

Seed imbibition and germination of *Plathymenia reticulata* Benth. (Fabaceae) affected by mercury: possible role of aquaporins

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ABSTRACT

Studies that evaluate the physiological and biochemical mechanisms of germination within forest species are needed in order to improve our understanding of such processes. Mercury and dithiothreitol are indicated as important tools in studies that assess the activity of aquaporins during imbibition and germination of seeds. To investigate the alterations caused by mercury in *Plathymenia reticulata* seeds, different doses of mercury were used in the presence and absence of dithiothreitol. Mercury had a dose-dependent effect on the seeds; in the most dilute solutions mercury partially inhibited the imbibition process, whereas in the most concentrated solutions it caused the death of the embryos. A delay in the hydration of the seeds may have caused decreased germination as a result of the reduced functionality of the aquaporins that were oxidized by mercury. In the presence of the reducing agent dithiothreitol, the activity of these proteins was restored and the germination process was re-established. These findings indicate the importance of aquaporins in the imbibition and germination stages of *P. reticulata* seeds, and they provide a better understanding of these important developmental events in plants.

Keywords: aquaporins, dithiothreitol, forest species, mercury, seed

Introduction

Germination is a crucial process in plant development required for the perpetuation of species, and it has three phases. Initially, rapid imbibition occurs because of the water potential gradient between the dry seed and external environment (phase I). Simultaneously, the metabolic activity of the seed is restored, which characterizes the beginning of the germination process. This stage is followed by a period of limited water absorption (phase II) and is the final stage for dead or dormant seeds. The last stage (phase III) is characterized by renewed hydration that culminates in the emission of a radicle and finalization of germination (Nonogaki *et al.* 2010).

Plant aquaporins constitute a multigenic family of water channel proteins, subdivided into five subfamilies: plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), NOD26-like intrinsic proteins (NIP), small basic intrinsic proteins (SIP), and X intrinsic proteins (XIP) (Danielson & Johanson 2010). Those are intrinsic proteins with six trans-membrane domains and, among other

functions, they facilitate water transport in biomembrane systems (Aroca *et al.* 2012).

In recent years, studies have indicated the importance of the aquaporins in the imbibition and germination processes of seeds (Schuurmans *et al.* 2003; Liu *et al.* 2007; Liu *et al.* 2013). The greater functionality of the aquaporins in seeds may be related to the water absorption process and activation of the metabolic system of the seed, which leads to higher germination rates (Liu *et al.* 2007).

Mercury is a heavy metal indicated as a primary inhibitor of aquaporin activity. Under physiological conditions, it interacts with the thiol groups of cysteine residues, oxidizing them and reversibly interrupting the activity of these proteins (Aroca *et al.* 2012). Because of its great efficiency in blocking the activity of aquaporins (Przedpelska-Wasowicz & Wierzbicka 2011), mercury chloride (HgCl₂) has been frequently used as a tool to study the action of these proteins in water transport at various stages of plant development.

Some authors have discussed the potentially toxic side effects of mercury on plant metabolism (Willigen *et al.* 2006; Gaspar 2011) and, for this reason, it is important to study

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the possible oxidative stress caused by this metal. It can be done by quantifying the activity of the main reactive oxygen species scavengers, such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX). While SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, CAT and POX detox hydrogen peroxide to water and oxygen, maintaining cellular homeostasis.

Reducing agents, including dithiothreitol (DTT) and β -mercaptoethanol, have also been used in conjunction with HgCl_2 in current studies that seek to evaluate the activity of aquaporins (Przedpelska-Wasowicz & Wierzbicka 2011; Obroucheva *et al.* 2012; Liu *et al.* 2014). They reduce the sulfhydryl groups that are oxidized by mercury, allowing these proteins to reassume their original conformation and functionality. Once other works have shown similar activity for both reducing agents (Jain *et al.* 2008), only DTT was used in the present study.

