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Germination of Seeds of *Melanoxylon brauna* Schott. under Heat Stress: Production of Reactive Oxygen Species and Antioxidant Activity

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Received: 9 August 2017; Accepted: 9 October 2017; Published: 25 October 2017

Abstract: In this article, the authors aimed to analyze the physiological and biochemical alterations in *Melanoxylon brauna* seeds subjected to heat stress. For this, seed germination, electric conductivity (EC), the production of reactive oxygen species (ROS), and the activity of antioxidant enzymes were assessed. Seeds were incubated at constant temperatures of 25, 35, and 45 °C. Independent samples were first incubated at 35 and 45 °C and then transferred to 25 °C after the intervals of 24, 48, 72, and 96 h. To evaluate EC, seeds were soaked for 0, 24, 48, and 72 h, at 25, 35, and 45 °C and then transferred to Erlenmeyer flasks containing 75 mL of deionized water at 25 °C, for 24 h. ROS production and enzyme activity were assessed every 24 h in seeds soaked at the aforementioned temperatures. Germination did not occur at 45 °C. Seeds soaked at 35 °C for 72 h and then transferred to 25 °C showed higher percentages of germination and a higher germination speed. Seed soaking at 45 °C increased peroxide production, which compromised the antioxidant enzyme system due to a reduction in the activity of enzymes APX, POX, and CAT, thus ultimately also compromising the cell membrane system.

Keywords: climate change; seeds; physiological quality; antioxidant enzymes

1. Introduction

The projections from the Brazilian Panel on Climate Change show that the global temperature will increase throughout the century. Such change might range from 1 to 5 °C until the end of this time period [1]. Considering the possibility of this temperature increase in the next years, the following questions remain: how will species adapt to such change and how can we interfere so that they do not disappear?

*Melanoxylon brauna (*Fabaceae-Caesalpinioideae), also known as brauna, is a native species to the Atlantic Forest, occurring in the Brazilian states of Bahia, São Paulo, Minas Gerais, Espírito Santo, Pará, and Rio de Janeiro [2]. The wood species is dense and highly used in the sailing industry, as well as in construction and the manufacture of light poles and furniture [3]. The species also has ornamental features, being used in afforestation and landscaping projects, as well as in folk medicine [2,4].

Brauna is currently included in the "Official List of Species from the Brazilian Flora Threatened with Extinction", under the 'vulnerable' category, according to the Brazilian Ministry of Environment [5]. In view of these factors, studies approaching seed physiology and germination represent starting points for the development of new strategies to preserve the brauna species [6].

Seed germination is influenced by environmental factors such as temperature, which can be manipulated to optimize the percentage, speed, and uniformity of germination, resulting in more vigorous seedlings and lower production costs [7,8]. Temperature affects water absorption by the seed and the biochemical reactions that regulate the entire seed metabolic process [9]. The temperature range in which germination occurs varies amongst species, and thus each species may have a base and an optimal germination temperature. Generally, the range of 20 to 30 °C is adequate for the germination of many subtropical and tropical species [10–12]. In brauna, for instance, the range between 25 and 30 °C is considered optimal for seed germination [13].

Heat stress increases the production and accumulation of reactive oxygen species (ROS) in seeds [14]. ROS include free radicals such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and molecules that are not considered free radicals, like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [15]. ROS are formed either due to excess energy in plants, specifically in chloroplasts, mitochondria, and plasma membranes; or as byproducts of metabolic pathways in different cell compartments [16]. Excess ROS are highly damaging, and when the levels of these molecules exceed the capacity of the defense mechanisms which scavenge them, cells undergo oxidative stress [17].

Plant cells have efficient enzymatic mechanisms for ROS removal, which enables them to remain undamaged by intoxication. Temperature affects the removal capacity of ROS, as it determines the activation and action of the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX), which are the main responsible agents for ROS scavenging [18].

Considering the ecological and economic importance of brauna and the influence of environmental conditions on seed germination, we aimed to evaluate the physiological and biochemical alterations that occur during the germination of *Melanoxylon brauna* seeds subjected to heat stress.

2. Materials and Methods

The experiments were performed between February and August 2016. *Melanoxylon brauna* fruits were collected in the Leopoldina municipality, in the State of Minas Gerais, southeastern Brazil (21°31′55″ S and 42°38′35″ W), in September 2015. Fruits were dried in the sun until opening and seeds were then extracted manually.

Seeds were incubated in water, in petri dishes, at 25, 35, and 45 °C under constant light. Another test was performed aiming to evaluate possible damage to the seeds after exposure to stressful temperatures. For that, independent samples were first incubated at 35 and 45 °C under the same previously described conditions, and then transferred to 25 °C after 24, 48, 72, and 96 h, after which they were evaluated for the germination percentage and germination speed index (GSI).

