Production of reactive oxygen species in *Dalbergia nigra* seeds under thermal stress[1]

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ABSTRACT – Seed germination is dependent on abiotic factors, temperature being one of the main ones, whose influence causes seed damage under extreme conditions. The aim of the present study was to investigate the effect of different temperatures during germination of *D. nigra* seeds and their physiological and biochemical implications. We assessed germination percentage and production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in seeds subjected to temperatures of 5, 15, 25, 35 and 45 ºC for different periods of time. Hydration is promoted at 45 ºC and inhibited at 5ºC, without germination in either, whereas it is minimal at 15 °C and at a maximum level at 25 °C. Superoxide production increases at higher temperatures (25 and 35 ºC) after 72 hours of hydration, coinciding with the beginning of radicle protrusion. Production of hydrogen peroxide decreases at all temperatures, except for 5 ºC, with values near each other at temperatures of 15, 25, and 35 ºC, where there was radicle protrusion.

Index terms: forest, physiology, biochemistry, rosewood.

Produção de espécies reativas de oxigênio em sementes de *Dalbergia nigra* sob estresse térmico

RESUMO - A germinação de sementes é dependente de fatores abióticos, sendo a temperatura um dos principais, cuja influência, em condições extremas, causa danos às sementes. O presente trabalho teve por objetivo investigar o efeito das diferentes temperaturas durante a germinação de sementes de *D. nigra* e suas implicações fisiológicas e bioquímicas. Avaliaram-se o porcentual de germinação e a produção de ânion superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2) em sementes submetidas às temperaturas de 5, 15, 25, 35 e 45 ºC por diferentes tempos. A hidratação é estimulada a 45 ºC e inibida a 5 ºC, não havendo germinação em ambas, enquanto é mínima a 15 ºC e máxima a 25 ºC. A produção de superóxido aumenta nas temperaturas mais altas (25 e 35 ºC) após 72 horas de hidratação, coincidindo com o início da protrusão radicular. A produção de peróxido de hidrogênio decresce em todas as temperaturas, à exceção da de 5 ºC, com valores próximos entre si nas temperaturas de 15, 25 e 35 ºC, onde houve protrusão radicular.

Termos para indexação: floresta, fisiologia, bioquímica, jacarandá-da-bahia.

Introduction

Dalbergia nigra (Vell.) Fr.All. ex Benth*, also known as rosewood, is a tree species that occurs in different Brazilian states, especially in areas of the Atlantic Forest Formation. Due to intense exploitation and the lack of reforestation programs, this species has been included as vulnerable on the Red List of the International Union for Conservation of Nature since 1998 (IUCN, 2013), with prohibition of its trade since the 1990s (CITES, 2008), as well as being included on the official list of endangered species of Brazilian flora (IBAMA, 2013). It is propagated through seeds and is indicated for programs of recovery of degraded areas, with high potential for sustainable forest management (Lorenzi, 2002).

Knowledge of seed physiology is fundamental. For each species, specific environmental conditions are necessary to ensure germination, as shown by Araújo Neto et al. (2003) and Rego et al. (2009) for the species *Acacia polyphylla* and *Blepharocalyx salicifolius*, respectively. The range of environmental adaptation is related to the cardinal temperatures (minimum, optimum, and maximum) that each species requires for germination, determining the distribution limits of the species (Orozco-Almanza et al., 2003; Borghetti, 2005; Oliveira and Garcia, 2005; Bewley et al., 2013). In relation to

the seeds of native forest species, the thermal range suitable for germination is frequently from 20 to 30 °C (Mello and Barbedo, 2007; Brancalion et al., 2010; Pimenta et al., 2010; Dousseau et al., 2013).

