LUCIANE PEREIRA REIS

EFFECTS OF HIGH TEMPERATURE ON MORPHOPHYSIOLOGICAL, ULTRASTRUCTURAL, AND BIOCHEMICAL PARAMETERS DURING THE GERMINATION OF TWO FOREST SPECIES

Thesis submitted to the Forest Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Genaina Aparecida de Souza
Danielle Santos Brito

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To my mother and grandmother (*in memorian*), with all my love and gratitude, for having fought for my dreams and supported me in all of them.
To my friend Jefferson Reis (*in memorian*), who could not experience this moment, but will be forever in my heart.

DEDICATE

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““*The fruits of tomorrow are the seeds of learning planted today*”.

Alexandre Willian

ABSTRACT

REIS, Luciane Pereira, D.Sc., Universidade Federal de Viçosa, September, 2021. **Effects of high temperature on morphophysiological, ultrastructural, and biochemical parameters during the germination of two forest species.** Adviser: Eduardo Euclydes de Lima e Borges. Co-advisors: Genaina Aparecida de Souza and Danielle Santos Brito.

Climate change resulting from anthropogenic actions has increased global temperature. These temperature increases will likely influence the survival of part of the species. Among the environmental factors, the temperature is one of the most critical for affecting the physiological, biochemical, and molecular factors of the seed. Temperature increases is considered one of the environmental factors that help in the overproduction of reactive oxygen species (ROS). The urgency of understanding seed responses to climate change is particularly important. A detailed overview of seed responses to heat stress can help formulate appropriate strategies for species conservation and preservation. Knowledge about the capacity of forest seeds to deal with temperature changes is still scarce. In this sense, the objectives of this work were: 1. To investigate the morphophysiological, biochemical, and ultrastructural changes during germination of *Melanoxylon brauna* seeds under heat stress; 2. Evaluate the effects of temperature on morphology, generation of ROS, antioxidant system, and mobilization of reserves in *Ormosia coarctata* seeds; 3. Characterize the effects of diphenyleneiodonium (DPI) on *M. brauna* germination, internal anatomy, hydrogen peroxide (H_2O_2) content, and activity of reserve enzymes and antioxidant enzymes under optimal conditions (25 °C) and stress by heat (40°C). To evaluate the internal morphology the seeds were radiographed. Ultrastructural and anatomical parameters were evaluated using transmission electron microscopy and light microscopy. ROS production, malondialdehyde (MDA) and glucose content, carbonyl proteins, and enzyme activity (superoxide dismutase - SOD, ascorbate peroxidase - APX, catalase - CAT, peroxidase - POX, glucose-6-phosphate dehydrogenase - G6PDH, lipase, α- and β-amylase and protease) were measured by spectrophotometric analysis. The high temperature causes a reduction in the percentage and speed of germination and affects the internal morphology of *M. brauna* and *O. coarctcta* seeds. In *M. brauna* seeds, heat stress decreases respiratory rates and compromises the structure of mitochondria. ROS content and protein carbonylation in seeds submitted to 40 °C increased in relation to 25 °C and 35°C. The activities of the enzymes SOD, APX, CAT, and POX were significantly reduced in seeds subjected to heat stress. Glucose content, G6PDH (*M. brauna*), and lipase activity also decreased in seeds exposed to high-temperature stress. Seed exposure to DPI decreased germination percentage at 25 °C and does not affect germination at 40 °C. DPI caused less reduction in the loosening of the outer layers of the integument and reduced the H_2O_2 content and activity of all tested enzymes. In summary, heat stress negatively affects germination, promotes oxidative stress, and induces damage to mitochondrial ultrastructure, seed morphology, and anatomy. The data obtained in this study contribute to a better understanding of the effects of high temperatures on the germination of native seeds, in addition to showing the contribution of ROS to germination.

Keywords: Climate change. Heat stress. *Melanoxylon brauna*. *Ormosia coarctata*.

RESUMO

REIS, Luciane Pereira, D.Sc., Universidade Federal de Viçosa, setembro de 2021. **Efeitos da alta temperatura sobre os parâmetros morfofisiológicos, ultraestruturais e bioquímicos durante a germinação de duas espécies florestais.** Orientador: Eduardo Euclydes de Lima e Borges. Coorientadores: Genaina Aparecida de Souza e Danielle Santos Brito.

As mudanças climáticas resultantes das ações antropogênicas tem aumentado a temperatura global. Esses aumentos na temperatura provavelmente influenciarão na sobrevivência de parte das espécies. Entre os fatores ambientais, a temperatura é um dos mais críticos por afetar os fatores fisiológicos, bioquímicos e moleculares da semente. O aumento da temperatura é considerado um dos fatores ambientais que auxiliam na superprodução de espécies reativas de oxigênio (ROS). A urgência em compreender as respostas das sementes às mudanças climáticas é particularmente importante. Uma visão geral detalhada das respostas da sementes ao estresse térmico pode ajudar a formular estratégias apropriadas para a conservação e a preservação das espécies. O conhecimento a respeito da capacidade das sementes florestais em lidar com as mudanças de temperatura ainda é escasso. Neste sentido, os objetivos deste trabalho foram: 1. Investigar as alterações morfofisiológicas, bioquímicas e ultraestruturais durante a germinação de sementes de *Melanoxylon brauna* sob estresse térmico; 2. Avaliar os efeitos da temperatura na morfologia, na geração de ROS, no sistema antioxidante e na mobilização de reservas em sementes *Ormosia coarctata*; 3. Caracterizar os efeitos do difenilenoiodônio (DPI) na germinação de *M. brauna*, na anatomia interna, no conteúdo de peroxido de hidrogênio (H_2O_2) e na atividade das enzimas de reservas e enzimas antioxidantes sob condições ótimas (25 °C) e estresse por calor (40 °C). Para avaliação da morfologia interna, as sementes foram radiografadas. Parâmetros ultraestruturais e anatômicos foram avaliados usando microscopia eletrônica de transmissão e microscopia de luz. A produção de ROS, conteúdo de malondialdeído (MDA) e glicose, proteínas carboniladas e atividade das enzimas (superóxido dismutase — SOD, ascorbato peroxidase — APX, catalase — CAT, peroxidase — POX, glicose-6- fosfato desidrogenase - G6PDH, lipase, α- e β-amilase e protease) foram medidos por análise espectrofotométrica. A alta temperatura causa redução na porcentagem e na velocidade de germinação e afeta a morfologia interna das sementes de *M. brauna* e *O.*

coarctcta. Nas sementes de *M. brauna* o estresse por calor diminui as taxas respiratórias e compromete a estrutura das mitocôndrias. O teor de ROS e carbonilação de proteínas nas sementes submetidas a 40 °C aumentam em relação a 25 °C e a 35°C. As atividades das enzimas SOD, APX, CAT e POX são significativamente reduzidas nas sementes submetidas ao estresse térmico. O conteúdo de glicose, G6PDH (*M. brauna*) e atividade da lipase também diminui nas sementes expostas ao estresse por alta temperatura. A exposição das sementes ao DPI diminui a porcentagem de germinação a 25 °C e não afeta a germinação em 40 °C. O DPI causa menor redução no afrouxamento das camadas externas do tegumento e reduz o conteúdo de H_2O_2 e a atividade de todas as enzimas testadas. Em síntese, o estresse por calor afeta negativamente a germinação, promove estresse oxidativo e induz danos na ultraestrutura das mitocôndrias, na morfologia e anatomia das sementes. Os dados obtidos neste estudo, contribuem para o melhor entendimento dos efeitos das altas temperaturas na germinação de sementes nativas, além de mostrar a contribuição das ROS para a germinação.

Palavras-chave: Mudanças climáticas. Estresse térmico. *Melanoxylon brauna. Ormosia coarctata.*

SUMMARY

INTRODUCTION

Germination is a process that begins with water absorption and is complete with the emergence of the primary root (Taiz et al., 2017). Under favorable conditions, quiescent seeds begin to germinate in response to environmental stimuli and the seed's intrinsic biochemical and molecular mechanisms (Nonogaki, 2019). Events such as the reactivation of metabolism, cell respiration, mitochondrial biogenesis, the DNA repair mechanism, and the mobilization of reserves are necessary for successful germination (Nonogaki, 2017).

The entry of water and oxygen into the seed cells initiates the reactivation of metabolism (Nonogaki, 2010). This reactivation requires energy. However, mitochondria in dry seeds are functionally and structurally deficient (Howell et al., 2006). The most accepted model for mitochondrial biogenesis is mitochondrial maturation. This model shows the existence of organelles called promitochondria (Plattner et al., 1970). Promitochondria in dry seeds does not have ridges (Howell et al., 2006). Studies on mitochondrial biogenesis during germination reveal that in seeds of *Zea mays* (Logan et al., 2001), *Oryza sativa* (Howell et al., 2006), *Arabidopsis thaliana* (Law et al., 2012), and *Melanoxylon brauna* (Reis et al., 2021), promitochondria develop into functional mitochondria after imbibition.

After changing from a quiescent to an energetic state, the seeds use their reserve compounds as substrates for respiration (Bewley et al., 2013). The energy supply and biosynthesis of new compounds involve the participation of several hydrolytic enzymes. α and β-amylase are the most reported in the mobilization of starch in seeds. The first is a calcium metalloenzyme (Pujadas and Palau, 2001). It works by cleaving α 1-4 glycosidic bonds, acting at random locations along the starch chain (Tiwari et al., 2015). β-amylase cleaves α1,4 glycosidic bonds at the non-reducing end of the chains (Suriya et al., 2016).

Lipids are another source of energy and biosynthesis for new compounds, in addition to proteins. In oilseeds, triacylglycerols stored in lipid bodies and free fatty acids are oxidized to produce acetyl-CoA (Kumar et al., 2021). Triacylglycerols are hydrolyzed during germination by the action of lipases (Kelly and Feussner, 2016). These catalyze the hydrolysis of ester bonds releasing, free fatty acids and glycerol (Choudhury and Bhunia, 2015). Proteins, in turn, are cleaved by proteases at N-terminal or C-terminal peptide bonds, providing amino acids for the construction of new proteins (Van der Hoorn, 2008).

Reactive oxygen species (ROS) also play important roles during germination processes. Studies have shown that ROS has a dual role in germination (Jeevan Kumar et al., 2015). Under basal levels, ROS are related to increased germination percentage (Ishibashi et al., 2010; Bahin et al., 2011; Zhou et al., 2018), in relieving dormancy (Cembrowska-Lech et al., 2015), in hormonal regulation (Li et al., 2018), in the weakening of the endosperm (Zhang et al. 2014), in the induction of the activity of antioxidant enzymes (Ellouzi et al., 2021) and reserve enzymes (Panngom et al., 2018).

Cellular ROS levels must undergo a rigorous control mechanism to fulfill their role in signaling (Bailly et al., 2019). Thus, antioxidant defenses also play key roles during germination. The main elimination pathways for ROS in seeds include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POX) (Govindaraj et al., 2017). Some compounds, as well as antioxidant enzymes, also play important roles in controlling ROS production. Carotenoids are lipophilic compounds inserted into membranes and exhibit antioxidant activity (Bast, Haenen, and Van den Berg 1998). They have the function of preventing the oxidation of lipids by oxidizing their double-bonded chains (Ziegler et al., 2016).

As sessile organisms, plants can face stressful conditions. Thus, ROS can surpass the antioxidant capacity causing a series of damages to cellular constituents. These free radicals can react with lipids, proteins, and nucleic acids causing oxidative stress (Hasanuzzaman et al., 2020). In general, exposure of plants to increased temperature causes overproduction of ROS (Essemine et al., 2012).

The last seven years were considered the hottest since 1880 (NOOA, 2021). As the temperature increases, the question arises about the tolerance of forest species to current environmental conditions. Among the species of great importance is *Melanoxylon brauna*. It is an endemic species to Brazil, occurring in the Atlantic Forest of the States of Minas Gerais, Rio de Janeiro, São Paulo, Bahia, and Espírito Santo (Lorenzi, 2009). It can reach 15 to 25 m in height and 40 to 80 cm in diameter, has yellowish flowers, panicles, dehiscent fruit, and dispersal of its seeds by the wind (Lorenzi, 2014). Considered is a wood of high density, quality, and durability (Campos Filho and Sartorelli, 2015), of great economic value and with potential for reforestation and urban afforestation (Brito and Oak, 2014). However, due to predatory exploitation, it is included in the list of species of Brazilian flora at risk of extinction (Martinelli and Moraes, 2013).

Another species of great importance is Ormosia coarctata Jacks (Fabaceae, Papilionoideae). In Brazil, it occurs in the states of Amazonas, Pará, Roraima and Mato Grosso, it is found in countries such as Guyana, French Guiana, Bolivia, Venezuela, Suriname and Colombia (Campos Filho and Sartorlli, 2015). It is popularly known as tento mulungu or goat eye (Campos Filho, 2012). It has compound, alternating, imparipinnate leaves and rust-colored hairiness on the branches and fruits (Rudd, 1965). It is used by the traditional peoples of Suriname for medicinal and spiritual purposes (Robert et al., 2004). It is indicated for silvopastoral systems (Cárdenas and Ramírez, 2004), used in ethnopharmacology (Hajdu and Hohmann, 2012), and ecological restoration projects (Isernhagen, 2015).

Plant growth and development are severely limited by temperature increase (Jagadish et al., 2021). In seeds, the increase in temperature harms the germination potential, resulting in low or no germination (Yuan and Wen, 2018; Reis et al., 2020; Lima et al., 2021). Studies have also shown that high temperature stress affects the activity of storage enzymes. In seeds of different species such as *M.brauna*, *Erythrina velutina*, and *O. coarctata*, the mobilization of reserves is impaired after heat stress (Ataíde et al., 2016; Felix et al., 2020; Reis et al., 2020).

Heat stress makes the antioxidant system inefficient. For example, in seeds of *Vigna aconitifolia* and *M. brauna*, heat stress significantly decreases the activity of SOD, CAT, APX, and POX enzymes (Harsh et al., 2016; Santos et al., 2017). With an ineffective defense system, there is increased oxidation of lipids and proteins. In lipid oxidation, one of the end products is malonaldehyde (MDA). While in protein oxidation, carbonyl proteins are formed (Hameed et al., 2011). Research has reported that heat stress can increase the content of MDA and carbonyl proteins in seeds of different species, such as *O. sativa* (Bhattacharjee, 2013), *Medicago sativa* (Wassie et al., 2019), and *Brassica napus* (Rashid et al., 2020). Exposure to high temperature also causes changes in the ultrastructure of organelles, impairing their functions (Bita and Gerats, 2013). Mitochondria, for example, from *Triticum spelta* and *B. napus* have a visibly swollen, underdeveloped, and deformed structure (Babenko et al., 2019; Rashid et al., 2020).

The urgency of understanding plant responses to climate is particularly important. In South America, 2020 was the second warmest year since pre-industrial levels, with temperatures above 40 °C (WMO, 2021). As a consequence of current climate change scenarios, the increase in temperature may compromise the development of germination of several species, including seeds of *M. brauna* and *O. coarctata* that have an optimal temperature range between 25 and 35 °C (Flores et al., 2014; Reis et al., 2020). Therefore, high temperature may cause negative effects at different levels (morphophysiological, ultrastructural and biochemical) during germination of *M. brauna* and *O. coarctata* seeds.

Therefore, it is essential to carry out further investigations into the role of ROS in germination and the effect of high temperature on parameters involving physiology, biochemistry, morphology, anatomy, and ultrastructure of cell organelles in forest seeds. Conducting studies on the high temperature stress condition for these two species will provide relevant information for their conservation and preservation.

OBJECTIVES

General objective

To analyze the physiological, morphological, anatomical, and ultrastructural effects of heat stress and reactive oxygen species on the germination of two forest species.

Specific objectives

1. To investigate the morphophysiological, biochemical, and ultrastructural changes during germination of *Melanoxylon brauna* seeds under heat stress.

2. To analyze the effects of temperature on morphology, generation of reactive oxygen species (ROS), antioxidant system, and mobilization of reserves in *Ormosia coarctata* seeds.

3. To characterize the effects of DPI on *Melanoxylon brauna* germination, internal anatomy, hydrogen peroxide (H_2O_2) content and activity of reserve enzymes and antioxidant enzymes under optimal conditions (25 °C) and heat stress (40 °C).

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CHAPTER I

Heat stress-mediated effects on the morphophysiological, biochemical, and ultrastructural parameters of germinating *Melanoxylon brauna* Schott. seeds

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ORIGINAL ARTICLE

Heat stress-mediated effects on the morphophysiological, biochemical, and ultrastructural parameters of germinating *Melanoxylon brauna* Schott. seeds

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Abstract

Key message **The present study showed that the heat stress (40 °C) caused changes in morphophysiological, biochemical, and ultrastructural parameters to the seeds Melanoxylon brauna, ultimately leading to loss of germination capacity.**

Abstract Temperature is an abiotic factor that influences seed germination. In the present study, we investigated morphophysiological, biochemical, and ultrastructural changes during the germination of *Melanoxylon brauna* seeds under heat stress. Seed germination was evaluated at constant temperatures of 25 and 40 °C. The samples consisted of seeds soaked in distilled and ionized water for 48 and 96 h at both temperatures. For the evaluation of internal morphology, the seeds were radiographed. Ultrastructural parameters were assessed using transmission electron microscopy (TEM). The production of reactive oxygen species (ROS), content of malondialdehyde (MDA) and glucose, carbonylated proteins, and activity of the enzymes (superoxide dismutase—SOD, ascorbate peroxidase—APX, catalase—CAT, peroxidase—POX, glucose-6-phosphate dehydrogenase—G6PDH, lipase, α- and β-amylase, and protease) were measured by spectrophotometric analysis. An 82% reduction in the germination of *M. brauna* seeds was observed at 25 °C, and 0% at 40 °C. TEM showed that seeds submitted to heat stress (40 °C) had poorly developed mitochondria and significantly reduced respiration rates. The content of ROS and protein carbonylation in seeds subjected to 40 °C increased compared to that at 25 °C. The activity of antioxidant enzymes, namely SOD, APX, CAT, and POX, was significantly reduced in seeds subjected to heat stress. Glucose content, G6PDH, and lipase activity also decreased when the seeds were exposed to heat stress. Conversely, α- and β-amylase enzymes and the protease increased due to the increase in temperature. Our data showed that the increase in temperature caused an accumulation of ROS, increasing the oxidative damage to the seeds, which led to mitochondrial dysfunction, ultimately leading to loss of germination.

Keywords Anti-oxidative enzymes · High temperatures · Mitochondria · Reserve enzymes · Respiratory rate

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Introduction

Global temperatures are heading towards an increase of 3.2 °C compared to pre-industrial levels, exceeding the global target of the Paris agreement of 1.5 °C (UNDRR 2020). In Brazil, projections indicate an increase of 2–6 °C from 2071 to 2100 (PBMC 2014). As a consequence of climate change, plant species are expected to face negative effects on development and growth, as their metabolism is temperature-dependent.

During the germination process, temperature is the main factor that affects water absorption, biochemical reactions, physiological processes, and the percentage and speed of germination (Bewley et al. 2013). Although plants are adapted to a wide range of temperatures, for many tropical species, the optimum temperature for germination is in the range of 25–35 °C (Gomes et al. 2016; Felix et al. 2018; Reis et al. 2020). Thus, temperatures above the ideal can negatively affect redox homeostasis, which is crucial for several cellular functions dependent on the signaling and accumulation of reactive oxygen species (ROS) (Liu et al. 2019; Ihsan et al. 2019).

ROS are versatile compounds that can have toxic or signaling effects on a wide range of living organisms, including seeds. Under optimal conditions, these compounds are constantly being eliminated by different antioxidant mechanisms (Ahmad et al. 2010, 2019; Kohli et al. 2019; Siddiqui et al. 2020). However, enzymes depend on an optimal temperature range for their activation and inactivation, and when the critical temperature is reached, there is a decline in antioxidant activity (Dai et al. 2019). For instance, high temperatures significantly decreased the activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) enzymes in different species (Harsh et al. 2016; Rashid et al. 2020; Hanif et al. 2021).