Seeds were considered germinated when the primary root emerged. GSI was calculated by Maguire's equation [19], with replicates of 20 seeds per treatment.

To evaluate electric conductivity (EC), seeds were soaked for 0, 24, 48, and 72 h at 25, 35, and 45 °C and then transferred to Erlenmeyer flasks containing 75 mL of deionized water at 25 °C for 24 h. EC of the solution was determined by a MICRONAL conductivity-meter, as described by Woodstock [20]. The variable was assessed in five replicates of 20 seeds and the results were expressed in $\mu S\ cm^{-1}\ seed^{-1}$.

The effect of temperature on ROS production, lipid peroxidation, and enzyme activity was evaluated throughout germination. The analyses were performed on the embryo axis of seeds soaked for 0, 24, 48, and 72 h at 25, 35, and 45 °C.

Superoxide was analyzed as described by Mohammadi and Kar [21]. Superoxide anion production was evaluated by determining the amount of accumulated adrenochrome [22], using a coefficient of molar absorptivity of $4.0 \times 10^3\ M^{-1}$ [23].

Samples of 50 mg of embryonic axis and micropylar endosperm used to quantify hydrogen peroxide were crushed and homogenized in 2.0 mL of 50 mM potassium phosphate buffer, followed by centrifugation at 8400× *g* for 15 min at 4 °C, after which the supernatant was collected [24]. Aliquots of 100 µL of the supernatant were added to the reaction medium, which consisted of 250 µM ferrous ammonium sulfate, 25 mM sulfuric acid, 250 µM xylenol orange, and 100 mM sorbitol, in a final volume

of 2 mL [25]. The mixture was then homogenized and kept in the dark for 30 min. Absorbance was determined by a spectrophotometer at 560 nm. Contents of H_2O_2 were quantified based on the calibration curve, using the peroxide concentration as a standard. Plant extracts were obtained from samples while analytical blanks were prepared in parallel.

Lipid peroxidation was evaluated by determining the TBA (thiobarbituric acid) concentration [26]. The results were expressed as mg MDA g^{-1} FW, after absorbance conversion [27]. Three replicates were used per treatment.

To evaluate enzyme activity, seeds were soaked at 25, 35, and 45 °C as previously described in the germination section, and samples were collected from seeds every 24 h. The embryonic axis was extracted, frozen in liquid nitrogen, and lyophilized. These samples were stored in a freezer (−20 °C) until analysis.

The enzyme extracts used to determine the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were obtained following the method described by Hodges [28], with adaptations. Samples of 50 mg were crushed and homogenized with 2.0 mL of a solution of 50 mM phosphate buffer pH 7.8 and 1% (*w/v*) polyvinylpolypyrrolidone (PVPP). Then, the extract was centrifuged at 19,000 *g* for 30 min at 4 °C and the supernatant was used as an enzyme extract. The entire procedure was conducted at 4 °C.

SOD activity: Superoxide dismutase activity was determined by an assay using 30 µL of extract and 2.97 mL of a reaction mixture comprised of 1500 µL of 100 mM phosphate buffer pH 7.5, 780 µL of 50 mM methionine, 225 µL of 1 mM p-nitro blue tetrazolium (NBT), 60 µL of 5 mM EDTA, 60 µL of 2 µM riboflavin, and 345 µL of distilled water [29]. The reaction was conducted at 25 °C in a reaction chamber under fluorescent light (15 W). After five min of light exposure, the blue formazan produced by NBT photoreduction was measured at 560 nm and the reading obtained at 560 nm was retrieved from the illuminated sample [30]. The absorbance at 560 nm of a reaction mixture equal to the other one, yet which was kept in the dark for an equal period, was used as the control. One SOD unit was defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50% [31].

APX activity: Ascorbate peroxidase activity was determined by an assay adapted from Ramalheiro [32], using 100 µL of enzyme extract and 1400 µL of a reaction mixture comprised of 700 µL of 50 mM phosphate buffer pH 7.8, 400 µL of 0.25 mM ascorbic acid containing 0.1 mM EDTA, and 300 µL of 0.3 mM H_2O_2. Enzyme activity was calculated based on the molar extinction coefficient of 2.8 mM^{-1} cm^{-1} [33]. One activity unity (U) was defined as the amount of enzyme needed to convert 1 nmol of substrate into product per min, per mL, under the assay conditions.

CAT activity: Catalase activity was determined by an assay adapted from Hodges et al. [34], using 100 µL of enzyme extract and 1400 µL of a reaction mixture constituted by 900 µL of 50 mM phosphate buffer pH 7.8 and 500 µL of 0.97 M H_2O_2. Enzyme activity was calculated using the molar extinction coefficient of 36 M^{-1} cm^{-1} [35]. One activity unit was defined as the amount of enzyme needed to convert 1 µmol of substrate into product per min, per mL, under the assay conditions.