Many studies have evaluated the effects of mercury on seed germination of cultivated species focusing on aquaporins activity. Among them, one can cite works with rice (Liu *et al.* 2013), tomato (Jain *et al.* 2008), broad bean (Novikova *et al.* 2014), and pea (Veselova & Veselovsky 2006). However, only one data on forest species is currently available (Obroucheva *et al.* 2012).

Plathymania reticulata (Fabaceae) is a neotropical arboreal species that is found in different Brazilian biomes. Its high economic and environmental potential makes it one of the most important species found in the Brazilian Cerrado (Carvalho 2003). Because additional studies are required that evaluate the stages of germination in forest seeds, the objective of this study was to investigate alterations in the imbibition and germination processes of *P. reticulata* caused by different doses of mercury, in the presence and absence of dithiothreitol.

Materials and Methods

Plant material and experimental conditions

Seeds of *Plathymania reticulata* Benth. were collected in August 2012 from nearly 30 trees located in the city of Guaraciaba (20° 34'S, 43° 00'W, Atlantic Forest Domain), Minas Gerais State, Brazil and they were stored in plastic bags placed in cold chamber at 20°C until the beginning of experiment (January 2013).

It was used 50 seeds to determine seed size and the results are described forward (Mean \pm SD): 11.2 \pm 2.5 mm length and 6.8 \pm 1.5 mm breadth. Seed dormancy was overcome by immersion in sulfuric acid for 10 minutes, followed by washing in distilled water. The seeds were dried in the shade at room temperature.

The seeds were then distributed on 90 mm diameter Petri dishes containing two sheets of Germitest paper (JProLab®) moistened with 4 mL of the tested solutions and 20 seeds per plate. The plates were sealed and maintained in a biochemical oxygen demand (BOD) incubator at 25 \pm

1°C with a 12 h photoperiod. Seeds that showed emergence of the primary root with a length greater than 1 mm were considered germinated.

Effect of HgCl_2 and DTT on seed germination

To evaluate the effect of HgCl_2 on germination, the seeds were soaked in distilled water and HgCl_2 solutions at concentrations of 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} M for 72 h, and daily evaluations of germination were performed. At the end of the experiment, the percentage of germination and germination speed index (GSI) (Maguire 1962) were evaluated along with the water content of the embryonic axes using the standard method in an oven at 105°C for 24 h (MAPA 2009).

The seeds were soaked only in distilled water or in solutions of 10^{-3} and 10^{-2} M HgCl_2 for 72 h and then washed in distilled water and transferred to Petri dishes containing distilled water for an additional 72 h. Germination was monitored daily over 144 h, and the water content of the embryonic axes was determined after 72 and 144 h.

To determine the relationship between the action of HgCl_2 and DTT on the germination mechanism, the seeds were soaked in combined solutions of HgCl_2 (10^{-3} and 10^{-2} M) and DTT (10^{-3} M) for 72 h. At the end of the test, the percentage of germination, GSI and water content of the embryonic axes were measured.

The DTT concentration used in the experiment was selected based on preliminary experiments (data not shown), in which different concentrations of DTT solutions were tested on the germination of *P. reticulata* seeds. The highest concentration that did not show an effect on the germination percentage and GSI relative to the control was selected.

Effect of HgCl_2 and DTT on imbibition of the embryonic axis

To evaluate the relationship between the effect of HgCl_2 and DTT on the hydration of the embryonic axes, seeds were soaked in distilled water, solutions of 10^{-5} , 10^{-4} and 10^{-3} M HgCl_2 and a solution of 10^{-3} M HgCl_2 combined with 10^{-3} M DTT. The fresh mass (g) of the embryonic axes was determined until 42 h at regular intervals of 6 h. The embryonic axes were extracted from the seeds using a stylus, and their fresh mass was determined using an analytical scale with a precision of 0.0001.

Activity of enzymes of the antioxidant system

The seeds were soaked in distilled water and the following solutions for 48 h: 10^{-3} M DTT, 10^{-3} M HgCl_2 and 10^{-3} M HgCl_2 + 10^{-3} M DTT. The activities of the enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) and POX (EC 1.11.1.7) were determined in the embryonic axes of the seeds after intervals of 24 and 48 h.

