MARCONE MOREIRA SANTOS

GERMINAÇÃO DE SEMENTES DE*Melanoxylon brauna* Schott.SOB ESTRESSE TÉRMICO E ASCONSEQUÊNCIAS FISIOLÓGICAS, ANATÔMICAS E BIOQUÍMICAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência Florestal, para obtenção do título de *Doctor Scientiae*.

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MARCONE MOREIRA SANTOS

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Dimas Mendes Ribeiro

Juraci Alves de Oliveira

Glauciana da Mata Ataíde

Genaina Aparecida de Souza

Eduardo Euclydes de Lima e Borges
(Orientador)

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BIOGRAFIA

Marcone Moreira Santos, filho de José Luiz Moreira dos Santos e Maria Geral de Oliveira Santos, natural de Caetanópolis, Minas Gerais, em 12 de abril de 1989.

Em agosto de 2007, ingressou na Universidade Federal dos Vales do Jequitinhonha e Mucuri, graduando-se Engenheiro Florestal em janeiro de 2012.

Em fevereiro de 2012, ingressou no Programa de Pós-graduação em Ciência Florestal, em nível de Mestrado, na Universidade Federal dos Vales do Jequitinhonha e Mucuri, concluindo os requisitos necessários para obter o título de Mestre em Ciências com ênfase em Melhoramento Florestal em fevereiro de 2014, com defesa da dissertação.

Em agosto de 2014, ingressou no Programa de Pós-graduação em Ciência Florestal, em nível de Doutorado, na Universidade Federal de Viçosa, concluindo os requisitos necessários para obter o título de *Doctor Scientiae* em fevereiro de 2018, com defesa da tese.

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SANTOS, Marcone Moreira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Germinação de sementes de *Melanoxylon brauna* Shott. sob estresse térmico e as consequências fisiológicas, anatômicas e bioquímicas**. Orientador: Eduardo Euclydes de Lima e Borges.

Estudos anatômicos, fisiológicos e bioquímicos durante a germinação de sementes de *Melanoxylon. brauna* sob estresse térmico são fundamentais diante da possibilidade do aumento da temperatura global, da importância econômica da espécie e do pouco conhecimento referente àquelas áreas específicas.Nesse sentido, o presente trabalho tem por objetivos:caracterizar alterações bioquímicas e fisiológicas durante a germinação sob estresse térmico. Também foram avaliadas as alterações anatômicas, reação de Fenton. Avaliou-se a germinação nas temperaturas constantes de 25, 35 e 45 ºC e em sementes embebidas por 24, 48 e 72 h em 35 e 45 ºC e posteriormente transferidas para 25 ºC. A CE, produção de EROs e atividade enzimática foram avaliadas em sementes embebidas por 0, 24, 48 e 72 h em 25, 35 e 45 ºC. A resistência micropilar foi avaliada em sementes secas e embebidas por 24 h em água e solução de H_2O_2 nas concentração de 20 e 40 µm.Os experimentos foram conduzidos no delineamento inteiramente casualizado (DIC).Para a germinação e CE foram utilizadas 5 repetições com 20 sementes cada. Para a produção de EROs e atividade enzimática foram utilizadas cinco repetições de 50 mg de eixos embrionários ou 100 mg de micrópilas. A força de rompimento região micropilar também foi avaliada em 3 repetições com 10micrópilas cada.A embebição das sementes em 35 ºC com posterior transferência para 25 ºC favorece a germinação. Em 45 ºC ocorre maiores danosao sistema de membranas, maior acúmulo de H_2O_2, comprometimento do sistema antioxidante e posterior morte das sementes. Após 16 h de embebição, foram observadas as primeiras alterações anatômicas na região micropilar e endosperma lateral. A presença de H_2O_2e redução da força de ruptura da região micropilar indicam provável ocorrência da reação de Fenton. A temperatura influenciou a atividade das enzimas, α-galactosidase, poligalacturonase (PG), pectina metil esterases (PME), pectina liase (PL), celulases totais, β-1,3 glicosidase e β-1,4 glicosidase e o estresse térmico não comprometeu essesistema enzimático, porém comprometeu a germinação em 45 ºC.

ABSTRACT

SANTOS, Marcone Moreira, D.Sc., Universidade Federal de Viçosa, February, 2018. **Germination of seeds of*Melanoxylon brauna* Shott. under termal stress and the physiological, anatomical and biochemical consequences**. Advisor: Eduardo Euclydes de Lima e Borges.

Anatomical, physiological and biochemical studies during the germination of *Melanoxylon brauna* seeds under thermal stress are fundamental to the possibility of global temperature increase, the economic importance of the species and the lack of knowledge related to those specific areas. In this sense, the present work has as objectives: to quantify germination, electrical conductivity (EC), production of reactive oxygen species (ROS) and enzymatic activity during germination under thermal stress. The anatomical alterations, Fenton reaction (H2O2 levels and Fe and Cu ion contents) and resistance of the micropillary region of the seeds during imbibition were also evaluated. The germination was evaluated at constant temperatures of 25, 35 and 45 ° C and in seeds soaked for 24, 48 and 72 h at 35 and 45 ° C and subsequently transferred to 25 ° C. EC, ERO production and enzymatic activity were evaluated in seeds soaked in 0, 24, 48 and 72 h at 25, 35 and 45 ºC. The micropylar resistance was evaluated in dry seeds and soaked for 24 h in water and H2O2 solution at the concentration of 20 and 40 μm. The experiments were conducted in a completely randomized design (DIC). For germination and CE, 5 replicates with 20 seeds each were used. For the production of EROs and enzymatic activity, five replicates of 50 mg of embryonic axes or 100 mg of micropiles were used. The force breaking micropillary region was also evaluated in 3 replicates with 10 micropiles each. The imbibition of the seeds at 35 ° C with subsequent transfer to 25 ° C favors the germination. At 45 ºC, there is greater damage to the membrane system, higher accumulation of H_2O_2, impaired antioxidant system and subsequent seed death. After 16 h of imbibition, the first anatomical changes were observed in the micropillary region and lateral endosperm. The presence of H_2O_2, Fe and Cu and reduction of the rupture force of the micropylar region indicate a probable occurrence of the Fenton reaction. The temperature influenced the activity of the enzymes, α-galactosidase, polygalacturonase (PG), pectin methyl esterases (PME), pectin lyase (PL), total cellulases, β-1,3 glycosidase and β-1,4 glycosidase. Thermal

stress did not compromise the enzymatic system, but compromised the germination in 45 ºC.

Introdução geral

As projeções do Painel Brasileiro de Mudanças Climáticas mostram, de forma geral, que haverá alta nas temperaturas do país no decorrer desse século. As mudanças na temperatura podem oscilar de 1 °C a 5 °C até o final do século dependendo do aquecimento global e da emissão de gases de efeito estufa. O nordeste da Mata Atlântica terá alta entre 2 °C e 3 °C e baixa pluviométrica entre 20% e 25% em meados do século. Na porção sul e sudeste da Mata Atlântica a temperatura deverá subir entre 2,5 °C a 3 °C nesse mesmo período. Embora haja alto grau de incerteza nas previsões das mudanças climáticas, dados de diferentes fontesa respeito mostram alterações na temperatura ao longo das décadas. As previsões indicam alterações nos biomas e comprometimento na variabilidade genética das espécies florestais (PBMC, 2013).

A temperatura é um dos componentes determinantes da ocorrência de determinada espécie em um bioma pela influência que exerce na germinação da semente e no crescimento da planta. Cochrante *et al.* (2014) demonstraram a vulnerabilidade das espécies às mudanças climáticas, sobretudo, em condições de temperaturas elevadas. Brancalion et al. (2010) relacionaram a temperatura ótima de germinação de sementes de 272 espécies florestais nativas com os biomas e com o grupo sucessional. Os resultados indicaram que 90,4% das espécies dos diferentes biomas requerem temperaturas de 20 a 30 ºC como ótimas para germinar. Esses mesmos autores relatam que reduz substancialmente a proporção de espécies que germinam em 35 ºC. A ocorrência de dada espécie em determinado bioma está relacionada à temperatura de origem, sendo, pois, adaptação fisiológica (Brancalion *et al*., 2010).

Tendo em vista a possibilidade da elevação da temperatura, a pergunta é como será a resposta adaptativa das espécies e como se pode interferir no processo para que não haja desaparecimento da(s) espécie(s)?

Dentre as espécies florestais nativas de importância ecológica e econômica tem-se a *Melanoxylon brauna*, popularmente conhecida como braúna, pertencente à família Fabaceae Caesalpinoidea.De ocorrência natural na floresta pluvial da encosta atlântica das regiões Nordeste e Sudeste, especialmente nos estados da Bahia, São Paulo e Minas Gerais(Lorenzi, 2008). A espécie é conhecida pela qualidade e durabilidade de suas madeiras, apresentam característica acastanhada, quase negra

nos espécimes mais velhos, sendo considerada uma madeira de lei de grande valor econômico(Ataíde *et al.,* 2016). No entanto, devido a intensa exploração, a espécie se encontra atualmente na lista de espécies ameaçadas de extinção (Brasil, 2014)

Apesar de a espécie ser conhecida pela qualidade e durabilidade de sua madeira, do grande valor econômico e ambiental, pouco tem sido feito no sentido de aprofundar investigações científicas a respeito da(s) estratégia(s) de propagação da espécie, em particular com ênfase na sua germinação, a qual constitui a via principal utilizada. Existem poucas pesquisas publicadas até o momento, envolvendo espécies florestais nativas, que identificam as alterações bioquímicas e fisiológicas que ocorrem nas sementes como reação ao estressse.

Somente o conhecimento da fisiologia e da bioquímica da germinação das sementes possibilitará a tomada de decisão acertada. Entretanto, o conhecimento gerado até o momento é praticamente nulo, especialmente, considerando a imensa quantidade de espécie somente no Bioma Mata Atlântica. As pesquisas, em suamaioria, determinam as temperaturas máximas e ótimas, sem, contudo, estabelecer o que foi alterado internamente na semente, sendo, portanto, resultados pontuais (Oliveira *et al.,* 2016; Silva *et al.,* 2016).

Dentre as várias consequências metabólicas causadas pelo estresse térmico durante a germinação, as espécies reativas de oxigênio (EROs), por meio do aumento na produção e acúmulo, são das que mais acarretam danos.Para evitar danos celulares irreversíveis, enzimas do sistema antioxidante entram em ação quando os níveis de EROs ultrapassam níveis normais.As enzimas peroxidase do ascorbato (APX), catalase (CAT), superóxido dismutase (SOD) e peroxidases (POX)têm importante papel na remoção do excesso de EROs nos diferentes compartimentos celulares. A temperatura é um dos fatores que determina capacidade de defesa quanto a remoção de tais substâncias, tendo em vista que essa determina a ativação e a ação das enzimas (Matos *et al.,* 2014).

Por outro lado, EROs como o H_2O_2 em níveis controlados, desempenham papel fundamental durante a germinação. As paredes celulares são compósitos fibrosos em que as microfibrilas de celulose são coextensivas permeadas por polissacáridos não celulósicos. Essas redes fibrosas são barreira à germinação, são enfraquecidas pela presença de peróxido de hidrogênio. Sua presença leva ao

aumento da atividade de enzimas responsáveis pela quebra e mobilização de compostos de reserva (Zhang *et al.*, 2014).

A presença do H_2O_2 pode contribuir ainda para ocorrência da reação de Fenton. Esta, definida como a geração catalítica de radicais hidroxilo (OH•) a partir da reação em cadeia entre os íons metálicos, normalmente de ferro (Fe^{2+}) e H_2O_2, em meio ácido, tem demonstrado ser bastante eficiente na oxidação de compostos orgânicos tóxicos e não biodegradáveis (Nogueira *et al.*, 2007). Nesse sentido a reação de Fenton pode contribuir para o enfraquecimento do endosperma micropilar, no entanto, ainda não há relatos sobre a ocorrência ou papel desse processo na germinação.

