



## 'Desiccation tolerance of *Rhamnidium elaeocarpum* Reissek (Rhamnaceae) seeds

Lílian Abadia da Silva<sup>1\*</sup>, Juliana de Fátima Sales<sup>1</sup>, João Almir Oliveira<sup>2</sup>, Édila Vilela de Resende Von Pinho<sup>2</sup>, Heloisa Oliveira dos Santos<sup>2</sup> and Marcos Antônio Soares<sup>3</sup>

<sup>1</sup>Programa de Pós-graduação em Ciências Agrárias, Instituto Federal de Educação, Ciência e Tecnologia Goiano, Km 1, Cx. Postal 66, 75901-970, Câmpus Rio Verde, Rod. Sul Goiana, Rio Verde, Goiás, Brazil. <sup>2</sup>Departamento de Agricultura (Setor de Sementes), Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil. <sup>3</sup>Departamento de Botânica e Ecologia, Universidade Federal do Mato Grosso, Cuiabá, Mato Grosso, Brazil. \*Author for correspondence. E-mail: lilianabadia5@gmail.com

**ABSTRACT.** This study aimed to investigate the desiccation tolerance of *Rhamnidium elaeocarpum* Reissek seeds through physiological and biochemical alterations. Fruit were collected from the municipality of Jaupaci, Goiás State, Brazil, when the water content of their seeds had a 37% wet basis (w.b.) and the seeds were subsequently kept in a drying oven with air circulation at a temperature of 35°C until a water content was reached of 20 to 12% (w.b.). The following parameters were evaluated: germination, germination speed index, emergence, emergence speed index, electrical conductivity and respiration rate. Furthermore, the electrophoretic profile of the isoenzymes: catalase, peroxidase and esterase, the enzymatic activities of endo- $\beta$ -mannanase and  $\alpha$ -amylase and the electrophoretic analysis of heat-resistant proteins were performed. A higher germination speed and respiratory rate was obtained for seeds with a water content of 12% w.b., and the activity of catalase isoforms was more pronounced in seeds with a higher degree of dehydration, which was in contrast to peroxidase, showed decreased activity. The seeds contained heat-resistant proteins of low molecular weight that ranged from 48.7 to 13.2 kDa. We found that the acquisition of drying tolerance in *R. Elaeocarpum* Reissek seeds is associated more with catalase than with esterase and peroxidase.

**Keywords:** drying, germination, isoenzymes, respiration rate, heat-resistant proteins.

### Tolerância à dessecação em sementes de *Rhamnidium elaeocarpum* Reissek (Rhamnaceae)

**RESUMO.** O trabalho teve como objetivo investigar a tolerância a dessecação em sementes de *Rhamnidium elaeocarpum* Reissek por meio de alterações fisiológicas e bioquímicas. Os frutos foram coletados no município de Jaupaci, Estado de Goiás, quando as sementes apresentavam teor de água de 37% base úmida (b.u.), sendo em seguida mantidas em estufa de secagem com circulação de ar à temperatura de 35°C até alcançarem os teores de água de 20 e 12% (b.u.). Foram avaliados os seguintes parâmetros: germinação; índice de velocidade de germinação; emergência; índice de velocidade de emergência; condutividade elétrica; taxa respiratória; perfil eletroforético das isoenzimas: catalase, peroxidase e esterase; atividade enzimática de endo- $\beta$ -mananase e  $\alpha$ -amilase e análise eletroforética de proteínas resistentes ao calor. Maior velocidade de germinação e taxa respiratória foi obtida para sementes com teor de água de 12% b.u. A atividade das isoformas de catalase foi mais pronunciada em sementes com maior grau de desidratação em relação à peroxidase que diminuiu sua atividade. As sementes apresentaram proteínas de baixo peso molecular resistentes ao calor, variando de 48,7 a 13,2 kDa. A aquisição de tolerância à secagem em sementes de *R. elaeocarpum* Reissek é mais associada à enzima catalase do que às enzimas esterase e peroxidase.

**Palavras-chave:** secagem, germinação, isoenzimas, taxa respiratória, proteínas resistentes ao calor.

#### Introduction

Desiccation tolerance is a complex phenomenon that results from biochemical and cellular adjustments that allow cells to withstand considerable water loss with minimal tissue damage (MARCOS FILHO, 2005). Substantial changes in the structure and organisation of cells occur in the late stages of dry matter accumulation and maturation drying (BERJAK; PAMMENTER, 2008;

WALTERS; KOSTER, 2007). Upon the beginning of drying, various mechanisms of differential expression of genes are activated that culminate in the expression and activity of important enzymes that are used in the process of tolerance acquisition (BUTINK et al., 2006; RAMANJULU; BARTELS, 2002).

In orthodox seeds, the expression of these genes occurs during development as part of the maturation programme. As a result, embryos become

considerably tolerant to desiccation before maturation drying (HOEKSTRA et al., 2001). Considering that the acquisition of desiccation tolerance is regulated during seed development, and possible tolerance mechanisms accumulate only at precise moments after fertilisation, plant tissues must respond to environmental signals to activate protective mechanisms for the entire plant. It is, therefore, conceivable that environmental signals of extreme water loss can activate an existing repertoire of specific protection genes in seeds and plant tissues (ILLING et al., 2005).

The stresses that are associated with extreme water loss include mechanical stress with a loss of turgor, oxidative stress by generation of reactive oxygen species (ROS) and the destabilisation or loss of macromolecular integrity (WALTERS et al., 2002). In organisms that survive the removal of water, its cell constituents are protected or can be repaired by enzymatic and non-enzymatic mechanisms (WALTERS et al., 2001). Desiccation tolerance seems to be mediated by protective systems that prevent the lethal damage of different cellular components such as membranes, proteins and the cytoplasm. Three important systems have been characterised: the conversion of reducing sugars into non-reducing, long-chain sugars; the ability to prevent, tolerate or repair ROS attack by enzymatic and non-enzymatic mechanisms; and the induction of "late embryogenesis accumulated" (LEA) proteins and resistance to thermal shock (BERJAK; PAMMENTER, 2008; LI; SUN, 1999; KERMODE; FINCH-SAVAGE, 2002).