When the balance between production and scavenging is disturbed, ROS levels exceed the antioxidant capacity, resulting in the oxidative modification of nucleic acids, lipids, and proteins (Ahmad et al. 2010, 2019; Kohli et al. 2019; Alamri et al. 2021). As a result of heat stress and overaccumulation of ROS, the attack on lipids and proteins is considered one of the causes of low survival (Bhattacharjee 2013), reduced membrane integrity (Santos et al. 2017), and loss of vigor in seeds (Rashid et al. 2018).

High temperatures also impair the function of organelles, mainly by inducing changes in their ultrastructure (Krishnan et al. 2020; Sun et al. 2020; Zhang et al. 2021). In heat-stressed seeds, increased mitochondrial volume and reduced mitochondrial ridges have been reported (Rashid et al. 2020). Mitochondria play important role in seed germination since they are a source of energy for cell growth and metabolism. In addition, the mitochondrial electron transport chain is a major source of ROS; therefore, they are directly involved in the maintenance of redox homeostasis (Dunn et al. 2015; Ratajczak et al. 2019).

The perspective on the effects of increasing temperatures as a result of climate change is worrying when considering the impacts of hyperthermia on plant species, especially for species at risk of extinction. *Melanoxylon brauna*, for instance, a species widely used in the shipbuilding and furniture industry (Carvalho 2010), has an optimum temperature that varies between 25 and 30 °C, and temperatures above this range decrease its germination. Due to intense exploitation and the lack of compensatory planting programs, this species is included in the vulnerable category of the List of Endangered Species from the Ministry of Environment of Brazil (Martinelli and Moraes 2013).

There are still gaps in knowledge of the effect of heat stress on different physiological, biochemical, and morphological mechanisms in *M. brauna* seeds during germination. Within this context, understanding these effects on forest seeds is of fundamental importance since this is the main route of propagation for most native species. In this study, morphophysiological, biochemical, and ultrastructural parameters were used to characterize the germination of *M. brauna* seeds under heat stress (40 °C).

Materials and methods

Plant materials and experimental conditions

M. brauna seeds were collected in the municipality of Leopoldina (21°31′ 55″ S and 42° 38′ 35″ W), in the state of Minas Gerais, Brazil. After collection and processing, seeds were stored in fiber drums in a cold chamber at 5 °C until the beginning of the experiment. For the germination test, four replicates of 25 seeds were sown under two sheets of germination paper and incubated under constant light at temperatures of 25 and 40 °C. Germination was defined through the daily count of seeds that had an emerged primary root.

Treatments for the physiological, morphological, and ultrastructural analyses consisted of seeds not soaked (control) and seeds soaked in distilled water for 48 and 96 h at temperatures of 25 and 40 °C. After the imbibition period, the embryonic axes were removed with the aid of a stylet for physiological and ultrastructural analyses.

Seed morphology

Ten seeds from each treatment and control were radiographed using an MX-20 specimen radiography system equipped with a 14-bit digital camera (Faxitron X-Ray Corp., Wheeling, IL, USA). The voltage and radiation time of the source were set at 35 kV for 10 s at a focal length of 5 cm. The digital images were processed using the ImageJ® software (U.S. National Institutes of Health, Bethesda, MD).

Glucose quantification and activity of reserve enzymes

The determination of glucose content was performed using a BIOCLIN monoreagent glucose kit (QUIBASA—Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil). The absorbance reading was performed at 505 nm (Evolution 60S UV–visible spectrophotometer; Thermo Scientific, Madison, WI, EUA). The glucose concentration was calculated based on the standard glucose curve and the results were expressed in mg^{-1} g^{-1} FW (fresh weight).

The activity of glucose-6-phosphate dehydrogenase (G6PDH) was determined according to the method of Ribeiro et al. (2007). The enzyme activity was evaluated at 30 °C for 30 min at 340 nm. G6PDH activity was defined based on the reduction of $NADP^+$ to NADPH, using the molar extinction coefficient of 6.22 mmol cm^{-1}; the results were expressed in mol min^{-1} μg^{-1} protein.

The activity of α- and β-amylase enzymes were determined according to the Bernfeld method (Bernfeld 1955) with modifications. A reaction mixture containing citrate buffer (pH 5.0 for α-amylase and pH 3.4 for β-amylase), starch, and enzyme solution were incubated at 30 °C for 5 min. The reaction was stopped by adding 500 μL of alkaline dinitrosalicylic acid solution. For the quantification of reducing sugars, the solution was placed in a water bath at 100 °C for 10 min. After cooling, it was diluted five times with distilled water. Enzymatic activity (α- and β-amylase) was measured at 540 nm and calculated using a standard maltose curve. Lipase activity was determined using a colorimetric test with a Bioclin kit (QUIBASA—Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil).

Protease activity was evaluated by the non-specific sigma protease assay method described by Cupp-Enyard (2008) with some modifications (reduction of four times the volume of the reference value of the assay). The reaction mixture was read at 660 nm. The tyrosine concentration was measured with the aid of a standard tyrosine curve obtained in the range of 10–100 μg/ml. A unit of protease activity was defined as the amount of enzyme that resulted in the release of 1 μg/ml tyrosine per min under the conditions of the assay. The protein content was determined by the Bradford method (Bradford 1976), using the standard curve consisting of bovine serum albumin (BSA), for all enzymatic activities described above.

Quantification of fatty acids

The seed oil was extracted according to the method of Folch et al. (1957). The seeds were dried in an oven at a temperature of 40 °C, weighed (500 mg), and macerated in chloroform–methanol solution (2:1 v/v) for 10 min. The homogenized suspension was filtered through a separating funnel coupled with a vacuum pump. The clear filtered volume was carefully mixed with 0.88% KCl in a separating funnel. The lower phase was removed, the solvents were removed with nitrogen gas, and the oil was weighed.

For the quantification of fatty acids, the samples were trans-esterified and, subsequently, 1 μL of the sample was injected in a gas chromatograph (SHIMADZU GC-14A) equipped with a flame ionization detector (FID), coupled to a recorder and integrator C-R6A Chromatopac. A capillary column from Shimadzu, of 50 m × 0.2 mm (diameter) of fused silica was used. The flow of carrier gas (H_2) was 30 ml min. The injector and detector temperatures were 220 and 230 °C, respectively. The column temperature was programmed to isothermal 190 °C for 60 s, followed by an increase of 2 °C per min, until reaching the maximum temperature of 200 °C.

Quantification of superoxide anion, hydrogen peroxide, MDA, and carbonylated proteins

Production of the superoxide anion ($O_2^{\bullet-}$) was measured according to Able et al. (1998) with modifications. The $O_2^{\bullet-}$ production rate was calculated using extinction coefficient 2.16×10^4 M^{-1} cm^{-1} at 470 nm for 5 min.

The determination of hydrogen peroxide (H_2O_2) was carried out according to Junglee et al. (2014). The absorbance of the supernatants was measured at 390 nm spectrophotometrically. The quantification of H_2O_2 content was calculated using a standard curve with standard H_2O_2 solutions.

The level of lipid peroxidation was measured according to the method of Heath and Packer (1968). Absorbance was recorded at 532 and 600 nm. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The content of carbonylated proteins was determined by derivatizing carbonyls from proteins using 2.4-dinitrophenylhydrazine (DNPH), as performed by Xia et al. (2016). The carbonyl content was calculated using the absorption coefficient of 22 000 M^{-1} cm^{-1} at 370 nm. The protein concentration was measured using a 2-D Quant Kit. The carbonyl content was expressed in nmol of carbonyl per mg of protein.

Antioxidant enzyme activity and carotenoid content

For the assay of the antioxidant enzymes SOD, APX, CAT, and POX, the frozen samples were homogenized in 2 ml of

50 mM phosphate buffer (pH 7.8) and 1% (p/v) of polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15,000×*g* for 20 min at 4 °C.

SOD activity was measured by inhibiting the photoreduction of nitro blue tetrazolium, as described by Del Longo et al. (1993). A unit of SOD was defined as the amount of enzyme needed to inhibit 50% of the photoreduction rate of tetrazolium nitro blue.

APX activity was determined by estimating the oxidation rate of ascorbic acid (Chen and Arora 2011). Enzymatic activity was calculated using a molar extinction coefficient of 2.8 mM cm^{-1}. A unit of activity (U) was defined as the amount of enzyme needed to convert 1 nmol of the ascorbate in product per min at 290 nm/ml.

CAT activity was determined by H_2O_2 decomposition at 240 nm for 1 min (Bailly and Kranner 2011). The enzymatic activity was calculated using the molar extinction coefficient of 36 M cm^{-1}. A unit of activity (U) was defined as the amount of enzyme needed to convert 1 mmol of the substrate into product per min, per ml, under the conditions of the assay.

POX activity was determined by the oxidation of pyrogallol, according to the methodology of Kar and Mishra (1976). The enzymatic activity was calculated using the molar extinction coefficient of 2.47 mM^{-1} cm^{-1} at 420 nm.

The content of β-carotene and lycopene was determined according to the methodology proposed by Nagata and Yamashita (1992). The pigments were extracted from 150 mg of embryonic axes in 10 mL of acetone/hexane (2:3, v/v). The reaction mixture was read at absorbances of 453, 505, 645, and 663 nm. To calculate the concentration of β-carotene and lycopene, the following equation was used: β-carotene (mg/100 mL) = $0.216_{A663} - 1.22_{A645} - 0.304_{A505} + 0.452_{A453}$; lycopene (mg/100 mL) = $-0.0458_{A663} + 0.204_{A645} + 0.372_{A505} - 0.0806_{A453}$. The results were expressed in mg/100 g FW.

Transmission electron microscopy and respiratory rate

The embryonic axes were fixed for 24 h in Karnovsky solution [2.5% glutaraldehyde; 2% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2)], washed in 0.1 M phosphate buffer (pH 7.2—3 times for 10 min), and then placed in 1% osmium tetroxide solution for 1 h. Subsequently, the sample was washed again (twice for 15 min), immersed in uranyl acetate for 12 h, and dehydrated in a gradient of increasing concentrations of acetone (30, 50, 70, 90, and 100%, 3 times). The specimen was placed in 1:1 mixture of absolute acetone and the final Spurr resin mixture for 8 h at room temperature, then transferred to 1:3 mixture of absolute acetone and the final resin mixture for 12 h and final Spurr resin mixture for 24 h. Next, the sample was assembled in molds and placed to polymerize in an oven at 7 °C for 48 h. The blocks were sectioned in a Leica EM UC6 ultramicrotome (Leica Microsystems, Germany) using a diamond blade. The sections were contrasted with uranyl acetate and lead citrate and observed under a TECNAI™ G2 12 Spirit BioTWIN transmission electron microscope (FEI, Eindhoven, Netherlands) at 120 kV.

Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments, Norfolk, UK) following the protocol described by Yoshida et al. (2007). For this purpose, 20 mg of embryos were used, which were incubated at 25 °C in the dark at a final volume of 2 ml solution, containing 100 mM sucrose, 50 mM HEPES, 10 mM MES, and 0.2 mM $CaCl_2$ (pH 6.6). The oxygen depletion in the buffer was maintained at less than 20% of the initial value.

Statistical analyses

The experiment was conducted in a completely randomized design (CRD) with five replications (150 mg of embryonic axis per repetition) for enzymatic analysis and oxygen consumption and 10 seeds per treatment for X-ray analysis. Data were evaluated by analysis of variance (ANOVA). The means of each treatment were compared by Tukey's test ($p \leq 0.05$).

The data obtained from the seed characteristics were submitted to principal component analysis (PCA) and permutational multivariate variance analysis (PERMANOVA) with 1000 permutations and Euclidean distance to explore patterns in different treatment groups (temperature and time). The multivariate dispersion homogeneity test (PERMDISP) was used to check the assumption of dispersion homogeneity. These data were normalized in a univariate way (subtract the mean and divide by the standard deviation), and the eigenvalues and eigenvectors were calculated from the covariance matrix. Statistical analyses were performed using R software (R core team, version 4.0.0).

Results

Heat stress inhibited the germination of *M. brauna* seeds

An 82% reduction in the germination of *M. brauna* seeds was observed at 40 °C. The emergence peak of the primary root occurred between the 5th and 6th day after sowing (Fig. 1). At 40 °C, no germination was observed at the end of the evaluation period (10 days).

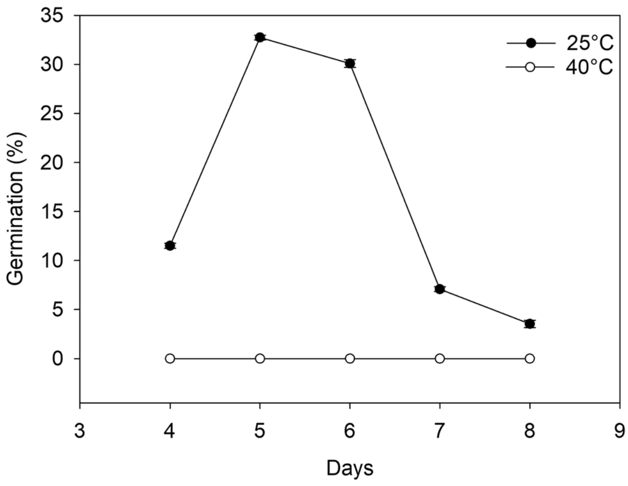


Fig. 1 Effect of temperature on the germination percentage of *Melanoxylon brauna* seeds. The seeds showing radicle extension of 2 mm were scored as having germinated. Values are expressed as the mean ± SD (standard deviation; $n = 4$) of 25 seeds each

Embryo morphology did not change with increasing temperature

It was possible to identify two regions in the *M. brauna* embryos: an ellipsoid portion, which corresponds to the cotyledons, and another curve and oblique, corresponding to the embryonic axis (Fig. 2). After 48 h of soaking at 25 and 40 °C, it was possible to identify the plumule, constituted by two leaf beginnings. After 96 h of imbibition at 25 °C, a 9.9% increase in the area of the embryonic axis and rupture of the micropyle was observed, which did not occur at 40 °C.

Heat stress reduced the glucose and unsaturated fatty acids content and the activity of reserve enzymes

The glucose content and activity of the G6PDH enzyme showed the same pattern of behavior (Fig. 3A, B). Higher glucose content and activity of G6PDH were observed at the optimum temperature (25 °C for 96 h) in comparison to

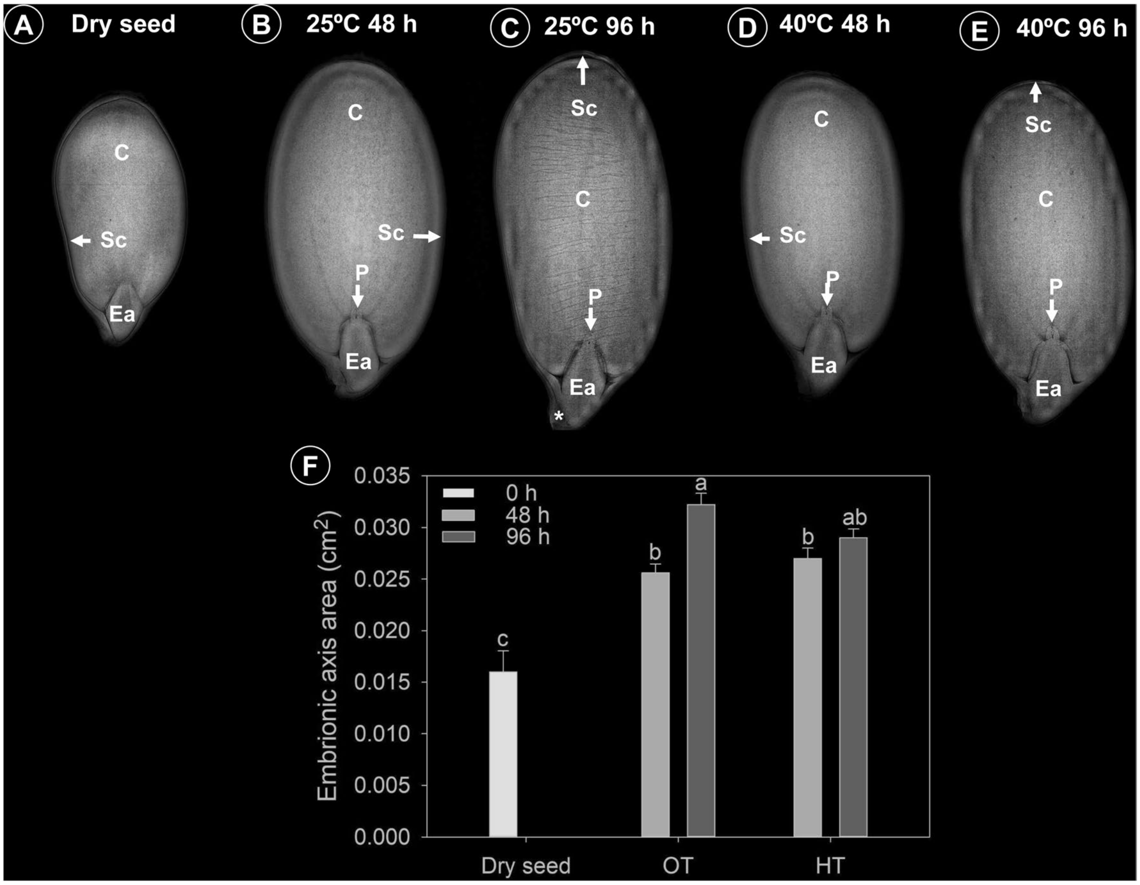


Fig. 2 Radiographic images of *Melanoxylon brauna* seeds under different temperatures and imbibition times. OT: optimum temperature; HT: heat stress; Sc: seed coat; C: cotyledon; Ea: embryonic axis; P: plumule. Asterisk (*) shows the emergence of the primary root. Means followed by the same letter do not differ statistically (ANOVA; Tukey's test, $p < 0.001$). Values are expressed as the mean ± SD (standard deviation; $n = 10$) per treatment

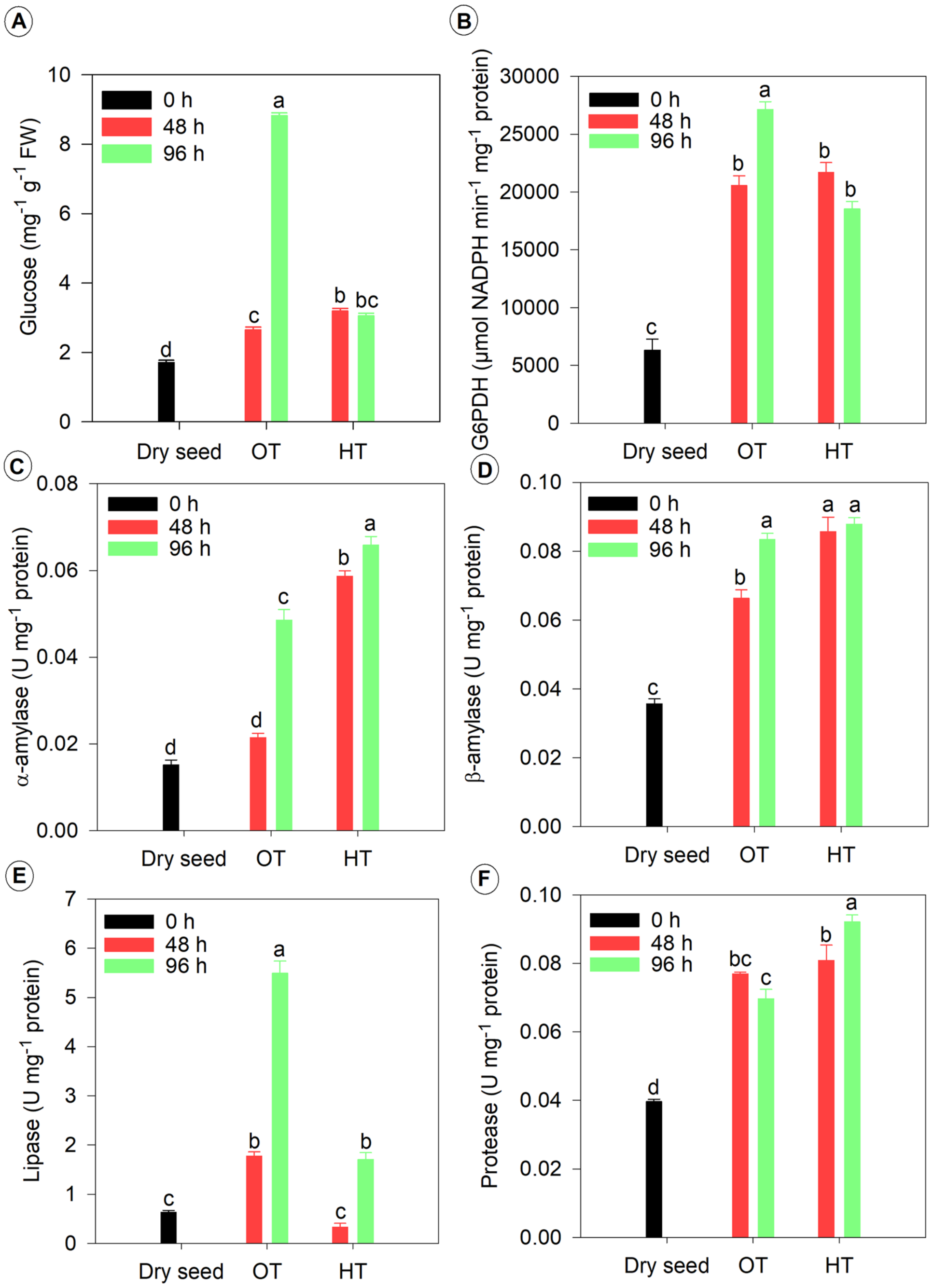


Fig. 3 Glucose content (**A**) and glucose-6-phosphate dehydrogenase (G6PDH) (**B**), α-amylase (**C**), β-amylase (**D**), lipase (**E**), and protease (**F**) activity in the embryonic axis of *Melanoxylon brauna* seeds in the treatments: dry seed, optimal temperature (OT—25 °C), and heat stress (HT—40 °C). Different letters indicate significant differences using the Tukey test ($p < 0.05$). Values are expressed as the mean ± SD ($n = 10$) per treatment

other treatments. The dried seeds, when hydrated at 25 °C, had increased glucose levels (37 and 69% after 48 and 96 h, respectively). Between the imbibition times, there were no significant differences for 40 °C ($F_{4,\,25} = 95.7$, $p = 0.06$).

