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# Physiological and biochemical changes during desiccation tolerance loss in millet (*Pennisetum glaucum* L.) seeds

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**ABSTRACT.** The aim of this study was to evaluate the physiological and biochemical changes related to desiccation tolerance loss in millet seeds. The studied points of the germination process were determined according to the seed imbibition curve of the millet hybrid ADRF6010: control (0h), 3h of imbibition, 1 and 3 mm radicle. The seeds were dried on silica gel for 72h at 20°C, followed by pre-humidification at 25°C for 24h. Seed physiological quality was evaluated by electrical conductivity and a germination test, and seed vigor was evaluated with a first germination count and a germination speed index. The experiment was performed in a completely randomized design, and means were compared by the Scott-Knott test at a 5% probability. The enzymatic systems of superoxide dismutase, catalase, peroxidase, and α-amylase, as well as the expression of heat-resistant proteins were evaluated. Enzymatic activity was quantified with the ImageJ® software. Millet seeds lost desiccation tolerance when the radicle reached 1 mm in length. According to enzymatic standards, peroxidase and α-amylase activity, as well as heat-resistant protein activity, were related to desiccation tolerance loss in millet seeds.
**Keywords:** desiccation sensitivity; germination; proteins; enzymes.

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## Introduction

Desiccation tolerance is the ability of some organisms to survive extreme water loss, at levels below 0.1 g $H_2O$ $g^{-1}$ of dry tissue, followed by subsequent rehydration without accumulation of lethal damage (Oliver, Tuba, & Mishler, 2000). In orthodox seeds, this survival mechanism is acquired during seed development, in the reserves accumulation period (Le et al., 2010; Verdier et al., 2013). During this period, seed moisture is gradually reduced, and molecular and metabolic changes occur that allow seed dispersion and tolerance to long periods without water (Angelovici, Galili, Fernie, & Fait, 2010; Huang & Song, 2013). On the other hand, recalcitrant seeds lack the genetic mechanisms required to tolerate drying, or if are present, these mechanisms are not functional (Berjak & Pammenter, 2008; Delahaie et al., 2013).

Orthodox seed cells become sensitive to desiccation during the germination process (Farrant, 2010). Thus, during germination, they behave similarly to recalcitrant seeds and can be used as a model in the study of dehydration sensitivity which affects longevity and seed storage (Berjak, Farrant, & Pammenter, 2008; Walters, 2015).

Desiccation tolerance in orthodox seeds is directly linked to changes in cellular components, to the activation of antioxidant enzyme systems, to oligosaccharides and to heat-resistant proteins (Berjak & Pammenter, 2008; Spanò, Bottega, Grilli, & Lorenzi, 2011; Walters, 2015).

Reactive oxygen species (ROS) are normal components of the aerobic metabolism whose production is enhanced under stress conditions, such as the dehydration process, that generate toxic products for the cells. Thus, ROS production and activity are controlled by antioxidant enzyme systems such as superoxide dismutase, catalase and peroxidase (Gill & Tuteja, 2010). Along with the enzyme systems, certain sugars act as signaling mechanisms. The synergistic interaction of sugars may contribute to stress tolerance, especially in tissues or organelles abundant in soluble sugars (Bolouri-Moghaddam, Le Roy, Xiang, Rolland, & Van den Ende, 2010). The α-amylase enzyme converts starch into sugars; thus, its expression is linked to reserve mobilization in seeds. The late embryogenesis abundant (LEA) and heat shock proteins (HSP) are accumulated under stress conditions, such

as high temperatures, and have an important role in the protection mechanism that ensures desiccation tolerance in seeds (Boswell, Moore, & Hand, 2014).

The identification of physiological and biochemical changes in the regulatory mechanisms of DT combined with the genetic improvement of plants can help develop cultivars better adapted to abiotic stresses (Shanker et al., 2014). Previous studies on agronomic species have sought a greater understanding of the changes that occur in seeds as they lose water to develop new seed drying methods (Veiga et al., 2007). However, little is known about the possible biochemical changes caused by DT loss in seeds of agronomic species.

Pearl millet (*Pennisetum glaucum* L.) is an African annual grass belonging to the *Poaceae* family that is able to produce seeds in extremely dry conditions and low soil fertility (Oumar, Mariac, Pham, & Vigouroux, 2008). The species is also resistant to drought and produces orthodox seeds. The aim of the present study was to evaluate the biochemical and physiological changes during the DT loss process in *P. glaucum*, to better understand these changes and to generate information for the improvement and development of the seed production and technology field.