The extracts were obtained by maceration of 0.1 g of the embryonic axis in 100 mM potassium phosphate buffer

(pH 6.8) containing 0.1 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1% (w/v) polyvinylpyrrolidone (PVPP) (Peixoto *et al.* 1999). The homogenized sample was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was used as the enzymatic extract. The protein concentration was determined using the Bradford method (Bradford 1976).

The SOD activity was evaluated by the ability of the enzyme to inhibit the photoreduction of nitrotetrazolium blue (NTB) in a reaction medium consisting of 50 mM sodium phosphate buffer (pH 7.8), 15 mM methionine, 75 µM NTB, 0.1 mM EDTA and 10 mM riboflavin (Del Longo *et al.* 1993). The reaction was conducted in a reaction chamber under illumination from a 15 W fluorescent lamp for 15 min. After this period, the absorbance at 560 nm was determined in an ultraviolet-visible (UV-Vis) spectrophotometer (Giannopolitis & Ries 1977). One unit of SOD was defined as the amount of enzyme needed to inhibit the photoreduction of NTB by 50% (Beauchamp & Fridovich 1971).

The CAT activity was determined in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 6.8) and 12.5 mM H₂O₂ (Havir & McHale 1987). The decrease in absorbance at 240 nm was measured in a UV-Vis spectrophotometer during the first two minutes of the reaction at 25°C. The enzyme activity was calculated using the molar extinction coefficient of 36 M cm⁻¹ (Anderson *et al.* 1995).

The POX activity was determined by the method of Kar & Mishra (1976) in a reaction medium consisting of 25 mM potassium phosphate buffer (pH 6.8), 20 mM pyrogallol and 20 mM H₂O₂. The increase in absorbance was evaluated during the first two minutes of the reaction at 420 nm in a UV-Vis spectrophotometer at 25°C. The enzymatic activity was calculated using the molar extinction coefficient of 2.47 mM⁻¹ cm⁻¹ (Chance & Maehly 1955).

Experimental design and statistical analysis

The experimental design was completely randomized, and there were five replicates of 20 seeds each for the germination percentage and germination speed; five replicates of 10 embryonic axes each for the water content; five replicates of 10 embryonic axes each for the hydration curve; and five replicates of 0.1 g of fresh mass of embryonic axes each for enzymatic activity. The data were subjected to an analysis of variance (ANOVA), and the means were compared using Tukey's test at $P < 0.01$ and Student's t -test at $P < 0.01$. The values in percentage and the IVG values were transformed into arcsin to obtain data normalization.

Results

The HgCl₂ doses caused different alterations in the germination of the *P. reticulata* seeds. The seeds soaked in 10⁻⁵ and 10⁻⁴ M HgCl₂ behaved in a similar manner to the seeds

soaked in water, and the water content in the embryonic axes and germination percentage and speed were not significantly different from the control. However, 10⁻³ M HgCl₂ caused a significant reduction in the water content of the embryonic axes and germination of the seeds. The seeds hydrated in 10⁻² M HgCl₂ did not germinate by the end of the evaluation; therefore, they are not shown in the graph (Fig. 1).

The transfer of seeds that had been soaked in 10⁻³ M HgCl₂ to water led to a rapid increase in germination and similar percentage values to those obtained for seeds soaked in water (Fig. 2). The water content of the embryonic axes of these seeds also increased significantly from 78% to 89% (Student's t -test, $P < 0.01$) after the transfer to water and achieved values similar to those of the embryonic axes whose seeds were soaked in water. However, the transfer of seeds soaked in 10⁻² M HgCl₂ to water had no effect on germination (Fig. 2), and the test performed with tetrazolium showed a loss of viability (data not shown).