A germinação é o resultado da expansão do eixo embrionário pelo efeito do turgor, e do enfraquecimento da parede celular da micrópila. Entretanto, o embrião ativo somente completará o processo quando a radícula conseguir romper a resistência mecânica exercida pelos tecidos envoltórios (Bewley *et al.*, 2013).

Diferentes enzimas participam no processo de germinação, tanto no eixo embrionário como na micrópila. A força de resistência do endosperma micropilar diminui pela ação de enzimas que degradam a parede celular, como exemplo: endo-β-mananase, α-galactosidase, poligalacturonase (PG), pectina metil esterases (PME), pectina liase (PL), celulases totais, β-1,3 glicosidase e β-1,4 glicosidase(Ataíde *et al.*, 2013; Han & Yang, 2015; Sainz *et al*., 2015; Scheler *et al*., 2015)

Considerando-se as alterações climáticas observadas e os consequentes aumentos na temperatura que atingirão as espécies da mata Atlântica; que a diversidade genética das espécies restantes nesse bioma é extremamente reduzida pela ação predatória do homem; que as mudanças climáticas podem comprometer a ocorrência das espécies nos biomas atuais, tornam-se fundamentais estudos envolvendo os efeitos do estresse térmico na germinação de sementes*de M. brauna*.

Embora as temperaturas médias nas regiões de ocorrência da espécie não ultrapassem 30 ºC, em diversas épocas do ano podem ocorrer temperaturas acima 40 ºC em algumas regiões específicas (Köppen e Geiger, 1936). Esses valores somados àqueles previstos pelas alterações climáticas podem comprometer o desenvolvimento de diversas espécies, inclusive o processo de germinação da *M. brauna*, cujo a temperatura ótima de germinação é 27 ºC e a temperatura máxima de germinação é 42 ºC (Flores *et al.*, 2014).

Dessa forma, propõe-se neste trabalho analisar as alterações fisiológicas, anatômicas e bioquímicas que ocorrem durante a germinação de sementes de *M. brauna* em diferentes temperaturas. As informações a serem buscadas, além de relevantes para a pesquisa básica, são essenciais para o melhor entendimento dos processos ecológicos de estabelecimento de plantas, sucessão e regeneração dentro das comunidades, constituindo ferramenta básica para a conservação da espécie, seja para cultivos comerciais ou para reflorestamento, e preservação da espécie.

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

Marcone Moreira Santos[1,*], Eduardo Euclydes de Limas e Borges[2], Glauciana da Mata Ataíde[3] and Genaina Aparecida de Souza[4]

[1] Department of Forest Engineering, Federal University of Viçosa, Viçosa, Brazil; marconemoreirasantos@hotmail.com
[2] Department of Forest Engineering, Federal University of Viçosa, Viçosa, Brazil; elborges@ufv.br
[3] Department of Crop Sciences, Federal University of São João Del Rei, Sete Lagoas, Brazil; glaucianadamata@yahoo.com.br
[4] Department of Plant Biology, Federal University of Viçosa, Viçosa, Brazil; genainasouza@yahoo.com.br
*****Correspondence: marconemoreirasantos@hotmail.com
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Abstract

In this article, the authors aimed to analyze the physiological and biochemical alterations in *Melanoxylon brauna* seeds subjected to heat stress. For that, seed germination, electric conductivity (EC), production of reactive oxygen species (ROS), and 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 during 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 germinationand higher germination speed. Seed soaking at 45 °C increased peroxide production, which compromised the antioxidant enzyme system due to 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 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 temperature to 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 to develop 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 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 production and accumulation of reactive oxygen species (ROS) in seeds [14]. ROS include free radicals such as 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 is highly damaging, and when the levels of these molecules exceed

the capacity of defense mechanisms to scavenge them, cells undergo oxidative stress [17].

Plant cells have efficient enzymatic mechanisms for ROS removal, which enables them to not be damaged by intoxication. Temperature affects the removal capacity of ROS, as it determines the activation and action of enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX), 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 germination of *Melanoxylon brauna* seeds subjected to heat stress.

2. Materials and Methods

The experiments were performed between February and August 2016. *M. 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 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 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 x 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 minutes 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 peroxide concentration as a standard. Plant extracts were obtained from samples while analytical blanks were prepared in parallel.

Lipid peroxidation was evaluated by determining TBA (thiobarbituric acid) concentration [26]. 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 necessary amount of enzyme to inhibit of 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 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 $protein^{-1}$).

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.

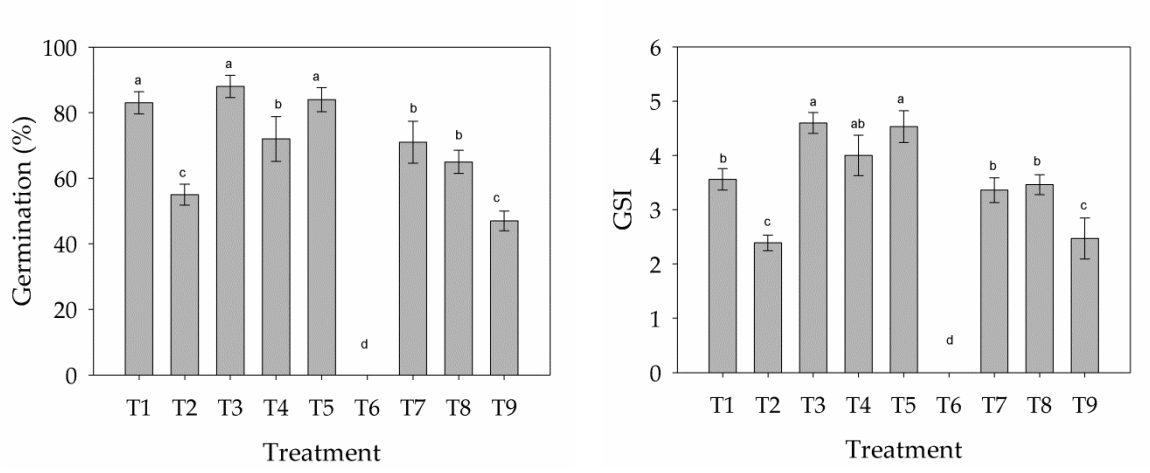
For all determinations, the statistical design was entirely randomized with five replicates. The data of germination was submitted to a variance analysis using the Statisca (Statsoft) program and the averages obtained for the treatments were compared by the Tukey test as a 5% significance. The data of EC, ROS and enzyme activity was submitted to a regression analysis ($p<0,05$).

Pearson correlation analysis was performed (GENES software) [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 loss of vigor. A significant difference in GSI was also observed. 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.



(a) **(b)**

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 differed from that at 35 °C only with a 72-h soaking period, the former showing a clear decrease.

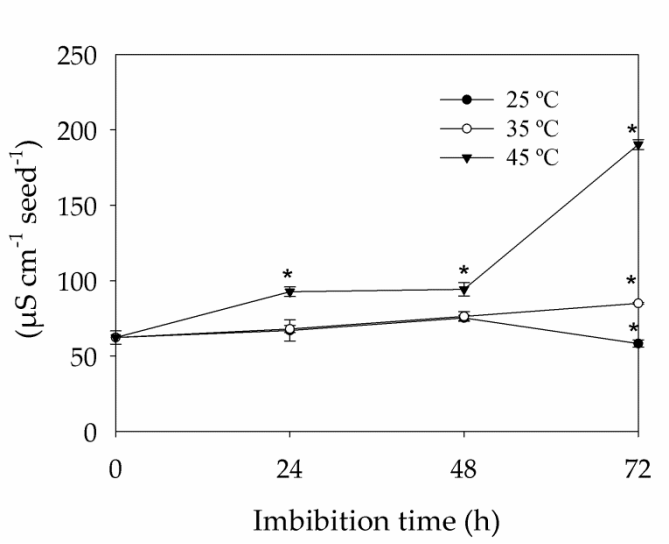
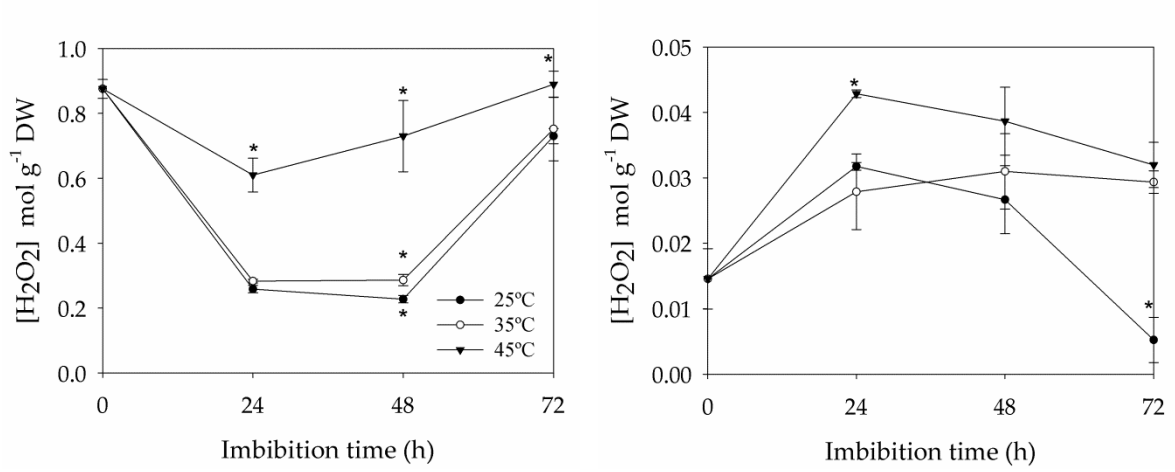


Figure 2: Electric conductivity in *Melanoxylon brauna* seeds under different temperatures and soaking periods. *Indicate statistical difference between means. Vertical bars = ±SE, n=5.

3.3.Superoxide anion and hydrogen peroxide

Superoxide anion was not detected by the adopted method under the tested conditions. 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 axiswas observed after any of the analyzed soaking times.



(**a**) (**b**)

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. *Indicate 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).

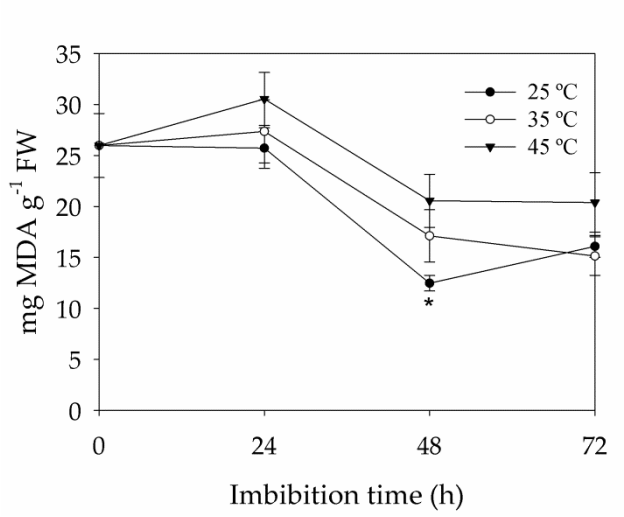


Figure 4: Malondialdehyde (MDA) concentration in *Melanoxylon brauna* seeds soaked at 25, 35, and 45 °C. *Indicate statistical difference between means. Vertical bars = ±SE, n=5.

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 these factors influenced enzyme activity in the embryo axis during 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 sampling effect. It is worth noting that seeds showed a wide range of maturation levels during the harvest period.

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).

