MERCURY AFFECTS AQUAPORINS ACTIVITY AND GERMINATION OF THE EMBRYONIC AXIS OF *Schizolobium parahyba* (Vell.) BLAKE (Fabaceae)

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ABSTRACT – Germination is a physiological process that begins with hydration. Specific channels known as aquaporins are responsible for water uptake through biological cell membranes. The mercuric chloride ($HgCl_2$) reversibly inhibits water transport during germination through the aquaporins and can changes deoxyribonucleic acid (DNA) integrity, which can result in aquaporins deficiency. The aim of this study was to evaluate the effect of $HgCl_2$ and dithiothreitol (DTT) on aquaporins activity and on the integrity/degradation of DNA in embryonic axes of *Schizolobium parahyba* (Vell.) Blake during the germination process. Isolated axes were exposed to different concentrations of $HgCl_2$ or DTT; and aquaporins activity, embryo hydration, and DNA integrity were evaluated during embryonic axes imbibition. Growth and changes in axis fresh weight were quantified. We found that the heavy metal affects seed hydration by inhibition of aquaporins activity and does not cause changes in DNA integrity.

Keywords: Aquaporins; Heavy metal; Forest seed

EFEITO DO MERCÚRIO NA ATIVIDADE DE AQUAPORINAS NA GERMINAÇÃO DO EIXO EMBRIONÁRIO DE Schizolobium parahyba (Vell.) BLAKE (Fabaceae)

RESUMO – A germinação é um processo fisiológico que começa com a hidratação. Canais específicos conhecidos como aquaporinas são responsáveis pela absorção de água através das membranas celulares biológicas. O cloreto mercúrico ($HgCl_2$) inibe reversivelmente o transporte de água pelas aquaporinas durante a germinação e pode alterar a integridade do ácido desoxirribonucleico (DNA), o que pode resultar na deficiência das aquaporinas. O objetivo deste estudo foi avaliar o efeito de $HgCl_2$ e ditiotreitol (DTT) sobre a atividade das aquaporinas e sobre a integridade/degradação do (DNA) em eixos embrionários de **Schizolobium parahyba** *(Vell.) Blake durante o processo de germinação. Os eixos isolados foram expostos a diferentes concentrações de $HgCl_2$ ou DTT; e a atividade de aquaporinas, hidratação do embrião e a integridade do DNA foram avaliadas durante a embebição do eixo embrionário. O crescimento e as mudanças no peso fresco do eixo foram quantificados. Descobrimos que o metal pesado afeta a hidratação das sementes por inibição da atividade das aquaporinas e não causa mudanças na integridade do DNA.*

Palavras-Chave: Aquaporinas; Metal pesado; Sementes florestais.

1. INTRODUCTION

Water represents more than 90% of the fresh weight of most plant tissues in the growth phase and it is essential for maintaining turgidity and cell expansion (Steinbrecher and Leubner-Metzger, 2017). In germination, the hydration process results from the difference in water potentials between cells and the medium, and this varies from one type of seed to another. This imbibition is fundamental for activating seed metabolism, directly affecting the physiological processes that precede root protrusion, such as respiration, mitochondrial multiplication and repair, mobilization of reserves, and DNA synthesis and repair (Nonogaki, et al., 2010).

Water movement in the tissues occurs through three routes: the apoplastic, symplastic, and transcellular pathways (Kshetrimayum, et al., 2017). In the apoplastic pathway, water passes through the intercellular spaces through pores in the cell wall. In the symplastic pathway, water movement occurs through plasmodesmata that interconnect the membranes of neighboring cells, creating cytoplasmic bridges. The transcellular pathway involves the movement of water through the cell membranes, in water selective channels known as aquaporins (Preston, et al., 1992; Martínez-Ballesta, et al., 2016; Qi, et al., 2016).

The involvement of aquaporins in seed hydration during germination has been shown through analysis of gene expression in different plant species, such as *Brassica napus* (Ge, et al., 2014), *Medicago truncatula* (Bouton, et al., 2005), *Arabidopsis thaliana* (Gattolin, et al., 2011), *Oryza sativa* (Liu, et al., 2013), *Spinacia oleracea* (Chen, et al., 2013), and *Vicia faba* (Novikova, et al., 2014).