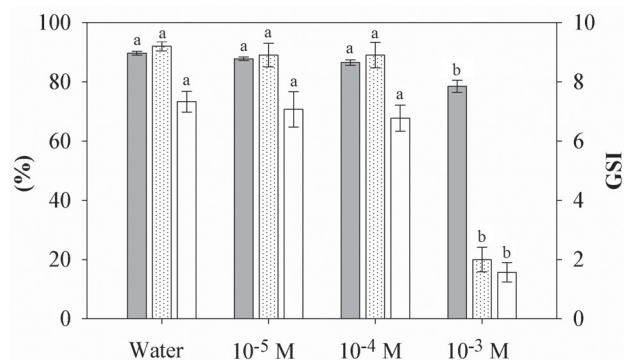


Figure 1. Water content of the embryonic axis (%) (gray bars), germination (%) (dotted bars) and GSI (white bars) for *P. reticulata* seeds soaked in water and solutions of HgCl₂ at different concentrations for 72 h. Bars followed by the same letters do not differ at the 1% level by Tukey test. Mean ± SE, n = 5.

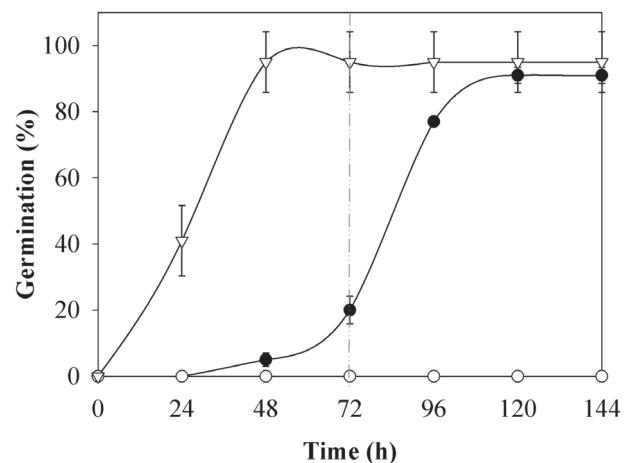


Figure 2. Germination (%) for *P. reticulata* seeds soaked only in distilled water (v) or in HgCl₂ at 10⁻³ M (●) and 10⁻² M (○) for 72 h and transferred to distilled water (indicated by the dashed line) until 144 h. Mean ± SE, n = 5.

The 10^{-3} M DTT solution did not significantly alter the water content of the embryonic axes or percentage and speed of germination of the seeds compared to water. However, when 10^{-3} M DTT was used in conjunction with 10^{-3} M HgCl_2 , there was an increase in the water content of the embryonic axes and germination of the seeds compared to 10^{-3} M HgCl_2 (Fig. 3 and Fig. 1 in supplemental material).

The seeds soaked in 10^{-2} M HgCl_2 + 10^{-3} M DTT did not germinate during the evaluation period, and the tetrazolium test demonstrated a loss of viability of these seeds under these conditions (data not shown).

The 10^{-5} and 10^{-4} M HgCl_2 solutions did not cause reductions in the hydration of the embryonic axes of the seeds relative to water, whereas 10^{-3} M HgCl_2 significantly reduced hydration (Tukey's test, $P < 0.01$) in the first 6 h (Fig. 4A). When 10^{-3} M HgCl_2 was applied in conjunction with 10^{-3} M DTT, hydration of the embryonic axes occurred in a similar manner to that of hydration in water (Fig. 4B).

Although 10^{-3} M HgCl_2 affected the hydration of the embryonic axes and germination of *P. reticulata* seeds, the solution did not cause an increase in the activity of the primary enzymes of the antioxidant system in the embryonic axes during the evaluation period. However, the 10^{-3} M HgCl_2 + 10^{-3} M DTT solution caused an increase in the activity of the three enzymes evaluated in the 48 h period and the 10^{-3} M DTT also boosted CAT activity at the same time (Fig. 5A-C).

Discussion

Different doses of mercury have different effects on seed germination. At high concentrations, the metal is

capable of causing the death of the seed embryos because oxidative stress is generated and important cellular components are damaged. At lower concentrations, mercury can alter the pattern of hydration and germination of the seeds (Munzuroglu & Geckil 2002; Jain *et al.* 2008).