POX activity: Peroxidase activity was determined by adding 30 µL of crude enzyme extract to 2.97 mL of a reaction mixture constituted by 25 mM potassium phosphate buffer pH 6.8, 20 mM pyrogallol, and 20 mM H_2O_2 [36]. Purpurogallin production was determined in a spectrophotometer by the increase in absorbance at 420 nm, at 25 °C, until the second minute of the reaction. Enzyme activity was calculated using the molar extinction coefficient of 2.47 mM^{-1} cm^{-1} [37].

Enzyme activities were expressed as specific activity (SOD: U SOD min^{-1} mg $protein^{-1}$; APX: nmol Asc min^{-1} µg $protein^{-1}$; CAT: µmol H_2O_2 min^{-1} mg $protein^{-1}$; POX: µmol min^{-1} mg $protein^{-1}$).

The protein concentration in samples was determined by the Bradford method [38], with a standard curve constructed using bovine serum albumin (BSA) at 2.5 to 50 µg protein.

For all determinations, the statistical design was entirely randomized with five replicates. The data of germination was submitted to a variance analysis using the SAS statistical software (version 9.2; SAS Institute, Inc., Cary, NC, USA) [39] and the averages obtained for the treatments were compared by the Tukey test as a 5% significance. The data of EC, ROS, and enzyme activity were submitted to a regression analysis ($p < 0.05$).

Pearson correlation analysis was performed (SAS statistical software (version 9.2; SAS Institute, Inc., Cary, NC, USA)) [39] on the evaluated variables. The results were interpreted as suggested by Mukaka [40], under the following criteria: a correlation coefficient of 0.9 to 1.0 (positive or negative) indicates strong correlation (***), of 0.7 to 0.9 (positive or negative) indicates high correlation (**), of 0.5 to 0.7 (positive or negative) indicates moderate correlation (*), of 0.3 to 0.5 (positive or negative) indicates low correlation, and of 0 to 0.3 (positive or negative) indicates negligible correlation.

3. Results

3.1. Germination and GSI

A significant difference was detected between the mean values of germination as a function of temperature. In general, seeds incubated at 35 °C for 24 and 72 h and then transferred to 25 °C and seeds incubated constantly at 25 °C showed higher germination values: 88%, 84%, and 83%, respectively. However, constant incubation at 45 °C caused seed death. Moreover, temperature increase prior to the transfer of seeds to 25 °C caused a loss of vigor. A significant difference in GSI was also observed. The soaking of seeds at 35 °C for 24, 48, and 72 h followed by their ulterior transfer to 25 °C favored germination speed (Figure 1). After a 96 h soaking at 45 °C, all seeds died.

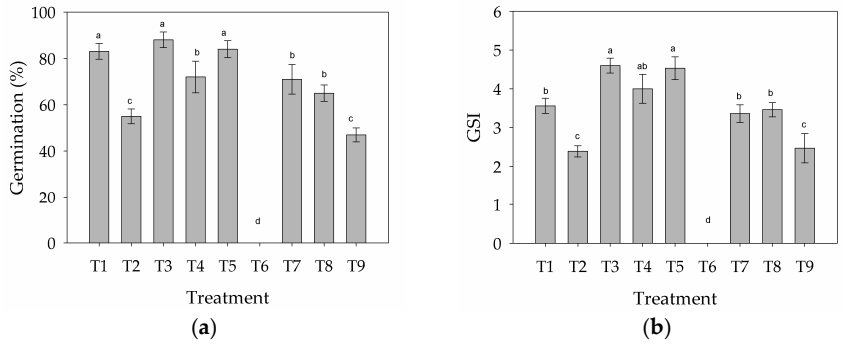

Figure 1. Germination percentage (**a**) and germination speed index (**b**) of *Melanoxylon brauna* seeds under different temperatures (T1: 25 °C; T2: 35 °C; T3: 35 °C/24 h; T4: 35 °C/48 h; T5: 35 °C/72 h; T6: 45 °C; T7: 45 °C/24 h; T8: 45 °C/48 h; T9: 45 °C/72 h). Vertical bars = ±SE, n = 5.

3.2. Eletric Condutivity

The interaction between temperature and soaking time was significant for EC, being highest at the highest temperature (Figure 2). EC at 25 °C only differed from that at 35 °C with a 72 h soaking period, with the former showing a clear decrease.