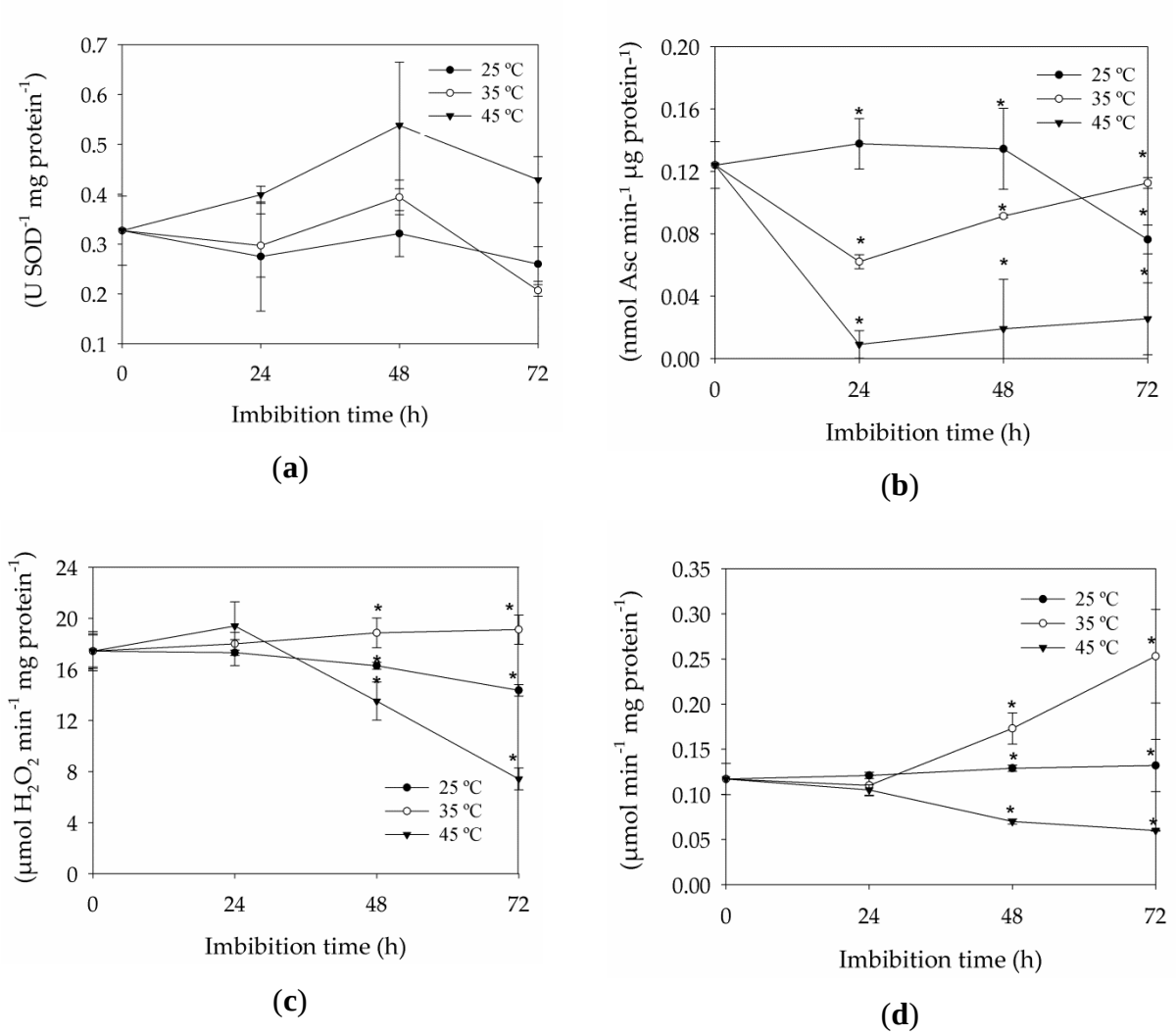


Figure 5: Specific activities of enzymes superoxide dismutase (SOD) **(a)**, ascorbate peroxidase (APX) **(b)**, catalase (CAT) **(c)**, and peroxidase (POX) **(d)** in the embryonic axis of *Melanoxylon brauna* seeds during the germination period, after soaking at 25, 35, and 45 °C. * Indicatestatistical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

Pearson correlation was assessed for variables: EC, H_2O_2 concentration in the embryo and micropylar endosperm, and 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 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 germination percentages of Braúna 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.

Soaking of seeds at 35 °C during 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 amount of leaked electrolytes in the solution, being therefore directly related to the integrity of cell membrane. Thus, high EC values indicate high leakage of solutes due to alteration in the integrity of 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 Braúna 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 along the first 24 h (Figure 3). Such increase is due to resumption of respiration. Increased amounts of H_2O_2 leads to weakening of the wall of seed coat cells, thus enhancing germination. The roles that ROS play in plants also include cell signaling, promotion of programed cell death, and 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 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, 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. Increased peroxidation of lipids, mediated by free radicals and peroxides, is a possible reason for 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 to 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 to SOD activity in *Medicago sativa* seeds, which showed constant behavior at 22 °C [48]. During 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 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 hours, 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 to not be detected by the enzyme.

The activity of enzymes APX, POX, and CAT decreased at 45 °C, which led to an increased concentration of H_2O_2 and a consequent damage to 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 need for 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 increased

concentration of H_2O_2 [13], may determine an increase in 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 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 a 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 absence of germination in *Melanoxylon brauna* seeds.

Table 1 shows that EC had a significant inverse correlation with H_2O_2 concentration in the embryo only at 25 °C. In that sense, it seems reasonable to presume that such correlation is associated with the production of ROS and their impact on cell membrane integrity. Increase in ROS contents would lead to damage to the cell membranes, while a reduction in those contents would not affect 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 during 72 h followed by 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.

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Biochemical and Anatomical Changes and Fenton reaction in the Micropilar Endosperm of *Melanoxylon brauna* Seeds During Germination.

Marcone Moreira Santos[1], Genaína Aparecida de Souza[2], Eduardo Euclydes de Lima e Borges[1], Edgard Augusto Toledo Picoli[2], Maicon Nascimento Araújo[2], Glauciana da Mata Ataíde[4].

[1] Department of Forest Engineering– Federal University of Viçosa, Viçosa, MG, Brasil, email: marconemoreirasantos@hotmail.com

[2]Departament of Plant Biology – Federal University of Viçosa, Viçosa, MG, Brasil

[3]Departament of Crop Sciences – Federal University of São João Del Rei, Sete Lagoas, MG, Brasil

*Correspondence author:marconemoreirasantos@hotmail.com

Abstract

Thisaimed wasevaluate anatomical and enzymatic changes in the micropilar endosperm of *Melanoxylon brauna* seeds during germination. Seeds were germinated at 25 ºC. Samples for evaluations of reactive oxygen species and activity of the enzymes endo-β-mannanase, α-galactosidase, polygalacturonase and pectinmetylesterase were collected with 0, 24, 48 and 72 h soaking. Seeds soaked at 25ºC for 0, 16, 24, 48 and 72 h were used for anatomical evaluation. During the first 72 h of soaking ocurred reduction of micropyle endosperm thickness and consumption of lateral endosperm cells. Also ocurred an increase of hydrogen peroxide in the micropyle during the first 24 h of soaking. There was no activity of the enzyme endo-β-mannanase under tissues and conditions evaluated. During the soaking process there was activity of the enzymes α-galactosidase and pectinmetylesterase in the micropilar region of the seeds, evidencing the importance of these enzymes in germination process.

Key words: Micropyle, reactive oxygen species, hydrolytic enzymes, cell wall.

1. Introduction

Melanoxylon brauna Schott. (Braúna) is a native species to the Atlantic Forest, occurring in the brazilian states of Bahia, São Paulo, Minas Gerais, Espírito Santo, Pará, Rio de Janeiro and Sergipe. Due to the exploration of its wood, the species is currently under the 'vulnerable' category on "Brazilian Official List of Flora Threatened Species", according to the Ministry of Environment. Therefore, information about seeds physiology and germination could contribute for the development of new strategies for the species conservation (Almeida *et al.,* 1998; Lorenzi, 2008; Brasil, 2014; Ataíde, 2016).

Seed germination involves a series of physiological and biochemical processes relatively complex (Nonogaki *et al.,* 2014). In the soaking process, changes occur in the embryo cells involving reactive oxygen species (ROS), proteins and enzymes capable of changing cell wall characteristics. The lower resistance of the cell wall due to the enzymatic activity contribute for the cell elongation, resulting in primary root protrusion (Zhang *et al.,* 2014).The mechanisms related to the micropilar region rupture, specially the weakening of involved tissues, are scarcely known (Wang *et al.,* 2014; Zhang *et al.,* 2014). However, it is known that the elongation of the hypocotyl-radicle axis and the weakening of the micropyle tissues are involved in germination. For the radicle to emerge from a seed, the balance of embryo growth potential and the mechanical resistance of the tissues that it must change (Bewley *et al.,* 2013).

ROS is a general concept used to describe oxygen reduced molecules, in the form of molecular oxygen "singlet" (O2), oxygen peroxide (H_2O_2), hydroxyl radical (OH) and oxygen (O). Although its accumulation causes damage to several cell compartments, under controlled levels ERO act as an important germination regulator given that it weakens the endosperm (Zhang *et al.,* 2014; Borges *et al.,* 2015).

A different hypothesis is the occurrence of Fenton Reaction during germination process. The catalytic generation of hydroxyl radicals (OH•) originated from the reaction between metal ions in acid medium, usually iron (Fe^{2+}) and hydrogen peroxide (H_2O_2), has proved to be very efficient at oxidizing toxic and non-biodegradable organic compounds (Nogueira *et al.,* 2007).

Simultaneously, endo-β-mannanase, α-galactosidase, polygalacturonase and pectinmetylesteraseare are hydrolytic enzymes and play an essential role in several species germination processes, contributing for weakening the micropilar endosperm (Bewley *et al.*, 2013).Primary root protrusion and loss of cell wall integrity is due to activity of hydrolases, transglycosylases, cellulases, hemicellulases such as: endo-β-mannase, α -galactosidase, polygalacturonase e pectin metyl esterase (PME) (Wang 2014; Nonogaki, 2014; Zhang 2014).

Based on the foregoing, this study aimedto evaluate the anatomical changes, quantify the presence of ROS and the activity of the enzymes endo-β-mannanase, α-galactosidase, polygalacturonase e pectinmetylesterase in the micropilar endosperm of *M. brauna* seeds during germination.

2. Material e methods

Melanoxylon brauna fruits were collected in the Leopoldina region, in the State of Minas Gerais (21º 31' 55" S and 42º 38' 35" W). After collection, the fruits were sun dried until its opening and the seeds were manually extracted. Seeds were benefited, conditioned in fiber drums and stored in cold room at 5 ºC and 60% RH, until the experiments were carried out. Subsequently, to stimulate germination, seeds were placed above two sheets of germitest paper moistened with distilled water at 25 ºC. Seeds with primary root emission were considered germinated, and results were expressed as mean percentage. Five replicate of 20 seeds were used for each treatment.

2.1. Anatomical changes

M. brauna seeds soaked at 25 ºC after 0, 16, 24, 48 and 72 h were fixed in FAA_{50} (formaldehyde: acetic acid and ethyl alcohol (50%, 5: 5: 90, v/v/v), for 48 h, and stored in 70% ethanol (Johasen, 1940). Then, the material were included in methacrylate (Historesin-Leica), according to the manufacturers recommendations. Samples were longitudinally sectioned in automatic advance rotary microtome (model RM2155, Leica microsystems Inc., Deerfield, USA), with 5 µm thickness and stained with Toluidine blue (O'brien *et al.*, 1964). Later, permanent slides were produced using synthetic resin (Permount®). Structural analysis images were obtained by optical microscope (model AX-70 TRF, Olympus Optical, Tokyo, Japan) coupled to digital camera (model Zeiss AxioCam HRc, Göttinger, Germany) and microcomputer with Axiovision Image Capture Program.

2.2. Reactive Oxygen Species

The production of reactive oxygen species (ROS) and enzymatic activity were evaluated during germination. The analysis were performed in micropyles of seeds soaked for 0 (totally dry seeds), 24, 48, 72 h at 25 ºC, subsequently freeze-dried and stored at -20 ºC. Enzymatic activity in micropyles extracted before soaking process was also evaluated.