Studies in respect to physiological and biochemical aspects during seed germination of tropical species under abiotic stress conditions, especially thermal stress, are highly relevant in the face of environmental adversities found in tropical ecosystems, and also through the lack of information in regard to the mechanisms involved in seed tolerance to determined levels of stress. Plants under abiotic stress conditions, such as drought, salinity, and high and low temperatures, produce reactive oxygen species (ROSs), as observed by Luo et al. (2011), in which low temperature continually increased the formation of superoxide anion and hydrogen peroxide in leaves of *Fragaria ananassa* Duch., Zoji and Toyonaka cultivars, up to 48 hours, decreasing after that. Airaki et al. (2012) observed an increase in the level of the non-enzymatic antioxidants ascorbate and glutathione and in the activity of the enzyme NADPH dehydrogenase during acclimatization of *Capsicum annum* L. plants, indicating the action of these substances in the cell antioxidant system. Even in storage under low temperature, there is production of superoxide anion and hydrogen peroxide, as observed by Pukacka and Ratajczak (2005) in *Fagus sylvatica* seeds stored at temperatures of 4, 20 and 30 ºC. The loss of viability that occurred in nine weeks under all conditions was related to the increase in the two compounds.

Studies show that the ROSs may not be as harmful to the seed life cycle as previously portrayed, but may have a key role in signaling in response to the different possible stresses during germination (Gomes and Garcia, 2013). The ROSs may be signalers of various biological processes, including responses to biotic and abiotic stresses and programmed cell death (Bailly, 2004; Mittler et al., 2004; Foyer and Noctor, 2005; Fujita et al., 2006). Seeds pass through an infection-sensitive period during germination, and it is believed that the ROSs play an important protective role against attacks from incompatible pathogens (Schopfer et al., 2001; Oracz et al., 2009). Thus, the ROSs play a key role in the seed germination process.

Accordingly, the study of the effect of different temperatures is important, especially those outside of the optimum range for germination, in the germination process and in the production of substances arising from thermal stress, which may result in loss of quality or death of the seeds. Thus, the aim of this study was to assess the germination of *Dalbergia nigra* seeds under thermal stress conditions in association with the production of reactive oxygen species.

Materials and Methods

The seeds were collected in September 2012 in the region of Viçosa, MG, Brazil, processed, and placed in cold storage (5 °C/60% relative humidity-RH).

Percentage of water gain (%) was calculated in relation to the initial seed weight of each treatment. For that purpose, the seeds were placed in a laboratory oven at 105 ± 3 ºC for 24 hours (Brasil, 2009), using three replications of 20 seeds. Calculation was made on a wet basis, with the degree of moisture expressed in percentage.

The seeds were weighed on a digital balance with 0.0001 g precision and subsequently placed for soaking in a Petri dish on two sheets of filter paper moistened with 4.0 mL of distilled water and kept under constant light at the temperatures of 5, 15, 25, 35 and 45 °C. The seeds were weighed at two-hour intervals in the first 12 hours and subsequently weighed at 12-hour intervals until they reached 50% germination or until the tenth day (240 hours) after the beginning of soaking. Before each weighing, the surface of the seeds was dried with absorbent paper, and then they were once more placed in Petri dishes with paper moistened with distilled water. Five replications of 20 seeds were used.

In the germination test, the seeds were immersed in 0.5% Captan® solution for three minutes and placed in Petri dishes on two sheets of paper moistened with 4.0 mL of distilled water and kept in a BOD type germinator at the temperatures of 5, 15, 25, 35 and 45 °C under constant light for 240 hours. Daily assessments were made, with protrusion of the primary root as the criterion of germination. Each treatment (temperature) consisted of five replications of 20 seeds.

The effect of temperature on production of reactive oxygen species (ROSs) was assessed by comparing production levels during germination. Seeds began primary root protrusion at 132 hours of soaking at the temperature of 25 ºC, used as the standard. That way, analyses were made of the embryonic axes of seeds that were soaked for 0 hours (dry seeds) and 24, 72 and 120 hours, which correspond to the end of phase I, and the middle and end of phase II, respectively. The same sample taking times were used for the temperatures of 5, 15 and 35 °C. For the temperature of 45 ºC, samples were taken at 8 and 24 hours since preliminary tests had indicated death of the seeds after 24 hours at that temperature. The seeds were placed under the same conditions of the germination test.