The effect of mercury also varies with species, and the concentrations are considered to be high or low depending on the tolerance shown by the plant. HgCl_2 at a concentration of 10^{-5} M has a toxic effect on tomato seeds that leads to death (Jain *et al.* 2008); however, we show that the same solution did not affect the germination of *P. reticulata* seeds. It demonstrates that the tolerance of different species of seeds varies when subjected to solutions of the metal.

A possible explanation to the found result may be the difference in seed size between these two species. This feature is appointed as a key determinant of evolutionary fitness in plants, and plays an important role in seed tolerance under stress conditions (Coomes & Grubb 2003). Once *P. reticulata* seeds are bigger than tomato ones, they may be able to germinate in an environment with higher concentrations of mercury.

The 10^{-2} M concentration is higher than the tolerance level of *P. reticulata* and causes the death of the seeds. The 10^{-3} M concentration can be overcome by *P. reticulata*, although it causes a delay in the hydration of the seeds during the first hours of soaking and reduces the water content in the embryonic axis, which may explain the drastic reduction in germination. Because mercury blocks the activity of the aquaporins and these proteins are responsible for the transmembrane transport of water (Aroca *et al.* 2012), the reduction in hydration in seeds treated with 10^{-3} M HgCl_2 might have been caused by the lower activity of these proteins.

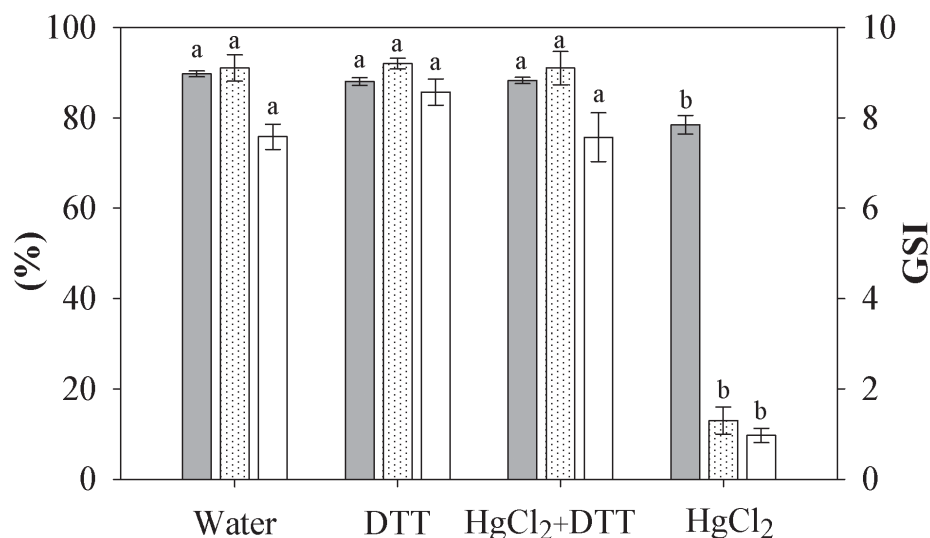


Figure 3. Water content of the embryonic axis (%) (gray bars), germination (%) (dotted bars) and GSI (white bars) for *P. reticulata* seeds soaked in water and solutions of 10^{-3} M DTT, 10^{-3} M HgCl_2 + 10^{-3} M DTT and 10^{-3} M HgCl_2 for 72 h. Bars followed by the same letters do not differ at the 1% level by Tukey test. Mean \pm SE, n = 5.