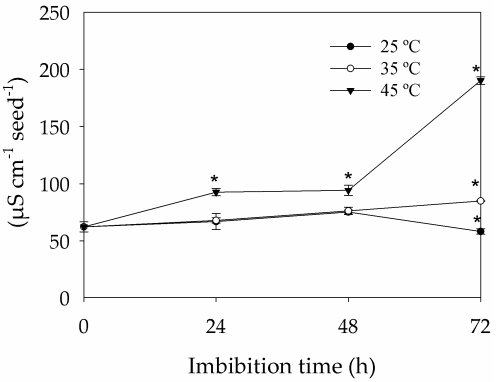

Figure 2. Electric conductivity in *Melanoxylon brauna* seeds under different temperatures and soaking periods. * Indicates statistical difference between means. Vertical bars = ±SE, n = 5.

3.3. Superoxide Anion and Hydrogen Peroxide

Superoxide anion was not detected by the adopted method under the tested conditions. The H_2O_2 concentration decreased during the first 24 h of soaking and increased from 48 h. At 45 °C, the embryonic axis and micropylar endosperm showed the highest H_2O_2 levels at all soaking times (Figure 3). At 25 and 35 °C, no difference in peroxide concentration in the embryonic axis was observed after any of the analyzed soaking times.

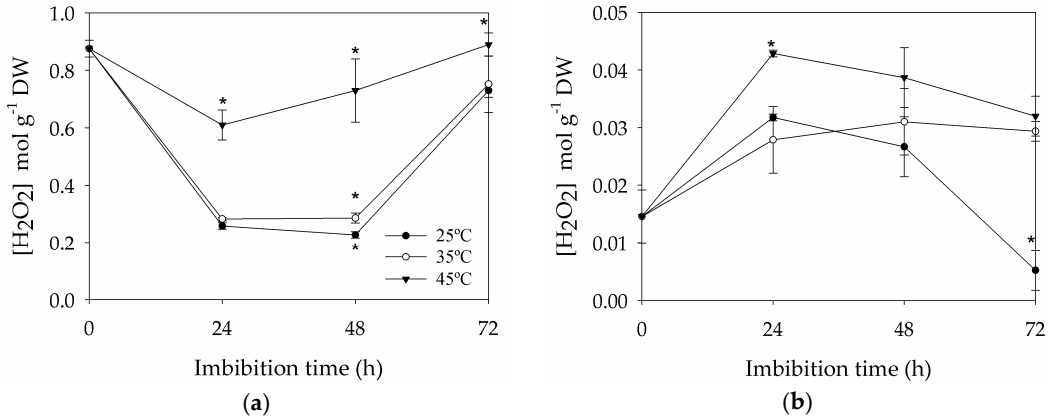

Figure 3. Hydrogen peroxide concentration in the embryonic axis (**a**) and micropylar endosperm (**b**) of *Melanoxylon brauna* seeds soaked at 25, 35, and 45 °C. * Indicates statistical difference between means. Vertical bars = ±SE, n = 5.

3.4. Lipid Peroxidation

Lipid peroxidation at 25 °C showed a decrease in the first 48 h of soaking, followed by an increase. At 35 and 45 °C, peroxidation increased during the first 24 h, with posterior reduction. The highest peroxidation levels were observed at 45 °C (Figure 4).

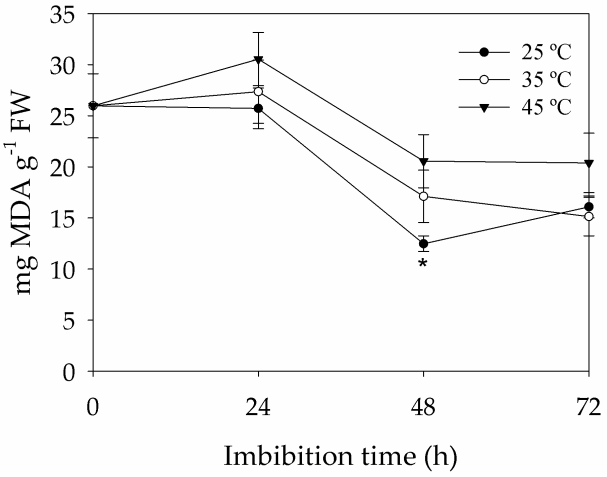

Figure 4. Malondialdehyde (MDA) concentration in *Melanoxylon brauna* seeds soaked at 25, 35, and 45 °C. * Indicates statistical difference between means. Vertical bars = ± SE, n = 5.

3.5. Specific Activity of Antioxidant Enzymes

A significant interaction was detected among the specific activity of enzymes APX, POX, SOD, and CAT at the different temperatures and soaking times, indicating that both of these factors influenced enzyme activity in the embryo axis during the germination of brauna seeds.