The content of fatty acids in the seeds is displayed in Table 1. The highest abundance was observed in the oleic acid content of dry seeds ($p < 0.001$). The effects of temperatures and soaking times on palmitic, oleic, and linoleic acid content were significant ($F_{4,\,25} = 161.8$, $p < 0.001$; $F_{4,\,25} = 53.81$, $p < 0.001$). There was no statistical difference in stearic acid content between treatments ($F_{4,\,25} = 1.157$, $p = 0.35$).

The α-amylase activity in relation to the control increased progressively at 25 °C; the values corresponded to increases of 2.82 mg^{-1} g^{-1} protein for 48 h and 15.16 U mg/protein after 96 h of imbibition (Fig. 3C). At 40 °C, α-amylase activity was significantly higher than other treatments ($F_{4,\,25} = 189.9$, $p < 0.001$).

In relation to dry seeds, β-amylase activity increased in all treatments (Fig. 3D). At 25 °C, significant changes were observed between soaking times (48 and 96 h), with values

Table 1 Content of fatty acids (mg/g) in the embryonic axis of *Melanoxylon brauna* seeds soaked at 25 and 40 °C

Fatty acids	Treatment	Mean	*F*	*p*	CV (%)
Palmitic acid (16:0)	Dry seed	0.288^a	8.73	0.0003	21.3
	25 °C-48 h	0.278^a			
	25 °C-96 h	0.171^b			
	40 °C-48 h	0.325^a			
	40 °C-96 h	0.176^b			
Stearic acid (18:0)	Dry seed	0.286^a	1.157	0.359	7.23
	25 °C-48 h	0.272^a			
	25 °C-96 h	0.284^a			
	40 °C-48 h	0.291^a			
	40 °C-96 h	0.267^a			
Oleic acid (18:1)	Dry seed	1.922^a	161.8	0.0006	20.66
	25 °C-48 h	0.734^b			
	25 °C-96 h	0.228^c			
	40 °C-48 h	0.187^c			
	40 °C-96 h	0.139^c			
Linoleic acid (18:2)	Dry seed	0.153^a	53.81	0.0002	25.23
	25 °C-48 h	0.138^{ab}			
	25 °C-96 h	0.108^b			
	40 °C-48 h	0.012^c			
	40 °C-96 h	0.008^d			

Different letters in each line indicate a significant difference at $p \leq 0.05$ according to the Tukey test. *F* value of *F*-statistics

CV (%) coefficient of variation

of 29.88 and 37.58 U mg/protein, respectively, on average, when compared to the control ($F_{4, 25} = 73.04$, $p < 0.001$). At 40 °C, no significant differences were observed between soaking times ($p = 0.97$).

Progressive increases in lipase activity were observed at 25 °C (Fig. 3E). At 25 °C, lipase activity increased from 1.77 after 48 h of imbibition to 5.49 U/mg/protein after 96 h. The highest lipase activity was observed at 25 °C after 96 h of imbibition. At 40 °C, the difference in lipase activity compared to seeds submitted to 25 °C was 81.1 and 68.8% lower in 48 and 96 h, respectively.

At a temperature of 25 °C, no significant changes in protease activity were observed at different imbibition times ($F_{4, 25} = 62.34$, $p = 0.27$) (Fig. 3F). Conversely, at 40 °C, protease activity was 5 and 24.4% (in 48 and 96 h, respectively) higher than in seeds at 25 °C.

Heat stress led to an increase in ROS

The $O_2^{\bullet -}$ levels were significantly higher under heat stress conditions (40 °C; 96 h) which corresponded to a six-fold increase over 25 °C for 96 h (Fig. 4A). At the optimum temperature (25 °C), the content increased by an average of 50% compared to dry seeds, followed by a decrease of 72.5% after 96 h.

A significant increase in the concentration of H_2O_2 was observed at 25 °C ($F_{4, 25} = 74.89$, $p < 0.001$, Fig. 4B). The maximum accumulation occurred at 25 °C after 96 h of imbibition. After 48 h, there was no significant difference between temperatures 25 and 40 °C. The lowest values of H_2O_2 were observed in dry seeds.

On average, MDA levels increased 42% when seeds were exposed to temperatures of 25 and 40 °C after 48 and 96 h, compared to dry seeds (0 h). However, MDA content did not show significant differences between temperatures and soaking times ($F_{4, 25} = 9.023$, $p > 0.05$) (Fig. 4C).

The lowest amount of carbonylated proteins was detected in dry seeds (Fig. 4D). At 25 °C, the amount was approximately four times greater than that of dry seeds; there were no significant changes in the carbonyl content between the two soak times. Conversely, heat stress (40 °C) significantly increased the content of carbonylated proteins in relation to the optimal temperature (25 °C) ($F_{4, 25} = 50.58$, $p < 0.001$).

Heat stress decreased the activity of anti-oxidative enzymes and altered the content of β-carotene

A significant decline in SOD activity occurred in seeds soaked for 48 h (Fig. 5A). The specific activity of SOD in the control (235.47 U min/mg/protein), decreased significantly at 25 (140.99 U min/mg/protein) and 40 °C (173.38 U min/mg/protein) when soaked for 48 h ($F_{4, 25} = 47.31$, $p < 0.001$). When the seeds were exposed to 96 h of soaking, there was an increase in activity for both temperatures (52.2 for 48 h and 37.1% for 96 h) in relation to 48 h. The maximum activities of SOD were observed at 25 °C after 96 h of imbibition, followed by 40 °C at the same time of imbibition.

APX activity was increased (approximately 43% for 48 h and 46% for 96 h) in seeds exposed to 25 °C when compared to 40 °C (Fig. 5B). The highest activity of APX in relation to all treatments was observed in response to 25 °C for 96 h. When subjected to a temperature of 40 °C for 48 h, the enzymatic activity of APX did not increase in relation to dry seeds.

CAT activity did not show significant changes when the seeds were exposed to 25 °C for 48 h ($F_{4, 25} = 49.18$, $p > 0.05$, Fig. 5C). The maximum activity of CAT occurred in seeds submitted to 25 °C for 96 h. At a temperature of 40 °C, CAT activity showed no differences between the control and imbibition for 48 and 96 h.

The maximum POX activity was found in seeds submitted to 25 °C for 96 h in relation to all treatments (Fig. 5D). Compared to the control, POX activity decreased by 66%, followed by an increase of approximately 46% in 96 h at

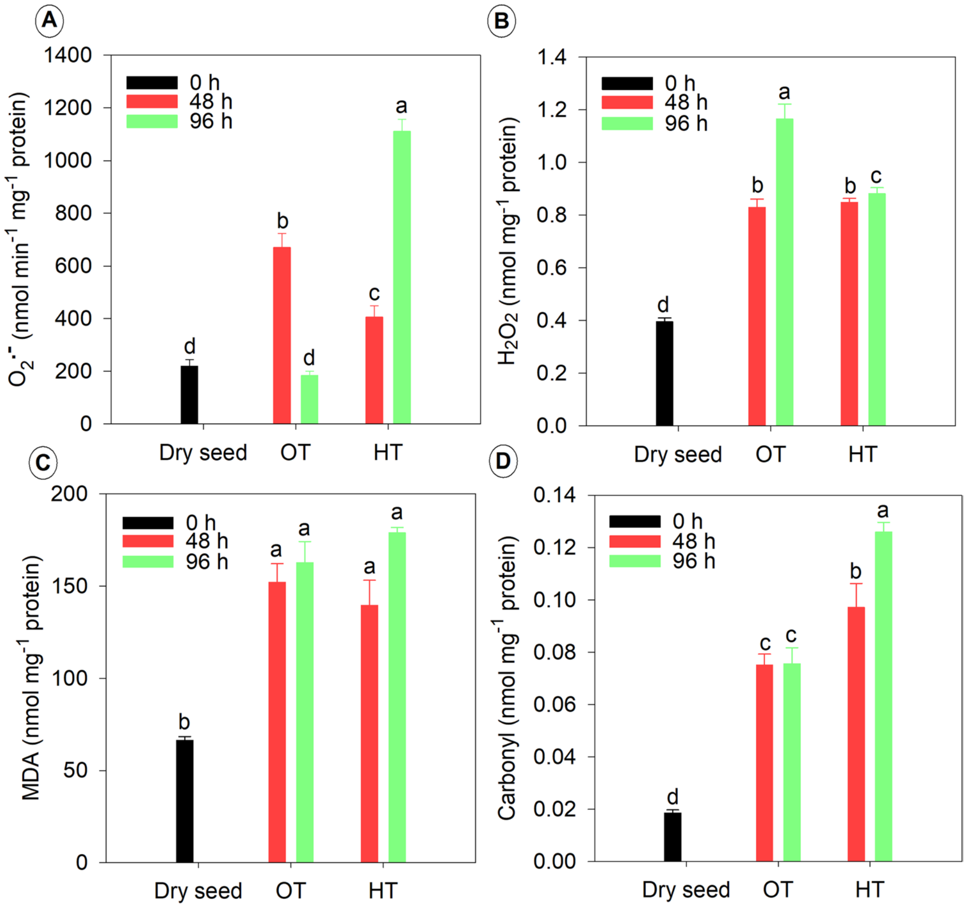


Fig. 4 Concentration of **A** superoxide anion, **B** hydrogen peroxide, **C** malondialdehyde, and **D** carbonylated proteins in embryos of *Melanoxylon brauna* seeds in the treatments: dry seed, optimal temperature (OT—25 °C), and heat stress (HT—40 °C). Black, red, and green bars correspond to dry seeds (0 h), 48 h, and 96 h, respectively. Different letters indicate significant differences according to the Tukey test ($p < 0.001$). Values are expressed as the mean ± SD ($n = 10$) per treatment

25 °C. At 40 °C, POX activity decreased significantly compared to 25 °C ($F_{4, 25} = 107.9$, $p < 0.001$).

Regarding carotenoids, the highest lycopene content was observed in dry seeds (Fig. 5E). After 48 h of imbibition, no accumulation of lycopene was detected at both temperatures. However, after 96 h at 25 and 40 °C, there was a tendency to increase the concentration of lycopene. In contrast, there was no significant change in its content at different temperatures ($F_{4, 25} = 77.63$, $p > 0.05$).

There was no β-carotene content in the dried seeds (Fig. 5F). Conversely, at 25 °C, an accumulation of β-carotene content (0.044 mg/100 g/FW) was found after 48 h, followed by a decrease (0.028 mg/100 g/FW) after 96 h. When the seeds were exposed to 40 °C for 96 h of imbibition, the β-carotene content decreased abruptly (up to 93%) in relation to the other treatments.

Heat stress caused damage to the mitochondria ultrastructure and decreased oxygen consumption

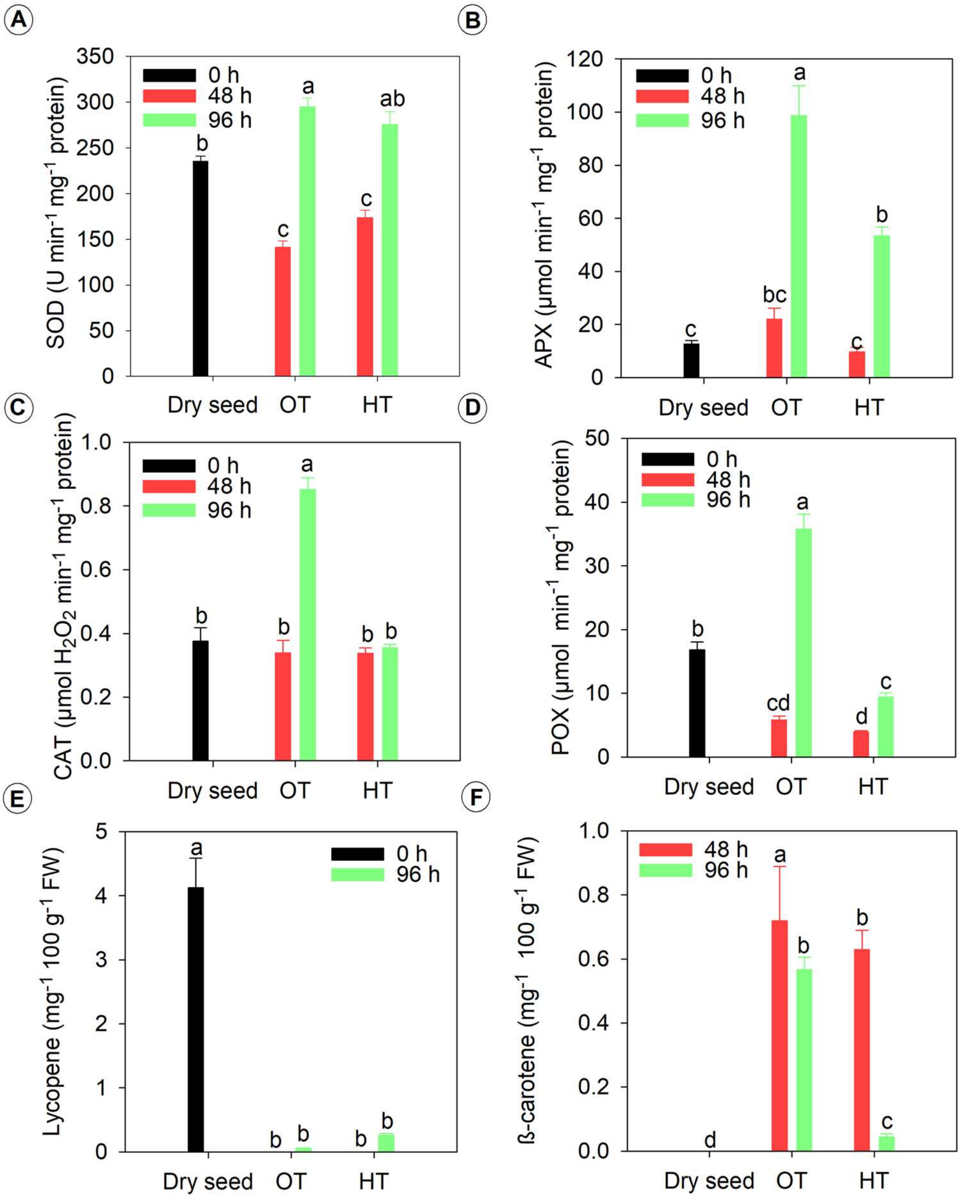
The mitochondria of embryonic axis cells showed considerable differences between treatments. In the dried seeds (0 h), the mitochondria showed an indiscernible internal structure (Fig. 6A). Conversely, when soaked for 48 and 96 h, they presented a distinguishable internal structure, with invaginations of the internal membrane and the presence of mitochondrial ridges, a typical structure of mature mitochondria (Fig. 6B, C). In contrast, when exposed to 40 °C for 48 h, the cells showed large organelles without organized mitochondrial ridges (Fig. 6D). At this temperature, after 96 h, smaller and condensed mitochondria were found (Fig. 6E, F). Regarding oxygen consumption, it was observed that the respiratory rate significantly differed between the imbibition times.

The O_2 consumption was 68% higher in seeds soaked at 25 °C for 96 h compared to 0 h (Fig. 7). However, with increasing temperature, oxygen consumption showed significant decreases ($p < 0.001$); the values corresponded to decreases of 39.31 and 51.73% after 48 and 96 h of imbibition, respectively, when compared to 25 °C.

Principal component analysis revealed low levels of antioxidant enzymes and high $O_2^{\bullet-}$ content in seeds subjected to heat stress

PCA was performed to explore how treatments differ from each other and what variables are primarily responsible for these differences. When considering the set of 16 variables,

Fig. 5 Activity of **A** superoxide dismutase, **B** ascorbate peroxidase, **C** catalase, and **D** peroxidase and content of **E** β-carotene and **F** lycopene in the embryonic axis of *Melanoxylon brauna* seeds in treatments: dry seed, optimal temperature (OT—25 °C), and heat stress (HT—40 °C). Different letters indicate significant differences by the Tukey test, $p < 0.001$. Values are expressed as the mean ± SD ($n = 10$) per treatment



two main components (PC1 and PC2) were extracted, from which they were responsible for 76.7% of the variance. The assumption for PERMANOVA was accepted (homogeneous dispersion) (PERMIDISP: $F_{4,\,20} = 2.5$, $p = 0.076$). PERMANOVA indicated a significant difference in seed variables in relation to treatment groups ($F_{4,\,20} = 62.3$, $p = 0.001$, $R^2 = 0.93$). The graphs showed that the group at 25 °C for 96 h was separated from the dry seeds (0 h) in principal component 1 (PC1), which explained 46.1% of the total variability (Fig. 8A). The second principal component (PC2) separated the dry seeds and 25 °C for 96 h from the other groups, 25 °C for 48 h, 40 °C for 48 h, and 40 °C for 96 h, which explained 30.6% of the total variability. The variables APX, CAT, POX, lipase, glucose, and G6PDH showed a greater association with the treatment at 25 °C for 96 h. There was less relation of the variables APX, CAT, POX, lipase, glucose, G6PDH, and lycopene in the seeds submitted to 25 and 40 °C for 48 h and 40 °C for 96 h. The variables that contributed most to these groups were $O_2^{\bullet-}$, β-amylase, and protease (Fig. 8B).