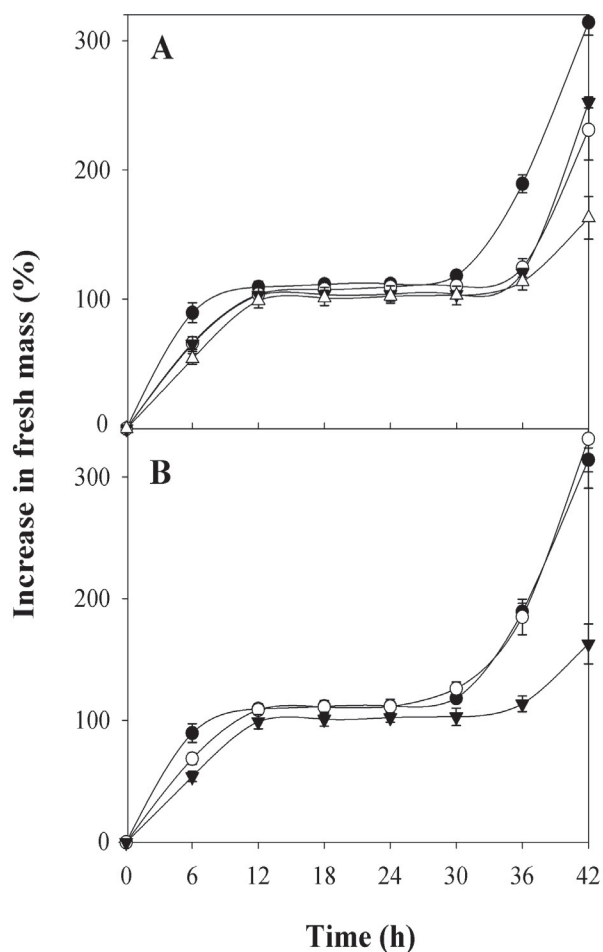


Figure 4. Increase in the fresh mass (%) of embryonic axes of *P. reticulata* seeds soaked in different solutions: (A) water (●) and HgCl_2 at concentrations of 10^{-5} M (○), 10^{-3} M (▼) and 10^{-3} M (△) and (B) water (●), 10^{-3} M HgCl_2 + 10^{-3} M DTT (○) and 10^{-3} M HgCl_2 (▼) until 42 h. Mean \pm SE, n = 5.

Transferring seeds from 10^{-3} M HgCl_2 to water allowed for an increase in hydration and germination, which demonstrates that 10^{-3} M HgCl_2 does not irreversibly affect the seed integrity of *P. reticulata*. Thus, prolonged contact with water and the synthesis of new aquaporins may have permitted the restoration of hydration and germination.

In evaluating the primary enzymes of the antioxidant system (SOD, CAT and POX), their activity was not altered by 10^{-3} M HgCl_2 , which indicates that mercury does not cause oxidative stress in the studied species at the evaluated concentrations and the reduced germination is not caused by the toxic effect of the metal. An evaluation of these results confirms that particularly in our study the drop in the germination of seeds treated with 10^{-3} M HgCl_2 was caused by lower seed hydration and not by possible oxidative stress, as some authors suggest (Willigen *et al.* 2006; Gaspar 2011).

The 10^{-3} M DTT applied in conjunction with 10^{-3} M HgCl_2 produced high germination rates because the solution can reverse the inhibitory effects caused by the 10^{-3} M