In quantification of superoxide anion, samples of 20 embryonic axes were weighed on a balance with 0.0001 g

precision and cut in half in the transversal direction in two segments and incubated in 2.0 mL of reaction medium consisting of disodium salt of 100 μM Ethylenediamine tetraacetic acid (Na$_2$EDTA), 20 μM β-Nicotinamide adenine dinucleotide reduced (NADH), and 20 mM sodium phosphate buffer, pH 7.8 (Mohammadi and Karr, 2001) in hermetically sealed tubes. The reaction was started by the introduction of 100 μL of 25.2 mM epinephrine in 0.1N HCl, using a chromatography syringe. Samples were incubated at 28 °C, remaining under shaking for 5 minutes. After that, the segments were removed and, as of the seventh minute, reading of absorbance at 480 nm was begun in a Thermo Scientific EVOLUTION 60S spectrophotometer, which was carried out for five minutes. The blank was performed under the same conditions, but without plant tissue. Production of superoxide anion was assessed by determination of the amount of accumulated adrenochrome (Misra and Fridovich, 1971), using the molar absorption coefficient of 4.0 x 10^3 M^{-1} (Boveris, 1984).

For quantification of hydrogen peroxide, samples of 20 embryonic axes were weighed on a 0.0001 g-precision balance, ground in liquid nitrogen, and then homogenized in 2.0 mL of 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM hydroxylamine, followed by centrifugation at 10,000 g for 15 minutes at 4 °C, and the supernatant was collected (Kuo and Kao, 2003).

Aliquots of 100 μL of the supernatant were added to the reaction medium consisting 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 (Gay and Gebicki, 2000), homogenized, and kept in the dark for 30 minutes. Absorbance was determined in a Thermo Scientific EVOLUTION 60S spectrophotometer at 560 nm, and quantification of H$_2$O$_2$ was made based on a calibration curve, using peroxide concentrations as a standard. Blanks for the reagents and plant extracts were prepared in a parallel manner and taken from the sample.

The data were subjected to analysis of variance (ANOVA). For germination, a single-factor completely randomized design (5 temperatures) was used, and subjected to regression analysis.

For ROS production data, a completely randomized design in a 4 x 4 factorial arrangement (5, 15, 25 and 35 °C x 0, 24, 72, and 120 hours) was used, plus 4 additional treatments (45 °C x 0, 8, 24 and 48 hours). The statistical model $Y_{ij}=m+t_i+e_{ij}$ was used for the 20 treatments, with one factorial with 15 degrees of freedom (5, 15, 25 and 35 °C x 0, 24, 72 and 120 hours), the additional treatments with 3 degrees of freedom (45 °C x 0, 8, 24 and 48 hours), and a contrast between factorial x additional with 1 degree of freedom, exhibiting the ANOVA tables (Tables 1 and 2). The regression models were chosen based on biological logic, at the significance of the regression coefficients, using the t test at 5% probability and in the coefficient of determination. The software *Statistica 8* (STATSOFT Inc., 2009) was used.

Table 1.　Summary of analysis of variance (ANOVA) for the data on production of superoxide anion (O$_2^-$) in embryonic axes of *Dalbergia nigra* seeds under different temperatures.

Sources of Variation	DF	SS	MS	F	p-value
Treatments	19	12846.73	676.14	329.46	0.0000
Factorial	15	10147.25	676.48	329.63	0.0000
Temperature (T)	3	33.63	11.21	5.46	0.0030
Period (P)	3	10028.39	3342.80	1628.83	0.0000
T x P	9	85.24	9.47	4.61	0.0003
Additional	3	2691.70	897.23	437.19	0.0000
Factorial vs Additional	1	7.78	7.78	3.79	0.0585
Error	40	82.0909	2.0523		
Total	59	12928.8242			

DF=degrees of freedom; SS=sum of squares; MS=mean square; F=value of the F test; p-value=probability of significance.