The loss of viability during the drying of various seeds susceptible to desiccation is accompanied by increased lipid peroxidation and the accumulation of free radicals. These free radicals can react with hydrogen peroxide to produce singlet oxygen and hydroxyl radicals (OH<sup>-</sup>) that are toxic to cells and can damage cellular constituents, such as proteins, DNA and membranes. Free radical removers accumulate because the systems do not function as effectively in dehydrated bodies (BOUDET et al., 2006). Desiccation tolerance may be related, at least in part, to the cell's ability to sequester reactive oxygen species and therefore avoid deleterious effects such as lipid peroxidation, which is caused by these reactive species (VERTUCCI; FARRANT, 1995). Therefore, antioxidant compounds and enzymes are of particular importance for seed development (GARNCZARSKA et al., 2009). The activity of antioxidant enzymes protects tissues against oxidative damage due to water loss in species that are tolerant to desiccation (ILLING et al.,

2005), and several reports have described the synergistic action of the simultaneous expression of free-radical remover enzymes on desiccation tolerance in seeds (KWON et al., 2002).

Changes in esterase enzyme patterns indicated that the occurrence of deteriorative events might contribute to a reduction in seed germination in addition to an adverse increase in the temperature and moisture content in the seeds because esterase is an enzyme involved in the hydrolysis of esters (SANTOS et al., 2004; SANTOS et al., 2005). According to Nkang et al. (2000), various enzyme systems process radicals, including superoxide dismutase (SOD), which catalyses the dismutation of superoxide (O<sub>2</sub><sup>-</sup>) to H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>, as well as enzymes involved in the detoxification of H<sub>2</sub>O<sub>2</sub> (catalase, glutathione reductase, ascorbate and other peroxidases).

A particular group of mRNAs and LEA proteins typically accumulate during the final stages of embryogenesis or in response to dehydration, low temperature, salinity or treatment with exogenous ABA, which indicates the capacity of the cell response to dehydration (RAMANJULU; BARTELS, 2002). LEA mRNA in embryonic tissues is expressed at the beginning of the desiccation process and becomes the most prevalent mRNA species in the desiccated state, gradually decreasing several hours after seed imbibition. LEA-type proteins are rich in glycine and other amino acids. They have few hydrophilic and hydrophobic residues when extracted under conditions of high temperature, and they exhibit no apparent catalytic activity (GOYAL et al., 2005).

The LEA proteins are generally able to protect other proteins or membranes, similar to the actions of sugars, acting as water replacement molecules (BOUDET et al., 2006). LEA proteins are widely distributed among species of monocots and dicots, and because of their amphipathic nature, these proteins are able to inhibit the denaturation of macromolecules and stabilise intracellular structures under stress conditions because they are associated with the desiccation tolerance of the seeds (BLACKMAN et al., 1995; CLOSE, 1997).

One of the most well-researched processes in the adaptation to stressful conditions is the induction of heat shock proteins (HSPs), which include several highly conserved protein families. The role of HSPs has been related to preserve and repair macromolecular structures during dehydration or rehydration, respectively. Although all organisms synthesise HSP in response to heat, the balance of the proteins synthesised and the relative importance

of the individual HSP families in terms of stress tolerance vary greatly among organisms (QUEITSCH et al., 2000).

The *Rhamnidium elaeocarpum* Reissek, which is popularly known as cafezinho or cafezinho-do-Cerrado, is a species that is widely distributed in Brazil and is found in nearly every state but is primarily found in riparian areas. The leaves and bark of this plant are popularly used in Brazil to reduce the itching of gums of children who are teething and for stomachaches. The methanolic extract of the leaves contains satisfactory amounts of flavonoids, alkaloids, saponins and tannins. Such substances can be responsible for the antiulcer activity that is displayed by this extract because these compounds have antioxidant activity (OLIVEIRA et al., 2010).

Because few studies have been conducted investigating the mechanisms of desiccation tolerance in seeds of native species, the present study aimed to investigate desiccation tolerance through enzymatic and biochemical patterns, changes in the electrophoretic pattern of heat-resistant proteins and the physiological quality of the seeds of *Rhamnidium elaeocarpum* Reissek after being subjected to drying.

## Material and methods

Ripe fruit were collected from the Padre Galileu farm, which was located in the municipality of Jaupaci, Goiás State, Brazil, from five matrices that were at least 100 m apart in December 2011. The study was conducted in the Laboratory of Seed Analysis of the Agriculture Department of the Federal University of Lavras (Minas Gerais State, Brazil). The process was manually performed and consisted of macerating the ripe fruit in a sieve (5 mm) under running water, separating the waste and placing the seeds under a paper towel until the excess water was eliminated. The moisture contents of four subsamples of 10 seeds were determined on a wet basis using an oven that was set at  $105 \pm 3^\circ\text{C}$  for 24 hours (BRASIL, 2009). The moisture contents of the seeds were expressed as percentages. After determining the initial water content, a portion of the seeds was wrapped in paper bags and left in the oven with controlled, forced-air circulation at a temperature of  $35^\circ\text{C}$ , and the mass was evaluated using a precision scale every hour until the mass corresponding to a water content of 20% to 12% of wet basis (w.b.) was obtained. The amount of water that was lost by the seeds during desiccation was determined by a formula that was recommended by the Rules for Seed Testing:

$$Pf = Pi \cdot \left( \frac{100 - T_{Ai}}{100 - T_{Af}} \right)$$

where:

Pf: final sample mass (g); Pi: initial sample mass (g); T<sub>Ai</sub>: initial water content of the seeds (% w.b.); and T<sub>Af</sub>: desired water content of seeds (% w.b.).