One of the possibilities for estimating the contribution of aquaporins in water transport in plant tissues is through use of agents that block these water channels, such as mercuric chloride ($HgCl_2$) (Daniels, et al., 1996; Javot and Maurel, 2002). The mercury (Hg) interacts with thiol groups of the cysteine residues, oxidizing them and blocking the activity of the aquaporins (Agre, et al., 1998; Aroca, et al., 2012). This blockage can be reversed using dithiothreitol (DTT) or 2-mercaptoethanol, which induce reduction in the sulfhydryl groups, allowing aquaporins to return to their original form and function (Jain, et al., 2008; Obroucheva, et al., 2012).

In addition to the damage that heavy metals like Hg cause to aquaporins, they are directly or indirectly associated with various types of cell damage, such as oxidative stress (Koivula and Eeva, 2010), which leads to oxidation of proteins and damage to the plasmatic membrane and to DNA (Gajewska and Slodowska, 2008; Ahmad, et al., 2010; Gill and Tuteja, 2010).

Heavy metal pollution from artisanal or small-scale mining operations is recognized as a significant environmental problem, with Hg being prominent in gold extraction process, especially in tropical developing countries (Adjei-Kyereme, et al., 2015). In Brazil, Hg pollution has been recorded in different areas. The contamination in the Southeast region decreased, but it increased in the North region in the last quarter of the 20th century (Hacon, et al., 2008).

Popularly known as guapuruvu or ficheira, *Schizolobium parahyba* (Vell.) Blake grows from Bahia to Rio Grande do Sul State, Brazil (Ferreira, et al., 2007). This species has a potential for restoration of riparian forests and for many other purposes, in which its wood and bark can be used (Coneglian, et al., 2016). With the characteristics of a pioneer species and rapid growth (Sereda, et al., 2008; Freire, et al., 2015) in native environment, this species can reach a volumetric production of 45 $m^3 . ha^{-1} . year^{-1}$, over 10 years (Narita, et al., 2018). It can be used in the making of diverse objects, such as toys (Lorenzi, 2002), furniture, packaging, and wooden boxes (Bortoletto Júnior and Belini, 2002), as well as for production of cellulose and paper (Nisgoski, et al., 2012).

Considering the importance of the species in the production of diverse products, lack of information on germination in soils contaminated by mercury, and the processes involved in seed hydration by aquaporins, the aim of this study was to evaluate the effects of $HgCl_2$ and DTT on aquaporins activity and on the integrity/degradation of DNA in embryonic axes of *S. parahyba* during the germination process.

2. MATERIALS AND METHODS

S. parahyba fruits were collected from 20 trees in the municipality of Viçosa, Minas Gerais, Brazil (20º45'14"S, 42º52'55"W) in September, 2015. A homogenous group of seeds was obtained from this

collection for subsequent analyses. Moisture content (MC) was determined by oven method at 103 °C for 17 h (ISTA, 2004) using five replications of 20 seeds. The viability of fresh seeds was evaluated by germination test (Ferreira, et al., 2007). After mechanical scarification using a sand paper, the seeds were placed in Petri dishes (Ø = 150 mm) over two moistened germination papers and incubated at 25 °C under constant light. Five replications of 20 seeds each were used, and the germination was scored daily.

The embryonic axes used in evaluation of aquaporins activity were obtained by cutting the seed coats laterally and crosswise using a micro circular saw blade coupled to a multipurpose rotary tool, and extraction of the embryonic axes with a tweezers.

The embryo weight and length were evaluated by using five replications of 10 embryos each, arranged in Petri dishes (Ø = 90 mm) between germitest papers, moistened with 2.5 mL of H_2O (control) or with the same amount of $HgCl_2$ or DTT. In all phases of the experiment, the Petri dishes were sealed with parafilm tape and incubated in the dark in biochemical oxygen demand (BOD) at 25 ºC and constant lighting provided by four 20 W bulbs with daylight type lighting. Elongation of the embryonic axes was measured through scaled images, registered by a digital camera coupled to a stereomicroscope (Zeiss) and processed with the aid of the software AxioVision Rel. 4.8. The gain in fresh weight of the embryonic axes was quantified on a digital analytical balance.