Table 2.   Summary of analysis of variance (ANOVA) for the data on production of hydrogen peroxide (H_2O_2) in embryonic axes of *Dalbergia nigra* seeds under different temperatures.

Sources of Variation	DF	SS	MS	F	p-value
Treatments	19	4730.26	248.96	73.92	0.0000
Factorial	15	3600.22	240.01	71.27	0.0000
Temperature (T)	3	44.98	14.99	4.45	0.0086
Period (P)	3	3453.93	1151.31	341.86	0.0000
T x P	9	101.31	11.26	3.34	0.0039
Additional	3	1125.19	375.06	111.37	0.0000
Factorial vs Additional	1	4.85	4.85	1.44	0.2371
Error	40	134.71	3.37		
Total	59	4864.97			

DF=degrees of freedom; SS=sum of squares; MS=mean square; F=value of the F test; p-value=probability of significance.

Results and Discussion

The *Dalbergia nigra* seeds were dispersed with moisture content of 8.86%, which was similar to the values of moisture content found by Ataíde et al. (2013) for two lots of seeds of the same species collected in 2010 and 2011 (7.92 and 8.98%, respectively) in the region of Viçosa, MG, Brazil. According to the imbibition curves, temperature stimulated the rate of water uptake; weight gain of the seeds increased through increase in temperature. At the temperature of 5 °C, the lowest rate of water uptake was observed, requiring 72 hours to reach phase II. At the temperature of 15 °C, 36 hours were necessary to characterize phase II, while at the temperatures of 35 and 45 °C, 24 hours were necessary. However, deterioration of the seeds began over the period at the temperature of 45 °C. The seeds remained at phase II at the temperatures of 5 and 45 ºC. For the temperature of 25 °C, the three phase imbibition pattern was observed, reaching phase III in 132 hours (Figure 1).

Significant effects of temperatures on germination of *D. nigra* seeds were observed (Figure 2). At 25 °C, the seeds reached the maximum estimated percentage of germination for the 12 days (94%). For the temperatures of 5 and 45 °C, there was no radicle protrusion during the period of observation. Seed imbibition at low temperatures has a harmful effect because it results in irreparable damage to the membranes and leaching of solutes (Castro et al., 2004). High temperatures may allow seed imbibition; however, they do not ensure embryo expansion and seedling establishment (Bradbeer, 1988). In regard to temperatures of 15 and 35 °C, the *D. nigra* seeds reached the maximum estimated percentages of germination at 12 days, with 5% and 39% of germination, respectively.
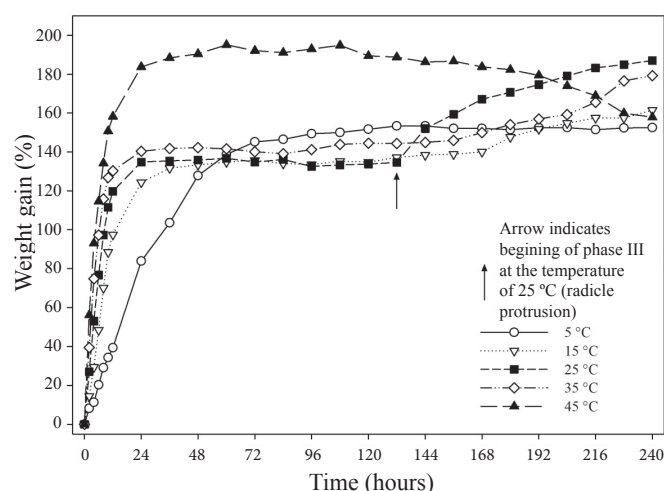