The highest values of SOD activity occurred in seeds subjected to 45 °C. At all temperatures, enzyme activity decreased after 48 h of soaking (Figure 5a). The differences between the two other temperatures were small, and such differences might have occurred due to a sampling effect. It is worth noting that the seeds showed a wide range of maturation levels during the harvest period.

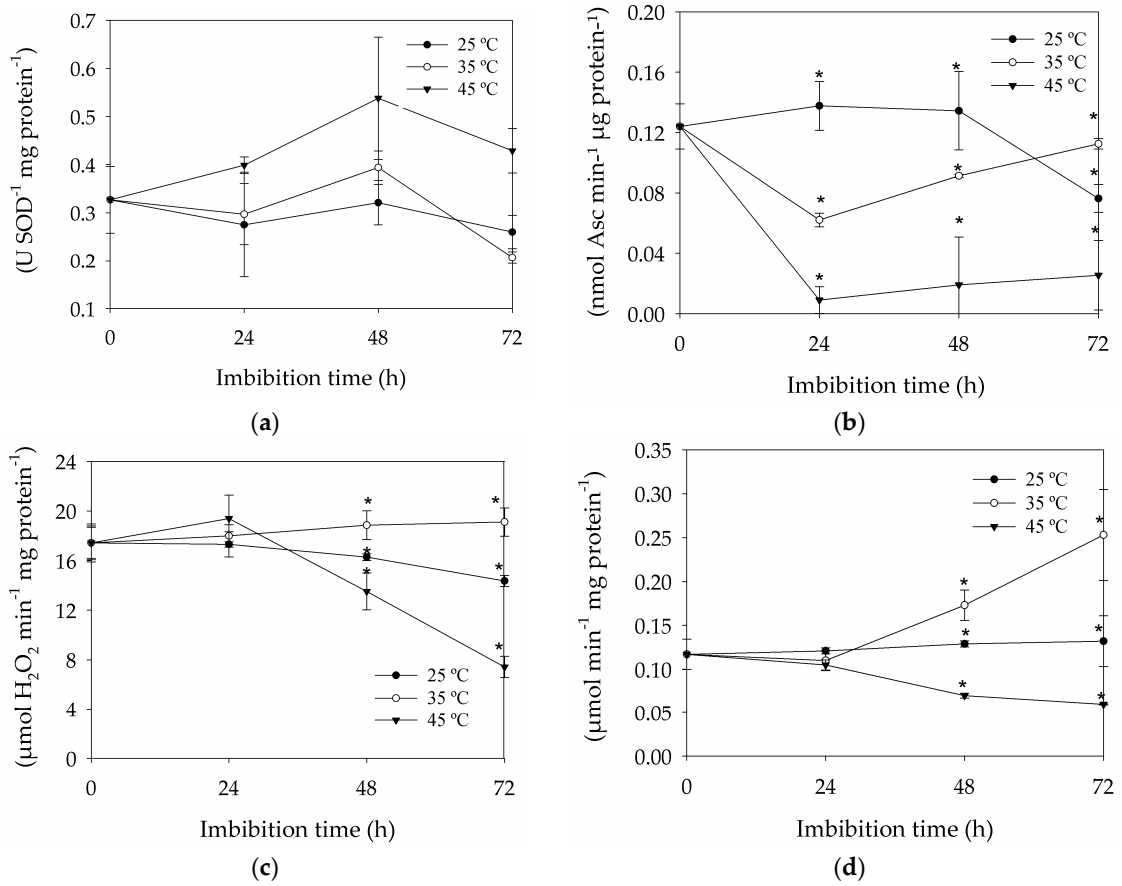

Figure 5. Specific activities of enzymes superoxide dismutase (SOD) (**a**), ascorbate peroxidase (APX) (**b**), catalase (CAT) (**c**), and peroxidase (POX) (**d**) in the embryonic axis of *Melanoxylon brauna* seeds during the germination period, after soaking at 25, 35, and 45 °C. * Mean statistical difference between means. Vertical bars = ±SE, n = 5.

Regarding APX specific activity at 25 °C, a slight increase was detected during the first 24 h, followed by a decrease. At 35 and 45 °C, a decrease in enzyme activity was observed during the first 24 h. After that period, an increase was observed at both temperatures, but more intensely in seeds incubated at 35 °C (Figure 5b).

CAT activity in the embryonic axis decreased after 24 h of soaking at 25 and 45 °C. At 35 °C, a small increase in enzyme activity was observed in the embryo during seed hydration. The differences in CAT activity among the three soaking temperatures were clearly highest after 72 h of hydration (Figure 5c).

POX activity was constant at 25 °C, but at 35 °C, it increased after 24 h of soaking. The opposite behavior was observed in seeds incubated at 45 °C, in which enzyme activity decreased during soaking (Figure 5d).