After drying, the seeds were placed in plastic bags and kept in a cold chamber (temperature of  $10^\circ\text{C}$  and 50-60% moisture) until analysis. The following parameters of the seeds were evaluated: germination (%), germination speed index, emergence (%), emergence speed index, electrical conductivity and respiration rate.

**Germination tests:** Seeding was performed on “germitest” paper sheets that were moistened with distilled water in amounts that were equivalent to 2.5-times the weight of the dry substrate (BRASIL, 2009), and four repetitions that included 50 seeds each were performed. The rolls, the paper towels, were kept in a germinator set at  $30^\circ\text{C}$ , and daily evaluations, where seeds with radicles  $\geq 0.5$  cm were recorded as having germinated, were performed until complete stabilisation occurred; the germination speed index (GSI) was calculated as reported by Maguire (1962). Only normal seedlings were considered for the germination percentage measurements (i.e., those that had all essential structures developed and lacked any damage).

**Emergence tests under controlled conditions:** For the emergence test, 200 seeds were used and divided into four replicates that each included 50 seeds. The seeding was performed in a plastic tray that measured 40 x 30 x 8 cm and contained a mixture of subsoil and sand in a proportion of 3:1 as a substrate. Seeds were placed 3cm deep into the substrate, and the substrate was watered until 70% of its field capacity was reached. The substrate was subsequently irrigated when necessary. The trays were maintained in a growth chamber (temperature of  $25^\circ\text{C}$ ) with alternating light and dark regimes (12 hours) for 30 days. Daily counts were performed to determine the emergence speed index (ESI), using emergence at 1.5 cm above the soil as the criterion for the emergence of seedlings according to the methods of Maguire (1962). The emergence percentage (%E) was evaluated at the end of 30 days, and only normal seedlings were analysed (i.e., those that had perfectly formed essential structures and lacked any damage).

**Electrical conductivity test:** The electrical conductivity test was performed on four subsamples of 50 seeds each that had been previously weighed on a scale with 0.001 g precision. Next, the seeds were placed in plastic cups that contained 75 mL of

deionised water and were kept in the BOD chamber at 25°C for 24 hours. After this period, the electrical conductivity was determined using a conductivity meter bench, Digimed equipment, model CD21A, and the results were expressed in  $\mu\text{S cm}^{-1} \text{g}^{-1}$ .

**Respiratory rate:** The respiratory rate was determined using a titration method according to methodology that was described by Crispim et al. (1994). Clear plastic boxes, gerbox-type, that contained fine screen trays that were used as support for the seeds were used. Four subsamples of 50 seeds each were used and weighed to obtain the dry weight of the seeds. At the bottom of each gerbox, 40 mL of a 0.1 N KOH solution was added, and two sheets of blotting paper were placed on the screen and moistened to 2.5 times their dry weight. The seeds were subsequently distributed on the screen to form an even layer. For each sample, a control was prepared without seeds and was referred to as the "blank". Next, the gerboxes were sealed with plastic film to avoid gas exchange and kept in a BOD-type chamber, growth for 24 hours at a constant temperature of 25°C. After this period, 25 mL of the 0.1 N KOH solution was removed from each box, and three drops of phenolphthalein were added to titrate with 0.1 N HCl. At the turning point, the volume of HCl that was used to neutralise the KOH solution in each of the tested samples was recorded. The final calculation of the respiration rate was carried out using the following equation:

$$\text{RR} = \frac{(\text{B} - \text{L}) \cdot \text{C}}{\text{MS}}$$

where:

B: was the blank reading; L: was the titration with the HCl reading; C: was the constant, denominated correction factor that had a value of 3.52, and MS: was the dry matter of the seeds that were subjected to evaluations. The results were expressed as mg of  $\text{CO}_2$  per gram of dry seed.

The following biochemical and molecular variables of the seeds were analysed: the enzymatic profiles of catalase, esterase, peroxidase and  $\alpha$ -amylase were evaluated; and the enzymatic activity of endo- $\beta$ -mannanase and the electrophoretic profile of heat-resistant proteins were determined.

**Determination of the isoforms of the  $\alpha$ -amylase enzyme:** The electrophoretic profile of  $\alpha$ -amylase was determined using a non-denaturing polyacrylamide gel in a discontinuous system. The seeds were subjected to the germination test for 48 hours. After this period, the seedlings were discarded, the reserve tissue was crushed in a crucible (on ice) in the presence of liquid nitrogen,

and the samples were stored in an ultrafreezer at -80°C until analysis. The enzyme extraction was processed by adding 200  $\mu\text{L}$  of extraction buffer (Tris-HCl, 0.2 mol  $\text{L}^{-1}$ , pH 8.0) to 100 mg of samples from each treatment. The homogenates were maintained for 12 hours in a refrigerator at a temperature of 5°C and centrifuged at 16,000  $\text{xg}$  at 4°C for 30 minutes, and 40- $\mu\text{L}$  volumes of the extracts were applied to the discontinuous system of polyacrylamide gels [4.5% (stacking gel) and 7.5% (separating gel - 5% soluble starch)]. The gel/electrode buffer was Tris-glycine, pH 8.9, and the electrophoretic run was performed at 4°C for four hours and at a constant voltage of 150V. The activity bands of the  $\alpha$ -amylase enzyme were developed according to Alfenas (1998), and after treatment with iodine, we evaluated the presence or absence of clear bands on a bluish background (negative developing) that resulted from the reaction with amylose and the relative intensity of the isoforms.