Discussion

This work showed that the temperature of 40ºC inhibited germination of the *M. brauna*, although the seeds showed embryonic growth (Figs. 1, 2). The inhibition of germination in *M. brauna* can be explained, at least in part, by the changes observed in the activity of reserve enzymes and deformations in the ultrastructure of mitochondria, with

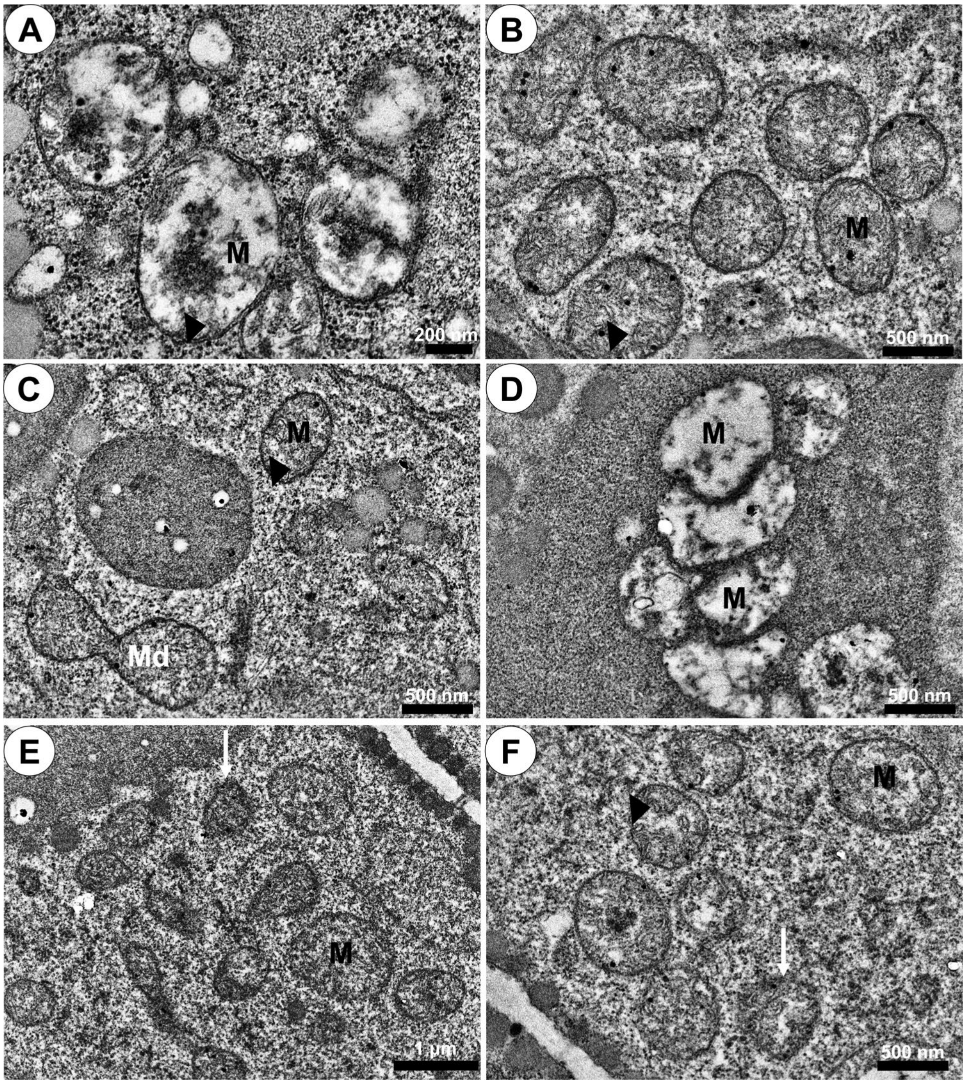


Fig. 6 Electron micrographs of mitochondria transmission from cells of the embryonic axis of *Melanoxylon brauna* seeds under different temperatures and imbibition times. **A** Dry seeds (0 h), **B** 25 °C for 48 h, **C** 25 °C for 96 h, **D** 40 °C for 48 h, **E**, **F** 40 °C for 96 h. M: mitochondria; Md: mitochondrial division; black arrows: mitochondria ridges; white arrows: condensed mitochondria

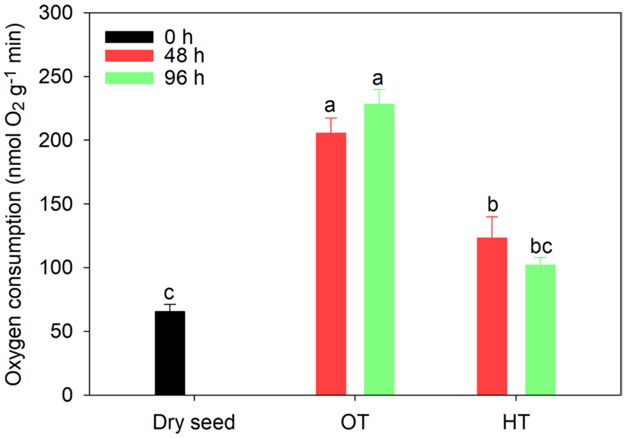


Fig. 7 Oxygen consumption in *Melanoxylon brauna* seed embryos in the treatments: dry seed, optimal temperature (OT—25 °C), and heat stress (HT—40 °C). Black bars correspond to dry seeds (0 h), red bars to seeds at 48 h, and green bars to 96 h. The means followed by the same letter do not differ significantly (ANOVA; Tukey, $p < 0.001$). Values are expressed as the mean ± SD ($n = 5$) per treatment

a consequent reduction in oxygen consumption and accumulation of ROS leading to the oxidation of biomolecules. Imbibition itself caused cell expansion. As this process does not depend on the metabolic activity of the seed since it is a purely physical process, the entry of water can occur in viable, dormant seeds in living or non-living tissues (Bewley et al. 2013). Therefore, the entry of water into the seed probably contributed to the generation of the initial growth potential of the embryo (Fig. 2). Conversely, the inhibition of germination at 40ºC may have been a consequence of the alteration of the enzymatic activity in the embryo of *M. brauna*. Heat stress can potentially alter enzyme activity, modifying metabolic reactions that can reduce embryo development, speeding up or slowing down seed metabolism (Laghmouchi et al. 2017). In good agreement, it was previously demonstrated that there is an increase in hydrolase activity at 45 °C in the micropyle of *M. brauna* seeds (Santos et al. 2020). Although hydrolases play a fundamental role in *M. brauna* seed germination, the increase in the activity of

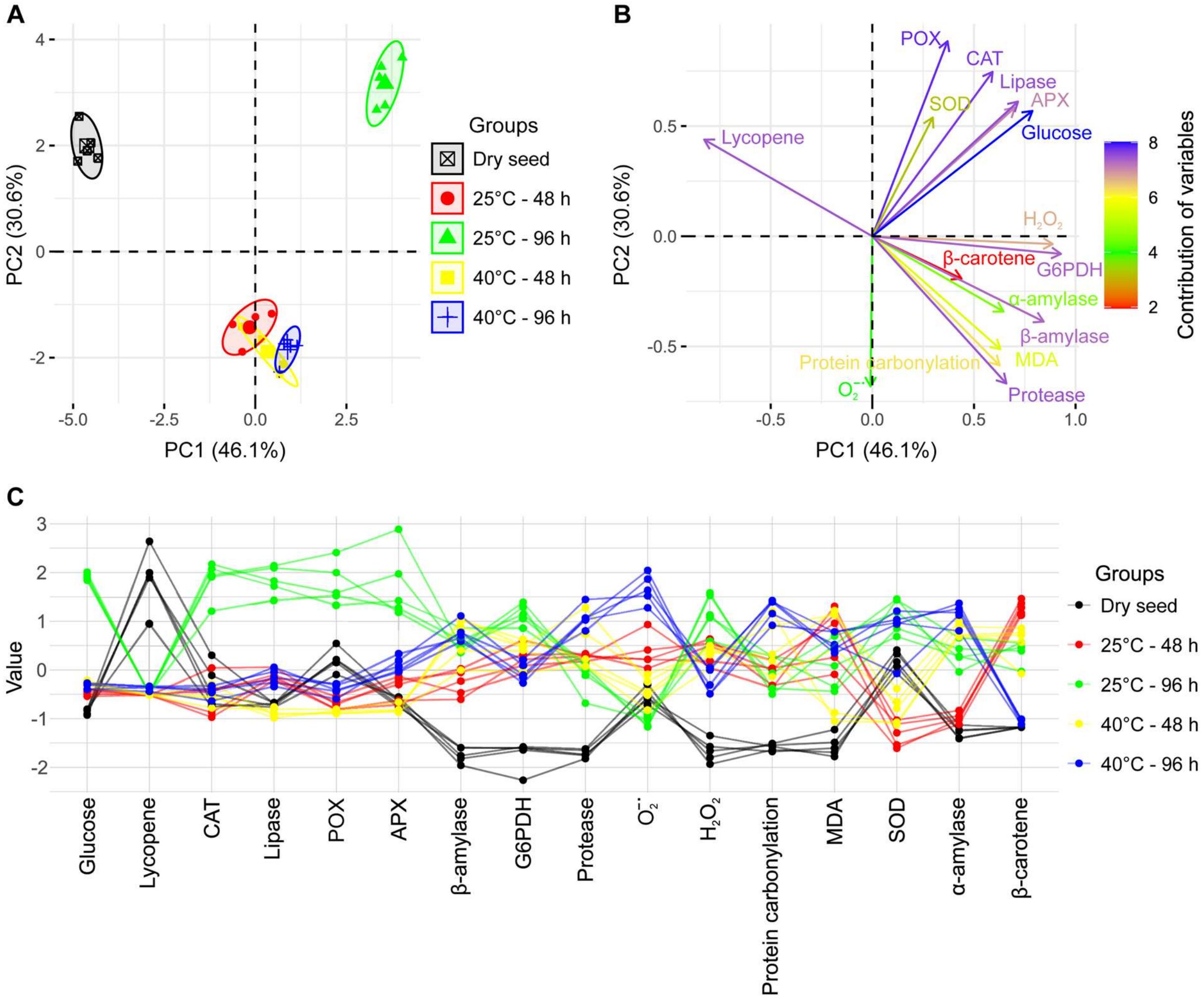


Fig. 8 Principal component analysis (PCA). **A** PCA ordering diagrams are categorized into different groups (temperatures and times). Confidence ellipses are based on treatment centers (95%). The percentage values on the axes indicate how much each component explains the total variance of the data. **B** Loads of principal components with color scale for resource contributions. The directions of the arrows show the relative loadings of the resources in the first and second principal components. **C** Parallel plot of normalized raw data (subtract mean and divide by SD). Counting all groups, 25 replicates were sampled ($n = 5$ per group)

these enzymes contributed to the deterioration of the seed (Santos et al. 2020).

The increase in the activity of reserve enzymes, in particular, α and β-amylase (Fig. 3C, D), can be explained by the fact that most amylases have optimal temperature activity between 50 and 60 °C (Janecek and Baláz 1992). In the case of *Eleusine coracana* and *Glycine max* seeds, the highest activity for α- and β-amylase were between 45 and 50 °C and 40 °C, respectively (Gimbi and Kitabatak 2002; Rani 2013). However, different species and different enzymes have optimal activity temperature ranges, and when exceeding the optimum temperature, incorrect folding and protein denaturation occurs (Zhou et al. 2014). In addition, some studies have demonstrated that metabolites derived from the hydrolysis of starch, besides supporting germination and plant growth, also provide compatible solutes to alleviate the effects caused by abiotic stresses (Krasensky and Jonak 2012; Dong and Beckles 2019). Therefore, high activity of α and β-amylase at 40 °C, hypothetically, could be an attempt to overcome heat stress by releasing protective metabolites, for instance, soluble sugars.

At least partly, exceeding the optimum temperature can explain the decrease of the lipase activity in *M. brauna* seeds exposed to 40 °C. Some species, such as *G. max* and *Solanum melongena*, have an optimum temperature for lipase activity equivalent to 25 and 30 °C; above these temperatures, there is a steady decline in activity (Gadge et al. 2011; Ozden et al. 2021). Additionally, heat stresses can induce lipid remodeling through the action of lipases (Lu et al. 2020). In this sense, during heat stress, plants decrease the ratio of unsaturated to saturated fatty acids to decrease the membrane's fluidity and prevent damage (Balogh et al.

2013), which is in accordance with our results; an increase in saturated fatty acids (palmitic and stearic) and a decrease in unsaturated acids (oleic and linoleic) (Table 1).

The uncontrolled production of ROS also damages proteins through changes in their structure, in which it induces proteins to be degraded by proteolytic systems (Ciacka et al. 2020). A significant increase in protease activity under heat stress strengthens the hypothesis of oxidative damage under this condition (Fig. 3F). According to He and Kermode (2010), the production of H_2O_2 was necessary for the activation of proteases similar to caspases in animal cells. Thus, we assumed that the increase in protease activity could be related, at least in part, to disorders in redox homeostasis, causing proteins to become susceptible to proteolysis.

Heat stress affected G6PDH activity (Fig. 3A, B). G6PDH is considered an important enzyme related to the functioning of the antioxidant system (He et al. 2020; Santiago et al. 2021). A decrease in the activity of this enzyme implies a compromise of the metabolic pathways responsible for producing reducing power, contributing to the elimination of ROS. ROS produced after imbibition are assumed to play a role in seed germination (Ishibashi et al. 2015; Bailly 2019; Ju et al. 2020). However, the accumulation of ROS causes disturbances in membrane systems through the oxidation of their constituents (Yalcinkaya et al. 2019; Dvorak et al. 2020). Our results showed that the high temperature increased the levels of $O_2^{\bullet -}$ and H_2O_2 in the seeds (Fig. 4A, B). It is likely that the increase in these free radicals due to heat stress caused oxidative damage during imbibition, and as a consequence, decreased the content of unsaturated fatty acids and induced the production of MDA and carbonylated proteins (Fig. 4C, D), causing a direct modification of lipids and proteins. Additionally, the enzyme system (SOD, APX, CAT, and POX) decreased with exposure to 40 °C, which can lead to an increase in the ROS content and an attack on cellular constituents. This corroborates studies with *Dalbergia nigra* and *Triticum aestivum* seeds in which the accumulation of ROS was associated with a reduction in antioxidant enzyme systems (Matos et al. 2014; Buttar et al. 2020). In this sense, seeds subjected to heat stress can be attacked by ROS due to the low activity of enzymatic antioxidant systems.

Another determining factor in protection against oxidative damage is lipophilic antioxidants that protect membranes by limiting lipid peroxidation (Wang et al. 2017). Our carotenoid data (β-carotene and lycopene) showed that the temperature increase only influenced the β-carotene content. Lycopene protects β-carotene against isomerization and degradation during oxidative reactions (Heymann et al. 2015). It is possible that the lycopene content may have acted initially as an inhibitor of free radicals, and in response to the increase in temperature (Fig. 5F), the cell used both carotenoids in defense against oxidative damage.

The poorly developed mitochondria, observed in dry seeds, indicated that imbibition influences the development of this organelle. These mitochondria, differentiated in their ultrastructure, are called pro-mitochondria (Logan et al. 2001; Carrie et al. 2013). To our knowledge, this is the first time that pro-mitochondria have been reported in forest seeds. The increase in temperature (40 °C) resulted in mitochondria similar to pro-mitochondria (Fig. 6D–F). It is likely that heat stress caused damage to the membranes and, consequently, the development of mitochondrial ridges, similarly to what has been reported in *G. max* seeds submitted to 40 °C (Xin et al. 2014). Increases in the content of MDA and ROS have been suggested to cause mitochondrial swelling and the loss of transmembrane potential in plant cells (Keunen et al. 2011). This was confirmed by observing the increase in the oxidation of lipids and proteins (Fig. 4C, D), which suggests that heat stress affect the constituents of the membrane and interfere with its integrity. Considering that mitochondrial membranes play an essential role in the respiratory process (Meyer et al. 2019), the consequences of heat stress may be associated with reducing in the glucose content observed in the seeds at 40 °C (Fig. 3A) and also with the decrease of oxidative phosphorylation efficiency since the respiratory rate was reduced under this condition (Fig. 7), implying that seeds subjected to high temperature stress reduce the energy supply necessary for germination.

Conclusion

Collectively, the results of this study demonstrate that exposure to heat stress (40 °C) caused the accumulation of ROS, increasing the oxidative damage in *M. brauna* seeds. Heat stress also increased (α-, β-amylase, and protease) and decreased (lipase) the reserve enzyme activity, caused oxidation of lipids and proteins, reduced respiratory rate, and induced changes in the integrity of mitochondrial structures. All of these events led to the loss of germination capacity. Finally, the results point to the possible environmental risks that heat stress can cause in forest tree seeds.

Understanding the physiological and morphological responses of seeds at heat stress during the development of germination may help to develop cultivars that are more resistant and adapted to climate change. Future research may also open new scenarios for programs of tolerance and adaptation of species to events that cause temperature extremes.

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Author contributions LPR and EDLB conceived and designed research. LPR and RSA conducted experiments. DSB contributed new reagents or analytical tools. LPR and RCB analyzed data. LPR wrote the manuscript. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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CHAPTER II

Heat stress negatively affects physiology and morphology during germination of *Ormosia coarctata* (Fabaceae, Papilionoideae)

Manuscript under review in the journal **Protoplasma**

Heat stress interferes negatively on physiology and morphology during germination

Abstract

Research on the morphophysiological behavior of forest seeds during germination concerning climate change is scarce. To date, there are no studies on biochemical or morphological aspects in *Ormosia spp*. In this study, we used seeds of *Ormosia coarctata* to investigate the effect of temperature on morphology, generation of reactive oxygen species (ROS), antioxidant system, and storage system. Analyses were performed on seeds exposed to 25, 35, and 40 °C for 48, 96, and 144 h. The morphology was evaluated through radiation in a Faxitron MX-20 device. The ROS production (superoxide anion and hydrogen peroxide), malonaldehyde (MDA), carbonylated proteins, antioxidant enzymes activity (superoxide dismutase - SOD, ascorbate peroxidase - APX, catalase - CAT, and peroxidase - POX), β-carotene, lycopene, glucose, and reserve enzymes activity (α and β amylase, lipase, and protease) were analyzed by spectrophotometry. Heat stress (40 °C) decreased germination by 76.2 and 78.1% (compared to 25 and 35 °C, respectively), caused damage to the external morphology of the seed, increased the content of ROS, MDA, and carbonylated proteins, and reduced APX, CAT, and POX activity. Furthermore, heat stress decreased glucose content and α-amylase activity. These results suggest that an increase of 5°C in temperature negatively affects germination, promotes oxidative stress, and induces deterioration in *O. coarctata* seeds.

Introduction

The increase in temperature is among the abiotic stresses that most threaten the productivity and growth of plant species (Hassan et al., 2020; Hu et al., 2020). In the context of climate change, heat stress episodes have been increasingly evident (WMO, 2021), which creates a greater risk of local extinction of species (Islam et al., 2021), reduced seed bank persistence (Ooi et al., 2009), changes in the spatial distribution of species (Agwu et al., 2020), and loss of seed germination capacity (Begcy et al., 2020; Lima et al., 2021).

Germination is controlled by a set of environmental factors and, among them, the heat stress is particularly important. Heat stress impairs the physiological and biochemical processes, affecting seed hydration (Cabrera-Santos et al., 2021), membrane permeability (Santos et al., 2017), mobilization of reserves (Felix et al., 2020; Reis et al., 2020), respiratory metabolism and enzymatic activity (Liu et al., 2019, Reis et al., 2021).

In general, plant exposure to increased temperature causes oxidative stress (Hassan et al., 2020). To avoid oxidative stress, the seeds have efficient antioxidant systems that comprise enzymatic components, for instance, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidases (POX) (Mei and Song, 2010; Rashid et al., 2020) and non-enzymatic such as ascorbic acid, tocopherols, glutathione, phenolics, flavonoids, and carotenoids (Ashraf et al., 2019).

An inefficient antioxidant system results in increased lipid and protein oxidation (Tiwari and Sarangi, 2015). One of the end products of lipid oxidation is malonaldehyde (MDA), while protein oxidation causes the formation of carbonylated proteins (Hameed et al., 2011); MDA and carbonylated proteins are used as indicators of oxidative damage in response to stress conditions (Li et al., 2018; Ren et al., 2020; Manafi et al., 2021).

Ormosia Jacks. (Fabaceae, Papilionoideae), comprises approximately 120 arboreal or shrub species distributed in tropical America, Southeast Asia, and Northern Australia (Pennington et al., 2005). Within this genus, the species *Ormosia coarctata* is especially important for silvopastoral systems (Cárdenas and Ramírez, 2004), ecological restoration projects (Isernhagen, 2015), and in ethnopharmacology (Hajdu and Hohmann, 2012).

Although the germination response to temperature increases has been extensively investigated for agricultural species (Akter and Islam, 2017; Kilasi et al., 2018; Malabarba et al., 2021), few research has focused on forest species. Studies on the germination of *O. coarctacta* at the morphophysiological level have not been characterized so far. Understanding the effects of temperature on seed germination, essentially on biochemical parameters, is critical for more heat-tolerant species.

Given the importance of forest seeds for forest restoration, we aimed to evaluate the impact of heat stress on *O. coarctata* seed germination. We investigated the consequences of heat stress on morphology, generation of reactive oxygen species - ROS (superoxide anion and hydrogen peroxide), antioxidant system (activity of the SOD, APX, CAT, and POX and carotenoids content), oxidative damage (MDA and carbonylated proteins), and mobilization of reserves (glucose content and activity of the α-amylase, β-amylase, lipase, and protease).