Pearson correlation was assessed for the following variables: EC, H_2O_2 concentration in the embryo and micropylar endosperm, and the activity of enzymes POX, SOD, APX, and CAT in *Melanoxylon brauna* seeds during soaking at 25, 35, and 45 °C. The obtained coefficients allowed for detecting significant correlations, both positive and negative, among the evaluated variables at all tested temperatures (Table 1).

Table 1. Pearson correlation coefficients for means of electric conductivity (EC), concentration of hydrogen peroxide in the embryo and micropylar endosperm, and activity of the enzymes peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) in *Melanoxylon brauna* seeds germinated after soaking at 25, 35, and 45 °C.

Temperature (°C)		Electric Conductivity	$[H_2O_2]$ Embryo
25	$[H_2O_2]$ embryo	−0.82 **	
	$[H_2O_2]$ micropyle		−0.85 **
	POX	0.06	−0.22
	SOD	0.51	0.05
	APX	0.75 **	−0.59 *
	CAT	0.38	−0.19
35	$[H_2O_2]$ embryo	−0.06	
	$[H_2O_2]$ micropyle		−0.70 **
	POX	0.95 ***	0.24
	SOD	−0.47	−0.47
	APX	0.07	0.90 ***
	CAT	0.97 ***	−0.26
45	$[H_2O_2]$ embryo	0.36	
	$[H_2O_2]$ micropyle		−0.78 **
	POX	−0.80 **	−0.21
	SOD	0.25	−0.29
	APX	−0.48	0.60 *
	CAT	−0.88 **	−0.61 *

* Moderate correlation (correlation coefficient of 0.5 to 0.7 (positive or negative)); ** high correlation (correlation coefficient of 0.7 to 0.9 (positive or negative)); *** strong correlation (correlation coefficient of 0.9 to 1.0 (positive or negative)) (following Mukaka [37]).

4. Discussion

The observed variation in the germination percentages of brauna seeds with different temperatures is in accordance with what was described by Flores et al. [12]. These authors verified that germination in this species occurs between 12.3 and 42.5 °C and that 27 °C is the optimal temperature for germination. Similar to what we observed in the present study, they also verified that no germination occurs at 45 °C, and increasing the soaking time of seeds at this temperature and then transferring them to 25 °C reduces seed germination potential.

The soaking of seeds at 35 °C for 24, 48, and 72 h followed by their transfer to 25 °C yielded higher percentages of germination and GSI. Studies on the germination physiology of other species indicate that temperatures near 35 °C provide higher GSI values than those obtained at 25 °C, even when the latter temperature yields higher germination percentages. The appropriate temperature for germination is different from the appropriate temperature for germination speed [41]. The same pattern has been observed in *Torresia acreana* and *Cecropia glaziovii* seeds. This phenomenon occurs because water absorption and biochemical reactions occur more quickly at higher temperatures [42,43].

Besides being a determining factor of seed germination, temperature affects the EC of seeds during soaking. The EC value is associated with the number of leaked electrolytes in the solution, thus being directly related to the integrity of the cell membrane. Thus, high EC values indicate a high leakage of solutes due to alteration in the integrity of the cell membranes, and thereby represent reduced seed vigor. Consequently, EC has been proposed as a parameter to be used in the assessment of seed physiological quality [44].

The observed EC values in brauna seeds incubated at 45 °C were higher than those from seeds incubated at 25 and 35 °C. This indicates that the damage to cell membranes was higher in seeds soaked at 45 °C. At elevated temperatures, membrane selective permeability is lost due to the inability of the membrane to resume its functions because of the disorganization of the lipid bilayer [13].

During soaking, there is an increase in H_2O_2 concentration in the micropylar endosperm during the first 24 h (Figure 3). Such an increase is due to the resumption of respiration. Increased amounts of H_2O_2 leads to the weakening of the wall of seed coat cells, thus enhancing germination. The roles that ROS play in plants also include cell signaling, the promotion of programed cell death, and an increase in the expression of genes that encode oxidative stress enzymes. However, at elevated concentrations, these free radicals may attack the cell membrane system, causing its disruption [45,46].

Regarding ROS production, our results showed that under the stress conditions of elevated temperatures, there is an increased production of H_2O_2. The increased concentration of this molecule might lead to the occurrence of lipid peroxidation and to an ulterior disruption of the cell membrane, as indicated by the increased leakage at 45 °C, which caused a gradual decrease in seed viability. Similar results were found in *Dalbergia nigra* seeds, which showed a gradual loss in viability at 45 °C [18]. With increasing stress, the formation of ROS is intensified, and their elimination must be constant to avoid oxidative stress. Therefore, the synchronized action of enzymes responsible for ROS removal provides a higher stress tolerance to plants subjected to elevated temperatures.