Figure 1.   Imbibition curves of *Dalbergia nigra* seeds under different temperatures.
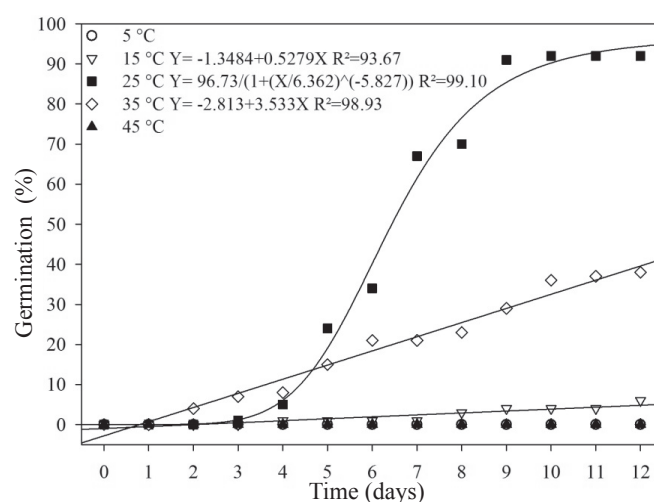

Figure 2.   Regression equations for germination of *Dalbergia nigra* seeds under different temperatures.

The balance between ROS production and the capacity of the defense system in their removal indicates the plant response to stress and reflects adaptation and/or tolerance to the different types of environmental conditions (Mittler, 2002; Apel and Hirt, 2004). The radical O_2^- may lead to cell death when there is no specific antioxidant defense system to remove it (Gill and Tuteja, 2010). The conversion of O_2^- to H_2O_2 is the natural route for removal of both substances due to the fact that the latter is less toxic than the former, as well as the possibility of being taken from the production location. Moreover, according to Gill and Tuteja (2010), the accumulation of H_2O_2 in the cells may cause damage to cell metabolism since this compound has the ability of oxidizing the thiol groups (-SH) of enzymes, deactivating them.

The production of O_2^- (34.01 $nmol.min^{-1}.g^{-1}$ FM) and H_2O_2 (34.20 $\mu mol.g^{-1}$ FM) in embryonic axes of dry *D. nigra* seeds was observed (Figures 3 and 4). In this case, the presence of ROSs may arise from non-enzymatic reactions, such as lipid peroxidation and the Amadori and Maillard reactions since the enzyme activity of the antioxidant system is probably reduced in this condition (Murthy et al., 2003). Pukacka and Ratajczak (2005) also found hydrogen peroxide production in dry *Fagus sylvatica* seeds. With the beginning of imbibition, there was a reduction in the concentration of O_2^- and H_2O_2 for all the temperatures assessed.

For the temperature of 5 °C, reduction in O_2^- production soon after imbibition was seen, when compared with the value of the dry seed, reaching minimum values up to 120 hours (Figure 3). Luo et al. (2011) observed a continual increase in the formation of superoxide anion in strawberry leaves under low temperature up to 48 hours, decreasing after that. During acclimatization of *Capsicum annum* seedlings to low temperature, Airaki et al. (2012) observed an increase in the level of the non-enzymatic antioxidants ascorbate and glutathione and in the activity of the NADPH dehydrogenase.

In relation to production of hydrogen peroxide, at the temperature of 5 °C, minimum production of this free radical was seen up to 70 hours of imbibition (9.6 $\mu mol.g^{-1}$ FM) and subsequent increase in this production up to 120 hours (20.5 $\mu mol.g^{-1}$ FM) (Figure 4). The high moisture content of the seeds associated with the increase in the hydrogen peroxide content compromised the germination process. Okane et al. (1996) observed that H_2O_2 production in calluses of *Arabidopsis thaliana* plants was greater at 4 °C than at 23 °C. Similar results were found by Sun et al. (2010), who observed accumulation of H_2O_2 in leaf tissue under low temperature conditions in *Nicotiana tabacum* seedlings. According to Torres and Dangl (2005), there was expression of various genes in different species when the plants were subjected to