## Material and methods

Millet seeds (hybrid ADRF6010) from the 2015 crop year were provided by the Sementes Adriana company. The seeds were stored in a cold room at 10°C and 50% RH before the beginning of the experiments.

The initial moisture content was determined on a wet basis using an oven set at 105 ± 3°C for 24 hours (Brasil, 2009) with 4 replicates (25 seeds in each). The calculation was performed, and the result was expressed as a percentage.

Imbibition curve**:** To evaluate the water absorption pattern of the seeds, 4 replicates with 25 seeds in each were used to determine the hydration process and obtain the imbibition curve. The seeds were soaked in germination boxes with two sheets of blotting paper as substrate saturated with distilled water and conditioned in incubators regulated at 25°C with a photoperiod of 12 hours. Every 3 hours, during 18 hours, the seeds were removed and weighed on a scale with an accuracy of 0.01 g.

Characterization of the desiccation tolerance loss: One-hundred seeds were divided into four replicates of 25 in each of the following points on the imbibition curve: control (0h), 3h of imbibition, 1 and 3 mm radicle. Hydration was performed in germination boxes containing 2 sheets of blotting paper saturated with water until the pre-established hydration periods were reached. Dehydration was performed in germination boxes sealed with a plastic film: the seeds were placed on a screen with a layer of 100 g of silica gel in the bottom and conditioned in incubators regulated at 20°C during 72 hours to keep the seeds in a prolonged stress situation. After dehydration, the seeds were pre-moistened in humid air (100% RH) during 24 hours in incubators regulated at 25°C to prevent any possible damage caused by subsequent imbibition (Crowe, Crowe, & Hoekstra, 1989). Then, a germination test was performed in germination boxes in incubators regulated at 25°C (Brasil, 2009).

The survival analysis was performed by identifying the seeds that returned to germination and produced normal seedlings as follows. Germination test**:** Four replicates of 50 seeds were distributed in germination boxes with two sheets of blotting paper moistened with distilled water until reaching 2.5 times the weight of the dry paper. The germination boxes were maintained in incubators regulated at 25°C with a photoperiod of 12 hours. Seven days after sowing, the number of normal seedlings was counted (Brasil, 2009), and the results were expressed as percentages. First germination count (FC)**:** During the germination test, the number of normal seedlings observed three days after sowing was counted and the results are expressed as percentages. Germination speed index (GSI)**:** The number of seedlings that returned to germination from the beginning until the last day of the test was counted daily. The GSI calculation was performed following Maguire (1962). Electrical conductivity**:** Four replicates of 25 seeds were used; the seeds were weighed with 0,01 g of accuracy and subjected to imbibition in plastic cups containing 50 mL of deionized water during 24 hours at 25°C (Vieira & Krzyzanowski, 1999). Subsequently, the conductivity was measured with a conductivimeter DIGIMED CD-21, and the results were expressed in $\mu S\ cm^{-1}\ g^{-1}$ of seed.

Biochemical analyses: In each of the selected points of the imbibition curve, the seeds were removed from the imbibition paper, and at this humidity condition, they were macerated in liquid nitrogen with polyvinylpyrrolidone (PVP) and stored in a deep freezer at -86°C. The methodology described in Alfenas (2006) was used for the extraction, electrophoretic run and quantification of superoxide dismutase isoenzymes, catalase, peroxidase and α-amylase. For the extraction of heat resistant proteins (HRP), 100 mg of each sample was used and placed in 1500 µL microtubes. After adding an extraction buffer solution (50 mM Tris-HCl, pH 7.5; 500 mM NaCl; 5 mM $MgCl_2$ and 1 mM PMSF) in a proportion of 1:10 (material weight: volume of extraction buffer), the samples were homogenized by vortexing. The homogenates were centrifuged at 13,000 rpm for 30 min. at 4°C. The supernatant was transferred to new microtubes, incubated in a water bath at 85°C for 15 min. and centrifuged again. The supernatant was then transferred to new microtubes, and the pellet was discarded. The quantification of enzymes and heat-resistant proteins was performed using the ImageJ® software (Schneider, Rasband, & Eliceiri (2012), where each gel photograph obtained in the electrophoresis was evaluated by the intensity and size of the isoforms. The results were expressed in $mm^2$.

Statistical analysis: The experiments were performed in a completely randomized design (CRD), with four replicates of 50 seeds for the germination test and four replicates of 25 seeds for the electrical conductivity test. The results were tested with analysis of variance and comparison of means by the Scott-Knott test at a 5% probability. The analyses were performed using the SISVAR® statistical software (Ferreira, 2011).