Superoxide Anion: Superoxide anion production was assessed by measurement of the rate of adrenochrome accumulation (Misra and Fridoovich, 1971), using the molar absorption of 4,0 x 103 M^{-1} (Boveries, 1984).

Hydrogen Peroxide: 100mg samples of micropyles were weighed, crushed and homogenized (Kuo and Kao, 2003). Determination of absorbance was obtained by spectrophotometer, in 560 nm, and the quantification of H_2O_2 was performed based in the calibration curve using peroxide concentration as pattern (Gay and Gebicki, 2000).

2.3. Reação de Fenton

Quantification of micronutrients: in the micronutrients extraction stage it was used Mehlich-1 extractor (Defilipo and Ribeiro, 1997). In the dosage stage, it was used an atomic absorption spectrophotometer and an optical emission spectrophotometer in induced plasma. It was evaluated Fe and Cu levels in dried micropyles, using five repetitions with 20 micropyles each.

Analysis of micropilar region resistance: The micropyles were kept in greenhouse at 80 ºC for 24 h to promote denaturation of all the enzymes. Then, micropyles were soaked for 0, 2 and 24 h in water or hydrogen peroxide in concentrations of 20 to 40 mM at 25 ºC. After soaking process, the micropyles were washed, dried on paper and positioned on texturometer (Stable Micro Systems Texture Analyzer), for evaluation of micropilar resistance. The following speeds were used: pre-test: 2,00 mm/s; test: 1,60 mm/s; post-test: 2,00 mm/s in a distance of 5 mm.

2.4. Enzymatic activity

Endo-β-mananase: Activity of the enzyme endo-β-mannanase was calculated according to Downie et al. (1994).

α-galactosidase:It was used the methodology described by Viana (2002). The enzyme unity was defined as the necessary amount of protein to produce 1 mol of ρ-NP per minute under experiment conditions.

Polygalacturonase: The enzymatic activity was determined by the dosage of reductive sugar produced according to the DNS method (3,5-dinitrosalicylic acid), adapted by Miller (1959). Results were expressed in polygalacturonase unities. Each polygalacturonase unity corresponds to the amount of galacturonic acid released (µM) by minute of reaction (U/mL).

PME: Extraction was performed according to the method described by Pinto et al. (2001), with a few modifications. The activity was quantified according do Grsic-Rausch and Rausch (2004), considering that each PME unity corresponds to 1 uM of NADPH formed per minute at pH 7,5 and 25ºC.

Protein concentration in samples was determined by the Bradford method, 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 Statisca (Statsoft) program and the averages obtained for the treatments were compared by the Tukey test as a 5% significance. The data of ROS and enzyme activity was submitted to a regression analysis ($p<0,05$).

3. Results

3.1. Anatomical changes during germination

Germination started after soaking for 72 h, reaching average of 83%. *M. brauna* seeds are formed by integument, endosperm and embryo. Therefore, it is an albuminous seed. It presents a hard and dark brown colored integument, besides a dense endosperm. It also presents a layer of macrosclereids with Malpighi cells, which are cells with thick walls, strongly connected, perpendicularly arranged in relation to the surface, constituting the exotesta. It was possible to observe differential thickness for that structure, being larger in the mycropyle than other regions. Internally to macrosclereids there is the hypoderme, constituted by a layer of osteo-sclereids which are sclerified, columnar and accented cells presenting hourglass shape (Figure 1A).

Soaking process caused reduction of micropilar endosperm thickness, initiated consumption of lateral endosperm cells and also caused breakingin the integument (Figure 1). After soaking for 24 h, it was observed consumption of cell wall material, macro and osteo-sclereids, and lateral endosperm cells (Figure 1E). During this period, it was also possible to observe the embryo development with hypocotyl-radicle axis elongation (Figure 1F), besides the formation of a protective region that will give rise to the root cap (Figure 6D). After soaking for 48 h, the tissue responsible for composing the outer layers of macro and osteo-sclereids was almost totally consumed in the micropilar region (Figura 1G). Meanwhile, even in the less consumed lateral regions, it was observed the presence of breaking(Figura 1I). The lateral endosperm was almost totally consumed with the presence of big empty spaces due to worn-out tissues, specially in the micropilar region (Figure 1H).

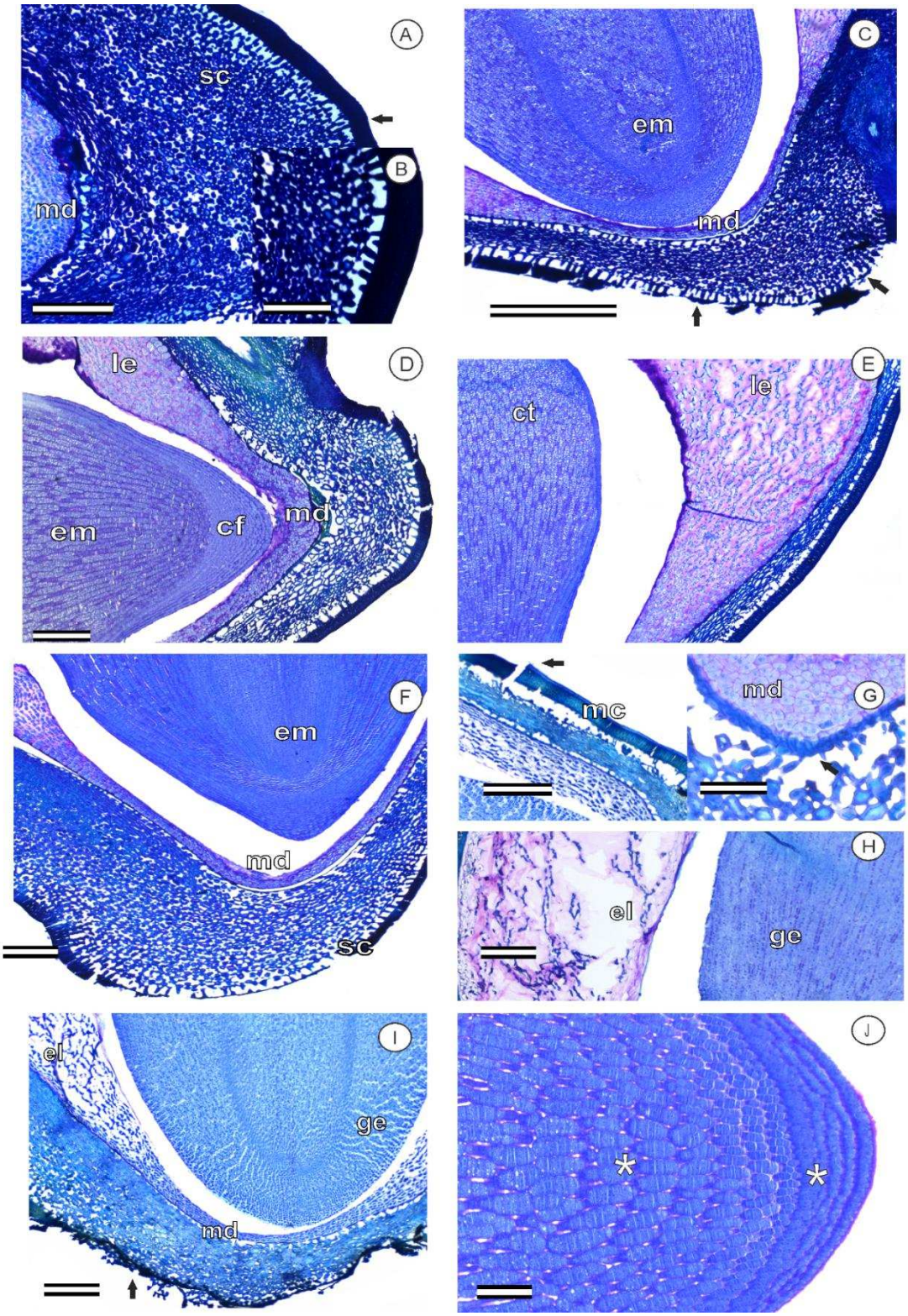

Figure 1: Anatomical changes during soaking process of *Melanoxylon brauna* seeds. A = Control group (0 h soaking); B = detail of intact integument (0 h); C = 16 h; D = 24 h; E = detail of lateral endosperm consumption (24 h); F = detail of embryo preparing for germination (24 h); G = 48 h; H = lateral endosperm consumption (48 h); I = integument detrition (72 h) and J = detail of embryonic axis cells (72 h). Arrows indicate weakening of endosperm during germination. Asterisks indicate

division and elongation of embryo cells. Em = embryo; le = lateral endosperm; mc = micropyle; md = endosperm. Bars = 300 µm; exception: B = 200 µm.

3.2. Metabolism changes

The presence of superoxide anion was not detected under tested conditions.Hydrogen peroxide concentration in micropyle endosperm region increased in the first 24 h of soaking, subsequently decreasing (Figure 7).

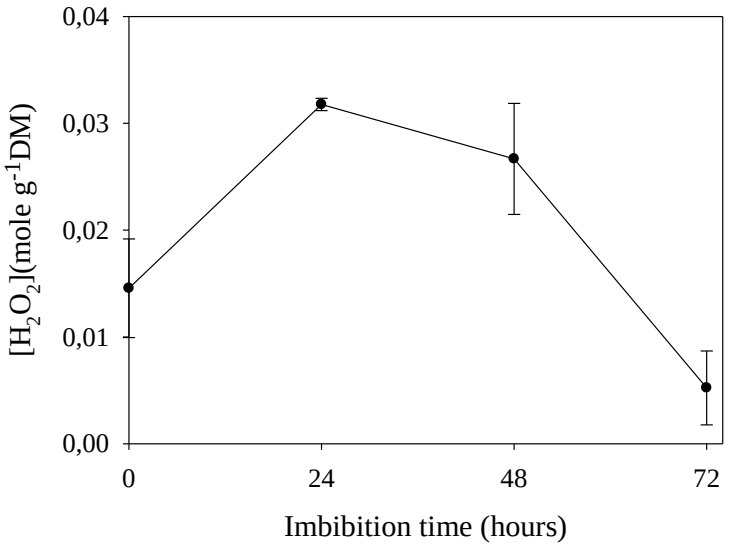

Figure 2:Hydrogen peroxide levels in micropyle endosperm of *M. brauna* seeds during (Vertical bars indicate standard error of the mean, n = 5).

It was detected the presence of iron (50,48 mg.Kg^{-1}) and copper (4,35 mg.kg^{-1}) in the micropyle of Braúna seeds.

The strength required to break up the micropilar region reduced after 24 h for all treatments. However, the addition of 40 mM of H_2O_2 had a significant reduction compared to other treatments (Figure 3).

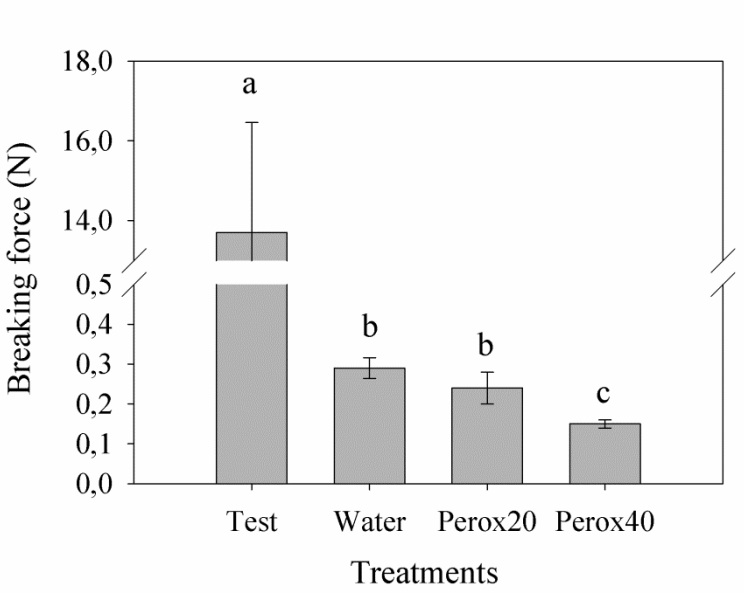

Figure 3: Força necessária para rompimento da região micropilar em micropilas secas (control) e embebidas durante 24 horas em água e solução de peróxido de hidrogênio nas concentrações de 20 (Perox20) e 40 mM (Perox 40). Barras verticais indicam erro padrão, n = 5.