Materials and methods

Germination of Ormosia coarctata

O. coarctata seeds were obtained in the municipality of Alta Floresta, Mato Grosso, Brazil. Seed dormancy was overcome with concentrated sulfuric acid for 45 minutes. After washing with distilled water, they were dried on absorbent paper and sterilized by immersion in captan 0.2% (w/v) fungicide for 5 min. In the germination test, 100 seeds from each treatment were transferred to five germitest paper rolls moistened with distilled water (2.5 times the dry paperweight) and placed inside perforated plastic bags. The test was conducted in a growth chamber with the controlled temperature at 25, 35, and 40 °C, under constant light. Germination was characterized by primary root protrusion. The germination rate was obtained by calculating the percentage (%) and the germination speed index was determined according to Maguire (1962).

The effects of temperature on both biochemical and morphological processes were evaluated throughout germination. The samples of each treatment consisted of five replicates of 20 seeds at times 48, 96, and 144 h of imbibition. Therefore, the treatments were dry seeds (T0); 25 °C for 48 h (T1), 96 h (T2), and 144 h (T3); 35 °C for 48 h (T4), 96 h (T5), and 144 h (T6); and 40 °C for 48 h (T7), 96 h (T8), and 144 h (T9).

Morphology

To analyze seeds morphology, 15 samples of each treatment were used. Each seed was subjected to radiation in a Faxitron MX-20 device (Faxitron X-ray Corp. Wheeling, IL, EUA), coupled to a computer. The voltage used was 35 kV for 10 seconds at a focal length of 5 cm. Radiographic images were saved in TIFF format (Tagged Image File Format) and posteriorly processed by ImageJ® software (U.S. National Institutes of Health, Bethesda, MD). We also used this software to calculate the total seed area.

Quantification of ROS and oxidative damage

The ROS were analyzed through the quantification of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide content (H_2O_2). To quantify $O_2^{\cdot-}$ production, 0.2 g of seeds were macerated in a 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 5,000xg for 10 min. The test was based on the reduction of a sodium tetrazolium dye,3'-(1-[phenylaminocarbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) by O_2 to a soluble formazan XTT (Able et al., 1998).

The H_2O_2 content was determined through 0.3 g of seeds homogenized in trichloroacetic acid (TCA) [0.1% (p:v)] and centrifuged at 12,000*x*g for 15 min at 4 ºC. The quantification of H_2O_2 was estimated using the method used by Junglee et al. (2014).

Oxidative damage was evaluated through the contents of MDA and carbonylated proteins. The MDA content was quantified with the same supernatant used for the determination of H_2O_2 and subsequently evaluated through the reaction with thiobarbituric acid (Heath and Packer, 1986). The carbonylated protein content was quantified through 0.3 g of seeds macerated in buffer containing Hepes-NaOH (10 mM, pH 7.5), protease inhibitor cocktail (0.1%), and β-mercaptoethanol (0.07%). The determination of carbonylated proteins was performed by derivatizing protein carbonyls using 2.4-dinitrophenylhydrazine (DNPH) (Xia et al., 2016).

Enzyme antioxidant activity assay

The extraction of antioxidant enzymes was performed from 0.3 g of seeds ground in liquid nitrogen and 2 mL of extraction buffer containing 50 mM sodium phosphate (pH 7.8) and 1% (w/v) of polyvinylpyrrolidone (PVP). Afterward, the samples were centrifuged at 15,000*x*g for 20 min at 4 °C. The supernatant was used as a crude extract for assaying enzymatic activities.

SOD activity (EC 1.15.1.1) was measured using the nitro blue tetrazolium (NBT) method. Crude extracts were homogenized in reaction medium containing 100 mM phosphate buffer, pH 7.5, 50 mM methionine, 1 mM p-nitro tetrazolium blue (NBT), 5 mM EDTA, 2µM riboflavin and distilled water. A unit (U) of SOD was defined as the amount of enzyme needed to inhibit by 50% of the photoreduction of NBT monitored at 560 nm (Beauchamp and Fridovich, 1971). APX activity (EC 1.11.1.11) was evaluated through crude enzymatic extract and reaction medium containing phosphate buffer (50 mM, pH 7, 0.25 mM ascorbic acid, 0.1 mM EDTA, and 0.3 mM H_2O_2). A U of APX activity was defined as 1 nmol of oxidized ascorbate per minute per mg of protein (Nakano and Asada, 1981). CAT activity (EC 1.11.1.6) was performed through the assay containing crude enzymatic extract and reaction medium constituted by phosphate buffer (50 mM, pH 7.8, and H_2O_2 0.97 M), adapted from Kar and Mishra 1976; the decrease in absorbance was measured at 240 nm and a U of CAT activity was defined as 1 mmol H_2O_2 per minute per mg of protein. POX activity (EC 1.11.1.7) was determined by adding 50 µL of crude enzymatic extract and reaction medium containing potassium phosphate buffer: 25 mM, pH 6.8, 20 mM pyrogallol, and 20 mM H_2O_2 (Kar e Mishra, 1976). Purpurogaline production was determined at 25 °C and reading at 420 nm.

Bradford's method was used to determine the amount of protein in the extracts from all enzymatic activities (Bradford, 1976). Bovine serum albumin was used to quantify protein concentrations through a standard concentration curve.

Carotenoid contents

Carotenoid contents (β-carotene and lycopene) were determined using 0.2 g of seeds in 10 mL of acetone/hexane (2:3). To calculate the concentrations, we use the following equations: β-carotene (mg/100 mL) = $0.216_{A663} - 1.22_{A645} - 0.304_{A505} + 0.452_{A453}$; lycopene (mg/100 mL) = $-0.0458_{A663} + 0.204_{A645} + 0.372_{A505} - 0.0806_{A453}$ (Nagata and Yamashita, 1992).

Glucose content and activity of α-amylase, β-amylase, lipase, and protease enzymes

To assess changes in glucose content, 1 g of seed was ground and cold defatted with hexane in a homogenizer. Subsequently, five 0.1 g samples from each treatment were kept in 80% alcohol at 80 ºC for 30 min and centrifuged at 10,000*x*g for 5 min: this process was repeated three times. After extractions, the supernatants were placed in an oven for 24 h at 45 °C and then resuspended with 1.0 ml of distilled water. Glucose concentration was determined with a BIOCLIN monoreagent glucose kit (QUIBASA - Química Basica Ltda, Belo Horizonte, Minas Gerais, Brazil).

To measure the activity of α-amylase (EC 3.2.1.1) and β-amylase (EC 3.2.1.2), 0.3 g of seeds from each treatment were extracted in 2 mL of ice-cold distilled water and centrifuged at 15,000*x*g for 20 min at 4 °C. The α-amylase activity was measured after β-amylase inactivation and vice versa (Kishorekumar et al., 2007).

To quantify lipase activity (EC 3.1.1.3), 0.2 g of sample from each treatment were macerated in liquid nitrogen with 2 mL of 50 mM Tris-HCl buffer, pH 8.0 and centrifuged at 9,000*x*g for 40 min at 4 °C. The supernatant was collected and the reagents were added according to the manufacturer's recommendations. Lipase activity was determined with a BIOCLIN kit (QUIBASA - Química Basica Ltda, Belo Horizonte, Minas Gerais, Brazil). Readings were taken at 410 nm.

Protease activity was performed using 0.3 g of seeds macerated in 50 mM sodium acetate buffer, pH 7.5, and centrifuged at 10,000*x*g for 10 min at 4 °C. Quantification was performed following the method described by Cupp-Enyard (2008).

Data analysis

Germination results, morphological measurements, quantification of biochemical products, and enzymatic activity were subjected to analysis of variance, considering treatment with different temperatures as an explanatory variable. When necessary, the means of each treatment were compared using the Tukey test ($p < 0.05$). Data normality was verified by the Shapiro-Wilk test.

The data obtained from the quantification of products biochemicals and enzymatic activity was also subjected to principal component analysis (PCA) to explore multivariate patterns in different groups of temperature treatments. Data were univariately normalized (subtract the mean and divide by the standard deviation), and the eigenvalues and eigenvectors were calculated from the covariance matrix. All analysis and resulting plots were performed in the R software with the integrated development environment RStudio (R core team, version 4.0.0).

Results

Germination

The increase in temperature significantly affected germination ($F_{4,20}= 190.4$, $p < 0.01$). On average, heat stress (40 °C) reduced the germination percentage by 76.2 and 78.1% compared to 25 and 35 °C, respectively. Germination at 25 °C (80%) and 35 °C (87%) did not differ significantly ($p = 0.082$, Fig. 1A). The germination speed index (GSI) was also significantly affected by different temperatures ($F_{4,20}= 131.6$, $p < 0.01$) and the values varied between 2.40 (35 °C) to 0.51 (40 °C, Fig. 1B).

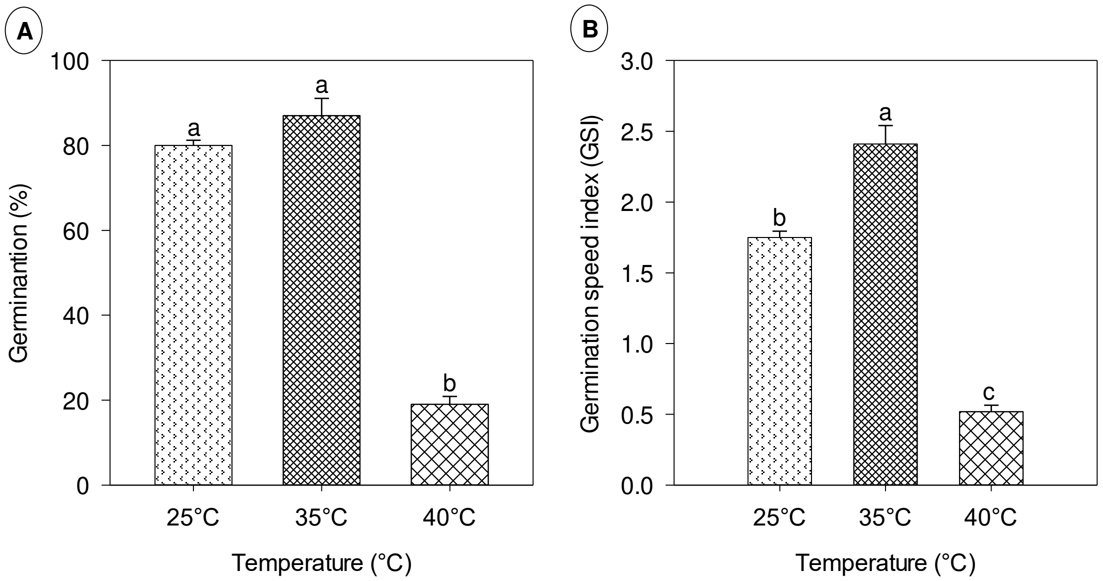

Fig. 1 Germination percentage (A) and germination speed index (GSI) (B) in *Ormosia coarctata* seeds submitted to different temperatures. Means followed by the same letter do not differ significantly from each other ($p < 0.05$). Values are expressed as the mean ± SD (standard deviation; n = 5) of 20 seeds each.

Seed morfologia

The area of the seeds did not undergo statistical variation ($F_{4,20} = 45.2$, $p > 0.05$) in T1 (1.48 cm^2), T4 (1.72), and T7 (1.82) (Fig 2A, 2E, and 2H, respectively). The largest areas were found in T5 (3.19) and T6 (3.46); at T6 there was primary root protrusion (Fig. 2G). The area of T8 (2.45) and T9 (2.54) were significantly larger than T7 at 25.7 ($p = 0.003$) and 28.4% ($p = 0.005$), respectively. Tegument deterioration was detected at T8 and T9 (Fig. 2I and J).

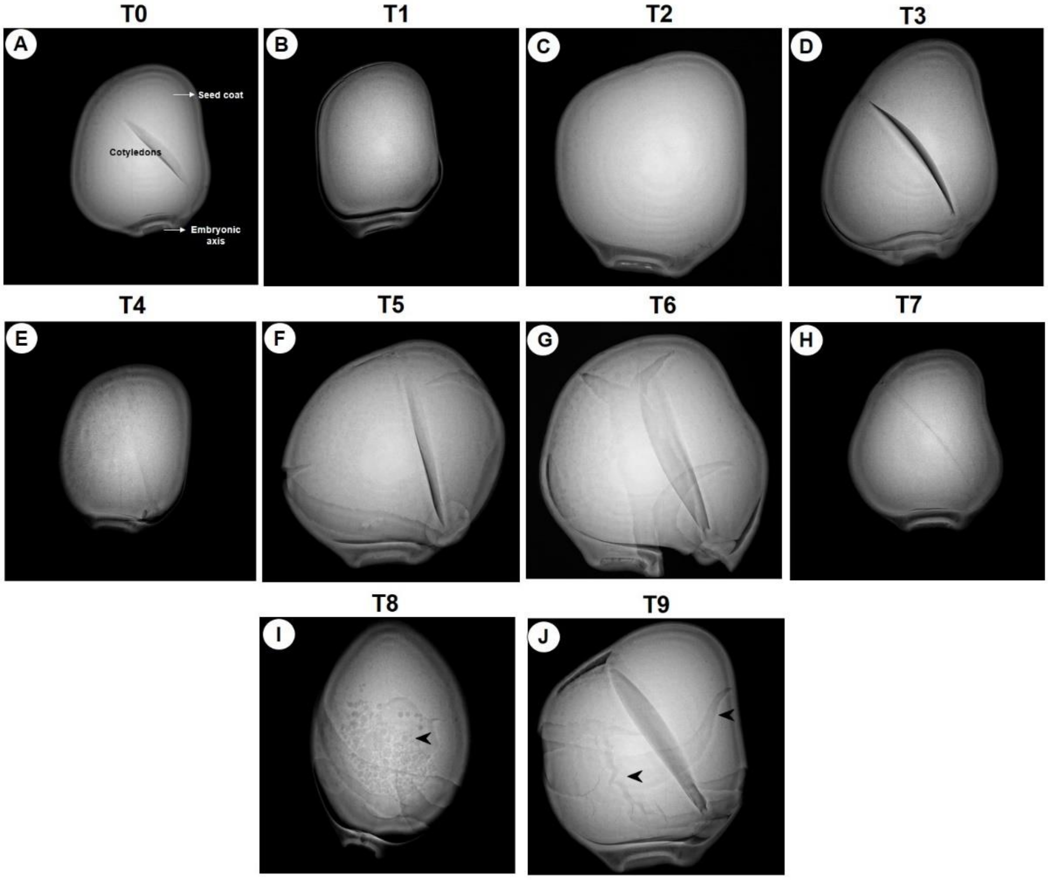

Fig. 2 X-ray photographs showing the internal and external morphology of *Ormosia coarctata* seeds submitted at 25, 35, and 40 °C for 48, 96, and 144 hours. (A) T0: dry seed; (B) T1: 25 °C 48 h; (C) T2: 25 °C 96 h; (D) T3: 25 °C 144 h; (E) T4: 35 °C 48 h; (F) T5: 35 °C 96 h; (G) T6: 35 °C 144 h; (H) T7: 40 °C 48 h; (I) T8: 40 °C 96 h; (J) T9: 40 °C 144 h. Arrows indicate deterioration of the tegument.

Superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide content (H_2O_2), malonaldehyde (MDA), and carbonylated proteins

There were significant differences ($F_{4,20} = 24.2$, $p < 0.01$) in the $O_2^{\cdot-}$ content of seeds exposed to different temperatures in relation to dry seed (Fig. 3A). Seeds kept in T1, T2, and T3 showed increases of 71.3, 78.5, and 77.2%, respectively, compared to T0. The $O_2^{\cdot -}$ content in T5 (1667.12 nmol/min/mgprotein) and T6 (1762.51) was significantly ($p < 0.01$) higher compared to T4 (1246.68). The highest means were observed in T7 (1911.42), T8 (1932.82), and T9 (2271.54).

The production of H_2O_2 progressively increased at different temperatures (Fig. 3B); in T7, T8, and T9 the increases corresponded to 55.9, 52.8, and 58.4%, respectively ($F_{4,20} = 16.3$, $p < 0.01$) compared to T0. The H_2O_2 content in T4 (2.09 nmol/mg/protein) was higher compared to T5 (1.60) and T6 (1.93). There was no statistical variation ($p > 0.05$) between T1 (1.70), T2 (1.58), T3 (1.56), and T5.

The MDA content in the different treatments increased significantly ($F_{4,20} = 73.8$, $p < 0.01$) compared to T0 (0.7139 nmol/mg/protein) and T1 (0.8935, Fig. 3C). There were no significant differences (p > 0.05) between T3 (2.4247), T4 (2.3978), T6 (2.6129), T7 (2.9193), and T8 (2.5488). The highest MDA content was observed in T9 (3.9784).

The carbonylated protein content increased statistically ($F_{4,20} = 31.6$, $p < 0.01$, Fig. 3D). There were no significant differences ($p > 0.05$) between T1 (0.0770 nmol/mg/protein), T2 (0.0608), T3 (0.0897), T4 (0.1045), T5 (0.0951), and T6 (0.1080). On the other hand, the maximum content of carbonyl proteins found in T9 (0.2161) was 36.8 and 35.3% higher compared to T7 and T8, respectively.

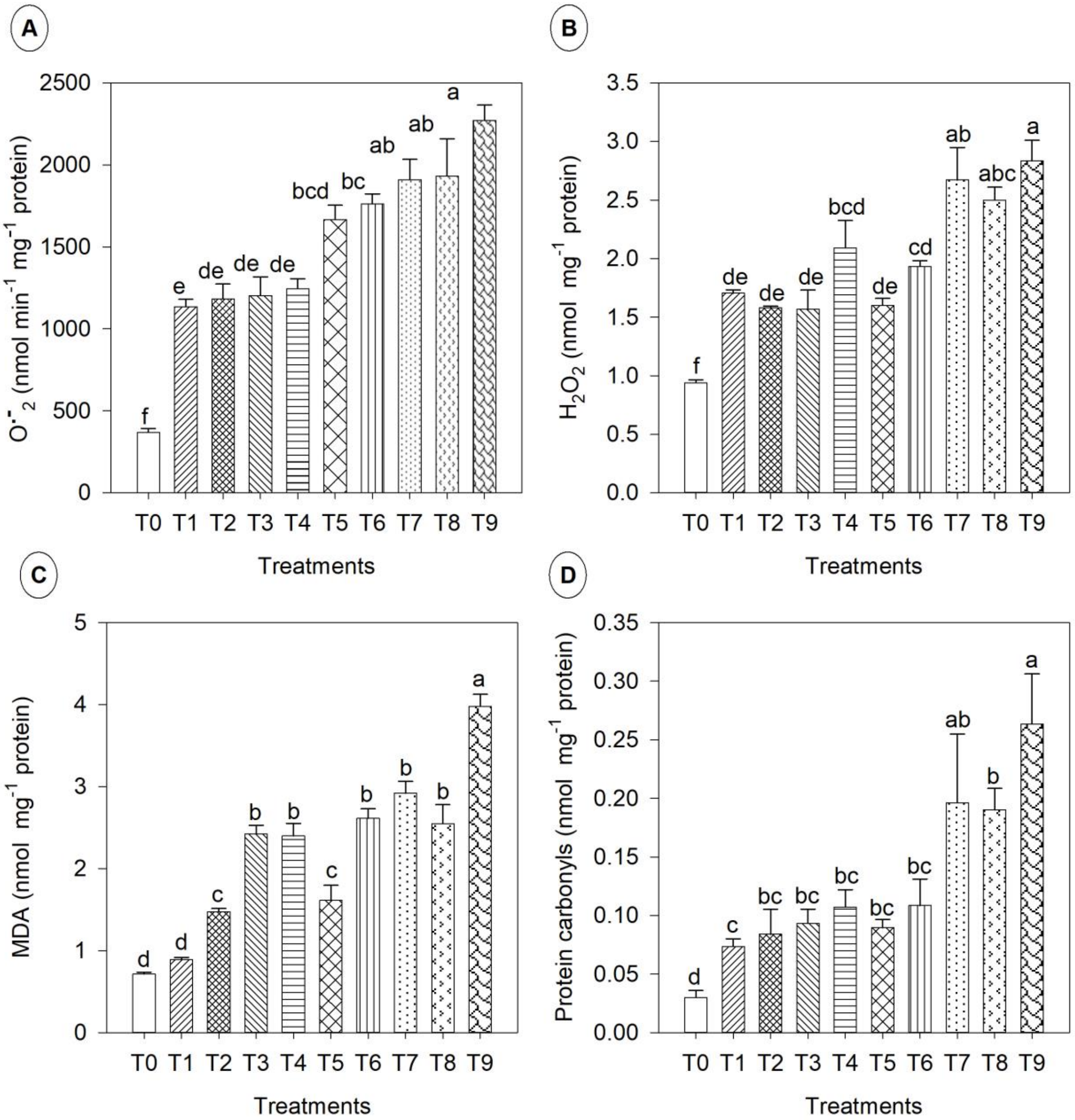

Fig. 3 Superoxide anion ($O_2^{\cdot -}$) (A), hydrogen peroxide (H_2O_2) (B), malondialdehyde (MDA) (C), and carbonylated proteins (D) in *Ormosia coarctata* seeds. T0: dry seed; T1: 25 °C 48 h; T2: 25 °C 96 h; T3: 25 °C 144 h; T4: 35 °C 48 h; T5: 35 °C 96 h; T6: 35 °C 144 h; T7: 40 °C 48 h; T8: 40 °C 96 h; T9: 40 °C 144 h. Different letters indicate significant differences by Tukey test, $p < 0.05$. Values are expressed as mean ± SD (n = 5) per treatment.

