 days of imbibition
D10	Dried seeds after 10 days of imbibition

Reverse transcriptase PCR

cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio Rad, Hercules, CA, USA), according to the manufacturer's protocol. Reactions were prepared by mixing the total RNA with iScript reaction mix and iScript reverse transcriptase and performed in a thermal cycler (iCycler, Bio Rad, Hercules, CA, USA), as follows: 5 min at 25°C; 30 min at 42°C and 5 min

at 85°C. A negative control for cDNA synthesis was also included, by omitting reverse transcriptase to the reaction.

Primer design

Degenerated primers were designed based on gene sequences available in database using the software Jellyfish version 3.2 (LabVelocity, Inc., Los Angeles, USA). Sequences amplified by PCR were cloned using pGemT vector (Promega, USA) following manufacturer's instructions and sequenced. Specific primers were designed with the software Vector NTI10 (Invitrogen Coorporation, USA), based on gene sequences obtained after cloning and sequencing of partial cDNA products.

Quantitative real-time PCR

Reactions were performed using an iCycler iQ instrument (Bio Rad, Hercules, CA, USA) with gene specific primers, cDNA and iQ SYBR green supermix (Bio Rad, Hercules, CA, USA). The amplification protocol consisted of 3 min at 95°C; then 40 cycles of 15s at 95°C followed by 1 min at 60°C. A primer for 18S gene was used as internal control to normalize the other products for analysis purposes. A negative control (negative cDNA) and a water control were used in every PCR plate. Real-time PCR evaluations were replicated four times for each treatment, deriving from 2 biological replicates repeated twice. Values of fold change in gene expression (mRNA) in relation to the control (fresh seeds) were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001), and plotted in graphs for comparison. Data were compared by Scott and Knott test ($p \leq 0.05$) (Scott & Knott, 1974).

Tab. 2. Genes studied during desiccation and imbibition of *Talauma ovata seeds*.

Gene Symbol	Gene name	Function
ABI3	Abscisic acid insensitive 3	Seed development
ACT2	Actin2	Cytoskeleton
CDC2-like	Cyclin-dependent kinase 2-like	Cell cycle
PKABA1	Abscisic acid induced kinase 1	Desiccation stress
2-CYS- PRX	2-Cys-Peroxiredoxin	Desiccation stress
sHSP17.5	Small heat-shock protein	Desiccation stress

Tab. 3. Specific primers used in the real-time PCR reactions.

Gene	Forward (sequences written 5' to 3')	Reverse (sequences written 5' to 3')
ABI3	GCTTCTTTCTTTGGCAACACAATCC	AACCGCCATGCAAACACAACAGC
ACT2	TGCTGTGATCTCCTTGNCTCATACG	GAAGCTGCTGGAATCCATGAGACC
CDC2-like	AGATGAGGGTGTTCCAAGCACAGC	CGTGGATTCTTGGCAAAATCTGG
PKABA1	GAGATTTAGCGAGGATGAGGCAAGG	GATTTTCAAGCGCGGAGCTGG
2-CYS- PRX	CCTACGCCGAAGAGTTCGAGAAGC	CCTTTTCGGGTCTGCTATTATCGG
sHSP17.5	AGGGCTGAAGAAAGAGGAAGTCAGG	TTTTCCATCGCTGCCTTCACG
18S	TGACGGAGAATTAGGGTTCG	CCTCCAATGGATCCTCGTTA

Results

Viability of *Talauma ovata* seeds after desiccation

The viability of fresh and mild dried *Talauma ovata* seeds was about 78%. However, the number of dormant seeds was reduced after a mild drying (0.25g H_2O • g^{-1} dw) (Tab 4). Drying seeds to water content below (0.10g H_2O • g^{-1} dw) led to a loss of viability, although some dormant seeds were detected at the end of the germination experiments.

Tab. 4. Effect of drying on germination and number of dormant seeds of *Talauma ovata*. Final germination was scored after 80 days of incubation at 20/10ºC. Lower case letters in the same column indicate significance of difference based on Scott-Knott test ($p \leq 0.05$).

Treatment	Drying time (hours)	Water content (g $H_2O \cdot g^{-1}$ dw)	Germination (%)	Dormant (%)
Fresh	0	0.28	51 ± 10.77 b	27 ± 2.85 a
Mild Dried	2	0.25	72 ± 8.03 a	7 ± 1.96 b
Dried	20	0.10	18 ± 7.71 c	13 ± 2.50 b

Expression of genes related to seed development, cell cycle and cytoskeleton during drying and imbibition of *Talauma ovata seeds*.