It was also not detected activity of the enzymes endo-β-mannanase or α-galactosidase, polygalacturonase and pectinmetylesterase in soaked micropyles without embryo. For complete soaked seeds, it was observed activity of these three enzymes in the micropilar endosperm.

Activity of α-galactosidase showed a decrease in the first 24 h of soaking, with posterior increase (Figure 4A). Polygalacturonase activity increased in the first 48 h of soaking (Figure 4B). Pectinmetylesterase activity decreased in the first 24 h of soaking, independente of temperature (Figure 4C). After 48 h occured a inexpressive increase in the activity of these enzymes.

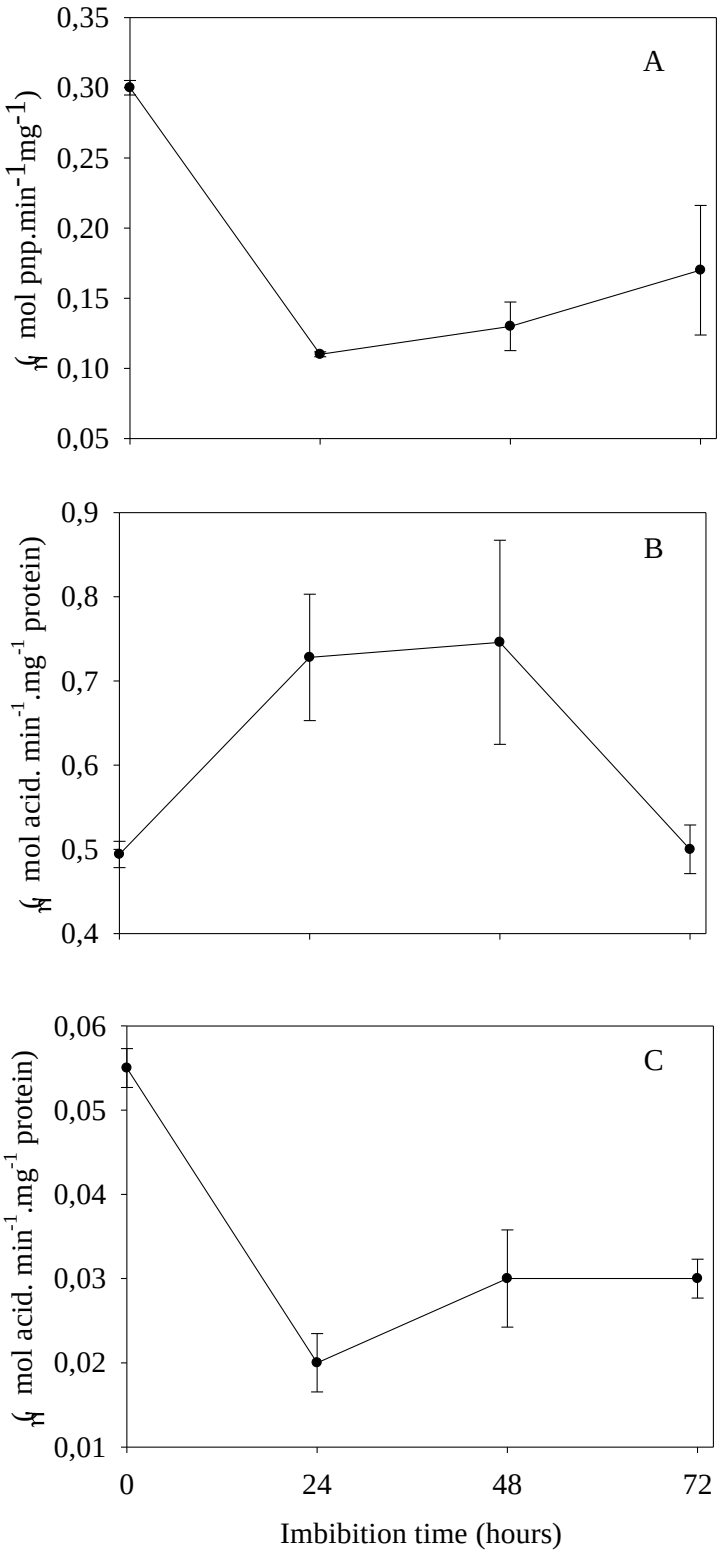

Figure 4: Specific activity of the enzymes α-galactosidase (A), polygalacturonase (B) and PME (C) in the micropilar endosperm of *M. brauna* seeds during soaking process. Vertical bars indicate standard error of mean, n = 5.

4. Discussion

The beginning of germination process was observed before the first 24 h of soaking, evidenced by the worn-out cells of tegument (Figure 1C). Embryo growth potential and weakening of surrounding tissue act concomitantly, contributing for primary root protrusion through surrounding tissues (Nambara *et al.*, 2010; Bewley *et al.*, 2013).

The sequence of events described in Figure 1 contradicts the hypothesis described in literature. This hypothesis affirms that micropylar endosperm consumption precedes lateral endosperm consumption in order to facilitate root protrusion (Muller *et al.*, 2006; Bewley *et al*, 2013; Yan *et al.*, 2014). However, in *M. brauna* seeds, lateral endosperm consumption is the first one to begin, and it also occurs concomitantly to micropilar endosperm consumption.

In other species, the micropilar endosperm is the first one to be consumed during soaking process. For example, it happens in tomato, wheat, barley, rice, watercress and tabacco seeds (Linkies *et al.*, 2009, Lee *et al.*, 2012; Yan *et al.*, 2014). This difference between both sequences of events might be associated to several factors, such as: integument thickness, soaking velocity, morphoanatomical characteristics and phytormonium activity.

During the first 24 h of soaking, there was an increase of hydrogen peroxide concentration (Figure 2). This compounds increase facilitates the weakening of cell walls that compose integument, mostly in the mycropyle region, helping to promote primary root protrusion. Hydrogen peroxide has been shown to be an important regulator of germination potential in seeds like Arabidopsis, by regulating the synthesis of hydrolytic enzymes that allow the breaking and mobilization of reserve compounds (Bethke *et al.*, 2007; Linkies *et al.*, 2009; Voegele *et al.*, 2011; Zhang *et al.*, 2014).

The reduction of hydrogen peroxide levels after 24 h soaking and the presence of Fe and Cu in micropilar region indicates that hydrogen peroxide might be consumed via Fenton Reaction. The reaction, defined as the catalytic generation of hydroxyl radicals (OH•) originated from the chain reaction between metal ions in acid medium, usually iron (Fe^{2+})and hydrogen peroxide (H_2O_2), has proved to be very efficient at oxidizing toxic and non-biodegradable organic compounds (Nogueira *et al.*, 2007).

The OH• radical is considered the most oxidizing among ROSs and its high reactivity results in fast and unspecific reactions with different substrates, potentially reacting with all types of biological molecules (Barbosa *et al.,* 2014). Although it is not described in literature the occurrence and effects of Fenton Reaction during germination, it is evident that moderated levels of hydroxyl radical act as an important compound regulating root growth and cell wall weakening resulting from the degradation of lignocellulosic and polysaccharide compounds (Mylona & Polidoros, 2011; Faure *et al.,* 2012; Barbosa *et al.,* 2014).

The occurrence of enzyme activity of α-galactosidase, polygalactunorase and pectinmetylesterase only in the micropilar endosperm of entire soaked seeds sugests that these enzymes are produced in the embryo and transfered to the micropyle during hidration. Such mechanism endorses the hypothesis that these enzymes act weakening cell walls and contributing for germination. Activity of α-galactosidase from the beginning of the soaking process suggests that the enzyme is formed since seed development and act during *M. brauna*germination. The observed results resemble to the results obtained by Bicalho et al. (2016), that verified similar behavior during germination of *Acrocomia aculeata* at 27ºC.

In most cases, α-galactosidase act by mobilizing polysaccharides of cell walls and raffinose series oligosaccharides. The enzyme degradation provides energy for the germination process (Buckeridge *et al.,* 2004; Ataíde *et al.,* 2013). The enzyme activity had an increase after the first 24 h, and did not presented expressive changes after 48 and 72 h. Thus, it might explain anatomical changes in integument and adjacent tissues to the embryo during advanced periods of germination process. It was reported increase in α-galactosidase activity during germination of *Glycine max* and *Lycopersicon esculentum*, evidencing the enzyme importance during the process (Furtado *et al.,* 2001; Guimarães *et al.,* 2001).

Polygalactunorase also act weakening the cell wall and consequently promoting root protrusion (Pontes, 2008). The enzyme is active even before soaking *Schizolobium parahyba* seeds, increasing its activity close to germination (Magalhães *et al.,* 2009). In *Lycopersicon esculentun* seeds, the enzyme activity increased during soaking process and even more in the moment of germination (Strit *et al.,* 1999). Detrition in tissues that composes integument caused in the previous 24 h also might be related to polygalacturonase activity. Although was not possible to

observe the highest activity of this enzyme in the beginning of hydration, the enzyme pre-exists in seed (Ataíde *et al.,* 2013). Thus, after initiating the soaking process, this enzyme act in the dissolution and loss of cohesion between cells, by catalyzing the hydrolysis of α 1-4 bond between galacturonic acid residues of the pectin chain (Figure 8B e 8C) (Fischer and Bennett, 1991). This way, polygalaturonic activy becomes fundamental to facilitate germination.

Studies using Arabidopsis (Iglesias-Fernandes *et al.,* 2013) and *Lepidium* (Morris *et al.,* 2011), for example, highlightened the role of enzymes that degrades cell wall components during seeds germination. The increase occurs around 24 h for polygalactunorase and 48 h for α-galactosidase and pectinmetylesterase (Figure 8). In both periods, it was possible do observe the highest degradations in tissues that composed integument, followed by lateral endosperm consumption and micropilar endosperm reduction (Figure 6D-6J).

Pectinmetylesterase (PME) are involved in deesterification of methyl esterified pectin, which can not be recognized by polygalactunorases. The enzyme play an important role in pectin degradation (Muller *et al.,* 2013), cell wall expansion and growth (Dixit *et al.,* 2016). PME activity presented high levels in the beginning of soaking process and declined in the first 24 h. From 24 h onwards, it was observed an intensification of its activity at 25, 35 and 45ºC. At 35ºC occurred activity decrease after 48 h. According to these results, it is possible to infer that the enzyme is preformed and it is associated with integument detrition, micropylar endosperm reduction and mobilization of lateral endosperm reservations, all events intensified after 24 h of soaking.

These results are consistent with results obtained by Borges et al. (2015), which verified increase in PME activity during hydration of *M. brauna* seeds at 30ºC. Scheler et al. (2015) detected the presence of PME during germination of *Lepidium sativum* seeds during germination, suggesting that the enzyme play a fundamental role in the teste rupture during root protrusion.

The role of H_2O_2 summed up to the activity of hydrolytic enzymes facilitate the cell expansion, due to turgidity, promoting the weakening of micropilar endosperm of *M. brauna* seeds during germination. Thus, the reduction of this compartment is visibly noticed, and it becomes as narrower as soaking time progresses (Figure 6). It

is also possible to observe the lateral endosperm consumption, which serves as an energy source for the embryo growth (Figure 6E and 6H).

5. Conclusions

Alterations in integument and lateral endosperm consumption begin in the first 24h of soaking, initiating lateral endosperm consumption and posterior weakening of micropilar endosperm.

There is a increase of H_2O_2 concentration during the first 24 h of soaking.

There are indications of occurrence of Fenton Reaction in micropilar endosperm of seeds.