**Determination of the isoforms of free-radical remover enzymes:** The seeds were crushed in the presence of the antioxidant PVP40 (polyvinylpyrrolidone) and immediately stored at -80°C until analysis. The material from each sample (100 mg) was placed into microtubules with 2.5-times its weight of extraction buffer (200 mM Tris-HCl, pH 8 and 0.001%  $\beta$ -mercaptoethanol). The samples from each treatment were refrigerated overnight and were centrifuged at 16,000 $\text{xg}$  for 30 minutes at 4°C. Subsequently, 60  $\mu\text{L}$  of the supernatant from each sample was applied to the discontinuous system of polyacrylamide gels [4.5% (stacking gel) and 7.5% (separating gel)]. The gel/electrode system buffer was Tris-glycine, pH 8.9, and the electrophoretic run was conducted at 4°C for four hours at a constant voltage of 150V. After the electrophoretic run, the gels were developed using the esterase and catalase enzyme system according to Alfenas (1998) and for peroxidase according to Tanksley and Orton (1983). After development, the gels were evaluated using a transilluminator, and the presence or absence of bands and the relative intensity of the different isoforms for each sample was determined.

**Activity of the endo- $\beta$ -mannanase enzyme:** To extract endo- $\beta$ -mannanase, the seeds from each treatment were pre-soaked in germitest paper that had been moistened with an amount of distilled water that was equivalent to 2.5-times the dry substrate weight of the seeds for 24h. The seeds were subsequently macerated in liquid nitrogen and PVP40, 200 mg of the seed extract from each treatment was weighed and 600  $\mu\text{L}$  of the extraction

buffer [100 mM Hepes, 500 mM NaCl and 0.5% ascorbic acid, pH8.0] was added. In the next step, the samples were centrifuged for 30 minutes at 10000 rpm, and 20  $\mu$ L of the supernatant was applied to a gel containing 6 mL of LBG (*locust bean gum*), 0.24 g of agarose and 24 mL of pH 5.0 buffer (1M Citric Acid/0.4M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ). The aliquots were applied into 2-mm holes that were made in the gel with the aid of a punch. To calculate the enzymatic activity, the activity of each sample was compared to a standard curve that was generated using the commercial endo- $\beta$ -mannanase of *Aspergillus niger* (Megazyme). The calculation of the enzymatic activity of endo- $\beta$ -mannanase was performed according to Downie et al. (1994), and the experiment was conducted following a completely randomised design that consisted of three repetitions.

**Electrophoretic profile of heat-resistant proteins:** The seeds were crushed in a crucible in the presence of liquid nitrogen, and an extraction buffer was subsequently added in the proportion of 10 parts of buffer per 1 part of crushed seeds as described by Alfenas (1998). The samples were centrifuged at 16,000 xg for 30 minutes at 4°C, and the supernatant was separated and incubated in a water bath at 85°C for 10 minutes and centrifuged again. Before applying the samples to a denaturing polyacrylamide gel, 40  $\mu$ L of sample buffer (ALFENAS, 1998) was added in 70  $\mu$ L of each extract, and this solution was incubated in a boiling water bath for five minutes. Subsequently, 50  $\mu$ L of each sample was applied into a denaturing and discontinuous polyacrylamide gel that consisted of stacking (6%) and separating (12.5%) gels. The run buffer was Tris-glycine+SDS, pH8.9, and the electrophoretic run was conducted using a vertical system at room temperature and with a constant voltage of 150V for four hours. To determine the molecular mass of the polypeptide chains, a mass marker was used that ranged from 10 to 220 KDa (Invitrogen®). After the run, the gels were stained using a 0.05% Coomassie Brilliant Blue solution for 24 hours and destained in a solution of ethanol: acetic acid: water [0.5:1:8.5 (v:v)] according to Alfenas (1998). The gels were evaluated using a transilluminator to determine the presence or absence of bands.

**Statistical design:** The experimental design for the germination and vigour measurements was completely randomised, with four replications for each of the three defined water levels. The data were interpreted statistically through variance analysis, and the means were compared using Tukey's test at 5% probability with the aid of Sisvar (System of

Variance Analysis) software for Windows (FERREIRA, 2000). The data that were expressed as percentages were transformed into the arcsine of the square root of  $x/100$ , where  $x$  was the percentage.

## Results and discussion

Table 1 describes the mean values of the germination speed index (GSI), percentage of germination (% G), emergence speed index (ESI) and percentage of emergence (% E) of *Rhazniidiiim elaeocarpum* Reissek seeds that were subjected to drying and had different water contents.

**Table 1.** Comparison of the mean values for the germination speed index (GSI); percentage of germination (G %); emergence speed index (ESI); and percentage of emergence (E %) in *Rhazniidiiim elaeocarpum* Reissek seeds with different water contents.

Water content (% w.b.)	GSI	G (%)	ESI	E (%)
37	10.29 c*	80.50 a	2.67 a	94.55 a
20	11.81 b	81.00 a	2.97 a	93.95 a
12	12.68 a	82.00 a	3.13 a	90.06 a
CV (%)	3.41	4.63	9.40	2.64

\*Means followed by the same letter in the column do not differ at a significance level of 5% probability by the Tukey's test.

Greater germination speed was obtained for the seeds that were subjected to drying and had a water content of 12% w.b., and no differences among the water contents of the other variables were observed. For *Coffea canephora* seeds, it was observed that as the water content decreased, a decrease occurred in seed germination and vigour values (ROSA et al., 2005b). Drying of *Magnolia ovata* seeds to a water content of below 0.10  $\text{g}\cdot\text{g}^{-1}$  led to the near complete loss of viability, although a portion of the seeds were dormant at the end of the germination test (JOSE et al., 2009). For *Anemone nemorosa* L. seeds, the germination was significantly greater for non-dried seeds than seeds that were subjected to drying during each sampling period (ALI et al., 2007).