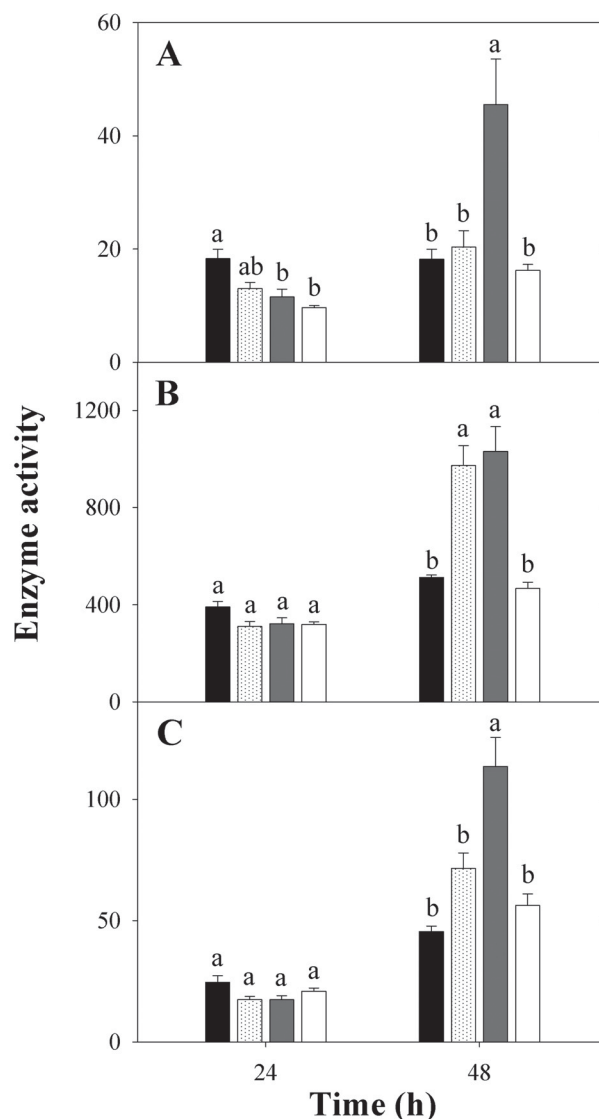


Figure 5. Enzyme activity of (A) SOD ($\text{U min}^{-1} \text{mg}^{-1}$ protein), (B) CAT ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) and (C) POX ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) in the embryonic axes of *P. reticulata* at 24 and 48 h after soaking of the seeds in water (black bars) and the following solutions: 10^{-3} M DTT (dotted bars), 10^{-3} M HgCl_2 + 10^{-3} M DTT (gray bars) and 10^{-3} M HgCl_2 (white bars). Among the four solutions evaluated at the same period (24 or 48 hours), bars followed by the same letters do not differ at the 1% level by Tukey test. Mean \pm SE, n = 5

HgCl_2 on the activity of the aquaporins (Jain *et al.* 2008; Przedpelska-Wasowicz & Wierzbicka 2011; Liu *et al.* 2014). Thus, the 10^{-3} M HgCl_2 + 10^{-3} M DTT solution produces higher hydration and germination rates than the 10^{-3} M HgCl_2 solution despite having a lower osmotic potential than the 10^{-3} M HgCl_2 solution, possibly by permitting the normal activity of the aquaporins.

It is also appointed that the greater activity of the ROS-scavenging enzymes caused by 10^{-3} M DTT applied in conjunction with 10^{-3} M HgCl_2 may explain the high germination rates founded in this treatment, once their higher activity improve seed germination performance (Chiu *et al.* 2006).

For *Arabidopsis* seeds, aquaporins do not act during the first phase of germination (Willigen *et al.* 2006). Also in the forest species *Aesculus hippocastanum* aquaporin gene expression is low during the beginning of imbibition and only at radicle emergence it is strongly activated. The authors suggest the presence of aquaporins in the seeds during imbibition phase, but in a closed state (Obroucheva *et al.* 2012; Obroucheva 2013).

For seeds from other species, such as pea and tomato (Schuurmans *et al.* 2003; Jain *et al.* 2008) the activity of these proteins is important in the initial phase of germination. Similarly, it is believed that these proteins show activity in the first stage of hydration of the seeds of *P. reticulata*, even though we consider that these proteins mainly act in the third stage in growing embryo axes. Further studies, including molecular tools, must be done in order to confirm this hypothesis.

Although the use of mercury can not by itself provide definitive evidence for aquaporins function, it allows us to indicate possible activity and function of these proteins (Willigen *et al.* 2006) and its use is still found in current works (Obroucheva *et al.* 2012; Liu *et al.* 2014). Novikova *et al.* (2014) say that in growing organs, and in the absence of suitable genetically altered materials, studies performed with mercury or other pharmacological blockers remain the only accessible technique for probing the functioning of aquaporins.

Heavy metals can affect plant physiology in different phases of a plant's development (Munzuroglu & Geckil 2002), and mercury has various effects on seed homeostasis. In *P. reticulata*, the dose dependent application of this metal is capable of altering the hydration process of the seeds and causing the death of the embryo.

Studies that evaluate the physiological alterations of plants when faced with heavy metals can help determine the mechanisms involved in the different stages of plant development; in addition, such studies can improve our understanding of the strategies that can be adopted to attenuate the damaging effects caused by the metals in contaminated locations. Thus, it is important that additional studies with mercury and varied species be performed to determine the physiological mechanisms affected by the metal.

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