ABI3 and *ACT2* showed a similar expression pattern after desiccation (Fig 1 A and B). Abundance of both did not change after drying the seeds (MD0 and D0 treatments) compared to the control (fresh seeds).

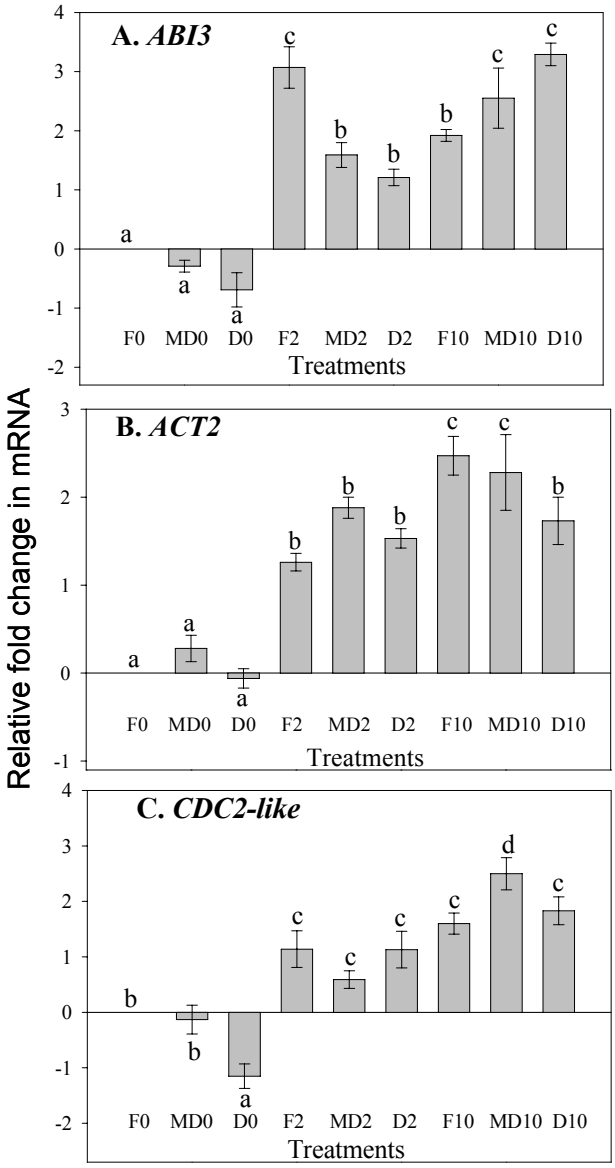

Fig. 1. Relative fold change in mRNA abundance of (A) *ABI3*, (B) *CDC2-like* and (C) *ACT2* in *Talauma ovata* seeds after drying to different water contents and subsequently imbibition, as compared to fresh seeds (F0). Data show the mean ± standard deviation of four replicates. Different lower case letters mean statistically significant difference based on Scott-Knott test ($p \leq 0.05$).

After imbibition, there was an increasing trend of the relative mRNA levels for both genes. *ABI3* expression was higher in fresh seeds imbibed for 2 days (F2) than in fresh seeds imbibed for 10 days (F10), whilst the relative levels of *ACT2* did not differ in dried seeds imbibed for 2 days (D2) and dried seeds imbibed for 10 days (D10). The relative abundance of *CDC2-like* in F0 and MD0 did not differ after drying (fig 1 C). It was down-regulated when seeds were dried to 0.10g $H_2O \bullet g^{-1}$ dw and up-regulated after the imbibition (2 and 10 days).

Expression of genes related to desiccation tolerance

The expression pattern of *PKABA1* and *sHSP17.5* showed the same behaviour after drying to different water contents. There was a trend in reduction in the expression after desiccation, but it did not differ statistically from fresh seeds (fig 2 A and C). The expression pattern of *PKABA1* was similar to *ABI3* expression (fig 1 A), showing no differences after drying and an increase in abundance after imbibition. In the same way, relative expression in fresh seeds imbibed for 2 days was higher than in those imbibed for 10 days. The relative levels of *sHSP17.5* increased with the progressing time of imbibition, with no differences between the drying treatments. After drying fresh seeds to 0.25g $H_2O \bullet g^{-1}$ dw (MD0) it was not detected differences in relative amounts of *2-Cys-PRX* mRNA (fig 2 B), however it was reduced after drying to 0.10g $H_2O \bullet g^{-1}$ dw (D0). There was no difference in mRNA levels before and after imbibition of fresh and mild dried seeds (F0, MD0, F2 and MD2), but it was reduced after 10 days of imbibition (F10 and MD10). In dried seeds, as mentioned above, the *2-Cys-PRX* levels were reduced. It remained at the same level after imbibition for 2 days (D2) and increased after imbibition for 10 days (D10) to the same level observed in fresh seeds (F0).