The concentration of $HgCl_2$ necessary to inhibit aquaporins activity was determined through variation in fresh weight, which was verified by the difference between the weight of the embryonic axes exposed to the concentrations of 0.01, 0.1, 1.0, and 5 mM and in water (control) for 72 hours. After this step, the concentration of 5 mM $HgCl_2$ was selected for the subsequent trials.

In restoration of aquaporins activity, the embryonic axes were soaked in 5 mM $HgCl_2$ for 48 hours and were then transferred to 5 mM DTT solution or H_2O. After 24 hours, resumption of weight gain was evaluated. The trials for evaluation of imbibition and restoration of aquaporins activity were performed with five replications of 10 embryonic axes.

To evaluate the integrity of the DNA, controls were used to simulate intact DNA (fresh leaves of *Copaifera langsdorffii*) and degraded DNA (embryonic axes of *S. parahyba* placed in boiling H_2O for two minutes). DNA extraction followed the protocol adapted from Masetto, et al. (2008) using hexadecyltrimethylammonium bromide (CTAB). The samples were ground in liquid nitrogen, transferred to 2 mL microtubes, to which were added 700 µL of the CTAB 2x buffer, and preheated to 65 °C, keeping the microtubes incubated at this same temperature for an hour, with homogenization every 10 minutes. After that, 600 µL of chloroform-isoamyl alcohol (24:1) was added to the microtubes, inverting them periodically in a 5-minute period. After this step, they were centrifuged at 12,000 rpm for 10 minutes at room temperature. Six hundred microliter of the supernatant was transferred to another microtube and 450 µL of cold isopropanol was added, keeping the tubes at -20 °C for 12 hours. The tubes were then centrifuged at 12,000 rpm at 4 °C for 10 min, and the supernatant was discarded. The residue was washed twice with 1 mL of 70% ethanol for 5 min and with 1 mL of 95% ethanol for 3 min. The residue was dried in a paper filter and dissolved in 50 µL of TE, pH 8.0 (10 mM Tris-HCl and 1 mM EDTA). The DNA samples were loaded on a 1% agarose gel with 0.01% GelRed™ and subjected to electrophoresis. The integrity of chromosomal DNA was evaluated using three replications of 10 embryonic axes.

A completely randomized design (CRD) was used in all experimental steps. Experimental evaluations were performed in 24-hour intervals for 72 hours. The results were subjected to ANOVA, followed by regression analysis. Similarity among the models ($p \leq 0.05$) was tested through the Model Identity Test (Regazzi, 1993). All statistical analyses were performed on software R for Windows® 2.12.0 (R Development Core Team, 2011). The figures were edited on SigmaPlot 11.0 software.

3. RESULTS

The moisture content (MC) of the seeds was 9.2% (fresh weight basis). Hydration of the excised embryonic axes kept in water (control) for 72 h resulted in fresh weight 5.51 times greater than weight observed before imbibition (Figure 1). Upon exposing the axes to 0.01, 0.1 and 1 mM $HgCl_2$, gains in fresh

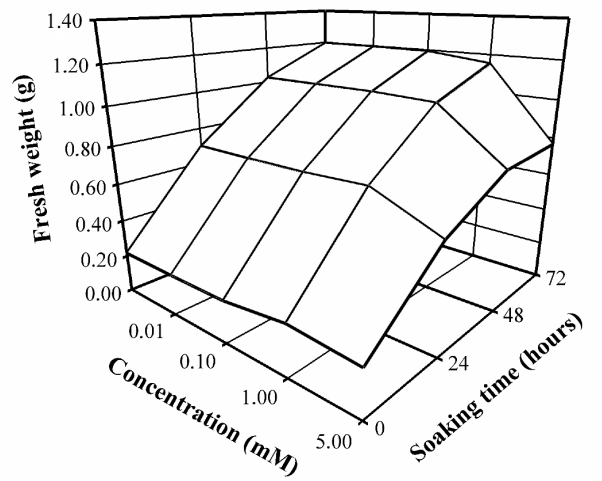

Figure 1 – Fresh weight (g) of the embryonic axes of *S. parahyba* over the period of 72 hours of soaking in H_2O (control) and $HgCl_2$ solutions at 0.01, 0.1, 1, and 5 mM.