Table 2 shows the mean values for the electrical conductivities and respiration rates of dried *Rhazniidiiim elaeocarpum* seeds.

**Table 2.** Comparison between the mean values for electrical conductivity (EC) and respiration rate (RR) of *Rhazniidiiim elaeocarpum* Reissek seeds with different water contents.

Water content (% w.b.)	EC ( $\mu\text{S cm}^{-1} \text{g}^{-1}$ )	RR (mg $\text{CO}_2/\text{g M.S.}$ )
37	3.48 a*	5.64 b
20	4.00 a	6.36 a
12	4.17 a	6.37 a
CV (%)	13.71	5.81

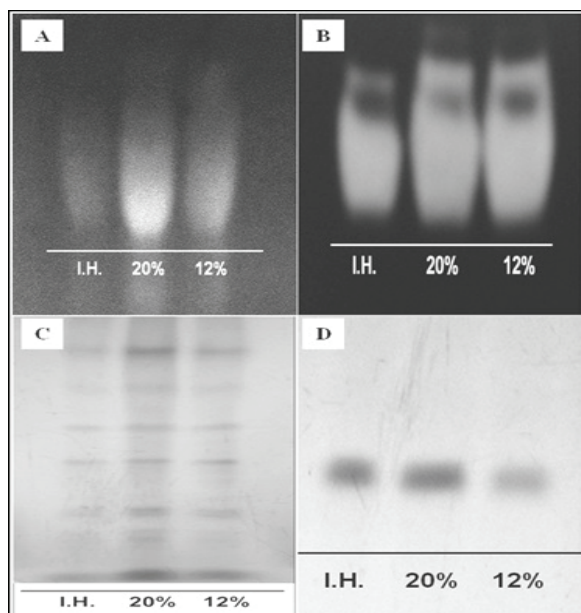
\*Means followed by the same letter in the column do not differ at a significance level of 5% probability by the Tukey's test.

No difference between the electrical conductivities of seeds with differing water contents was evident. However, lower electrical conductivity

values were verified in soybean seeds that were not subjected to drying. The seeds that were subjected to drying showed a decrease in the values of electrical conductivity, which indicated that the seeds that were harvested with higher water content were less tolerant of desiccation (VEIGA et al., 2007).

Dried seeds showed higher respiration rate values than non-dried seeds. Thus, an increase in the CO<sub>2</sub> that was released during the soaking process occurred with decreasing moisture content of the seeds of up to 12% (w.b.), which indicated a lower degree of deterioration in relation to the other water contents. The activity and integrity of the mitochondria of viable embryos increased after soaking. Therefore, ATP production and oxygen consumption were increased, and the production of carbon dioxide consequently was elevated (BEWLEY; BLACK, 1994). In lupine seeds, the respiratory rate decreased during desiccation of mature seeds, and this value represented only 10% of the value that was obtained for early embryos (GARNCZARSKA et al., 2009).

The present study evaluated the electrophoretic patterns of  $\alpha$ -amylase, catalase, esterase and peroxidase in *Rhannidium elaeocarpum* Reissek seeds that had been dried at 35°C and had different water contents (see Figure 1).



**Figure 1.** Electrophoretic patterns of different enzymes in *Rhannidium elaeocarpum* Reissek seeds that were subjected to drying at 35°C and with different water contents. A –  $\alpha$ -amylase; B – catalase; C – esterase; D – peroxidase. (I.H. – initial humidity - 37% w.b.; 20% w.b.; 12% w.b.). Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavras, Minas Gerais State, 2012.

Enzymatic activities of all evaluated enzymes were detected in non-denaturing polyacrylamide gels. In the evaluated conditions, the seeds of *R. elaeocarpum* presented one, two, six and one isoform (s) of  $\alpha$ -amylase, catalase, esterase and peroxidase, respectively (Figure 1). The determination of an electrophoretic profile for an isoenzyme is a useful tool for estimating and elucidating the genetic variability structure in natural populations and to determine the gene flow between species and hybridisation processes in different biological models (FERREIRA; GRATAPAGLIA, 1995). From an agricultural point of view, evaluation of the isoform pattern contributes to the determination of genetic linkage, identification of cultivars and indirect selection for traits of interest (TANKSLEY; ORTON, 1983).

In addition to these applications, the visualisation of enzymatic isoforms enables one to correlate the effects of biotic and abiotic factors to the enzyme expression profile and can be controlled at different times of gene expression (i.e., pre- or post-transcriptional and translational control).

Reducing the moisture in the seeds did not change the profiles of the  $\alpha$ -amylase, catalase, esterase or peroxidase isoenzymes. This result is indicative of the importance of the evaluated isoforms in the entire process of water loss by *R. elaeocarpum* seeds.

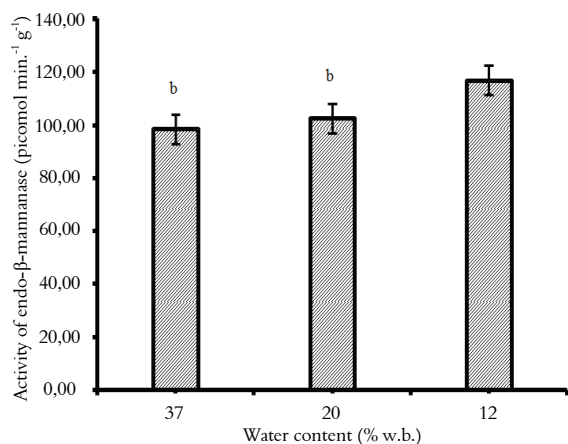
Dehydration of plant tissues is associated with increased ROS generation, which synergistically results in differential expression of genes and causes numerous deleterious effects (LEPRINCE et al., 1993; VERTUCCI; FARRANT, 1995). The main ROS detoxification system involves the assembly of superoxide dismutase, catalase enzymes and glutathione ascorbate cycle enzymes (SCANDALIOS, 1997).