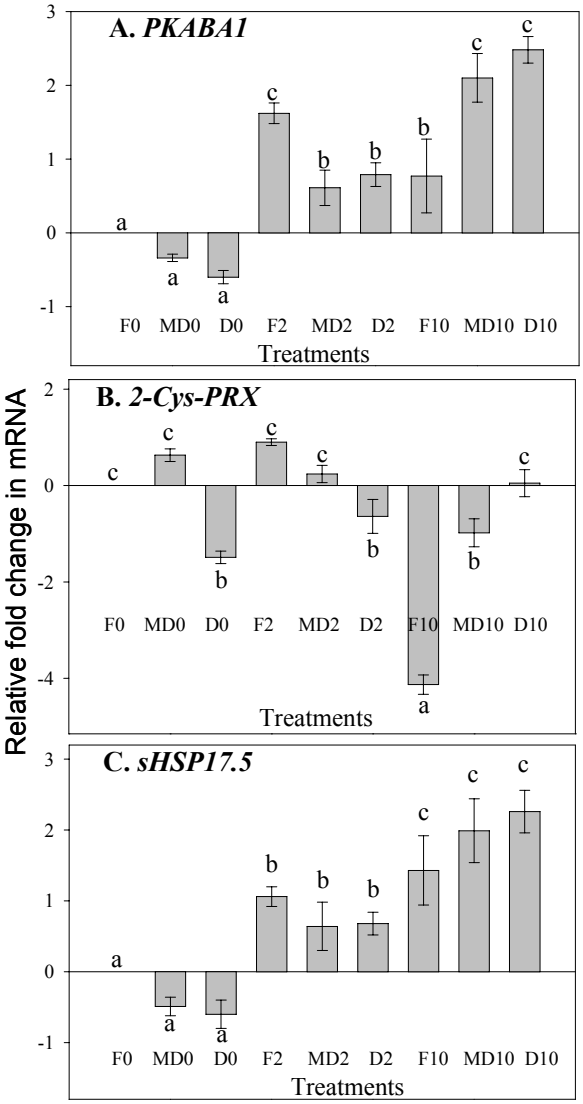

Fig. 2. Relative fold change in mRNA abundance of (A) *PKABA1*, (B) *2-Cys-PRX* and (C) *sHSP17.5* in *Talauma ovata* seeds after drying to different water contents and subsequently imbibition, as compared to fresh seeds (F0). Data show the mean ± standard deviation of four replicates. Different lower case letters mean statistically significant difference based on Scott-Knott test ($p \leq 0.05$).

Discussion

In orthodox seeds, the final maturation stage of the development is characterized by desiccation. Concomitantly, seeds of some species enter a state of dormancy, which allows survival under unfavorable environmental conditions and ensure dispersal (Ingram & Bartels, 1996). Molecular studies of seed maturation in model species reveal that it is driven mainly by three genes, ABA-Insensitive3 (*ABI3*), Fusca3 (*FUS3*), and Leafy Cotyledon1 (*LEC1*) (Parcy et al., 1997; Wobus & Weber, 1999). The expression pattern of *ABI3* in *Arabidopsis thaliana* seeds is characterized by a continuous increase throughout the development and transient expression during the first week of germination (Parcy et al., 1994). On the other hand, Bassel et al. (2006) studying *ABI3* expression in tomato (*Lycopersicon esculentum*) and Arabidopsis (*A. thaliana*) seeds found high expression of this gene during seed imbibition, before the completion of germination, but no expression in dry seeds. The results shows that the relative levels of *ABI3* transcripts remain constant during drying (Fig 1.A), and they increase in abundance after imbibition up to 10 days in all treatments. This suggests the participation of *ABI3* in events taking place during rehydration. It possible happens at different times according to the initial water content before imbibition. In fresh seeds higher levels of *ABI3* were found already 2 days after imbibition, while in mild-dried seeds and dried seeds, higher abundance was found at 10 days of imbibition. It is important to notice, however that even with high expression of *ABI3* in dried seeds after imbibition, the vast majority of the seeds did not germinate. These results are in accordance with studies developed by Faria et al. (2006) comparing changes in *ABI3* expression during the loss and re-establishment of desiccation tolerance in germinated seeds of *Medicago truncatula*. In that case, desiccation intolerant radicles (with 3mm length) showed a difference of 15-fold of *ABI3* levels compared with radicles

treated and untreated with polyethylene glycol (PEG 8.000). In both cases, after PEG treatment, dehydration led to loss of viability.