Figura 1 – *Peso fresco (g) dos eixos embrionários de* ***S. parahyba*** *durante o período de 72 horas de embebição em H_2O (controle) e soluções de $HgCl_2$ a 0,01, 0,1, 1 e 5 mM.*

weight were found analogous to the control treatment (Figure 1). Delays in weight gain in the embryonic axes were exhibited after 6 hours of soaking in 5 mM $HgCl_2$, extending up to 72 hours (Figure 1 and 2).

The observed gain in fresh weight was the same as observed for the control treatment upon soaking the embryonic axes in 5 mM DTT, showing that there was no effect of DTT on hydration of the axes during the soaking process (Figure 2A). In addition, when testing lower DTT concentrations (0.01, 0.1 and 1 mM) this same tendency was observed (data not shown).

Restoration of the aquaporins activity in the embryonic axes soaked for 48 hours in $HgCl_2$ (5 mM) followed by exposure for 24 hours to DTT (5 mM) is shown by resumption of imbibition (Figure 2B). Thus, embryonic axes transferred to DTT and H_2O showed gain in fresh weight of 47% and 14%, respectively, greater than that obtained in the embryonic axes that remained soaking in $HgCl_2$. Significant change was also found in the pattern of expansion of the embryonic axes exposed to $HgCl_2$ (Figure 3).

These changes resulted in reduction in the elongation of the embryonic axis in all the periods evaluated, especially after 72 h of soaking in the $HgCl_2$ solution (Figure 4A). Upon transferring

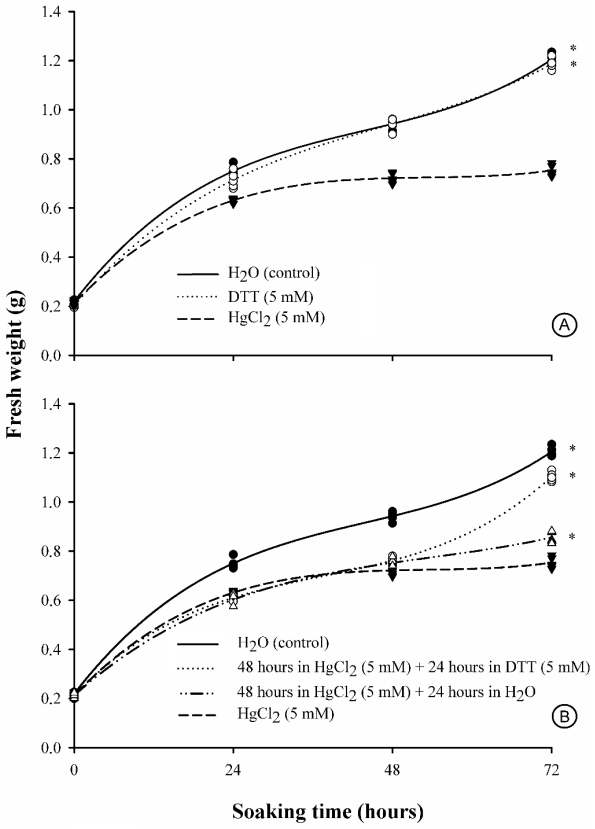

Figure 2 – Hydration curves of the embryonic axes of *S. parahyba* over the period of 72 hours of soaking. (A) Inhibition of aquaporins activity and (B) reversal of inhibition of aquaporins activity. * represents difference of equations in relation to the axes soaked in $HgCl_2$, at a significance level of 5% probability.

Figura 2 – *Curvas de hidratação dos eixos embrionários de* ***S. parahyba*** *durante o período de 72 horas de embebição. Inibição da atividade das aquaporinas (A) e reversão da inibição da atividade das aquaporinas (B). * representa a diferença de equações em relação aos eixos embebidos em $HgCl_2$, a um nível de significância de 5%* de probabilidade.

the embryonic axes kept in $HgCl_2$ solution to the DTT and H_2O solution, significant resumption in elongation of the embryonic axis was observed (Figure 4B) after 24 hours of exposure, achieving values 44% and 9% greater, respectively, than those that remained soaking in $HgCl_2$.