There was no activity of the enzyme endo-β-mannanase in the tested conditions.

The weakening of micropilar endosperm due to activity of the enzymes α-galactosidase, polygalacturonase and PME is indispensible to the end of germination by endosperm rupture.

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Atividade enzimática e produção de EROs no endosperma micropilar de sementes de *Melanoxylon Brauna* Schott. (Brauna) durante a germinação sob estresse térmico.

Marcone Moreira Santos [1*], Eduardo Euclydes de Lima e Borges[1], Glauciana da Mata Ataíde[3], Genaina Aparecida de Souza[4].

[1]Departamento de Engenharia Florestal – Universidade Federal de Viçosa, Viçosa, MG, Brasil,

[2]Departamento de Ciência Agrária – Universidade Federal de São João Del Rei, Sete Lagoas, MG, 12 Brasil

[3]Deparatamento de Biologia Vegetal – Universidade Federal de Viçosa, Viçosa, MG, Brasil

*email para correspondência: marconemoreirasantos@hotmail.com

Resumo

O presente trabalho tevepor objetivos avaliara germinação ea atividade enzimática na região micropilar de sementes de *M. brauna* durante a germinação sob estresse térmico**.** A germinação foi avaliada nas temperaturas de 25, 35 e 45 °C. Os níveis de ânion superóxido, peróxido de hidrogênio e atividade enzimática foram avaliadas em micrópilas de sementes embebidas por 0, 24, 48 e 72 horas a 25, 35 e 45 °C. Houve maior germinação em 25 °C. Não foi detectada atividade da endo-beta-mananase. A temperatura de embebição influênciou na atividade das enzimas α-galactosidase(α-Gal), poligalacturonase (PG), pectinametilesterase (PME), pectinaliase (PL), celulases totais, β-1,3 glicosidase e β-1,4 glicosidase na região micropilar de sementes de *M. brauna*indicando a importância de tais enzimas na germinação.

Palavras-chave:germinação, temperatura, espécies reativas de oxigênio, parede celular, enzimas.

Abstract

This work aims to quantify the germination, presence of ROS, possible occurrence of Fenton reaction and enzymatic activity in the micropylar region of *M. brauna* seeds during a germination under thermal stress. A presence of Fe and Cu ions in dry

micropiles and a bursting force in dry microgels impregnated with water and H_2O_2 peroxide solution (20 and 40 mM) for 24 h were evaluated. Germination was evaluated at temperatures of 25, 35 and 45 ° C. Superoxide anion, hydrogen peroxide and enzymatic activity levels were evaluated in 0, 24, 48 and 72 hours soaked micropipettes at 25, 35 and 45 ° C. There was greater germination at 25 ° C. There is a probable occurrence of Fenton reaction in the micropylar region. Endo-beta-mannanase activity was not detected. An imbibition temperature influenced the activity of α-galactosidase (α-Gal), polygalacturonase (PG), pectin methyl esterase (PME), pectinase (PL), total cellulases, β-1,3 glycosidase and β-1,4 glycosidase micropylar region of M. brauna seeds indicating the importance of such enzymes in germination.

Key words: germination, temperature, reactive oxygen species, cell wall, enzymes.

1. Introdução

Melanoxylon brauna Schott. (Braúna) é uma espécie arbórea nativa da Mata Atlântica, de ocorrência no sudeste e nordeste brasileiro. Possui madeira de ótima qualidade e sua casca e seiva são amplamente utilizadas na indústria e medicina popular (Lorenzi, 2008). No entanto, devido à intensa exploração de sua madeira, a espécie encontra-se atualmente na "Lista oficial das espécies da flora brasileira ameaçadas de extinção", na categoria vulnerável, segundo o Ministério do Meio Ambiente. Portanto, informações quanto a fisiologia e germinação das sementes podem contribuir para o desenvolvimento de novas estratégias para conservação da espécie (Brasil, 2014).

A germinação envolve uma série de processos físicos, fisiológicos e bioquímicos relativamente complexos. Na embebição ocorrem alterações nas células do embrião envolvendo espécies reativas de oxigênio (EROs), proteínas e enzimas capazes de alterar as características da parede. Entretanto, em muitas espécies, a região micropilar constitui uma barreira para a extensão e posterior emergência da radícula. Nesse caso, a penetração da radícula nesse tecido é precedida pelo enfraquecimento do endosperma na região micropilar da semente, por onde ocorre a emergência radicular (Yan *et al.,* 2014).

Os mecanismos relacionados à ruptura da região micropilar, especialmente o enfraquecimento dos tecidos envolvidos, são ainda pouco conhecidos. Porém, é aceito que o alongamento do eixo hipocótilo radícula e o enfraquecimento dos

tecidos da micrópila estão envolvidos na germinação. Esses dois processos atuam conjuntamente para que haja protrusão da raiz primária. Ambos processos requerem a perda da integridade da parede celular devido a atividade de hidrolases, transglicosilases, celulases, hemicelulases e a presença de espécies reativas de oxigênio (Borges *et al.*, 2015; Koen *et al.*, 2017; Singh *et al.*, 2017)

Enzimas como β-mananases, α-galactosidases (α-Gal), poligalacturonases (PG), pectina metil esterases (PME), pectina liase (PL) e celulases são ativadas no início da hidratação das sementes e são responsáveis pela degradação dos polissacarídeos das paredes celulares, contribuindo assim para o enfraquecimento do endosperma micropilar, possibilitando a protrusão da raiz primária (Betts *et al.*, 2017; Mascher *et al.*, 2017). Dentre os fatores que influenciam a atividade enzimática durante a germinação, a temperatura pode promover redução, aumento ou até mesmo inibição dessa atividade (Laghmouchi *et al.*, 2017).

Nesse sentido, o estudo da temperatura tem papel fundamental para a compreensão do processo germinativo, pois este fator é determinante no acelerar ou desacelerar do metabolismo. Além disso, é um importante fator físico que influencia na velocidade de embebição, modificando a velocidade das reações químicas que promovem a mobilização de reservas e a síntese de substâncias necessárias ao crescimento de plântulas, assim, a temperatura é um fator determinante para ocorrência de uma espécie em dado local (Medina *et al.*, 2016).

Estudos indicam que, devido ao agravamento do efeito estufa, a temperatura do planeta aumentará ao longo do século numa escala entre 1 e 5 °C (PBMC, 2013). Considerando a possibilidade desse aumento de temperatura nos próximos anos questiona-se: como as espécies se adaptarão e como podemos interferir para que elas não desapareçam perante tal mudança?

Diante da importância da *M. brauna*, das previsões de mudanças climáticas, da influência da temperatura na germinação e da escassez de informações sobre eventos bioquímicos na região micropilar, esse trabalho teve por objetivos: quantificar a germinação, a presença de EROs, a ocorrência da reação de Fenton e as atividades das enzimas endo-β-mananase, α-galactosidase, poligalacturonase, pectina metil esterase, pectina liase, celulases totais, β-1,3 glicosidase e β-1,4 glicosidase no endosperma micropilar de sementes de *M. brauna* durante a germinação sob estresse térmico.

2. Material e métodos

Os frutos foram coletados na região de Leopoldina, Minas Gerais. Após a coleta, os frutos foram secos ao sol até a sua abertura, sendo as sementes extraídas manualmente. As sementes foram beneficiadas, acondicionadas em tambores de fibra e armazenadas em câmara fria a 5 °C e 60% UR, até o momento de realização dos experimentos.

2.1. Germinação

As sementes foram colocadas para germinar em placas de Petri sobre duas folhas de papel germitest umedecidas com água destilada nas temperaturas constantes de 25, 35 e 45 °C. Foram consideradas germinadas as sementes que apresentaram emissão de raiz primária, sendo os resultados expressos em porcentagem média. Foram utilizadas cinco repetições de 20 sementes para cada tratamento.

2.2. Alterações no metabolismo

A produção de espécies reativas de oxigênio (EROs) foi quantificada em micrópilas isoladas de sementes embebidas por 0 (sementes secas), 24, 48 e 72 h em 25, 35 e 45 °C. Após a obtenção das micrópilas, estas foram liofilizadas e armazenadas à – 20 °C até o início das quantificações.

2.2.1. Produção de EROs

Ânion superóxido: Para avaliar a produção de ânion superóxido foram utilizadas amostras de 100 mg de micrópilas. A quantificação de $O_2^{\bullet-}$ foi realizada através da quantidade de adenocromo acumulado (Misra and Fridoovich, 1971), utilizando-se a absortividade molar de 4,0 x 103 M^{-1} (Boveries, 1984).

Peróxido de hidrogênio: Amostras de 100 mg de micrópilas foram pesadas, trituradas e homogeneizadas (Kuo and Kao, 2003). A determinação da absorbância foi feita em espectrofotômetro, em 560 nm, e a quantificação de H_2O_2 foi realizada com base em curva de calibração utilizando-se de concentrações de peróxido como padrão (Gay and Gebicki, 2000).

2.3. Atividade enzimática*:*

As análises enzimáticas foram realizadas em micrópilas de sementes embebidas por 0 (sementes secas), 24, 48 e 72 h em 25, 35 e 45 °C posteriormente liofilizadas e armazenadas à – 20 °C. Também foi avaliada a atividade enzimática

nas micrópilas isoladas e posteriormente embebidas nas condições descritas anteriormente.

A atividade da enzima endo-β-mananase foi calculada de acordo com Downie et al. (1994).

Utilizou-se a metodologia descrita por Viana (2002) na quantificação da α-galactosidase. A unidade de enzima foi definida como a quantidade de proteína necessária para produzir 1 mol de PNP por minuto nas condições do ensaio.

A atividade da PG foi determinada pela dosagem de açúcar redutor produzido segundo o método do DNS (3,5 dinitrossalicílico), adaptado de Miller (1959). Os resultados foram expressos em unidades de poligalacturonase, onde uma unidade corresponde à quantidade de ácido galacturônico liberado (μM) por minuto de reação (U/mL).

A extração da PME foi realizada de acordo com o método descrito por Pinto et al. (2011). A atividade foi quantificada de acordo com Grsic-Rausch e Rausch (2004), sendo considerado que uma unidade de PME corresponde a 1 uM de NADPH formado por minuto em pH 7,5 a 25 °C.

A atividade da PL foi determinada pelo método espectofotometro a 235 nm, conforme descrito por Albersheim e Kilias (1962), utilizando a absorvidade molar de 5550 $mol^{-1}.L.cm^{-1}$ para o cálculo (Albersheim, 1996).

A atividade da celulase em papel de filtro foi determinada no caldo de cultivo obtido no final da incubação, usando-se uma fita de papel de filtro de 6 cm^2 em um tubo de ensaio contendo 0,5 mL do caldo e 1,0 mL de tampão acetato de sódio 50 mM, pH 5,0. a reação foi interrompida pela adição de 1 mL do reagente DNS. Os açúcares redutores foram determinados em espectrofotômetro, utilizando-se comprimento de onda (λ) de 540 nm (Ghose, 1987).

A atividade da β-glicosidase foi avaliada utilizando-se o substrato sintético ρ-nitrofenil-β-D, glicopiranosídeo (β-1,3) e CMC (β-1,4,) em tampão fosfato 50 mM, pH 6,0. A atividade enzimática foi expressa em U/g do substrato nas condições do ensaio (Singhania *et al.,* 2013).

A concentração de proteínas para todas as amostras foi determinada pelo método de Bradford (1976), utilizando-se curva padrão construída com albumina sérica bovina (BSA), de 2,5 a 50 µg de proteína.

Para todas as análises, foi utilizado o delineamento inteiramente casualizado (DIC) com cinco repetições. Os dados foram submetidos a análise de variância eregressão utilizando o software estatístico SAS (versão 9.2; SAS Institute, Inc., Cary, NC, EUA) e as médias obtidas para os tratamentos foram comparadas pelo teste de Tukey 5% de significância.