low temperature. Thus, there is a clear presence of ROSs as a reaction to temperature stress for *D. nigra* seeds.
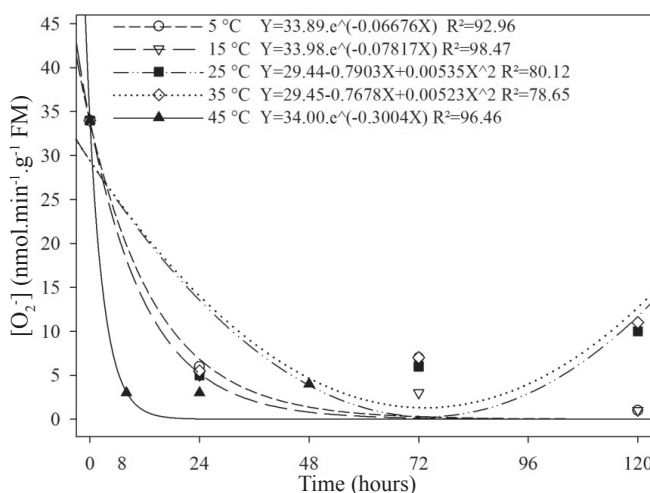

Figure 3. Superoxide anion (O_2^-) production in embryonic axes of *Dalbergia nigra* seeds during germination under different temperatures.
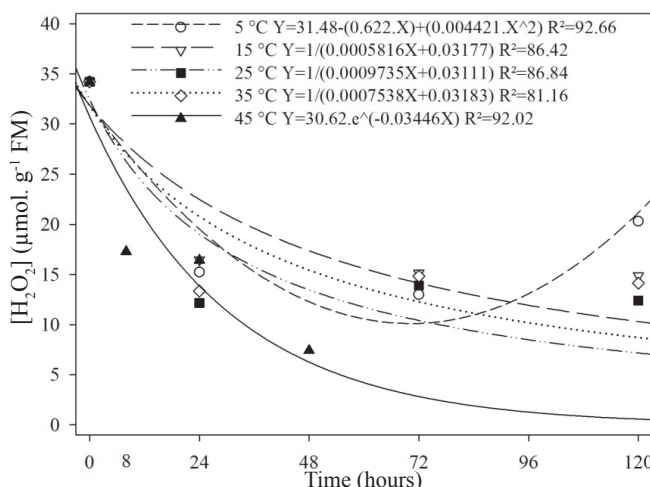

Figure 4. Hydrogen peroxide (H_2O_2) production in embryonic axes of *Dalbergia nigra* seeds during germination under different temperatures.

There was continual reduction in O_2^- production at the temperature of 15 °C up to 120 hours (Figure 3), showing effective action of the enzymes responsible for elimination of the ROSs. The concentration of hydrogen peroxide also decreased up to 120 hours (Figure 4). As it was not under optimum germination conditions (5% radicle protrusion in 120 hours), the production of hydrogen peroxide was still a reaction to thermal stress. It may be seen that the concentration of H_2O_2 at this temperature (9.84 $\mu mol.g^{-1}$ FM) is greater than

at 25 °C (6.75 µmol.g^{-1} FM) and at 35 °C (8.17 µmol. g^{-1} FM) up to 120 hours of imbibition. Germination at this temperature indicates that the respiratory system in the mitochondria is occurring, with consequent production of superoxide anion, effectively dismuted by the SOD enzyme.

Minimum production of superoxide anion at the temperature of 25 ºC occurred at 74 hours of imbibition (0.27 nmol.min^{-1}.g^{-1} FM), rising afterwards up to 120 hours (11.70 nmol.min^{-1}.g^{-1} FM) (Figure 3), when the seeds reached 19% radicle protrusion. Hydrogen peroxide, after a decrease during imbibition in relation to the control, remained relatively high up to 120 hours (Figure 4). The production of ROSs acts against pathogens during radicle protrusion, and also acts as a component of cell growth when it induces the depolymerization of components of the cell well, permitting the cellular expansion (Bailly, 2004; Muller et al., 2009).