The higher levels of H_2O_2 at 35 and 45 °C led to an increased production of malondialdehyde, which in turn is an indicator of high rates of lipid peroxidation. An increased peroxidation of lipids, mediated by free radicals and peroxides, is a possible reason for the loss of viability in seeds soaked at 45 °C.

Superoxide dismutase (SOD), the first enzyme of the antioxidant system to act, doing so by dismutating superoxide radical (O_2^-) into H_2O_2, showed low activity. However, such activity was higher in seeds soaked at 45 °C, indicating a possible detoxification. This is one of the possible explanations for the higher H_2O_2 concentration detected at that temperature. Our results are in accordance with what was reported by Flores et al. [13], who observed increased SOD activity in brauna seeds subjected to high temperatures. The behavior of SOD at 25 °C is similar to that found in *Picea omorika* seeds, in which enzyme activity remained constant during germination at 25 °C [47]. Similar results have also been reported for SOD activity in *Medicago sativa* seeds, which showed constant behavior at 22 °C [48]. During the soaking of *Dalbergia nigra* seeds, SOD activity is higher at 45 °C than at 25 °C [18].

Nevertheless, Kumar et al. [49] observed an increase in SOD activity until 40 °C in maize and rice genotypes, followed by a decrease after 45 °C. Although the production of SOD is one of the first responses to abiotic stress, the action of this enzyme must not be evaluated individually, since APX and/or CAT, for instance, eliminate H_2O_2, which permeates the membrane easily and is toxic to cells [50]. APX and CAT belong to different classes of antioxidant enzymes due to their different affinities for H_2O_2, in the orders of μM and mM, respectively. While APX is responsible for the refined modulation of ROS for cell signaling, CAT is responsible for removing the excess ROS generated during stress conditions [51,52].

The activity of SOD is stimulated at 45 °C in 48 h, when the highest H_2O_2 concentrations were detected. SOD activity is lower at 25 and 35 °C, indicating that peroxide levels at those temperatures are safe, being sufficiently low as to not be detected by the enzyme.

The activity of the enzymes APX, POX, and CAT decreased at 45 °C, which led to an increased concentration of H_2O_2 and consequent damage to the cell membranes (Figure 2), which ultimately affected seed germination (Figure 1).

There was no correlation between APX activity and H_2O_2 scavenging at 35 °C. The higher activity of this enzyme at 25 °C kept H_2O_2 concentrations at low levels. The decreased enzyme activity after 48 h and increased concentrations of H_2O_2 might be due to the affect of this compound on the weakening reactions of the wall of micropyle cells [45]. On the other hand, APX might also act on different organelles where H_2O_2 is produced at unsafe levels.

APX can scavenge H_2O_2 from cells using ascorbate as an electron donator for the reaction [53]. Sun et al. [54], after evaluating seeds and seedlings of wild plants and of two mutant linages of *Nicotiana tabacum* for the ATtAPX genes, observed that seeds from the wild genotype had lower percentages of germination at 42 °C, thus proving the importance of APX under stress conditions.

Hence, we suggest that APX is dependent on temperatures near the one that is ideal for germination of brauna seeds.

CAT activity at 25 °C was constant during the first 24 h of soaking, with a slight decrease during this period. At 35 °C, the activity of this enzyme increased as a function of soaking time, indicating that CAT acts under pre-stress conditions, avoiding ROS accumulation. However, at 45 °C, a slight decrease in CAT activity occurred after 24 h of soaking. Moreover, at 35 °C, both POX and CAT showed increased activities, especially the former. In the case of POX, the breakdown of storage lipids in peroxisomes results in the increase of H_2O_2 concentration. Additionally, even increased respiration in mitochondria, which results in an increased concentration of H_2O_2 [13], may determine an increase in the activity of both CAT and POX at 35 °C.

These results demonstrate that CAT has an important role in regulating ROS levels, acting in accordance with other metabolic cycles, such as that of ascorbate/glutathione. At 25 °C, APX is apparently responsible for H_2O_2 degradation, since at that temperature, neither CAT nor POX is important in such a process. This is justified by the fact that CAT acts at elevated H_2O_2 concentrations, which is not the case during the periods of 24 and 48 h. Despite acting on a micromolar scale, CAT seems to have not been essential in determining the existing H_2O_2 levels, which in turn did not cause major damage to seed cell metabolism.

Our results indicate that the low ROS production and the antioxidant enzyme activity at 25 °C maintained the physiological quality of seeds, thus favoring the occurrence of germination. Pre-soaking at 35 °C followed by posterior transfer to 25 °C increased seed metabolism and did not compromise seed viability, therefore enabling quicker and even more germination. Soaking at 45 °C, which stressed seeds, compromised antioxidant enzyme activity and membrane systems, resulting in increased H_2O_2 production and causing vigor loss and the absence of germination in *Melanoxylon brauna* seeds.