3. Resultados

A temperatura de 25 °C resultou nos maiores valores de germinação, apresentando média de 83%, enquanto a 35 °C ocorreu redução para 55%. Não ocorreu germinação a 45 °C, indicando a morte das sementes.

3.1. Espécies reativas de oxigênio

Não foi detectada a presença de ânion superóxido nas condições testadas. Para todas as temperaturas houve aumento na concentração de peróxido de hidrogênio nas primeiras 24 horas de embebição no endosperma micropilar das sementes. De modo geral, em 45 °C os níveis de H_2O_2 foram superiores em todos os tempos de embebição (figura 3, capítulo 1), com valor significativamente maior que nas outras duas temperaturas em 24 horas de hidratação. Em 25 °C os valores foram significativamente inferiores em relação às outras duas temperaturas.

3.2. Atividade Enzimática

Não foi detectada atividade enzimática nas micrópilas sem o embrião após 24 h de embebição. Em sementes embebidas inteiras, não foi detectada a atividade da enzima endo-β-mananase.

Houve diferença significativa na atividade das demais enzimas nas diferentes temperaturas. A α-Gal apresentou maior atividade nas temperaturas de 25 e 35 °C (figura 1A). As enzimas PG, PME e PLapresentaram maiores atividades após 72 h de embebição a 45 °C (figura 1B, 1Ce 1D).

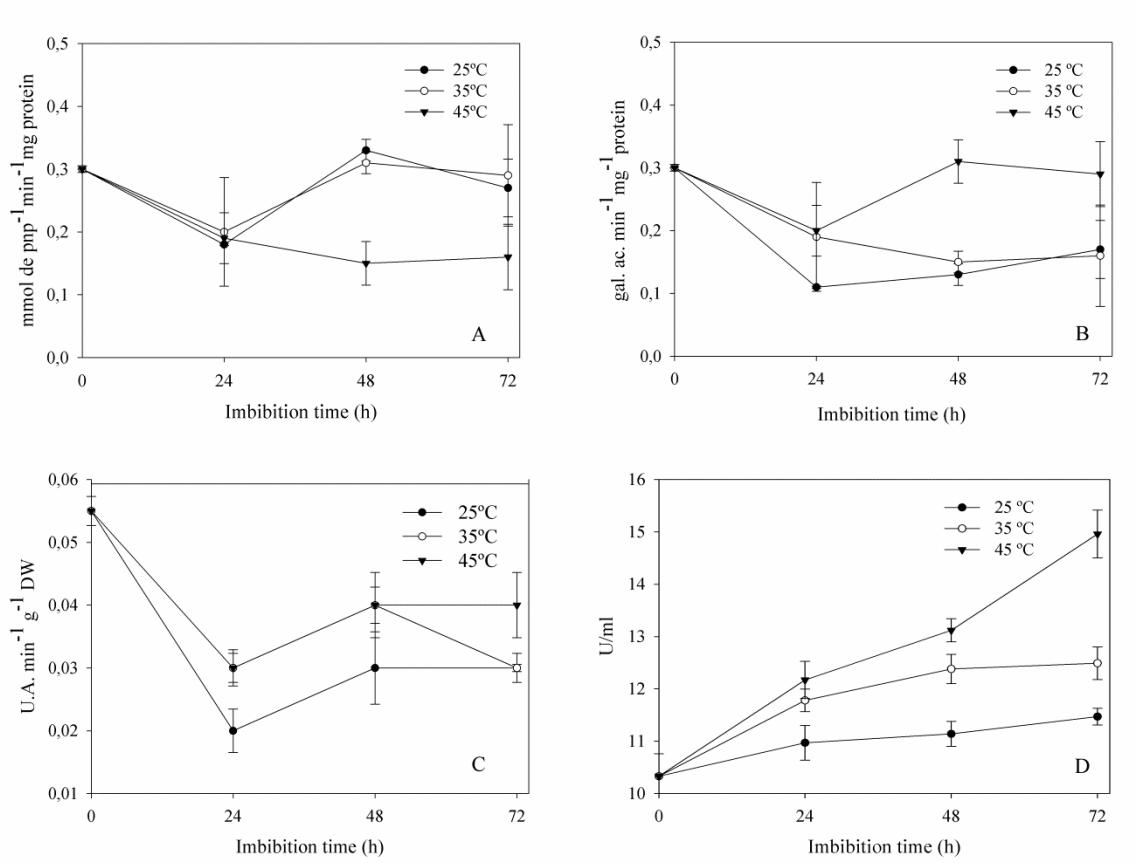

Figura6: Atividade específica das enzimas α-galactosidase (A), poligalacturonase (B), pectina metil esterase (C), pectina liase (D), * Indicam diferença estatística entre as médias. Barras verticais indicam o erro padrão, n = 5**.**

As atividades das celulases totais e da β- 1,4. glicosidase foram superiores em 35 º C após 24 h de embebição (figura 2A e 2C). A embebição a 35 °C também favoreceu a atividade da β- 1,3 glicosidase até o final do período de 72 h (figura 2B).

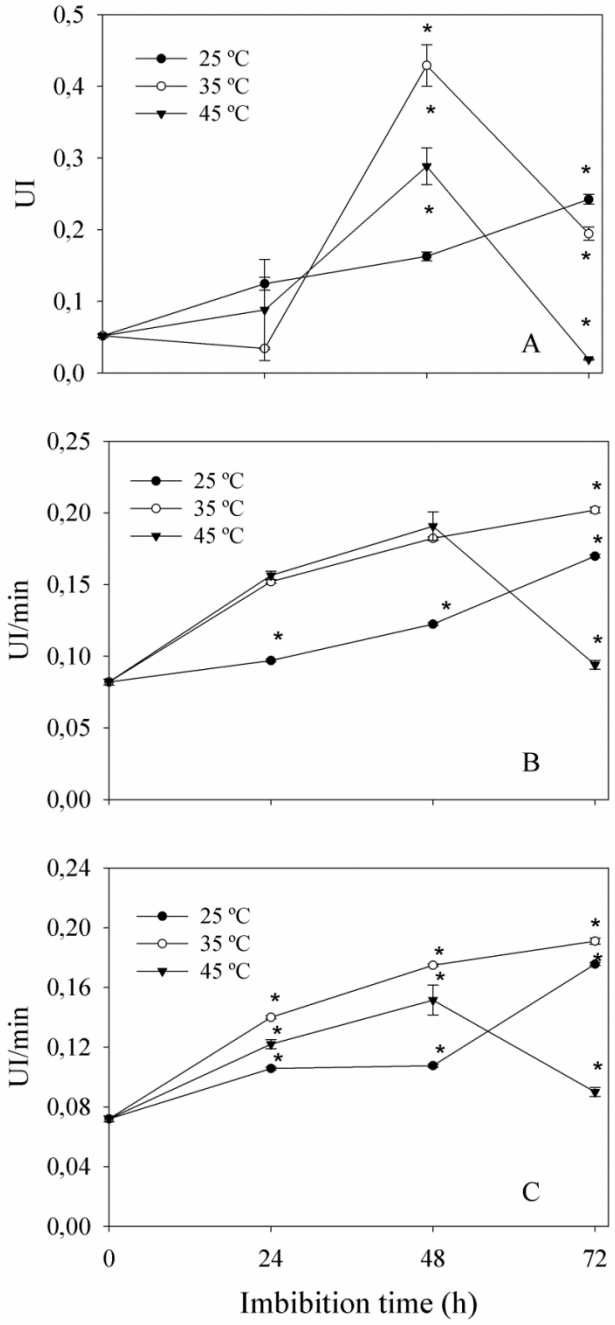

Figura 2: Celulases totais(A), β- 1,3. glycosidase (B) e β- 1,4. glicosidase (C) na região micropilar de sementes *M. braunad*urante a embebição em 25, 35, e 45 °C. * Indica diferença estatística entre as médias. Barras verticais indicam o erro padrão, n = 5.

4. Discussão

A temperatura é um fator que exerce grande influência no processo germinativo, regulando a absorção de água e as reações bioquímicas envolvidas em

todo o processo metabólico (Bewley *et al.*, 2013; Graeber et. al., 2014). De modo geral, a faixa entre 20 e 30°C é a adequada para a germinação de grande número de espécies subtropicais e tropicais (Oliveira *et al.*, 2016; Silva *et al.*, 2016).

Em sementes de Brauna, o comportamento da germinação nas diferentes temperaturas está em conformidade ao descrito na literatura. Flores *et al.*, 2014, verificaram que a germinação ocorre entre 12,3 e 42,5 °C, sendo 27°Ca temperatura ótima e sua ausência a 45 °C. A embebição por mais de 72 h a 45 °C causa danos irreversíveis às sementes de Brauna, impossibilitando a germinação, mesmo após transferidas para 25 °C (Santos *et al.*, 2017). Outro fato observado é que sementes submetidas a 35 e 45 °C sofrem maior ataque de micro-organismos.

Os níveis de peróxido de hidrogênio também variaram em função das temperaturas de embebição. Em altas concentrações, os radicais livres atacam o sistema de membranas celulares causando sua desestruturação (Borges et al.; 2015; Karkonen e Kuchitsu, 2015, Santos *et al.*, 2017). Nesse sentido, as maiores concentrações de H_2O_2 em 45 °C pode ser apontado como uma das causas da morte das sementes por estresse oxidativo. As EROs podem causar danos estruturais e funcionais às células (Prodanovic *et al.*, 2007). Os danos peroxidativos decorrem, principalmente, da deterioração oxidativa de ácidos graxos insaturados das membranas pela ação de EROs presentes no interior das células (Flores et al., 2014).

Embora o H_2O_2 em altas concentrações seja prejudicial, em níveis controlados esse tem se mostrado um importante regulador da germinação. As paredes celulares são compósitos fibrosos em que as microfibrilas de celulose são coextensivas permeadas por polissacáridos não celulósicos. Essas redes fibrosas que são barreira à germinação, são enfraquecidas pela presença de peróxido de hidrogênio,cuja presença leva ao aumento da atividade de enzimas hidrolíticas, responsáveis pela quebra e mobilização de compostos de reserva (Linkies *et al.*, 2009; Voegele *et al.*, 2011; Yan *et al.*, 2014; Zhang *et al.*, 2014).

Deacordo com os resultados, houve atividades das enzimas α-galactosidase, PG, PME, PL, celulases totais, β-1,4 glicosidase e β-1,4 glicosidase apenas no endosperma micropilar de sementes embebidas inteiras. Tais resultados sugerem que essas são produzidas no embrião e transferidas para a micrópila durante a hidratação.

Tal mecanismo reforça a hipótese que essas atuam no enfraquecimento da parede celular, contribuindo assim para a germinação.

A ação dessas enzimas tem sem mostrado importante reguladora da germinação através do enfraquecimento da parede celular, permitindo a protrusão da raiz primária (Borges *et al.,* 2015; Bicalho *et al.,* 2016). As α-galactosidases atuam diretamente na quebra e mobilização de polissacarídeos de parede celular e em oligossacarídeos da série rafinoses, fornecendo assim energia para germinação (Farias *et al.,* 2015). A maior atividade da enzima ocorreu em 25 °C, temperatura com maior germinação, demonstrando sua importância ao processo.

Bicalho *et al.,* 2016 verificaram comportamento semelhante durante a germinação de sementes de macaúba (*Acrocomia aculeata*) a 27 °C. Em sementes de *Dalbergia nigra* a maior atividade da α-Gal ocorre a 25 °C(Ataíde *et al.,* 2013). A redução da atividade em 45 °C indica que as isoformas de α-Gal presente em sementes de Brauna são mais sensíveis ao estresse térmico e tendem a potencializar sua atividade em temperaturas próximas às ótimas de germinação.

Assim como a α-Gal, a PG tem se mostrado fundamental na germinação de diversas espécies. A PG cataliza a hidrólise das ligações α 1-4 entre os resíduos de ácido galacturônico da cadeia de pectina. Sua atividade tem sido investigada e se mostrado importante durante a investigação de várias espécies, como *Schizolobium parahyba* e Arabidopsis (Magalhães *et al.,* 2009; Han & Yang, 2015; Scheler *et al.,* 2015).