Interestingly, evaluation of the relative intensities of the catalase and peroxidase isoforms in *R. elaeocarpum* Reissek seeds indicates a more effective role of the former as the moisture content of the seeds approaches 12%. The decrease in intensity of the only peroxidase isoform is clearly noticed (Figure 1D) compared to the increase in the intensity of the two catalase isoforms (Figure 1B). Dehydration of seeds is an abiotic stress that results in cell damage and can generate reactive oxygen species, such as hydrogen peroxide. Catalase and peroxidase converts hydrogen peroxide into compounds that are non-toxic to cells. The efficiency of the enzymatic, detoxification activity of the reactive species, especially of catalase, increases during seed development and is closely linked to the moisture content in sunflower seeds (BAILLY et al.,

2004). In contrast, the activities of peroxidase and glutathione reductase decreased during desiccation of *Triticum durum* seeds (DE GARA et al., 2003).

A similar result was found by Rosa et al. (2005a) while studying desiccation tolerance in maize seeds. In that case, catalase activity was increased in seeds that were tolerant to drying at 50°C, were submitted to periods of preconditioning that were equal or greater than 24 hours and exhibited polymorphisms. In lupine seeds, catalase activity was increased in the embryos of dry, mature seeds compared to that observed in young embryos (GARNCZARSKA et al., 2009).

Figure 2 indicates that an increased activity of endo- $\beta$ -mannanase is observed that correlates to a greater degree of dehydration of the seeds. Endo-enzymes have been identified as important for germination (before radicle protrusion) and in the post-germination events of mobilisation of the reserves (after radicle protrusion) in the endosperm cell walls (DIRK et al., 1995).

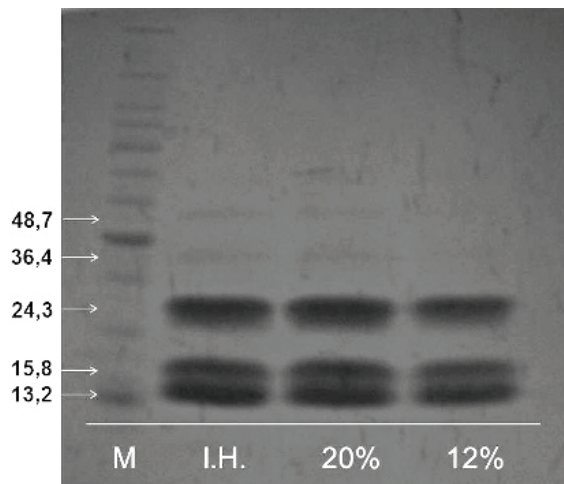


**Figure 2.** Activity of endo- $\beta$ -mannanase in *Rhannidium elaeocarpum* Reissek seeds that were dried at 35°C and had different water contents. (37% w.b.; 20% w.b.; 12% w.b.).\*Means followed by the same letter in the column do not differ at a significance level of 5% probability by the Tukey's test. Bar: Mean standard error. Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavra, Minas Gerais State, 2012.

Plant cells, when under different biotic or abiotic stresses, contain a set of proteins that are resistant to thermal shock and have different functions such as aiding in obtaining the native conformation of other proteins, i.e., enzymes. A particular group of these proteins, known as small heat shock proteins (sHsp), is quite diverse in plants, is located within different regions and cellular organelles and is synthesised during various stages of plant development. The expression of these proteins can be induced by different agents, such as oxidative stress, low temperature, heavy metals or radiation.

Furthermore, these proteins' expression has suggested that the roles of these proteins are important in different stressful conditions and in thermal stress (MORROW; TANGUAY, 2012).

The results of the electrophoretic analysis of heat-resistant proteins in *R. elaeocarpum* Reissek seeds that were dried and had different water contents are shown in Figure 3.



**Figure 3.** Electrophoretic patterns of heat-resistant proteins in *Rhannidium elaeocarpum* Reissek seeds that were dried at 35°C and had different water contents. (M – marker; I.H. – initial humidity - 37% w.b.; 20% w.b.; 12% w.b.). Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavras, Minas Gerais State, 2012.

The electrophoretic profile of these proteins demonstrated the presence of five polypeptide chains that ranged from 48.7 to 13.2 kDa in seeds with different water contents. The chains with lower molecular weights (24.3, 15.8 and 13.2 kDa) were the most abundant in seeds that were evaluated during the different treatments.

A low molecular weight protein of 22 kDa that was resistant to heat shock (sHsp) was found in *Fagus sylvatica* L. seeds, and its concentration was proportional to the storage time of those seeds (KALEMBA; PUKACKA, 2008). In contrast, soybean seeds that were dried in the field and harvested with a 13% water content had more intense bands or a greater number of heat-resistant proteins, which indicated that drying induced the synthesis of these proteins in seeds that were harvested with high water contents (VEIGA et al., 2007). Several mechanisms have been associated with the maintenance of desiccation tolerance, which confers protection against the consequences of water loss. Although seeds can accumulate heat-resistant proteins during their development, their presence alone does not seem to be responsible for

tolerance to high-temperature drying; therefore, other factors may influence this trait (JOSE et al., 2005). The functional role of heat-resistant proteins in *R. elaeocarpum* Reissek seeds must be investigated.

## Conclusion

Drying *R. elaeocarpum* Reissek seeds to a 12% water content enhanced germination, the activity of endo- $\beta$ -mannanase and development of seedlings. Those seeds exhibited  $\alpha$ -amylase, catalase, esterase and peroxidase activity because of the presence of different isoforms of those enzymes. The acquisition of desiccation tolerance by *R. elaeocarpum* Reissek seeds may be associated with catalase and the presence of low-molecular-weight, heat-resistant proteins, especially the 24.3, 15.8 and 13.2KDa polypeptide chains.