## Results and discussion

The increase in the fresh weight of millet seeds (Figure 1) indicated water absorption following the three-phasic pattern proposed by Bewley, Bradford, Hilhorst, and Nonogaki (2013).

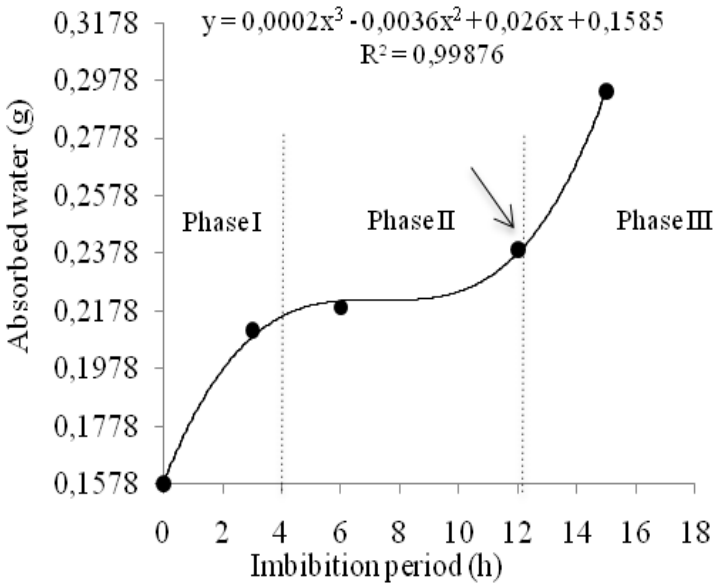


**Figure 1.** Imbibition curve of millet seeds (*Pennisetum glaucum* L.). The arrow indicates the moment of radicle protrusion.

The initial water content of the seeds was 11% and reached approximately 46% after 18h of imbibition.

Phase I of germination lasted approximately 4h and was characterized as a physical phenomenon of water absorption causing seed metabolism activation (Bewley et al., 2013). Similar to what was observed by Bewley (1997), Phase II of millet seed germination lasted longer (approximately 8h) and was characterized by slow water absorption, involving a series of metabolic events before radicle protrusion, which occurred at the beginning of Phase III (after 12 hours of imbibition).

A significant effect was observed for all treatments in the physiological tests of DT loss in millet seeds (Table 1).

**Table 1.** Mean values of first germination count (FC), germination (G), germination speed index (GSI) and electrical conductivity (EC) in millet seeds subjected to different imbibition periods, followed by dehydration and subsequent rehydration.

| Treatments | FC (%) | G (%) | GSI | EC ($\mu S\ cm^{-1}\ g^{-1}$) |
|---|---|---|---|---|
| Control (0h) | 81 a | 93 a | 42.67 a | 158.96 a |
| 3h of imbibition | 47 b | 76 b | 46.59 a | 128.72 a |
| 1 mm radicle | 4 c | 8 c | 4.54 b | 240.6 b |
| 3 mm radicle | 0 c | 0 c | 0.54 b | 450.52 c |
| CV (%) | 27.88 | 22.56 | 10.96 | 14.58 |

*Means followed by the same lowercase letter in a column do not differ from each other according to the Scott-Knott test at a 5% probability.

The highest percentage of germination in the first count was obtained from control seeds. For seeds subjected to three hours of imbibition followed by dehydration and rehydration, the percentage of normal seedlings at the first count decreased, showing a negative effect on seed vigor.

For seeds with 1 and 3 mm radicles, the mean FC was practically null and did not differ significantly between them. Similar results were observed for the final germination percentage. Masetto, Faria, and Fraiz (2015) observed that germinated seeds of *Sesbania virgata* with a 1 mm radicle still maintained their desiccation tolerance and that total loss of this capacity only occurred when the radicles reached 2 mm. Similar results of DT loss after radicle protrusion were found by Guimarães, Faria, Oliveira, and Silva, (2011) in *Peltophorum dubium*.