Upon evaluating the integrity of the DNA in the embryonic axes soaked in water, 5 mM $HgCl_2$, or in DTT, degradation was not observed over the period of 72 hours of soaking (Figure 5), maintaining the same pattern in the treatments in which the reversal of inhibition was evaluated (Figure 5)

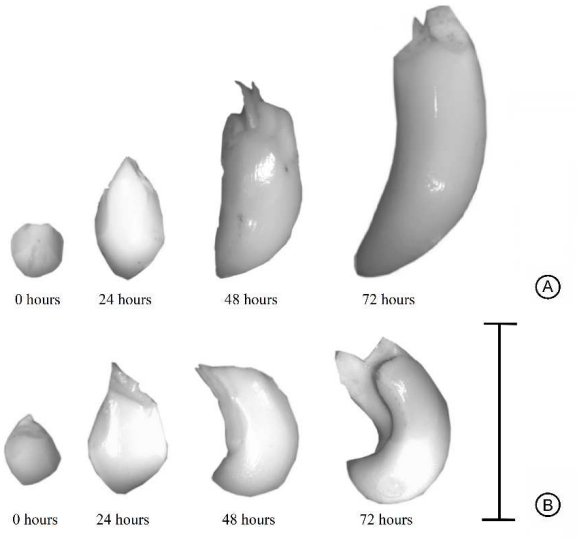

Figure 3 – Growth of the embryonic axes of *S. parahyba* over the period of 72 hours of soaking in water (A) and 5 mM $HgCl_2$ (B). Scale bar: 1 cm.

Figura 3 – Crescimento dos eixos embrionários de ***S. parahyba*** durante o período de 72 horas de embebição em água (A) e 5 mM de $HgCl_2$ (B). Barra de escala: 1 cm.

4. DISCUSSION

The moisture content (MC) of *S. parahyba* seeds is normally under 10%, according to data of Ferreira, et al. (2007), who found values of 8.2% and 7.8%, and by Cherobini, et al. (2010), who obtained values of 8.2, 6.3, and 7.1% in an evaluation of three seed lots. Studies have shown that *S. parahyba* seeds have high viability after dispersion, achieving germination percentages higher than 85% when dormancy is broken (Ferreira, et al., 2007; Magalhães, et al., 2010). In this study, the seeds had 95% germination after mechanical scarification, beginning root protrusion on the seventh day.

Concentrations of $HgCl_2$ up to 1 mM proved to have no effect on the imbibition process of the embryonic axes, indicating that significant damage to the aquaporins that would result in blocking the passage of water did not occur (Figure 1). It is possible that at lower concentrations the Hg is not enough to inhibit all the aquaporins in the plasmatic membranes and the tonoplast. In addition, part of the Hg could be distributed and adsorbed in the outermost cell layers, not affecting the activity of enzymes that act in expansion of the axis or even not inhibiting aerobic metabolism. Thus, imbibition related to aquaporins activity would not be impeded in a detectable manner.

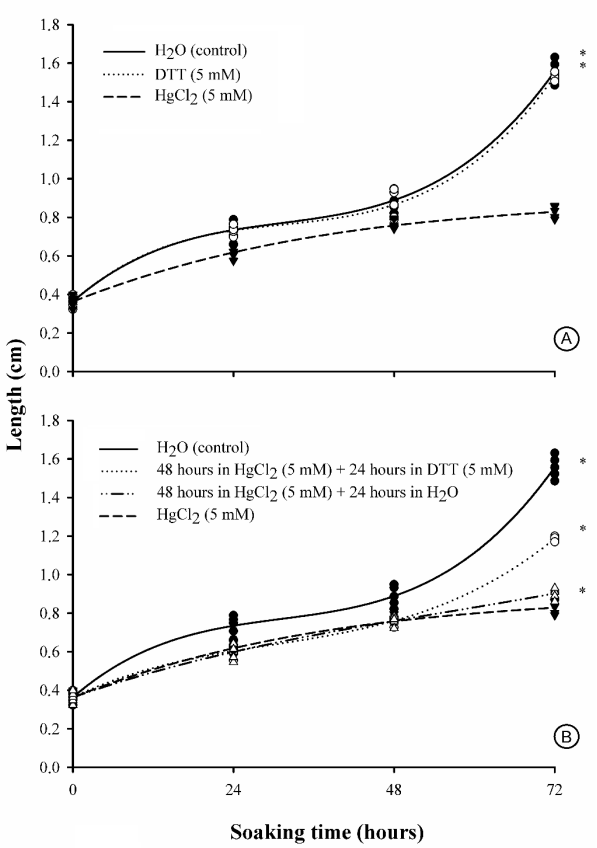

Figure 4 – Elongation of the embryonic axes of *S. parahyba* over the period of 72 hours of soaking. (A) Inhibition of aquaporins activity and (B) reversal of inhibition of aquaporins activity. * represents difference of equations in relation to the axes soaked in $HgCl_2$, at a significance level of 5% probability.