At the temperature of 35 °C, minimum production of O$_2^-$ was observed at 73 hours of imbibition (1.27 nmol.min^{-1}.g^{-1} FM) (Figure 3), increasing continually up to 120 hours (12.62 nmol.min^{-1}.g^{-1} FM), when 15% protrusion of the primary root was reached. There were small variations in the values of hydrogen peroxide during the period, which were lower than those at the temperature of 15 ºC, but greater than those at 25 ºC (Figure 4). As the percentage of germination was only 40% after 240 hours (94% germination at the temperature of 25 °C) and the production of superoxide anion was greater among the temperatures assessed, it is clear that at 35 ºC there is metabolic damage to the seeds. As the ROSs are produced in the mitochondria, in the glyoxysome, and in the plasmatic membrane, the system for control of the levels of superoxide anion and hydrogen peroxide could be in any one of them. As the increase in temperature corresponds to the increase in respiration, the production of ROSs on a greater scale in the mitochondria would have the consequence of change in their membranes and reduction in production of ATP and death of the seeds. The presence of H$_2$O$_2$ could be in the stress signaling system, when at 5 ºC or at 35 ºC, or in growth and protection at the temperature of 25 ºC. This is more certain in the last case since there was no interference in germination.

At the temperature of 45 °C, a 91% reduction was observed in O$_2^-$ production up to 8 hours of imbibition when compared to the value of the dry seeds, until reaching values near zero at 120 hours of imbibition (Figure 3). The H$_2$O$_2$ concentration reduced 99% in 8 hours of imbibition when compared with the value of the dry seeds, until reaching 0.49 µmol.g^{-1} FM after 120 hours of imbibition (Figure 4). Duan et al. (2009) established 40 ºC as the optimum temperature for activity of the enzyme NADPH oxidase in rice seeds. Thus, at the temperature of 45 °C, there may still be activity

of this enzyme, which may have an effect on the production of superoxide anion and a consequent harmful effect on seed viability. Maintenance of the H$_2$O$_2$ may be by the action of NADPH oxidase since, according to Sagi and Fluhr (2006), the production of hydrogen peroxide between the cell membrane and wall could return in the form of hydroperoxide, formed by the effect of the 5.0 extracellular pH, and, that way, would enable the action of the SOD in catalyzation of the formation of hydrogen peroxide. Thus, it is possible to suppose action of the enzyme already in the initial periods of germination. Kranner et al. (2010) observed an increase in O$_2^-$ production with an increase in temperature during experiments carried out with *Pisum sativum* seeds. Wang et al. (2012) observed an increase in production of ROSs, especially hydrogen peroxide and an increase in lipid peroxidation in soybean seeds under high temperature and moisture conditions. According to Bhattacharjee (2008), high temperature induced a significant increase in the superoxide anion and hydrogen peroxide content in *Amaranthus livindus* seeds and seedlings when compared to the control group. The involvement of other mechanisms in the effects of temperature on the reduction of viability or death of seeds may not be dismissed. According to Larkindale et al. (2005), heat resistance or the acquisition of thermotolerance is a complex multigenic process.

Conclusions

Hydration is stimulated at 45 ºC and inhibited at 5 ºC, without germination in either, whereas it is minimal at 15 ºC and at a maximum level at 25 ºC.

Superoxide production increases at higher temperatures (25 and 35 ºC) after 72 hours of hydration, coinciding with the beginning of radicle protrusion.

Hydrogen peroxide production decreases at all temperatures, except for 5 ºC, with values near each other at the temperatures of 15, 25 and 35 ºC, where there was radicle protrusion.

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