Antioxidant enzyme activity

SOD activity did not show significant variations ($p > 0.05$) between T1 (0.1077 U min.mg.protein), T2 (0.1127), T3 (0.1399), and T4 (0.1411); these treatments were significantly ($F_{4,20} = 11.2$, $p < 0.01$) smaller than T5 (0.1835), T6 (0.1736), T7 (0.2012), T8 (0.1731), and T9 (0.1953, Fig. 4A). The highest activity of this enzyme was observed in T7, but without significant differences ($p > 0.05$) when compared to T5, T6, T8, and T9.

APX activity, as well as CAT and POX activity, was not detected at T0. Regarding APX, T3 was 25.8 and 33.8% higher when compared to T1 and T2, respectively (Fig. 4B). The highest APX activity was verified in T6 (24144.8 µmol min mg protein) and was significantly higher ($F_{4,20} = 57.4$, $p < 0.01$) compared to the other treatments; there were no statistical variations ($p > 0.05$) between T4, T5, T7, T8, and T9. CAT activity values in T1 (585.57 µmol H_2O_2 min mg protein), T2, T4, T5, T7, T8, and T9 were similar, but these values were significantly lower ($F_{4,20} = 73.2$, $p < 0.01$, Fig. 4C) compared to T3 and T6. The highest CAT activity occurred at T6 (1877.81). POX activity at T3 (78156.00 µmol min mg protein) was significantly ($F_{4,20} = 27.5$, $p < 0.01$) higher compared to T2, T4, T5, T7, T8, and T9, but did not differ ($p > 0.05$) of T1 and T6 (Fig. 4D). POX activity in T7, T8, and T9 (43753.8, 33011, and 39075.6, respectively) presented the lowest values.

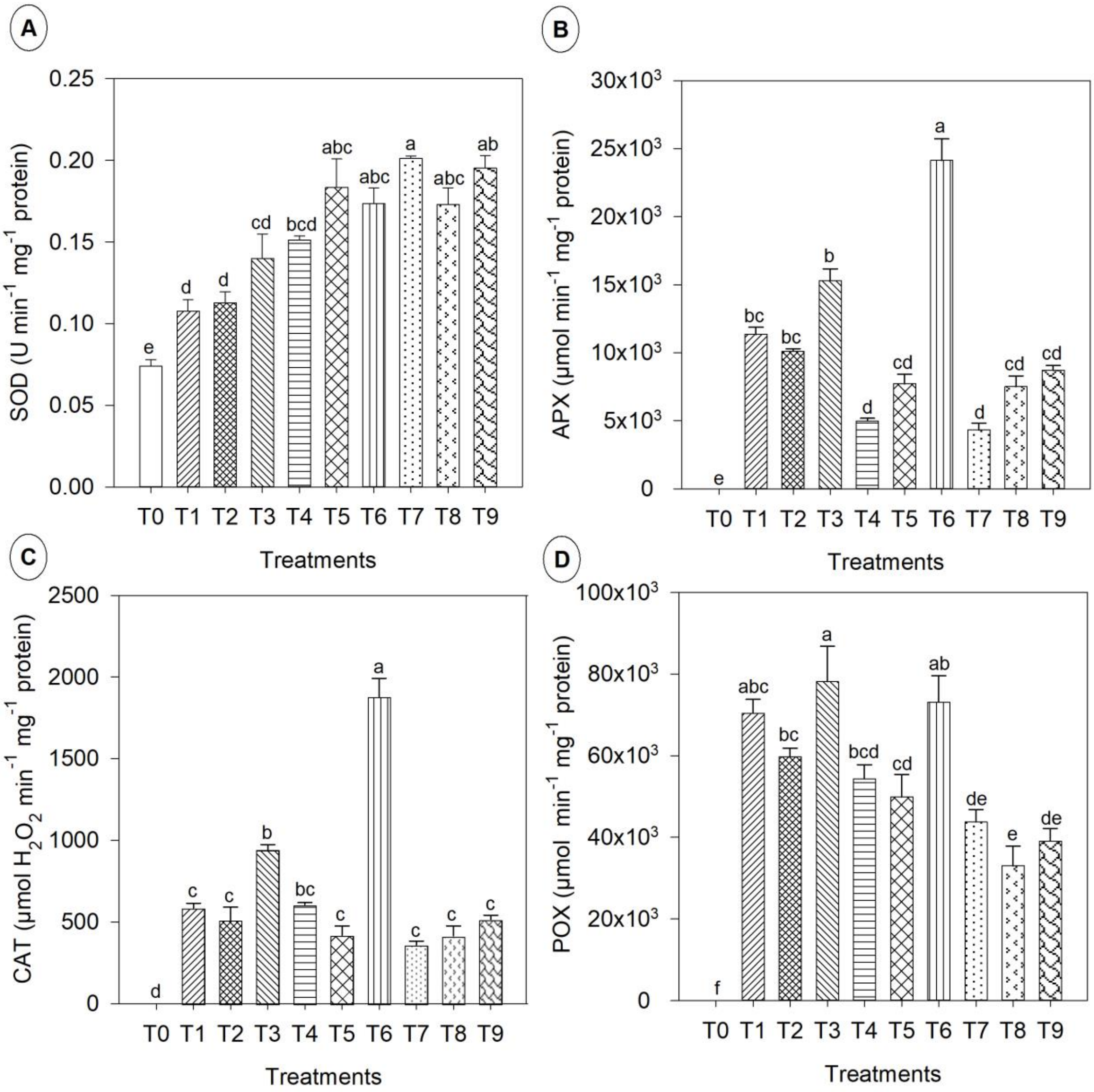

Fig. 4 Activity of superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B), catalase (CAT) (C), and peroxidase (POX) (D) in *Ormosia coarctata* seeds. T0: dry seed; T1: 25 °C 48 h; T2: 25 °C 96 h; T3: 25 °C 144 h; T4: 35 °C 48 h; T5: 35 °C 96 h; T6: 35 °C 144 h; T7: 40 °C 48 h; T8: 40 °C 96 h; T9: 40 °C 144 h. Different letters indicate significant differences by Tukey test, $p < 0.05$. Values are expressed as mean ± SD (n = 5) per treatment.

β-carotene and lycopene contents

The β-carotene content at T0 (0.0095) was significantly lower ($F_{4,20} = 34.9$, $p \leq 0.01$) than T1, T4, and T7 (68.3, 72.3, and 72.4%, respectively, Table 2). There were no significant variations ($p > 0.05$) in β-carotene content between T2, T3, T5, and T9. On the other hand, the lycopene content was only detected at T0, T1, and T8, with T1 being significantly ($p < 0.05$) higher in relation to the other two.

Table 2 β-carotene and lycopene contents in dry seeds (T0); 25 °C for 48 h (T1), 96 h (T2), and 144 h (T3); 35 °C for 48 h (T4), 96 h (T5), and 144 h (T6); 40 °C for 48 h (T7), 96 h (T8) and 144 h (T9).

Treatments	β- carotene	Lycopene
T0	0.0095^{e}	0.0032^{c}
T1	0.0300^{ab}	0.0071^{a}
T2	0.0218^{cd}	0.0000^{d}
T3	0.0269^{bc}	0.0000^{d}
T4	0.0344^{a}	0.0000^{d}
T5	0.0249^{bc}	0.0000^{d}
T6	0.0165^{de}	0.0000^{d}
T7	0.0345^{a}	0.0000^{d}
T8	0.0098^{e}	0.0050^{b}
T9	0.0222^{cd}	0.0000^{d}
P	0.001	0.001
F	18.14	68.3
CV(%)	18.53	44.9

Values are expressed as a mean (± SD). Different letters indicate significant diferences among samples (ANOVA, Tukey´s post hoc test $p < 0.05$).

Glucose content and storage enzyme activity

Seed exposure to different treatments had significantly affected glucose content ($F_{4,20} = 439.6$, $p < 0.01$, Fig. 5A). The values of T0 (0.0606 mg.g.protein), T1 (0.0674), T7 (0.1052), and T8 (0.0598) were similar and significantly ($p < 0.01$) lower compared to the other treatments. T2 (0.2562), T3 (0.2560), and T9 (0.2499) also did not present significant variations ($p > 0.05$) between them, but their values were higher than T7 and T9. The highest glucose content was observed in T6 (0.7623).

The activities of α-amylase, β-amylase, lipase, and protease showed significant changes ($p < 0.01$) in all treatments compared to T0. Seeds under T1, T2, and T3 had the highest α-amylase activity, while T7, T8, and T9 had the lowest (Fig. 5B). For this enzyme, the value of T6 (0.6428 U mg.g.protein) was significantly ($F_{4,20} = 72.2$, $p < 0.01$) higher than in T4 (0.4343) and T5 (0.4868).

The β-amylase activity in T1, T2, and T3 increased by 75.4, 80.9, and 71.6%, respectively, compared to T0 (Fig. 5C). At T5 (0.2281) and T6 (0.2289) the β-amylase activity was significantly ($F_{4,20} = 24.3$, $p < 0.01$) higher than at T4 (0.1273). In T9, in turn, the activity was higher compared to T7 (24.9%, $p = 0.17$) and T8 (25.3%, $p = 0.15$), but without significant differences between them.

Lipase activity in T3 (0.444 U mg.g.protein) increased significantly ($F_{4,20} = 38.9$, $p = 0.001$) compared to T2 (0.252), however, it was similar to T6 ($p = 0.98$) and T7 ($p = 0.97$, Fig. 5D). Seeds submitted to T4, T5, and T8 had the highest lipase activities, representing increases of 79.3, 81, and 79.2%, respectively, in relation to T0. Lipase activity observed in T9 was 63.2% lower than T7 and 73.5% lower than T8.

As for the protease activity, there was a significant increase ($F_{4,20} = 46.2$, $p < 0.01$) of 72.6% for T1, 70.7% for T2, and 72.7% for T3 compared to T0. There were no statistical differences ($p > 0.05$) between T4 (0.862 U mg.g.protein), T5 (0.764), T7 (0.829), T8 (0.822), and T9 (0.792). The highest protease activity was observed in T6 (0.915), but without significant differences when compared to T4 ($p = 0.90$), T7 ($p = 0.38$), and T8 ($p = 0.28$).

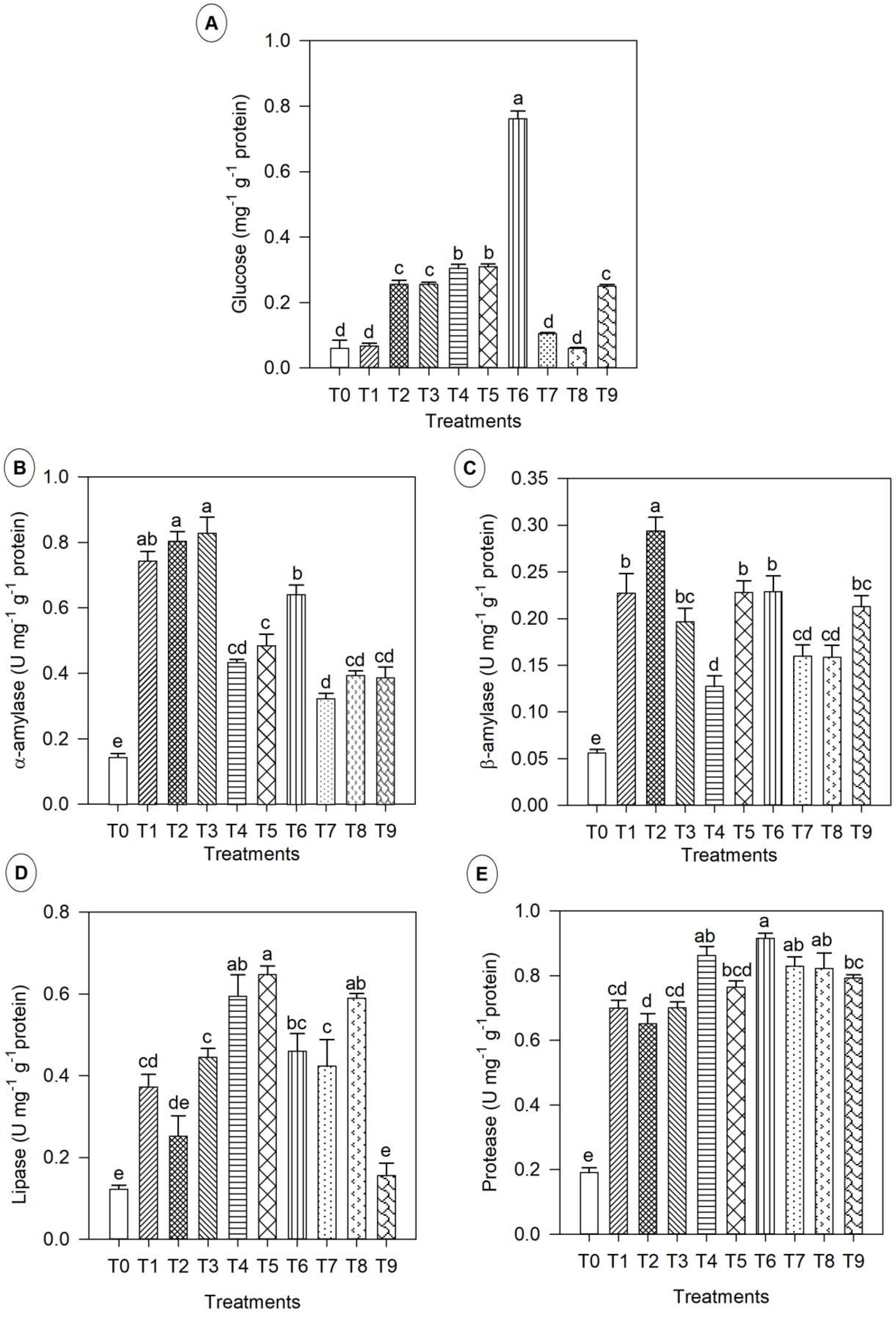

Fig. 5 Glucose content (A) and activity of the α-amylase (B), β-amylase (C), lipase (D), and protease (E) in *Ormosia coarctata* seeds. T0: dry seed; T1: 25 °C 48 h; T2: 25 °C

96 h; T3: 25 °C 144 h; T4: 35 °C 48 h; T5: 35 °C 96 h; T6: 35 °C 144 h; T7: 40 °C 48 h; T8: 40 °C 96 h; T9: 40 °C 144 h. Different letters indicate significant differences by Tukey test, $p < 0.05$. Values are expressed as mean ± SD (n = 5) per treatment.

Multivariate effect of temperature on products biochemicals and enzymatic activity of Ormosia coarctata *seeds*

The data from products biochemicals and enzymatic activity were submitted to PCA analysis. The first two main components explained 49.1% of the original variation among seed descriptors with 32.6% and 16.5% for PC1 and PC2, respectively. There was subtle separation among seeds according to temperatures (Fig. 6A) and imbibition times (Fig. 6B). Besides, there was remarkable separation among seeds according to treatment groups (from T0 to T9), evidencing of the joint effect of temperature and imbibition times in products biochemicals and enzymatic activity of *O. coarctata* seeds (Fig. 6C). The most important variables were associated with carbohydrate metabolism (i.e., glucose and α-amilase) and with enzymes associated with oxidative stress (i.e., CAT, APX, and POX) (Fig. 6D), which demonstrates the relationship of these parameters with the physiological quality of seeds. Moreover, there was similar behavior of the variables APX, CAT, Glucose, and α-amylase associated with treatment T6 (35 °C and 144h), indicating that this treatment had higher values to these variables (Fig. 6C and 6D). Differently, the treatment T0 (dry seed) exhibited higher values for H_2O_2 and Lycopene. Finally, the treatments associated with 40 °C (T7, T8, and T9) exhibited higher values mainly for POX, protease, protein carbonylation, and β-carotene, regardless of imbibition times.

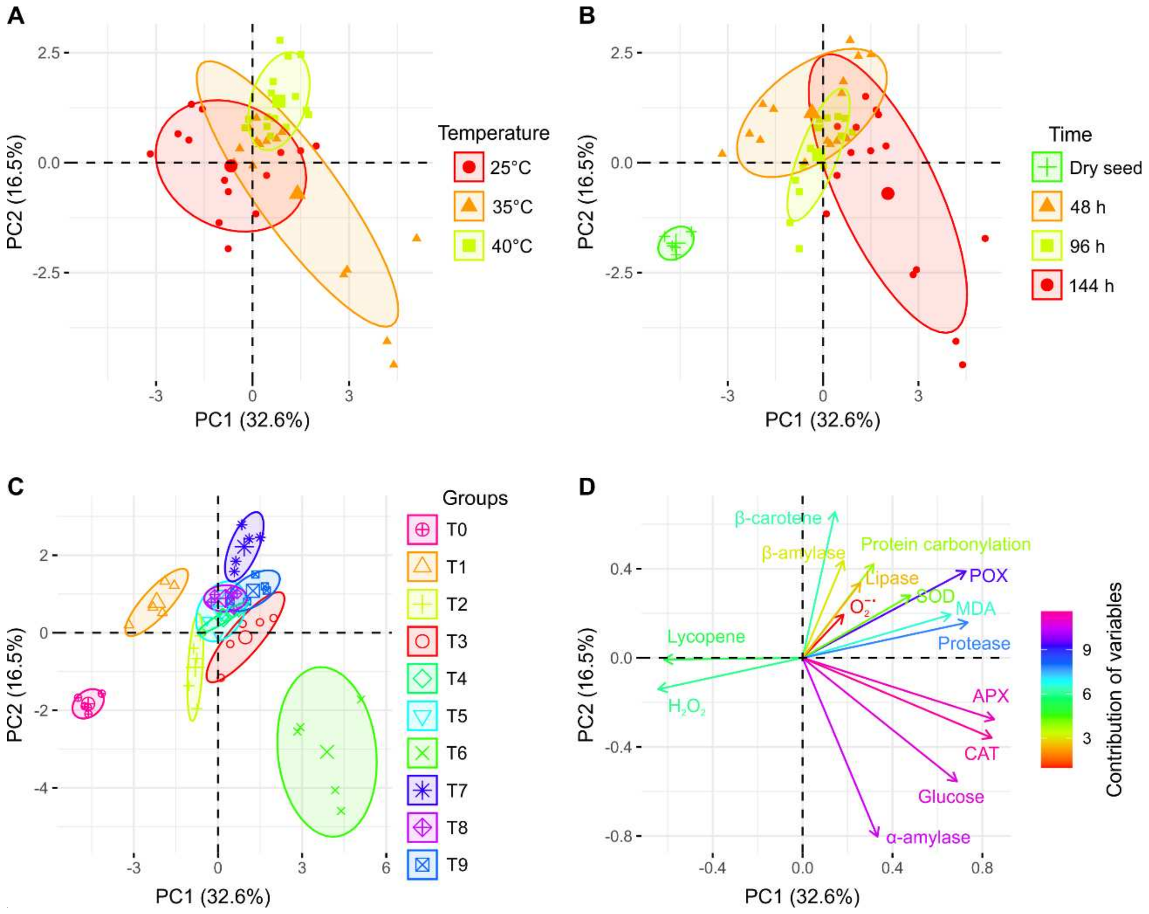

Fig. 6 Biplots of principal component analysis from products biochemicals and enzymatic activity data of the *Ormosia coarctata* seeds submitted to different (A) temperatures, (B) imbibition times, and (C) groups of temperature and time. The ellipses represent the confidence interval (95%) around the centroid of each group in biplots. T0: dry seed; T1: 25 °C 48 h; T2: 25 °C 96 h; T3: 25 °C 144 h; T4: 35 °C 48 h; T5: 35 °C 96 h; T6: 35 °C 144 h; T7: 40 °C 48 h; T8: 40 °C 96 h; T9: 40 °C 144 h. (D) Principal component loadings with color scale to contribution of variables. The directions of the arrows show the relative loadings of the variables on the first and second principal components. Percentage values on the axes indicate how much each component explains the total variance of the data.