Figura 4 – *Elongamento dos eixos embrionários de* ***S. parahyba*** *durante o período de 72 horas de embebição. Inibição da atividade das aquaporinas (A) e reversão da inibição da atividade das aquaporinas (B). * representa a diferença de equações em relação aos eixos embebidos em $HgCl_2$, a um nível de significância de 5% de probabilidade.*

At higher concentration (5 mM), however, inhibition of aquaporins activity was observed, with reduction in fresh weight gain (Figures 1 and 2) and in elongation of the embryonic axis of *S. parahyba* (Figures 3 and 4) were observed after 6 hours of soaking. Such results indicate that water transport through the aquaporins during phase I of imbibition was seriously affected by the presence of Hg. Similar results were found in *Plathymenia reticulata* seeds

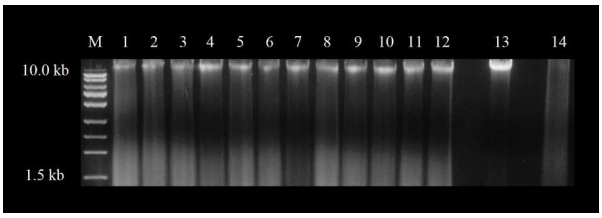

Figure 5 – Integrity of the chromosomal DNA in the embryonic axes of *S. parahyba* during inhibition and reversal of inhibition of aquaporins activity. M - marker, 1 - embryonic axes not soaked, 2 - 24 hours in H_2O (control), 3 - 48 hours in H_2O (control), 4 - 72 hours in H_2O (control), 5 - 24 hours in $HgCl_2$ (5 mM), 6 - 48 hours in $HgCl_2$ (5 mM), 7 - 72 hours in $HgCl_2$ (5 mM), 8 24 hours in DTT (5 mM), 9 - 48 hours in DTT (5 mM), 10 - 72 hours in DTT (5 mM), 11 - 48 hours in $HgCl_2$ (5 mM) + 24 hours in DTT (5 mM); 12 - 48 hours in $HgCl_2$ (5 mM) + 24 hours in H_2O, 13 - positive control (*Copaifera langsdorffii* leaves), 14 - positive control (dead embryonic axes of *S. parahyba*, after two minutes in water at 98 ºC).

***Figura 5** – Integridade do DNA cromossômico nos eixos embrionários de **S. parahyba** durante a inibição e reversão da inibição da atividade das aquaporinas. M - marcador, 1 - eixos embrionários não embebidos, 2 - 24 horas em H_2O (controle), 3 - 48 horas em H_2O (controle), 4 - 72 horas em H_2O (controle), 5 - 24 horas em $HgCl_2$ (5 mM), 6 - 48 horas em $HgCl_2$ (5 mM), 7 - 72 horas em $HgCl_2$ (5 mM), 8 - 24 horas em DTT (5 mM), 9 - 48 horas em DTT (5 mM), 10 - 72 horas em DTT (5 mM), 11 - 48 horas em $HgCl_2$ (5 mM) + 24 horas em DTT (5 mM), 12 - 48 horas em $HgCl_2$ (5 mM) + 24 horas em H_2O, 13 - controle positivo (folhas de **Copaifera langsdorffii**), 14 - controle positivo (eixos embrionários mortos de S. parahyba, após dois minutos em água a 98 ºC).*

(Cardoso, et al., 2015) and embryonic axes of *Aesculus hippocastanum* (Obroucheva, et al., 2012) and *Vicia faba* (Novikova, et al., 2014), where hydration kinetics were inhibited by $HgCl_2$ in the initial hours of imbibition.