For the germination speed index, the highest values were observed for the control treatment and for seeds subjected to 3h of imbibition with subsequent drying on silica gel and rehydration. The rapid drying technique on silica gel allows drying to a sufficiently low water content without adverse effects on seed viability and vigor (Varghese & Naithani, 2008). However, Reis et al. (2013), who studied seed conditioning, stated that rapid drying could be harmful to seed vigor. For millet seeds dried on silica gel in the present study, the germination speed index of the seeds soaked for 3h did not differ from the control treatment (0h). GSI values were observed for seeds with 3 mm radicles that were dried on silica gel with subsequent rehydration, even with null germinations. This result is due to the abnormal seedlings (4%) accounted in the determination of the GSI. Under these study conditions, millet seeds only developed a shoot part and were considered abnormal (Brasil, 2009). In a study on the reestablishment of DT in germinated seeds of *Arabidopsis thaliana,* Maia, Dekkers, Provart, Ligterink, and Hilhorst (2011) observed that different parts of the seed had different sensitivities to drying. Furthermore, seedlings that did not return to root growth frequently developed a cotyledon or hypocotyl, as observed in millet seedlings in low proportion in the present study.

In the electrical conductivity test, higher values were observed for seeds with radicle protrusion (1 and 3 mm). The germination process is followed by the release of sugars, amino acids and electrolytes in different amounts depending on the state of organization of the membrane system (Torres, Paiva, Almeida, Benedito, & Carvalho, 2015). In the present study, the rapid drying process applied to germinated millet seeds resulted in damage to the cell membranes and a subsequent rise in these solutes.

In a study on DT loss during seed germination of pioneer neotropical species, Daws, Bolton, Burslem, Garwood, and Mullins (2007) concluded that this tolerance was gradually lost during germination. Rapid drying with subsequent rehydration affected the physiological performance of millet seeds at the germination stages studied, and desiccation tolerance was lost when the radicle reached 1 mm. This result indicates the potential of millet seeds for studies on DT loss. In addition, millet is a species resistant to abiotic stresses (Kholová, Hash, Kakkera, Kočová, & Vadez, 2010), with a relatively short germination period and a growing agricultural interest in Brazil.

In addition to the evaluation of seed physiological quality and vigor, possible biochemical changes were evaluated through the analysis of enzyme systems: Superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POX), and α-amylase with heat-resistant proteins (HRP). The SOD enzyme activity (Figure 2) in millet seeds during imbibition and with 1 and 3 mm of radicle presented a constant expression among the treatments. Xin, Jing, Liu, and Song (2010), in a study on the viability loss of *Antiaris toxicaria* embryonic axes under rapid dehydration and its relation with oxidative damages, concluded that viability loss occurred due to mechanical and physical damages with no relation to the metabolic effects studied, such as SOD expression. In millet seeds, the expression of this enzyme did not differ significantly among the treatments.

CAT enzymatic expression was higher for seeds with a 3 mm radicle (Figure 3), indicating higher oxidative stress. Silva et al. (2015) concluded that the presence of CAT might be associated with the acquisition of desiccation tolerance in *Rhamnidium elaeocarpum* seeds. Their results indicated an increase in the content

of oxidative free radicals that are harmful to membranes, confirmed by the increase in electrolyte solutes and the loss of germination capacity in desiccation-intolerant seeds. Similar results were observed in the present study for millet seeds with a 3 mm radicle that did not tolerate desiccation, had high electrical conductivity values and null germination.

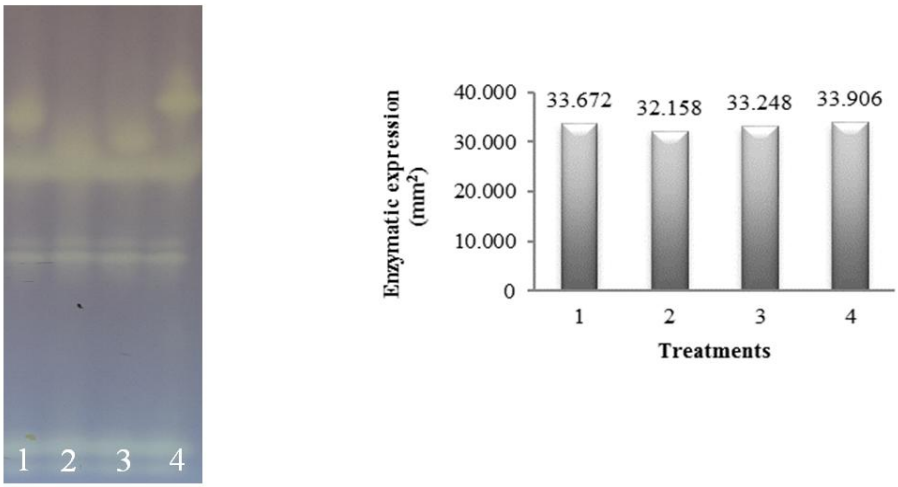


**Figure 2.** Electrophoretic patterns and quantification of SOD enzyme activity in millet seeds *(Pennisetum glaucum* L.) submitted to different imbibition periods, followed by dehydration and subsequent rehydration. Treatments: 1: control (0h); 2: 3h of imbibition; 3: seeds with a 1 mm radicle; 4: seeds with a 3 mm radicle.