## Acknowledgements

The authors acknowledge the financial support of CNPq-CAPES and FAPEG for granting the scholarship of the first author.

## References

- ALFENAS, A. C. **Eletrforese de isoenzimas e proteínas afins**: fundamentos e aplicações em plantas e microrganismos. Viçosa: UFV, 1998.
- ALI, A.; PROBERT, R.; HAY, F.; DAVIES, H.; STUPPY, W. Post-dispersal embryo growth and acquisition of desiccation tolerance in *Anemone nemorosa* L. seeds. **Seed Science Research**, v. 17, n. 3, p. 155-163, 2007.
- BAILLY, C.; LEYMARIE, J.; LEHNER, A.; ROUSSEAU, S.; COME, D.; CORBINEAU, F. Catalase activity and expression in developing sunflower seeds as related to drying. **Journal of Experimental Botany**, v. 55, n. 396, p. 475-483, 2004.
- BERJAK, P.; PAMMENTER, N. W. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. **Annals of Botany**, v. 101, n. 2, p. 213-228, 2008.
- BEWLEY, J. D.; BLACK, M. **Seeds**: physiology of development and germination. 2nd ed. New York; London: Plenum Press, 1994.
- BLACKMAN, S. A.; OBENDORF, R. L.; LEOPOLD, A. C. Desiccation tolerance in developing soybean seeds: the role of stress proteins. **Physiologia Plantarum**, v. 93, n. 4, p. 630-638, 1995.
- BOUDET, J.; BUITINK, J.; HOEKSTRA, F. A.; ROGNAUX, H.; LARRE, C.; SATOUR, P.; LEPRINCE, O. Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. **Plant Physiology**, v. 140, n. 4, p. 1418-1436, 2006.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. **Regras para análise de sementes**. Brasília: Secretaria de Defesa Agropecuária; Mapa/ACS, 2009.

BUITINK, J.; LEGER, J. L.; GUISELE, I.; VU, B. L.; WUILLEME, S.; LAMIRAULT, G.; LE BARS, A.; LE MEUR, N.; BECKER, A.; KÜSTER, K.; LEPRINCE, O. Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seeds. **The Plant Journal**, v. 47, n. 5, p. 735-750, 2006.

CLOSE, T. J. Dehydrins: A commonality in the response of plants to dehydration and low temperature. **Physiologia Plantarum**, v. 100, n. 2, p. 291-296, 1997.

CRISPIM, J. E.; MARTINS, J. C.; PIRES, J. C.; ROSOLEM, C. A.; CAVARIANI, C. Determinação da taxa de respiração em sementes de soja pelo método da titulação. **Pesquisa Agropecuária Brasileira**, v. 29, n. 10, p. 1517-1521, 1994.

DE GARA, L.; PINTO, M. C.; MOLITERNI, V. M. C.; D'EGIDIO, M. G. Redox regulation and storage processes during maturation in kernels of *Triticum durum*. **Journal of Experimental Botany**, v. 54, n. 381, p. 249-258, 2003.

DIRK, L. M. A.; GRIFFEN, A. M.; DWNIE, B.; BEWLEY, J. D. Multiple isozymes of endo- $\beta$ -mannanase in dry and imbibed seeds. **Phytochemistry**, v. 40, n. 4, p. 1045-1056, 1995.

DOWNIE, B.; HILLHORST, H. W. M.; BEWLEY, J. D. A new assay for quantifying endo- $\beta$ -mannanase activity using congo red dye. **Phytochemistry**, v. 36, n. 1, p. 829-835, 1994.

FERREIRA, D. F. **Sistema de análise estatística - SISVAR**. Lavras: UFLA, 2000.

FERREIRA, M. E.; GRATTAPAGLIA, D. **Introdução ao uso de marcadores RAPD e RFLP em análise genética**. Brasília: Embrapa-Cenargen, 1995.

GARNCZARSKA, M.; BEDNARSKI, W.; JANCELEWICZ, M. Ability of lupine seeds to germinate and to tolerate desiccation as related to changes in free radical level and antioxidants in freshly harvested seeds. **Plant Physiology and Biochemistry**, v. 47, n. 1, p. 56-62, 2009.

GOYAL, K.; WALTON, L. J.; TUNNAcliffe, A. LEA proteins prevent protein aggregation due to water stress. **Biochemical Journal**, v. 388, n. 15, p. 151-157, 2005.

HOEKSTRA, F. A.; GOLOVINA, E. A.; BUITINK, J. Mechanisms of plant desiccation tolerance. **Trends in Plant Science**, v. 6, n. 9, p. 431-438, 2001.

ILLING, N.; DENBY, J. K.; COLLETT, H.; SHEN, A.; FARRANT, J. M. The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. **Integrative and Comparative Biology**, v. 45, n. 5, p. 771-787, 2005.