Studies show that seeds of different plant species vary widely in terms of response to soaking time and to concentrations of $HgCl_2$ in the medium, which is related to whether aquaporins activity is inhibited or not. In *Solanum esculentum* seeds, imbibition was affected after 12 hours of exposure to 10, 20, and 30 µM $HgCl_2$ (Jain, et al., 2008), whereas in embryonic axes of *Aesculus hippocastanum* (Obroucheva, et al., 2012) and *Vicia faba* (Novikova, et al., 2014), hydration decreased after 15 hours of soaking at 0.75 and 0.5 mM $HgCl_2$, respectively.

The toxic action of Hg on the protein structures occurs predominantly by oxidation of the thiol groups, resulting in modification of the three-dimensional shape of the aquaporins and, consequently, in reduction in water transport (Daniels, et al., 1996; Tyerman, et al., 1999; Savage and Stroud, 2007; Aroca, et al., 2012). The operability of these structures, however, can be reestablished through reducing agents, such as DTT, which acts to protect and reduce the sulfhydryl groups, allowing resumption of water transport (Maurel, et al., 1993; Javot, et al., 2002; Jain, et al., 2008). Thus, the results obtained in *S. Parahyba* indicate that DTT was able to reestablish aquaporins activity in the embryonic axes during the germination process. Such results indicate that resumption of water uptake as a result of restoration of aquaporins activity was sufficient to modify cell turgor pressure and promote expansion of embryonic axes.

The use of DTT to unblock aquaporins inhibited by $HgCl_2$ during the germination process has already been shown in *Aesculus hippocastanum* and *Vicia faba* from results obtained after 15 hours of exposure to DTT (Obroucheva, et al., 2012; Novikova, et al., 2014). When DTT was used simultaneously with $HgCl_2$ (both 1 mM) in *Plathymenia reticulata* seeds, it was found that there was no inhibition of aquaporins activity during the germination process (Cardoso, et al., 2015).

Cell expansion is assisted by the combination of increase in cell turgor pressure, due to resumption of water uptake, and by reduction in the retaining forces in cell walls (Nonogaki, et al., 2010; Steinbrecher and Leubner-Metzger, 2017). Since they are involved in water transport during the germination process, aquaporins also play a crucial role in cell expansion. This participation was clear in the results obtained in embryonic axes of *S. parahyba*, soaked in 5 mM $HgCl_2$ up to 72 hours, with significant reduction in elongation (Figure 4A). Results that show the role of aquaporins in the seed germination process were also observed in *Arabidopsis thaliana* seeds, in which elongation of the embryonic axis during the germination process was less in seeds soaked in 5 µM $HgCl_2$ compared to seeds soaked in water (Willigen, et al., 2006).

Aesculus hippocastanum seeds (Obroucheva, et al., 2012) soaked in 0.5 mM $HgCl_2$ solution did not undergo a reduction in elongation of the embryonic axis. However, the solution brought about reversible delays in the initial growth of the embryonic axes after root protrusion. Likewise, in seeds of *Vigna radiata*

(Jagatheeswari and Ranganathan, 2012), *Platanus occidentalis*, *Pinus echinata* and *Pinus taeda* (Jean-Philippe, et al., 2012), *Linum usitatissimum* (Jain, 2013), and *Plathymenia reticulata* (Cardoso, et al., 2015), root protrusion was also negatively affected upon exposing seeds to mercury compounds during the germination process.

During the germination process, the activation of cellular metabolism induces a series of events, such as DNA repair and synthesis, both crucial for seed germination and initial seedling growth (Nonogaki, et al., 2010). Under stress conditions, such as the presence of heavy metals, cell metabolism induces high production of reactive oxygen species that can promote DNA cleavage, modifying from 10,000 to 100,000 base pairs in a single cell per day (Kranner and Colville, 2011; Štolfa, et al., 2015). The embryonic axes of *S. parahyba* exposed to Hg, however, did not exhibit damage to DNA, which may be a result of the effective action of the cell antioxidant system, promoting elimination or reduction in the concentrations of reactive oxygen species.

5. CONCLUSION

We conclude that the exposure time and the concentration of $HgCl_2$ were not sufficient to induce damage to DNA, but were able to reduce gains in fresh weight and elongation of the embryonic axis during germination, possibly by toxic action on aquaporins activity. This effect was partially reversed using agents that protect the thiol groups, such as DTT.

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