Actin2 expression did not change during drying, and showed an increase during imbibition. The abundance remained constant when comparing dried seeds imbibed for 2 and 10 days, with lower levels than those of fresh and mild dried seeds at 10 days of imbibition. It is partially in accordance with *ACT7* gene expression in Arabidopsis seeds, where no expression was detected in developing embryos, but it was high at seed maturity (after drying). This high expression persisted during imbibition of Arabidopsis seeds (McDowell et al., 1996).

In vegetative tissues, water deficit causes a decrease in the mitotic activity, which is attributed to the regulation of *CDC2* genes and other cyclin-dependent kinase (*CDK*) proteins (Schuppler et al., 1998). The authors propose that water-stress signal acts on *CDC2* by increasing phosphorylation of tyrosine, causing a shift to the inhibited form and slowing cell production. In *T. ovata* there was a reduction in the levels of *CDC2* mRNAs when seeds were dried to low water contents (D0). However, when seeds are imbibed, the transcripts levels increase even in dried seeds, which have low viability.

The expression of *ABI3*, *ACT2* and *CDC2* alone do not explain the germination behaviour of *T. ovata* seeds. It seems that the seeds, irrespective to the initial water content, perform in the same way during the initial period of germination and the deleterious effects of desiccation will take place latter, since the germination of *T. ovata* seeds is very slow, starting at 40 days after imbibition. These results suggest that seeds of this species may be not sensitive to desiccation to a water content of 0.10g H_2O • g^{-1} dw. Instead, seed may become sensitive to imbibitional stress, what was also observed in neem seeds (*Azadirachta indica*) (Sacandé, 2000).

PKABA1 is involved in a signaling pathway by which ABA suppresses the induction of gibberellin responsive genes in cereal aleurone layers (Gomez-Cadenas et al., 1999, 2001). Its expression is up-regulated by ABA, dehydration, cold, and osmotic stress in other plant tissues (Holappa & Walker-Simmons, 1995). In wheat seeds, during the development, the levels of *PKABA1* mRNA increase, but decrease during imbibition of nondormant seeds and transiently increase during imbibition of dormant seeds (Johnson et al., 2002). *PKABA1* was not up-regulated during drying of *T. ovata* seeds, but its expression was induced after imbibition, even in fresh seeds (F2 and F10, fig 2 A). It suggests the participation of some ABA-signaling process during the imbibition of *T. ovata* seeds. Seeds of some temperate recalcitrant species like *Aesculus hippocastanum* (Tompsett & Pritchard, 1993) and *Aesculus pseudoplatanus* (Hong & Ellis, 1990) have been reported to have dormancy at shedding. Fresh seeds of *T. ovata* showed a high number of dormant seeds after 80 days of imbibition (Tab. 4), what is in accordance with high expression of *PKABA1* at 2 and 10 days of imbibition.

PKABA1 and *sHSP17.5* are genes related to desiccation tolerance, but in the present study they did not show relationship with drying. The abundance of these genes remained constant during drying, but was up-regulated after imbibition. It does suggest the participation of these genes in protective mechanism during imbibition. The *ABI3* gene product is thought to activate expression of genes coding for specific small heat shock proteins during seed development (Rojas et al., 1999; Wehmeyer & Vierling, 2000). Comparing the expression pattern of *ABI3* (Fig 1 A) and *sHSP17.5* (Fig 2 C) a similar trend can be observed, with increasing expression during imbibition, what confirms, in part, the preposition of ABA signaling control of *PKABA1* and *sHSP17.5* expression.

Peroxiredoxins may protect barley embryos and aleurone cells against desiccation-induced free radical damage during late seed development and early imbibition (Stacy et al., 1996). The peroxiredoxin gene has previously been shown to be up-regulated during seed maturation and down-regulated during germination in Arabidopsis (Haslekås et al., 1998) and in *Brassica oleracea* (Soeda et al., 2005) and its expression is mediated by *ABI3* (Haslekås et al, 2003). In *B. oleracea*, expression of this gene differed between fast and slow-dried osmoprimed seeds. These results show no differences in gene expression between fresh (F0) and mild-dried seeds (MD0). However, its expression was down-regulated when seeds were dried to 0.10g $H_2O \bullet g^{-1}$ dw of water content Fig 2 B). This gene seems to have some role in fresh and mild dried seeds (F2 and MD2) only during the first days of germination, since the expression was up-regulated in F2 and MD2 treatments, while in dried seeds (D10) an increase in abundance of *2-Cys-PRX* mRNA happened only after 10 days of imbibition.

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