JOSE, A. C.; LIGTERINK, W.; DAVIDE, A. C.; SILVA, E. A. A.; HILHORST, H. W. M. Changes in gene expression during drying and imbibition of desiccation



- sensitive *Magnolia ovata* (A. St.-Hil.) spreng. **Revista Brasileira de Sementes**, v. 31, n. 1, p. 270-280, 2009.
- JOSE, S. C. B. R.; PINHO, E. V. R. V.; PINHO, R. G. V.; SILVEIRA, C. M. Padrão eletroforético de proteínas resistentes ao calor em sementes de milho. **Pesquisa Agropecuária Brasileira**, v. 40, n. 2, p. 115-121, 2005.
- KALEMBA, E. M.; PUKACKA, S. Changes in late embryogenesis abundant proteins and small heat shock protein during storage of beech (*Fagus sylvatica* L.) seeds. **Environmental and Experimental Botany**, v. 63, n. 1-3, p. 274-280, 2008.
- KERMODE, A. R.; FINCH-SAVAGE, W. E. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. In: BLACK, M.; PRITCHARD, H. W. (Ed.). **Desiccation and survival in plants: drying without dying**. Wallingford: CABI Publishing, 2002. p. 149-184.
- KWON, S. Y.; JEONG, Y. J.; LEE, H. S.; KIM, J. S.; CHO, K. Y.; ALLEN, R. D.; KWAK, S. S. Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. **Plant, Cell and Environment**, v. 25, n. 7, p. 873-882, 2002.
- LEPRINCE, O.; HENDRY, G. A. F.; MCKERSIE, B. D. The mechanisms of desiccation tolerance in developing seeds. **Seed Science Research**, v. 3, n. 4, p. 231-246, 1993.
- LI, C.; SUN, W. Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. **Seed Science Research**, v. 9, n. 3, p. 209-217, 1999.
- MAGUIRE, J. D. Speed of germination aid in selection and evaluation for seedling emergence and vigor. **Crop Science**, v. 2, n. 2, p. 176-177, 1962.
- MARCOS FILHO, J. **Fisiologia de sementes de plantas cultivadas**. Piracicaba: Fealq, 2005.
- MORROW, G.; TANGUAY, R. M. Small heat shock protein expression and functions during development. **International Journal of Biochemistry e Cell Biology**, v. 44, n. 10, p. 1613-1621, 2012.
- NKANG, A.; OMOKARO, D.; EGBE, A. Effects of desiccation on the lipid peroxidation and activities of peroxidase and polyphenoloxidase in seeds of *Telfairia occidentalis*. **Seed Science and Technology**, v. 28, n. 1, p. 1-9, 2000.
- OLIVEIRA, T. B.; SOARES, R. L.; TRESVESOL, L. M. F.; BRANQUINHO, D. S. P.; BARA, M. T. F.; FIUZA, T. S.; REZENDE, M. H.; PAULA, J. R. Caracterização das folhas e cascas de *Rhamnidium elaeocarpum* Reissek. **Latin American Journal of Pharmacy**, v. 29, n. 4, p. 534-541, 2010.
- QUEITSCH, C.; HONG, S. W.; VIERLING, E.; LINDQUIST, S. Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. **The Plant Cell**, v. 12, n. 4, p. 479-492, 2000.
- RAMANJULU, S.; BARTELS, D. Drought-and desiccation-induced modulation of gene expression in plants. **Plant Cell and Environment**, v. 25, n. 2, p. 141-151, 2002.
- ROSA, S. D. V. F.; PINHO, E. V. R. V.; VIEIRA, E. S. N.; VEIGA, R. D.; VEIGA, A. D. Enzimas removedoras de radicais livres e proteínas *lea* associadas à tolerância de sementes de milho à alta temperatura de secagem. **Revista Brasileira de Sementes**, v. 27, n. 2, p. 91-101, 2005a.
- ROSA, S. D. V. F.; JUNIOR, D. S. B.; PINHO, E. V. R. V.; VEIGA, A. D.; SILVA, L. H. C. Effects of different drying rates on the physiological quality of *Coffea canephora* Pierre seeds. **Brazilian Journal of Plant Physiology**, v. 17, n. 2, p. 199-205, 2005b.
- SANTOS, C. M. R.; MENEZES, N. L.; VILLELA, F. V. Alterações fisiológicas e bioquímicas em sementes de feijão envelhecidas artificialmente. **Revista Brasileira de Sementes**, v. 26, n. 1, p. 110-119, 2004.
- SANTOS, C. M. R.; MENEZES, N. L.; VILLELA, F. A. Modificações fisiológicas e bioquímicas em sementes de feijão no armazenamento. **Revista Brasileira de Sementes**, v. 27, n. 1, p. 104-114, 2005.
- SCANDALIOS, J. G. **Oxidative stress and the molecular biology of antioxidant defenses**. New York: Cold Spring Harbor Laboratory Press, 1997.
- TANKSLEY, S. D.; ORTON, T. J. **Isozymes: developments in plant genetic and breeding**. New York: Elsevier, 1983. (Parte A (IA))
- VEIGA, A. D.; ROSA, S. D. V. F.; SILVA, P. A.; OLIVEIRA, J. A.; ALVIM, P. O.; DINIZ, K. A. Tolerância de sementes de soja à dessecação. **Ciência e Agrotecnologia**, v. 31, n. 3, p. 773-780, 2007.
- VERTUCCI, C. W.; FARRANT, J. M. Acquisition and loss of desiccation tolerance. In: KIGEL, J.; GALILI, G. (Ed.). **Seed development and germination**. New York: Marcel Dekker, 1995. p. 237-271.
- WALTERS, C.; FARRANT, J. M.; PAMMENTER, N. W.; BERJAK, P. Desiccation and Damage. In: BLACK, M.; PRITCHARD, H. W. (Ed.). **Desiccation and survival in plants**. Wallingford: CABI Publishing Drying without dying, 2002. p. 263-291.
- WALTERS, C.; KOSTER, K. L. Structural dynamics and desiccation damage in plant reproductive organs. In: JENKS, M. A.; WOOD, A. J. (Ed.). **Plant desiccation stress**. Ames: Blackwell Publishing, 2007. p. 251-282.
- WALTERS, C.; PAMMENTER, N. W.; BERJAK, P.; CRANE, J. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. **Seed Science Research**, v. 11, n. 2, p. 135-148, 2001.

Received on December 12, 2012.

Accepted on April 6, 2013.

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.