Discussion

The temperature increase (40 ºC) caused a decrease in both germination percentage and GSI, in addition to the damage in external morphology of *O. coarctata* seeds, which is indicative of tissue deterioration, even for a species adapted to warm climate regions. These data corroborate investigations that point to a reduction in the germination percentage and GSI in forest seeds (*Melanoxylon brauna* and *Dalbergia spruceana*) exposed to temperatures above 35 ºC (Santos et al., 2020; Lima et al., 2021) evidencing the risk of global warming for such species. Although few studies have addressed the impact of high temperature on damage related to seed morphology during germination, in *M. brauna* heat stress (40 ºC) resulted in significant changes in the internal morphology, which was associated with an increase ROS (Reis et al. 2021). Thus, the deterioration observed in *O. coarctata* seeds exposed to high temperature can have occurred due to the accumulation of ROS ($O_2^{\cdot -}$ and H_2O_2), that may have caused the loss of cell membrane functions through the generation of free radicals (Berni et al., 2019), causing tissue damage.

The production of $O_2^{\cdot -}$ and H_2O_2 under optimal temperatures (25 and 35 °C) shows that ROS act as messengers triggering events that result in high germination rates. The accumulation of ROS, especially H_2O_2, increases the carbonylation rate of proteins, allowing the supply of reducing power (NADPH), promotes the activation of the thioredoxin system, and influences the hormonal balance, favoring germination (Barba-Espín et al., 2011; Bailly, 2019; Considine and Foyer, 2021). Therefore, increases in the content of $O_2^{\cdot -}$ and H_2O_2 offer beneficial effects on germination when there is an antioxidant system in balance.

Heat stress caused oxidative stress in the seeds through the accumulation of $O_2^{\cdot -}$ and H_2O_2; consequently, increased MDA in seeds submitted to prolonged stress (T9) and carbonylated proteins (Fig. 3). The level of oxidative stress in seeds has been demonstrated through the determination of lipid peroxidation and carbonylated protein content (Zheng et al., 2018; Rashid et al., 2020). However, the MDA content did not show significant effects in predicting oxidative damage when observed in a shorter time of exposure to heat stress. Interpretation of MDA data depends on the functioning of the redox regulation, as increases in the MDA content may indicate stress acclimatization (Morales and Munné-Bosch, 2019). In this sense, seeds exposed to short periods of stress presented defense systems similar to the optimal temperatures, such as the activity of the enzymes APX, CAT, and POX and a decrease in the levels of β-carotene and lycopene, giving the seeds thermotolerance up to certain limits of exposure to heat.

In seeds exposed to heat stress, glucose levels decreased been accompanied by a reduction in α and β-amylase enzyme activities. In *M. brauna*, heat stress also reduced the activity of α and β-amylase after a period of 48 h of imbibition (Ataíde et al., 2016). All these data show that the supply of sugars was affected after forest seeds (*O. coarctata* or *M. brauna*) to suffer heat stress. As the mobilization of carbohydrates is of fundamental importance for germination since it provides energy for the construction of new cells and tissues (Bewley et al., 2013), a decrease in this mobilization results in reduced germination.

The increase in lipase activity observed at optimal temperatures is a favorable factor because the mobilization of stored lipids will serve to boost post-germination growth. On the other hand, the increase in lipase activity under 40 °C in the initial times of imbibition (48 and 96h) can intensify the production of ROS, through free fatty acids and ß-oxidation (Kumar et al., 2015) since the antioxidant system is in low activity. The reduction in lipase activity in seeds submitted to 40 ºC for 144 h justifies, at least in part, the non-germination of *O. coarctata* seeds at this time.

Protease activity increased in seeds subjected to 35 and 40 ºC (Fig. 5E). At optimal temperatures, this protein is related to the degradation of storage proteins that will support embryo development (Szewińska et al., 2017; Martinez et al., 2019). However, our data show that at 40 ºC there was a reduction in the germination of *O. coarctata*, suggesting that the activity of this protein can be associated with another physiological process. Considering that plant's protease can act in regulating and signaling molecules for increase environmental stress-tolerance (Sharma and Gayen, 2021), the increased activity of the protease at 40 ºC is evidence that this protein is acting to minimize heat stress in the *O. coarctata* seeds.

Conclusion

The percentage of germination and GSI is higher in *O. coarctata* seeds exposed to 25 and 35 °C. Heat stress (40 °C) induced the production of ROS, increased the content of carbonylated proteins, reduced the activity of the enzymes APX, CAT, POX, and α-amylase, and decreased the glucose content. Additionally, heat stress promoted oxidative stress and induced seed deterioration. Furthermore, prolonged heat stress (40 °C for 144 h) decreased lipase activity and increased lipid oxidation. To our knowledge, this is the first time that morphophysiological parameters during germination are analyzed in *Ormosia spp.* subjected to different temperatures. These data, therefore, reinforce the possible environmental risks of temperature increase in forest species, including *O. coarctata*.

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CHAPTER III

Effects of diphenylene iodonium on the germination of Melanoxylon brauna (Fabaceae: Caesalpinioidae) under different temperature conditions

Manuscript in the journal's norms **Environmental and Experimental Botany**

Effects of diphenyleneiodonium chloride on the germination of *Melanoxylon brauna* (Fabaceae: Caesalpinioidae) under different temperature conditions.

Abstract

Reactive oxygen species (ROS) play a dual role in germination. Although ROS have been extensively considered dangerous, their generation in a controlled manner helps to regulate germination. In this work, we examined the effect of diphenyleneiodonium chloride (DPI) on *Melanoxylon brauna* seeds. The study objective is to explore the effect of DPI on germination, internal anatomy, hydrogen peroxide (H_2O_2) content, and the activity of α- and β-amylase enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POX) under conditions optimum (25 °C) and heat stress (40 °C). The analyzes were performed on seeds soaked in distilled water (control) and in DPI for 48 and 96 h. Anatomical and histological parameters were evaluated using light microscopy. H_2O_2 content and enzyme activity were measured by spectrophotometric analysis. Seed exposure to DPI at 25 °C decreased the germination percentage by 27% and the germination speed index by 35.4%. At 40 °C the seeds did not germinate in both the control and DPI treatments. DPI caused smaller loosening of the outer layers of the integument under optimal conditions, reduced the content of H_2O_2 and the activity of all tested enzymes. In general, treatment with DPI under heat stress conditions does not affect germination and anatomy but decreases the H_2O_2 content and enzyme activity. Finally, DPI does not act to mitigate the effects of heat stress on *M. brauna* seeds.

Keywords: DPI, reactive oxygen species, seeds, amylases, heat stress.

Introduction

Reactive oxygen species (ROS) are unavoidable by-products of aerobic metabolism (Considine and Foyer, 2021). Although they have been recognized as harmful molecules for many years, they can play a dual role in organisms. (Jeevan Kumar et al., 2015). According to the concept of the oxidative window for germination, cellular events are delimited by lower and upper limits of ROS (Bailly et al., 2008). At low concentrations, signaling is not activated and, at high levels, oxidative stress occurs, which can negatively affect the development of the embryonic axis. (Bailly, 2019).

In different species, basal levels of ROS are related to increased germination percentage (Ishibashi et al., 2010; Bahin et al., 2011; Zhou et al., 2018), as it participates in dormancy relief (Cembrowska-Lech et al., 2015), in hormonal regulation (Li et al., 2018), in the weakening of the endosperm (Zhang et al. 2014), in inducing the activity of reserve enzymes (Panngom et al., 2018), and of antioxidant enzymes (Ellouzi et al., 2021). On the other hand, high levels cause damage to cell constituents. These molecules can react with lipids, proteins, and nucleic acids causing oxidative stress (Hasanuzzaman et al., 2020).

The increase in temperature is considered one of the environmental factors that help in the overproduction of ROS (Firmansyah and Argosubekti, 2020). Heat stress leads to increased ROS production in seeds (Santos et al., 2017; Campobenedetto et al., 2020), alters respiration and mitochondrial morphology (Reis et al., 2021), induces lipid peroxidation (Lima et al., 2021) and promotes programmed cell death (Malabarba et al., 2021).

Diphenyleneiodonium (DPI), inhibitor of enzymes containing flavin oxidoreductases (O’Donnell et al., 1993), has been frequently used to inhibit the production of ROS in seeds. (Ben Rejeb et al., 2015; Zhang et al., 2018; Sun et al., 2019). Its action is related to blocking the flow of electrons from NADPH to molecular oxygen (Hancock and Jones, 1987). Among the enzymes inhibited by the action of DPI are NADPH oxidases (Ellis et al., 1988), nitric oxide synthase (Stuehr et al. 1991), xanthine oxidase (Zhang et al., 1998) and the mitochondrial respiratory chain complex I and II enzymes (Ozsvari et al., 2017). However, these studies have been limited to species such as *Arabidopsis thaliana* (Müller et al., 2009), *Hordeum vulgare* (Ishibashi et al., 2015), *Oryza sativa* (Li et al., 2017; Guha et al., 2021), *Vigna radiata* (Singh et al., 2017) and *Nicotiana tabacum* (Li et al., 2018).

Melanoxylon brauna Schott (Fabaceae - Caesalpinioideae) is a tree native to Brazil (Carvalho, 2010). It is considered a wood of high density, quality, and durability (Campos Filho and Sartorelli, 2015), of great economic value, and with potential for reforestation and urban afforestation (Brito and Carvalho, 2014). Due to predatory exploitation, it is included in the list of species of Brazilian flora at risk of extinction (Martinelli and Moraes, 2013).

The increase in temperature as a result of climate change will be a threat to populations of *M. brauna*, because germination, physiological, morphological, and ultrastructure quality of seed mitochondria are severely affected by heat stress (Santos et al., 2017; Reis et al., 2021). The mechanism of action of DPI in *M. brauna* seeds is still unknown. Accordingly, here we examine the effect of DPI on germination, internal anatomy, hydrogen peroxide (H_2O_2) content, α and β-amylase activity, and antioxidant enzymes at 25 and 40°C.

Materials and Methods

Plant material and experimental conditions

The experiment was carried out under laboratory conditions using *Melanoxylon brauna* seeds as study material. Seeds were obtained in the municipality of Leopoldina (21 ° 31 ′ 55 ″ S and 42 ° 38 ′ 35 ″ W), in the state of Minas Gerais. After processing, the seeds were stored dry at 5 °C and 60% relative humidity. The treatments consisted of seeds soaked in water or in diphenyleneiodonium chloride (DPI). The seeds were placed in Petri dishes with filter paper, applying 5 ml of water or 5 ml of 1mM DPI for each test. The concentration of the DPI solution was determined according to Ishibashi et al. (2010). Plates were sealed and incubated in the dark at 25°C and 40°C. Germination (based on primary root protrusion) was recorded daily. The experimental design was completely randomized, with five replications of 20 seeds. The germination percentage (G%) and the germination speed index (IVG) were calculated (Maguire, 1962). Seeds soaked for 48 and 96 h at 25 and 40 °C in the presence of water or DPI were dissected and the embryos were used for anatomical analysis, determination of H_2O_2 content, and enzymatic activities. The detection of H_2O_2 was performed immediately after collecting the seed samples.

Anatomical Analysis

The regions of the micropyle were fixed in FAA50 (formaldehyde, acetic acid, 50% ethanol - 1: 1: 18 - v:v) for 48h under vacuum and then stored in 70% ethanol (Johansen, 1940). Subsequently, the plant material was dehydrated in an ethanol series (70, 85, 95%) and placed in pure resin and 95% alcohol (1:1) for 7 days. After this procedure, they were transferred to pure resin for 30 days. Finally, included in methacrylate (Historesin-Leica), according to the manufacturer's recommendations. The samples were sectioned 5 µm thick on an advanced automated rotary microtome (model RM2155, Leica microsystems Inc., Deerfield, USA). Longitudinal sections were stained with toluidine blue in acetate buffer, pH 4.7. Then permanent slides were mounted with synthetic resin (Permount®). The samples were photographed with a light microscope (model AX-70 TRF, Olympus Optical, Tokyo, Japan), coupled to a digital camera (Zeiss AxioCam model HRc, Göttinger, Germany).

Quantification of hydrogen peroxide (H_2O_2)

The production of H_2O_2 was determined according to the method described by Junglee et al. (2014). Embryos (100 mg) were ground in liquid nitrogen and homogenized in a solution of 0.1% trichloroacetic acid, 1M KI, and 10 mM potassium phosphate buffer for 10 min at 4˚C. The homogenate was centrifuged at 12,000 × g for 15 min at 4 ˚C. The supernatant was incubated at 20 °C for 20 min. Samples and blanks were analyzed in rejoinders. Absorbance was measured at 350 nm. A calibration curve obtained with standard solutions of H_2O_2 prepared in 0.1% TCA was used.

Activity of α and β-amylase enzymes

The activities of the hydrolytic enzymes α-amylase and β-amylase were determined using the colorimetric method of 3,5-dinitrosalicylic acid (Kishorekumar et al., 2007). 100 mg of embryos were ground in liquid nitrogen and homogenized in ice-cold distilled water and centrifuged at 15,000 x g for 30 min at 4°C. The supernatant for α-amylase analysis was measured after β-amylase inactivation by incubation in 3 mM $CaCl_2$ at 70ºC for 5 min. Then, a reaction mixture containing citrate buffer (0.1 mM; pH 5.0), 2% soluble starch solution (w/v), and hot enzyme extract was prepared and incubated at 30 ºC for 5 min. The reaction was stopped by adding color reagent (3,5-dinitrosalicylic acid in NaOH 2M, potassium sodium tartrate, and distilled water) to the sample. Samples were incubated with color reagent at 100°C for 10 min. After cooling, it was diluted five times with distilled water. The β-amylase activity was determined after α-amylase inactivation at pH 3.4 with 0.1 M EDTA. A reaction mixture containing citrate buffer (0.1 mM; pH 3.4), soluble starch (2%), and enzyme extract treated with EDTA was incubated for 5 min at 30 °C. The reaction was stopped by adding color reagent as described above. Enzyme activity (α- and β-amylase) was measured at 540 nm and calculated using a standard glucose curve.

Extraction and assay of antioxidant enzymes

Embryos were ground in liquid nitrogen and enzymes extracted with 0.1 M sodium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% polyvinylpolypyrrolidone (w/v). The homogenate was then centrifuged at 15,000 g for 10 min at 4 °C. The supernatant was used as crude enzyme extract.

SOD activity was evaluated by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Del Longo et al. 1993. The crude extract was added to the reaction medium containing 50 mM sodium phosphate buffer pH 7.8, 13 mM methionine, 75 µM nitroblue tetrazolium (NBT), 0.1 mM EDTA and 2 µM riboflavin. The mixture was exposed to fluorescent light for 10 min. Samples with and without illumination were used as a control. The absorbance of the solution was measured at 560nm. One unit of SOD inhibits the reduction of NBT by 50%.

APX activity was determined by ascorbate oxidation at 290 nm according to the method described by Chen and Arora 2011. The crude extract was added to the reaction medium containing 0.05 M sodium phosphate buffer pH 7.8, ascorbic acid 0.25 mM, 0.1 mM EDTA and 0.3 mM H2O2 (v/v). The molar absorption coefficient of ascorbic acid (2.8 mM cm^{-1}) was used to calculate the enzyme activity. One unit (U) was defined as the amount of enzyme needed to convert 1 nmol of the substrate to product per minute, per ml, under the assay conditions.

CAT activity was evaluated by measuring the rate of decrease of H_2O_2 at 240 nm according to the method described by Bailly and Kranner, 2011. The reaction was performed in 50 mM potassium phosphate buffer (pH 6.8), enzyme extract, and 100 mM H_2O_2. The change in absorbance at 240 nm was measured for 1 min and used to determine the rate of decomposition of H_2O_2. One unit of CAT breaks down 1 µmol of H_2O_2 per minute.

The POX activity was determined through the oxidation of pyrogallol, according to the methodology of Kar and Mishra (1976). The measurement was carried out through the reaction mixture containing crude enzyme extract, 25 mM potassium phosphate buffer, pH 6.8, 20 mM pyrogallol, and 20 mM H_2O_2. Purpurogaline production was determined by the increase in absorbance at 420 nm at 25 °C. Enzyme activity was calculated using a molar extinction coefficient of 2.47 mM^{-1} cm^{-1}.

Protein content for all enzyme samples and H_2O_2 content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

Experimental design and statistical analysis

The treatments were distributed in a completely randomized design in a 2 x 2 x 2 triple factorial scheme, corresponding to two imbibition mediums: water and DPI; two temperatures: 25 and 40 °C and two soaking times: 48 and 96 hours. For statistical and graphical analysis, Sigmaplot 12.5 and RStudio programs were used. For all parameters, an analysis of variance (ANOVA) and Tukey's test were performed. The significance level adopted was $P < 0.05$.

Results

The use of DPI has no positive effect on seed germination at high temperature

Under optimal temperature (25ºC), the seeds treated with DPI showed a significant reduction in the germination rate (F4.20=243.4, P < 0.01) and in the germination speed index (F4.20=251.4, P < 0.01) (Figure 1A). This reduction was 27% and 35.4 % (germination and IVG, respectively) compared to seeds treated with distilled water (Fig. 1A and 1B). Under 40°C, seed germination was not observed in both treatment conditions (Figures 1A and 1B).

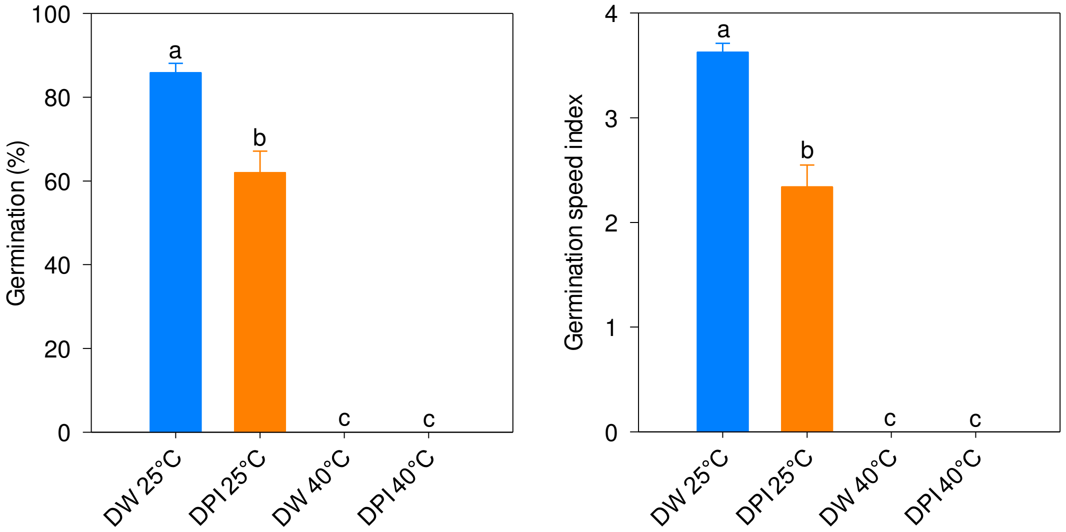

Figure 1: Effect of DPI on germination percentage (A) and germination speed index (GSI) (B) in Melanoxylon brauna seeds at temperatures 25 and 40 °C. Means followed by the same letter do not differ significantly from each other (P < 0.05). Values are expressed as the mean ± SD (standard deviation; n = 5) of 20 seeds each.