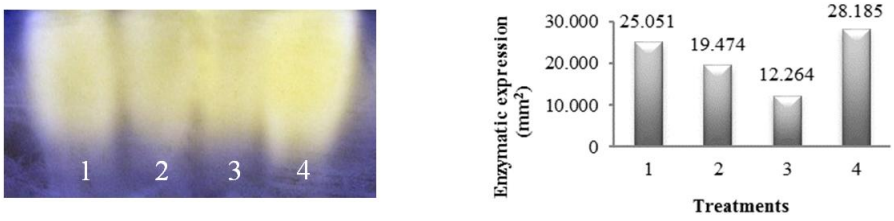


**Figure 3.** Electrophoretic patterns and quantification of CAT enzyme activity in millet seeds *(Pennisetum glaucum* L.) submitted to different imbibition periods, followed by dehydration and subsequent rehydration. Treatments: 1: control (0h); 2: 3h of imbibition; 3: seeds with a 1 mm radicle; 4: seeds with a 3 mm radicle.

POX enzymatic expression was null for the control, for seeds subjected to 3 hours of imbibition, and for seeds with a 1 mm radicle (Figure 4). A higher expression was observed in seeds with a 3 mm radicle that corresponded to the lowest results in the physiological tests. Coelho, Figueiredo, Clemente, Coelho, and Rosa (2015), who studied biochemical changes in coffee seeds exposed to different drying methods, observed that in seeds subjected to rapid drying, the activity of peroxidase increased as water content decreased. According to (Berjak et al., 2008), desiccation damages to membranes can be caused by oxidation, which promotes phospholipid esterification or lipid peroxidation. These results reiterate the high POX activity observed in seeds with a 3 mm radicle in our study.

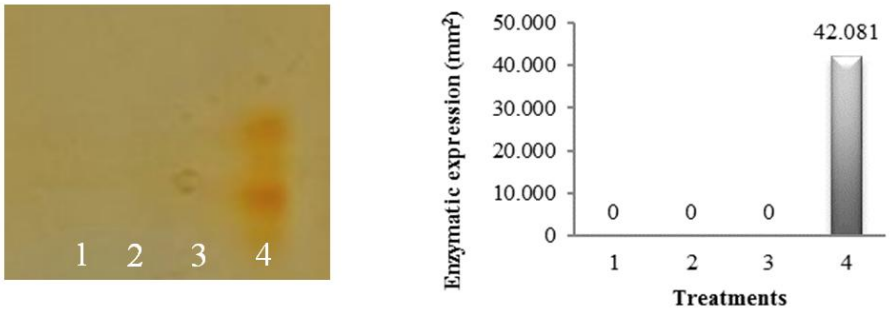


**Figure 4.** Electrophoretic patterns and quantification of POX enzyme activity in millet seeds *(Pennisetum glaucum* L.) submitted to different imbibition periods, followed by dehydration and subsequent rehydration. Treatments: 1: control (0h); 2: 3h of imbibition; 3: seeds with a 1 mm radicle; 4: seeds with a 3 mm radicle.

The production of oxygen-reactive species (ROS) is directly associated with sugar metabolism, acting as stress indicator molecules mainly in tissues or organelles with abundant soluble sugars (Smeekens, Ma, Hanson, & Rolland, 2010). Sugars that accumulate during dehydration have membranes and macromolecule protection and eliminate oxygen-reactive species (Zhang, Song, & Bartels, 2016). In millet seeds, a greater activity of α-amylase was observed in seeds with 3 mm radicles, indicating a higher supply mobilization. Overall, the activity of this enzyme remained constant for the other treatments (Figure 5).