Após 24 h de embebição a PG apresentou maior atividade em 45 °C, se mantendo superior em 48 e 72 h. Tais resultados evidenciam que a temperatura ótima de germinação nem sempre coincide com a temperatura ótima de atividade das enzimas que participam do processo. Em sementes de *Dalbergia nigra* os picos de atividade de PG acontecem em 40, 45 e 60 °C, enquanto as maiores taxas de germinação ocorrem em 25 °C(Pontes et al., 2008). Em frutos de *Uapaca kirkiana, Ziphus mauritiana, Tamarindus ind*a e *Berchemia*, a faixa de temperatura ótima esteve entre 25 e 37°C(Muchuweti *et al.,* 2005). Em frutos de *Prunnuspérsica* a maior atividade ocorre próximo aos 35 °C(Sainz *et al.,* 2015).

A atividade das PG ocorre de forma concomitante a das PME, sendo que essas catalisam a desesterificação substâncias pécticas, hidrolizando grupos metil-

éster, produzindo pectinas de menor grau de metilação que servirão de substrato às PG (Sainz *et al.*, 2015). A atividade da PME se apresentou alta no início da embebição e reduziu nas primeiras 24h. A partir desse período, houve uma intensificação na sua atividade em 25, 35 e 45 °C. Em 35 °C ocorreu queda na atividade após 48h. De acordo com esses resultados, é possível inferir que esta enzima é pré-formada e está associada com o desgaste do tegumento, redução do endosperma micropilar e mobilização de reservas.

Esses resultados estão em conformidade àqueles obtidos por Borges *et al.* (2015), que verificaram aumento da atividade da PME durante a hidratação de sementes de brauna a 30 °C. Durante a germinação de sementes de *Lepidium sativum*, ocorre atividade da PME, sugerindo que essa desempenha papel importante no rompimento da testa durante a protrusão radicular (Scheler *et al.*, 2015).

Durante a hidratação de sementes de brauna, ocorre aumento da atividade de PL em todas as temperaturas, sendo que em 45 °C a atividade é intensificada. As PLs atuam na quebra de oligogalacturonídeos de parede celular e tem se mostrado importante durante a germinação, pois atuam no enfraquecimento do endosperma lateral e na indução da síntese de expansinas (Zhao *et al.*, 2008; Cao, 2012; Sainz *et al.*, 2015).

As celulases totais, β-1,3 glicosidase e β-1,4 glicosidase apresentaram maior atividade em 35 e 45 °C. No entanto, após 48 h de embebição, ambas as enzimas reduziram a atividade em 45 °C. Tal fato pode estar relacionado com a perda de vigor das sementes acarretada pelo estresse térmico o que gera a perda de atividade e desnaturação de diversas enzimas (Santos *et al.*, 2017).

As celulases são enzimas responsáveis pela degradação da celulose, principal composto presente nas células vegetais. Dentre as celulases totais, as β-glicosidases quebram a ligação química existente entre as duas unidades de glicose que formam a celobiose, liberando unidades de glicose (livres). Nesse sentido a atividade de tais enzimas também favorecem o enfraquecimento do endosperma micropilar e expansão da radícula, fato confirmado em sementes de *Coffea arábica*, *Lactuca sativa* e *Lepidium sativum* (Castro e Pereira, 2010; Chen, *et al.*, 2016; Ogorék, 2016).

De modo geral, a temperatura influenciou na atividade enzimática. No entanto, não é possível estabelecer uma relação entre a atividade enzimática e a

germinação nas diferentes temperaturas. Nem todas as enzimas investigadas potencializaram sua atividade na temperatura ótima de germinação, porém todas, exceto a β-Man apresentaram atividade em 25 °C evidenciando importância no processo. Vale ressaltar que em 45 °C não ocorre comprometimento do sistema enzimático analisado e assim outros fatores contribuíram para redução da germinação em 35 °C e morte das sementes em 45 °C.

É provável que a maior atividade enzimática em 35 e 45 °C gera maior enfraquecimento da parede, facilitando a ocorrência de danos as membranas e demais compartimentos celulares devido ao acúmulo de EROs (Santos *et al.*, 2017) e ataque de microorganismos.

5. Conclusões

A temperatura de 25 °C favorece a germinação, enquanto em 45 °C ocorre a morte das sementes.

Em 45 °C há maior acúmulo de H_2O_2 na região micropilar.

Não há atividade da β-Man na região micropilar.

As maiores atividade da α-GAL ocorrem em 35 e 45 ºC.

A temperatura de 45 ºC favorece a atividade das enzimas PG, PME e PL.

O pico de atividade das celulases totais ocorre em 48 h de embebição na temperatura de 35 ºC.

As maiores atividades da β-1,3 e β-1,4glicosidase ocorrem em 35 e 45 ºC.A partir de 48h de embebição há redução da atividade em 45 ºC.

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Conclusões gerais

A temperatura de 25 ºC e a embebição por até 72 h em 35 ºC com posterior transferência para 25 ºC favorecem a germinação de *M. Brauna.*

Em 45 ºC ocorre maiores danos ao sistema de membranas e maior acúmulo de H_2O_2,

Em 45 ºC ocorre comprometimento do sistema antioxidante com queda na atividade das enzimas SOD, APX, CAT e POX.

Ocorre a morte das sementesem 45 ºapós 72 h.

Alterações anatômicas tem início nas primeiras 16 h de embebição.

Após 24 h de embebição há início do consumo do endosperma lateral e posterior enfraquecimento do endosperma micropilar.

A atividade da endo-β-mananase nas condições testadas é ausente.

O enfraquecimento do endosperma micropilar pela ação das enzimas alfa-galactosidase, poligaltacturonases e PME é imprescindível para a conclusão da germinação por ruptura da região micropilar.

A presença de H_2O_2, íons de Fe e Cu e redução da força de ruptura da região micropilar após embebição, indicam provável ocorrência da reação de Fenton.

A temperatura influencia na atividade das enzimas, α-Gal, PG, PME, PL, celulases totais, β-1,3 glicosidase e β-1,4 glicosidase, porém, o estresse térmico não compromete esse sistema enzimático.

ANEXOS

Tabela 2: Análise de variância dos dados de germinação (Artigo 1).

FV	*gl*	*QM*	*F*	*valor-P*	*F crítico*
Entre	8	4427,66	64,06	4,9E-22	2,208518
Dentro	36	69,11			
Total	44				

Tabela 3: Análise de variância dos dados de IVG (Artigo 1)

FV	*gl*	*QM*	*F*	*valor-P*	*F crítico*
Entre	8	10,00744	32,10823	3,77E-14	2,208518
Dentro	36	0,311678			
Total	44				

Tabela 4: Análise de regressão para os dados de condutividade elétrica em 25 ºC (Artigo 1)

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,673445	0,673445	0,008449	0,935142
Resíduo	2	159,4182	79,70912		
Total	3	160,0917			

Tabela 5: Análise de regressão para os dados de condutividade elétrica em 35 ºC (Artigo 1)

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	291,3898	291,3898	231,3425	0,004295
Resíduo	2	2,51912	1,25956		
Total	3	293,9089			

Tabela 6: Análise de regressão para os dados de condutividade elétrica em 45 ºC (Artigo 1)

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	7436,682	7436,682	8,107563	0,104384
Resíduo	2	1834,505	917,2525		
Total	3	9271,187			

Tabela 7: Análise de regressão para os dados de níveis de H_2O_2 no eixo embrionário em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,010974	0,010974	0,070044	0,816051
Resíduo	2	0,313343	0,156671		
Total	3	0,324317			

Tabela 8: Análise de regressão para os dados de níveis de H_2O_2 no eixo embrionário em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,006752	0,006752	0,048078	0,846785
Resíduo	2	0,280878	0,140439		
Total	3	0,28763			

Tabela 9: Análise de regressão para os dados de níveis de H_2O_2 no eixo embrionário em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,001315	0,001315	0,051288	0,841877
Resíduo	2	0,05129	0,025645		
Total	3	0,052605			

Tabela 10: Análise de regressão para os dados de níveis de H_2O_2 na região micropilar em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	5,48E-05	5,48E-05	0,291969	0,643085
Resíduo	2	0,000375	0,000188		
Total	3	0,00043			

Tabela 11: Análise de regressão para os dados de níveis de H_2O_2 na região micropilar em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,000113	0,000113	3,933046	0,18581
Resíduo	2	5,74E-05	2,87E-05		
Total	3	0,00017			

Tabela 12: Análise de regressão para os dados de níveis de H_2O_2 na região micropilar em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,000115	0,000115	0,656722	0,502815
Resíduo	2	0,000351	0,000176		
Total	3	0,000467			

Tabela 13: Análise de regressão para os dados de níveis de MDA nos eixos embrinários em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	92,12692	92,12692	3,815135	0,190019
Resíduo	2	48,29549	24,14775		
Total	3	140,4224			

Tabela 14: Análise de regressão para os dados de níveis de MDA nos eixos embrinários em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	91,38924	91,38924	8,103282	0,104431
Resíduo	2	22,55611	11,27805		
Total	3	113,9453			

Tabela 15: Análise de regressão para os dados de níveis de MDA nos eixos embrinários em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	35,78535	35,78535	2,031666	0,290122
Resíduo	2	35,22759	17,61379		
Total	3	71,01293			

Tabela 16: Análise de regressão para os dados da atividade da enzima SOD em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,001201	0,001201	1,132453	0,398732
Resíduo	2	0,002122	0,001061		
Total	3	0,003323			

Tabela 17: Análise de regressão para os dados da atividade da enzima SOD em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,003458	0,003458	0,473491	0,562477
Resíduo	2	0,014608	0,007304		
Total	3	0,018067			

Tabela 18: Análise de regressão para os dados da atividade da enzima SOD em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,009901	0,009901	1,505722	0,344634
Resíduo	2	0,013152	0,006576		
Total	3	0,023053			

Tabela 19: Análise de regressão para os dados da atividade da enzima APX em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,001072	0,001072	1,57769	0,335937
Resíduo	2	0,001359	0,00068		
Total	3	0,002431			

Tabela 20: Análise de regressão para os dados da atividade da enzima APX em 35 ºC (Artigo 1).

	gl	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	1,35E-06	1,35E-06	0,001213	0,97538
Resíduo	2	0,002222	0,001111		
Total	3	0,002223			

Tabela 21: Análise de regressão para os dados da atividade da enzima APX em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,00408	0,00408	1,807148	0,311035
Resíduo	2	0,004515	0,002257		
Total	3	0,008594			

Tabela 22: Análise de regressão para os dados da atividade da enzima CAT em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	5,239808	5,239808	12,66859	0,07067
Resíduo	2	0,827212	0,413606		
Total	3	6,067021			

Tabela 23: Análise de regressão para os dados da atividade da enzima CAT em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	1,758245	1,758245	55,26466	0,017618
Resíduo	2	0,06363	0,031815		
Total	3	1,821875			

Tabela 24: Análise de regressão para os dados da atividade da enzima CAT em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	64,47641	64,47641	6,719067	0,122152
Resíduo	2	19,19207	9,596035		
Total	3	83,66848			

Tabela 25: Análise de regressão para os dados da atividade da enzima POX em 25 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,00014	0,00014	65,32558	0,014965
Resíduo	2	4,3E-06	2,15E-06		
Total	3	0,000145			

Tabela 26: Análise de regressão para os dados da atividade da enzima POX em 35 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,011092	0,011092	10,91361	0,080693
Resíduo	2	0,002033	0,001016		
Total	3	0,013125			

Tabela 27: Análise de regressão para os dados da atividade da enzima POX em 45 ºC (Artigo 1).

FV	*gl*	*SQ*	*QM*	*F*	*F de sig.*
Regressão	1	0,002122	0,002122	36,51979	0,026307
Resíduo	2	0,000116	5,81E-05		
Total	3	0,002238			