Table 1 shows that EC only had a significant inverse correlation with H_2O_2 concentration in the embryo at 25 °C. In that sense, it seems reasonable to presume that such a correlation is associated with the production of ROS and their impact on cell membrane integrity. An increase in ROS contents would lead to damage to the cell membranes, while a reduction in those contents would not affect the membrane structure. Hence, at 25 °C, the H_2O_2 levels and EC values were safe, thus causing neither oxidation damage nor damage to the membrane system (Figures 2 and 3).

The H_2O_2 concentrations in the micropyle and embryo showed a significant inverse correlation at all temperatures. Peroxide contents increased with leakage from the embryonic axis. Such leakage may possibly act on weakening the micropyle by means of the Fenton reaction [55]. This phenomenon occurs due to the high capacity of H_2O_2 to cross cell membranes, through protein channels that have important physiological roles in the capture, translocation, sequestration, and extrusion of this molecule [56]. Transport of H_2O_2 through protein channels might occur in response to the increased concentration of this ROS in the micropyle [56]. The increase in H_2O_2 concentration from the occurrence of respiratory activity in the embryo during germination is explained by the kinetic features of this ROS. Such features enable the binding of the substrate to aquaporins, due to the molecule dipole moment of 2.26×10^{-18} esu (H_2O_2) vs. 1.85×10^{-18} esu (H_2O), the dielectric constant of 73.1 (H_2O_2) vs. 80.4 (H_2O), a molecular diameter of 0.25–0.28 nm (H_2O_2) vs. 0.275 nm (H_2O), and the capacity to form hydrogen bonds [57]. The role played by H_2O_2 is not restricted to oxidative stress; during seed germination, this ROS is also responsible for softening micropyle tissues and signaling both apoptosis and cell proliferation [55].

Regarding the enzymes, no relevant correlation of SOD, CAT, or POX activity with EC and H_2O_2 levels was detected in the embryo or micropyle at 25 °C. APX showed a strong correlation with H_2O_2 concentration in the micropyle, as well as with EC. Although APX activity was constant during the first 48 h of soaking, such activity was enough to keep H_2O_2 concentrations at safe levels. These results reinforce the hypothesis that APX has its activity potentialized at temperatures near the optimal temperature for germination.

At 35 °C, POX and CAT activities showed a strong correlation with EC, while APX showed a higher correlation with H_2O_2 in the micropylar endosperm (Table 1). Our results indicate an initial oxidative stress and compromise of the membrane system. Thus, the increased enzyme activity at 35 °C enabled the elimination of excess H_2O_2 and the consequent reestablishment of metabolic routes related to seed germination. In general, heat stress becomes evident at 35 °C; however, at that temperature, the antioxidant system and the cell membrane system were not entirely compromised, which explains the occurrence of germination under those conditions (Figure 1).

A strong correlation of POX and CAT activities with EC was observed at 45 °C. APX showed a strong negative correlation with H_2O_2 levels in the embryo (Table 1). The decreased enzyme activity at 45 °C might have compromised the antioxidant system due to heat stress. Consequently, there was a higher accumulation of H_2O_2, the excess of which attacked the cell membrane system, causing its disruption due to increased EC and ultimately leading to seed death.

Unlike existing studies, we tried to analyze the seed tissues separately. By evaluating the behavior of the micropillary region and the embryo as such, we were able to interpret changes in each tissue separately.

5. Conclusions

Soaking at 35 °C for 72 h followed by the transfer to 25 °C favors germination and germination speed in brauna seeds.

At 45 °C, on the other hand, there is an increased production and accumulation of H_2O_2, and the antioxidant system at that temperature is then compromised. Catalase is the enzyme with the highest activity among peroxidases. The temperature of 45 °C had a deleterious effect on the peroxidases, while it was a stimulant for SOD. In general, enzyme activities vary between the temperatures and during the period of germination.

Consequently, EC is significantly increased, as the membrane system of seed cells is compromised with these conditions. As a result, no germination occurs at 45 °C. Additionally, soaking seeds at this temperature leads to seed death.

Acknowledgments: Authors are thankful to the Department of Forest Engineering at the Federal University of Viçosa, FAPEMIG, and CNPq for financing the project and productive scholarship.

Author Contributions: Marcone Santos, Eduardo Borges, Glauciana Ataíde, and Genaína Souza conceived and designed the experiments; Marcone Santos and Glauciana Ataíde performed the experiments; Marcone Santos, Genaína Souza, and Eduardo Borges analyzed the data; Marcone Santos, Glauciana Ataíde, and Genaína Souza wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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