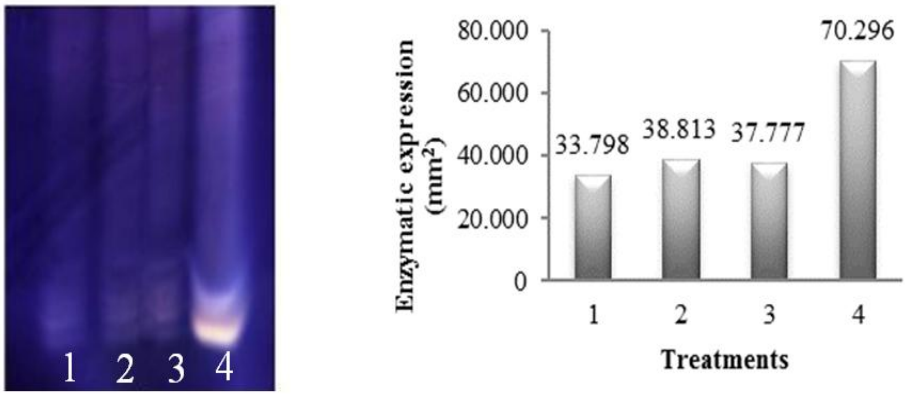


**Figure 5.** Electrophoretic patterns and quantification of α-amylase enzyme activity in millet seeds *(Pennisetum glaucum* L.) submitted to different imbibition periods, followed by dehydration and subsequent rehydration. Treatments: 1: control (0h); 2: 3h of imbibition; 3: seeds with a 1 mm radicle; 4: seeds with a 3 mm radicle.

The lowest activity of heat-resistant proteins was observed in seeds with 3 mm radicles (Figure 6). Studies with other species have shown a reduction in the HRP stripe intensity derived from DT loss, supporting the hypothesis that HRPs are involved in this process (Boudet et al., 2006). HRPs contribute to the stabilization of macromolecules and the prevention of cell protein denaturation through connections between water molecules and their surface, maintaining the stability of other protein membranes and adjusting the osmotic cell pressure (Mohammadkhani & Heidari, 2008).

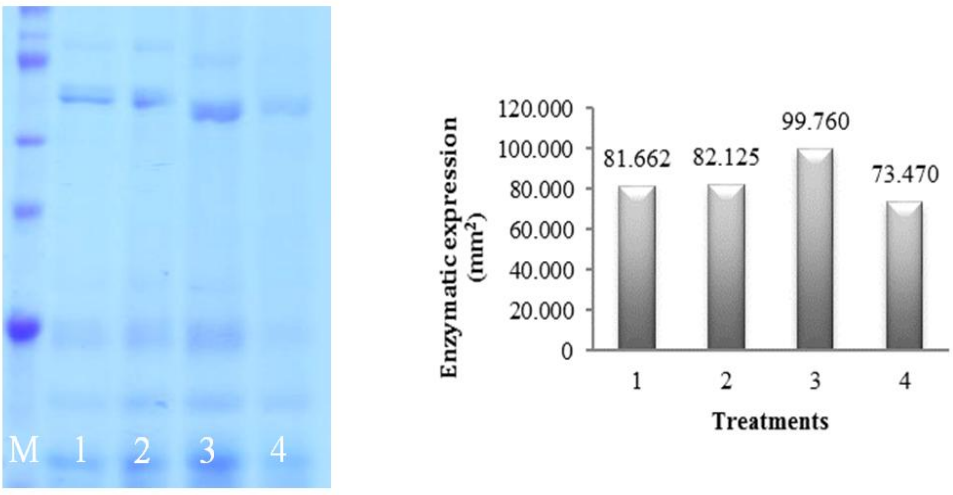


**Figure 6.** Electrophoretic patterns and quantification of heat-resistant proteins in millet seeds *(Pennisetum glaucum* L.) submitted to different imbibition periods, followed by dehydration and subsequent rehydration. Treatments: 1: control (0h); 2: 3h of imbibition; 3: seeds with a 1 mm radicle; 4: seeds with a 3 mm radicle. M: molecular marker.

Previous research on seed drying showed that an increase in the heat-resistant protein stripe intensity was related to water loss, indicating that the drying process leads to HRP synthesis in seeds with a low humidity content (Abreu, Veiga, Von Pinho, Monteiro, & Rosa, 2014). In our study, a greater expression of HRPs was observed in seeds with 1 mm radicles. A higher activity of HRPs was also observed by Abreu et al. (2016) who subjected less tolerant corn lineage seeds to different water stress potentials during germination.

Mishra and Grover (2016) determined that certain proteins from the HRP group had important roles in the response to dehydration.

## Conclusion

In millet seeds (*Pennisetum glaucum* L.), the desiccation tolerance is lost after germination when the radicle reaches 1 mm in length.

The desiccation tolerance loss in millet seeds is associated with the expression of peroxidase, α-amylase and heat-resistant proteins and less related to the expression of superoxide dismutase and catalase.

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