Anatomical analysis revealed that the seeds treated with DPI had thicker tegument

Through anatomical analysis, it was possible to observe the consumption of the lateral endosperm, both in the control (DW) and under DPI treatments (Figure 2). Seeds under DW showed greater wear of the tegument. There was a greater presence of cracks, rupture of the outermost layer of the tegument, composed of and macrosclereids. Observed loosening of the layer composed of osteosclereids was observed, at temperatures of 25 and 40º C in 48 and 96 hours of soaking (Figure 2A, 2B, and 2C). On the other hand, seeds exposed to DPI, presented thicker tegument, with less wear of the two layers already mentioned, which was evidenced not only by the thickness of the tegument but also by the intense blue color that marks the greater presence of lignin and cellulose

Seeds under DW and DPI after 48h at 25°C, the beginning of the elongation of the embryonic axis was observed (Figure 2A and 2D). However, the seeds under DW for 96h at 25 ºC, presented a more intense elongation of the axis (Figure 2B). The elongation of the embryonic axis was also observed in seeds treated with DPI (Figure 2E) for 96h at 25ºC, however, in a less intense way. To seeds incubated at 40 °C for 96h, both those treated with DW and DPI, consumption of components of the tegument, lateral endosperm, and cotyledon was observed, evidenced by depigmentation of the blue color, changing to purple. However, there was no elongation of the embryonic axis (Figures 2C and 2F).

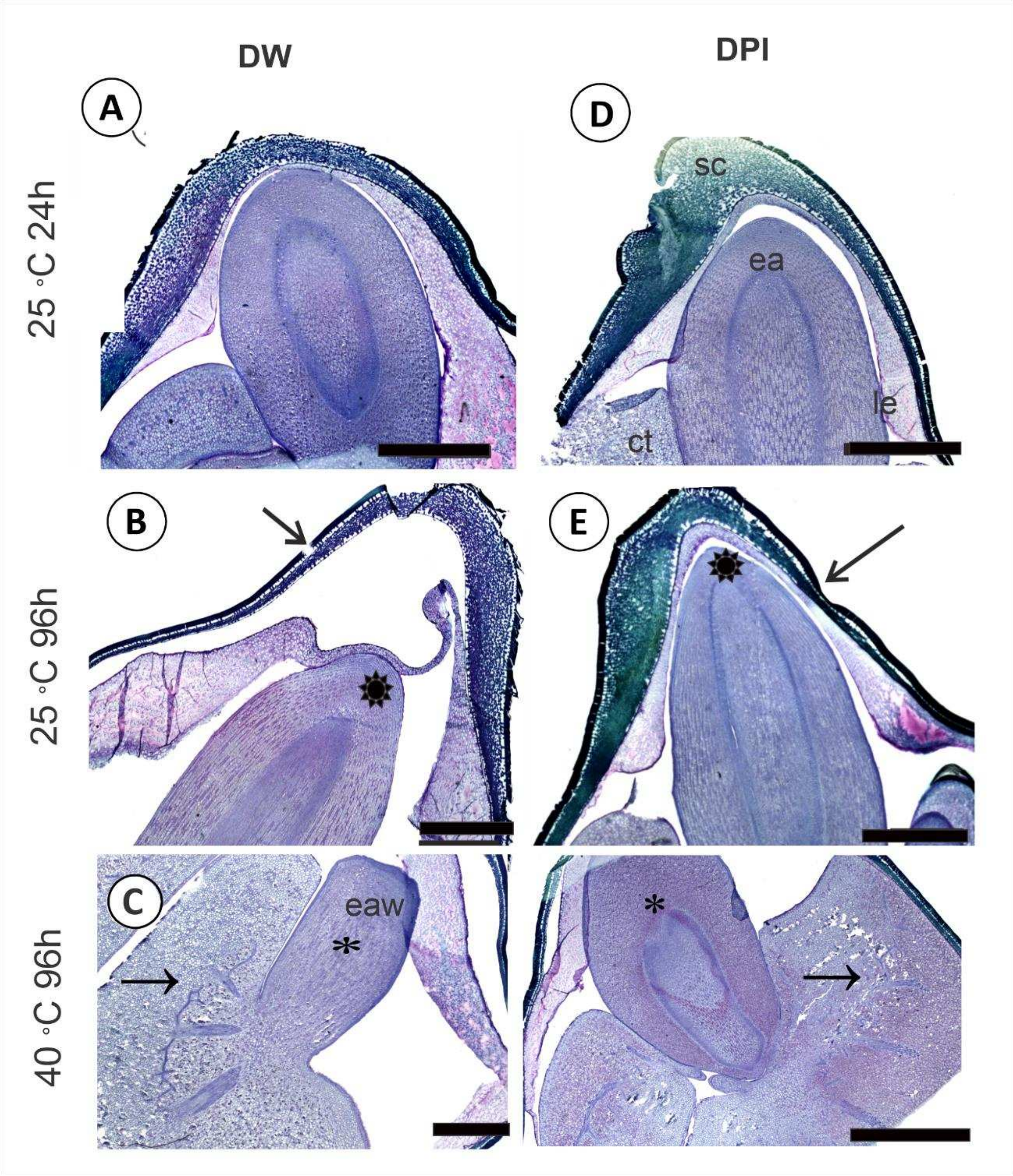

Figure 2: Longitudinal sections of Melanoxylon brauna seeds stained with toluidine blue. (A), seeds incubated at 25 °C for 48 hours in water; (B), seeds incubated at 25 °C for 48h in DPI; (C) seeds incubated at 25°C for 96h in water; (D), seeds incubated at 25 °C for 48h in DPI; (E), seeds incubated at 40 °C for 96h in water and (F), seeds incubated at 40 °C for 96h in DPI. sc, seed coat; os, osteosclereids; ma, macrosclereids; ea, embryonic axis in elongation; ct, cotyledon; le, lateral endosperm; me, micropylar endosperm; eaw, embryonic axis without elongation. The arrows indicate the consumption of the lateral endosperm and cotyledon reserves and show

the elongation of the embryonic axis, the stars indicate elongation and the ∗ the absence of elongation. Bars: A, B, C and D = 500µm and E and F = 650µm.

Seeds treated with DPI showed a reduction in H_2O_2

The production of H_2O_2 was significantly higher ($P < 0.01$) in water treatments (DW) at temperatures of 25 and 40 °C, in 96 hours of hydration (Table 1). Seed permanence on DPI significantly reduced hydrogen peroxide production at any time or temperature.

Table 1: Effect of DPI on hydrogen peroxide (H_2O_2) content in Melanoxylon brauna seeds at temperatures 25 and 40 °C. Data in nmol mg^{-1} protein.

Imbibition médium	Temp (°C)	Soaking time (hours)		Imbibition medium x Temp (°C)
		48	96	
DW	25	5.29 Ab	7.00 Aa	6.14 A
	40	5.60 Ab	6.22 Aa	5.91 A
Imbibition medium x Soaking time (DW)		5.44 A	6.61 A	
DPI	25	4.17 Bb	4.50 Ba	4.33 B
	40	3.99 Ba	2.30 Bb	3.14 B
Imbibition medium x Soaking time (DPI)		4.08 B	3.40 B	
CV (%)	10.29			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's $P > 0.05$).

α-β-amylase activities were lower in seeds treated with DPI.

α-amylase activity increased significantly when in water or DPI at 96h at any time/temperature combination (Table 2). In the DPI and water treatments, the two temperatures did not show significant differences ($P > 0.05$), except soaking in water for 48 h, with an increase in activity at 40 ºC. Overall, the soaking in water treatment enzyme activity at both temperatures was significantly higher. The exception occurred in the DW/48h combination where the temperature rise increased the activity.

Table 2: Effect of DPI on α-amylase activity in *Melanoxylon brauna* seeds at temperatures 25 and 40 °C. Data on U mg^{-1} g^{-1} protein.

Imbibition médium	Temp (°C)	Soaking time (hours)

		48	96	Imbibition medium x Temp (°C)
DW	25	0.15 Bb	0.38 Aa	0.26 B
	40	0.31 Ab	0.39 Aa	0.35 A
Imbibition medium x Soaking time (DW)		0.23 A	0.38 A	
DPI	25	0.11 Bb	0.14 Ba	0.12 B
	40	0.16 Bb	0.24 Ba	0.20 B
Imbibition medium x Soaking time (DW)		0.13 B	0.19 B	
CV (%)	6.48			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's P > 0.05).

The β-amylase did not change significantly (P > 0.05) in 48h of imbibition in the DW and DPI treatments at 25 °C (Table 3). On the other hand, in 96h there was a significant decrease in DPI treatment compared to DW. The β-amylase activity did not show a significant reduction under 40 °C in 48 h, however, in 96 h the activity was significantly higher in DW when compared to DPI.

Table 3: Effect of DPI on β-amylase activity in *Melanoxylon brauna* seeds at temperatures 25 and 40 °C. Data on U mg^{-1} g^{-1} protein.

Imbibition medium	Temp (°C)	Soaking time (hours)		Imbibition medium x Temp (°C)
		48	96	
DW	25	0.25 Bb	0.34 Aa	0.29 B
	40	0.37 Ab	0.42 Aa	0.39 A
Imbibition medium x Soaking time (DW)		0.31 A	0.38 A	
DPI	25	0.22 Ba	0.24 Ba	0.23 B
	40	0.38 Aa	0.23 Bb	0.30 A
Imbibition medium x Soaking time (DPI)		0.30 A	0.23 B	
CV (%)	8.07			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's P > 0.05).

Seeds treated with DPI showed reduced activity of antioxidant enzymes

SOD activity was significantly higher at temperatures of 25 and 40 °C in DW treatments compared to DPI. The activity verified in 96h was significantly higher in 48h, in water or DPI.

Table 4: Effect of DPI on superoxide dismutase activity in *Melanoxylon brauna* seeds at temperatures 25 and 40 °C. Data on U mg^{-1} g^{-1} protein.

Imbibition médium	Temp (°C)	Soaking time (hours)		Imbibition medium x Temp (°C)
		48	96	
DW	25	633.8 Ab	838.7 Aa	736.2 A
	40	624.0 Ab	884.9 Aa	754.51 A
Imbibition medium x Soaking time (DW)		628.9 A	861.8 A	
DPI	25	419.2 Bb	764.8 Ba	592.0 B
	40	251.5 Bb	698.4 Ba	474.94 B
Imbibition medium x Soaking time (DPI)		335.3 B	731.6 B	
CV (%)	5.89			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's P > 0.05).

APX activity was significant between treatments (Table 5). The lowest value was obtained in the DPI/40 ºC/96h treatment and the highest in DW/25 ºC/96h. The activity at DPI/25 ºC/48h differed significantly from the other treatments. The variations in 96h were clearer, with statistical similarity only between DW/40 ºC and DPI/25 ºC. Among soaking times, 48h was significantly shorter in all treatments compared to 96h, except for the DPI/40°C/96h treatment.

Table 5: Effect of DPI on ascorbate peroxidase activity in *Melanoxylon brauna* seeds at temperatures 25 and 40 °C. Data on U mg^{-1} g^{-1} protein.

Imbibition medium	Temp (°C)	Soaking time (hours)		Imbibition medium x Temp (°C)
		48	96	
DW	25	14.44 Bb	71.58 Aa	43.0 A
	40	10.71 Bb	33.72 Ba	22.2 B
Imbibition medium x Soaking time (DW)		12.51B	52.65 A	
DPI	25	17.38 Ab	31.18 Ba	24.2 B
	40	5.70 Bb	7.11 Cb	6.40 C

Imbibition medium x Soaking time (DPI)		11.54 B	19.14 B	
CV (%)	9.26			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's $P > 0.05$).

The CAT activity showed a significant reduction at the temperature of 25 ºC when the seeds were treated with DPI for 48h. The opposite occurred at a temperature of 40 ºC, where there was a significant increase in activity at the same time (Table 6). In 96h of hydration, the temperature of 25 ºC remained significantly higher, both in water and in DPI. The enzyme activity showed a significant increase at 96h of hydration in water, while at 40 ºC there were no significant variations. In the treatment with DPI, the activity remained similar to that of water between the two soaking times at 25 ºC. At 40 ºC the activity decreased significantly in 96h.

Table 6: Effect of DPI on catalase activity in *Melanoxylon brauna* seeds at temperatures 25 and 40 °C. Data in U min mg^{-1} protein.

Imbibition medium	Temp (°C)	Soaking time (hours)		Imbibition medium x Temp (°C)
		48	96	
DW	25	0.75 Ab	1.48 Aa	1.11 A
	40	0.61 Bb	0.77 Bb	0.69 B
Imbibition medium x Soaking time (DW)		0.68 B	1.12 A	
DPI	25	0.66 Bb	1.25 Aa	0.95 A
	40	0.76 Aa	0.54 Bb	0.65 B
Imbibition medium x Soaking time (DPI)		0.71A	0.89 A	
CV (%)	8.01			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's $P > 0.05$).

POX activity at 25 °C was significantly higher ($P < 0.01$) compared to soaking at 48 and 96h (Table 7). It is noteworthy the lower activity in the DPI/40 ºC/96h treatment. At 96 h the activity remained significantly higher compared to 48h, except in the DPI/40 ºC/96h treatment when the activity reached the lowest values.

Table 7: Effect of DPI on peroxidase activity in Melanoxylon brauna seeds at temperatures 25 and 40 °C. Data in U min mg^{-1} protein.

Imbibition medium	Temp (°C)	Soaking time (hours)

		48	96	Imbibition medium x Temp (°C)
DW	25	13.52 Ab	41.32 Aa	27.42 A
	40	6.75 Bb	15.79 Ba	11.27 B
Imbibition medium x Soaking time (DW)		10.13 B	28,55 A	
DPI	25	11.81 Bb	21.73 Ba	16.77 B
	40	10.21 Ba	5.57 Cb	7.89 B
Imbibition medium x Soaking time (DPI)		11.01 B	13.65 B	
CV (%)	7.22			

Means followed by the same uppercase letter in the columns and lowercase letter in the rows do not differ significantly (Tukey's *P* > 0.05).

Discussion

ROS plays a crucial role in the germination of *M. brauna*. This was demonstrated by exposing the seeds to DPI, which reduced germination and germination speed index (Figure 1A and 1B), as well as in seeds of *Nicotiana tabacum* and *Oryza sativa* (Li et al., 2017; Li et al., 2018; Guha et al., 2021). These results indicate that ROS under controlled conditions are essential to guarantee the speed and percentage of germination.

The increase in cracks, rupture of the outermost layer of the integument, and the elongation of the embryonic axis in control seeds at 25 ºC coincide with the increase in the amount of H_2O_2 during imbibition. In species such as *Lepidium sativum*, *Pisum sativum*, and *Lactuca sativa* the increase in ROS also corresponds to the loosening of the wall and the elongation (Muller et al., 2009; Kranner et al., 2010; Yang et al., 2020). The participation of ROS is even more evident due to the thicker integument and the lesser wear of macrosclereids and osteosclereids in seeds exposed to DPI (Figura 2E). This demonstrates that ROS help to loosen cell walls and contribute to the loss of cell delineation, allowing the elongation of cells. All of these events increase the potential growth of the axis, facilitating the occurrence of germination.

Under conditions of high temperature stress, both in control seeds and in those treated with DPI, there was a reduction in the components of the integument, lateral endosperm, and cotyledon. It is assumed that ROS are part of the main factors in the degradation of these components. The high temperature would participate in cell growth by increasing the activity of the enzymes polygalacturonase, pectin methylesterase, pectin lyase in M. brauna seeds (Santos et al., 2020), contributing to the degradation of micropyle wall components. The enzymatic cleavage resulting from the effects of heat stress could contribute to the reduction of seed components and not only the participation of ROS. However, high temperature has a negative role in root protrusion, the final part of the germination process.

H_2O_2 is one of the oxidative compounds that are related to the germination of *M. brauna* seeds. In some species such as Hordeum vulgare and *Oryza sativa*, H_2O_2 is suggested as essential to ensure speed and percentage of germination (Ishibashi et al., 2010; Li et al., 2017). In our study, its reduction through the use of DPI coincides with the decrease in the germination percentage under 25°C (Figure 1 and 2). H_2O_2 acts to regulate germination by signaling a series of metabolic events (Bailly et al., 2019; Silva et al., 2019; Yang et al., 2020). Thus, the decrease in H_2O_2 content through the exposure of seeds to DPI at 25°C, may have been one of the reasons for the reduction in speed and percentage of germination in this study.

Heat stress is known to increase seed ROS production (Santos et al., 2017; Liu et al., 2019; Reis et al., 2021) and this result was supported by the current study. The increase in ROS concentration can inhibit germination, and consequently cause damage to cell constituents (Bailly et al., 2019; Medina et al., 2021). Thus, the reduction in ROS production would prevent its accumulation under stressful temperatures. H_2O_2 levels were reduced in seeds exposed to DPI at 40°C (Figure 2). These results demonstrate that DPI is efficient in reducing the amount of H_2O_2, even under conditions of oxidative stress. However, this reduction does not alleviate heat stress. The reduction in H_2O_2 levels under stressful temperature could be favorable, however, the data observed in percentage and anatomy reveal that high temperature is the main factor in the loss of germination capacity.

ROS induce α-amylase activity in seeds (Sarath et al., 2007; Panngom et al., 2018). It is evident in our results that the activity of this enzyme was markedly reduced in seeds exposed to DPI at both temperatures. This reduction was also observed in seeds of *Hordeum vulgare* and *Zea mays* (Ishibashi et al., 2010; Patel et al., 2017). DPI treatment also harmed β-amylase activity. Studies have shown that H2O2 increases β-amylase activity in seeds (Wei et al., 2009; Hajihashemi et al., 2020).

Thus, it is proposed that DPI acts to reduce the activity of both enzymes, affecting the availability of cellular energy nutrients.

The development of germinative capacity is related, at least in part, to the ability to activate different detoxification systems (Hasanuzzaman et al., 2020). DPI treatment reduced SOD activity, suggesting the role of DPI in reducing $O_2^{\bullet-}$ production. Since H2O2 is considered a signaling molecule, the importance of its synthesis by SOD is clear. DPI reduced SOD activity, which is not interesting for the cell.

In this study, the activities of the enzymes SOD, APX, CAT, and POX were lower in seeds treated with DPI. The smaller amount of H_2O_2 generated influences the lower activity of these enzymes, as they use this compound as a substrate. It is supposed that the negative influence of DPI on the activities of the antioxidant enzymes studied has a lesser effect on the metabolism of ROS due to the possibility that these species are within the optimal window of action. On the other hand, the temperature had a significant effect on germination itself and less on metabolic activity.

Conclusion

In this work, we demonstrate through the use of DPI that ROS increases the percentage of germination and facilitates the weakening of the micropyle and the elongation of the embryonic axis in M. brauna seeds. We also demonstrate that ROS are essential for the activity of α- and β-amylase enzymes, as well as the antioxidant enzymes SOD, APX, CAT, and POX.

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GENERAL CONCLUSIONS

Exposure to heat stress (40°C) in *M. brauna* seeds causes ROS accumulation and increases oxidative damage, also affecting reserve enzymes. It increases α- and -β-amylase and protease activity and decreases lipase activity. High temperature causes oxidation of lipids and proteins, reduces respiratory rate, and induces alterations in the integrity of mitochondrial structures. All these events led to the loss of germination capacity.

The high temperature reduces the germination of *O. coarctata* seeds and promotes damage to the internal tissues of the seed. Heat stress (40 °C) induces the production of ROS, increases the content of carbonyl proteins, and reduces the activity of the enzymes APX, CAT, POX. Additionally, prolonged heat stress (40 °C for 144h) decreases lipase activity and increases lipid oxidation.

The exposure of *M. brauna* seeds to DPI causes a decrease in germination percentage and seed velocity index at 25 °C. On the other hand, it does not affect 40 °C. DPI causes a reduction in the loosening of the outer layers of the integument and reduces the H_2O_2 content. Furthermore, the activity of α- and β-amylases enzymes and antioxidant enzymes (SOD, APX, CAT, and POX) were reduced with exposure to DPI.

In general, the data obtained contribute to a better understanding of the role of ROS in germination and the effect of high temperature on parameters involving physiology, biochemistry, morphology, anatomy, and ultrastructure in native forest seeds. Therefore, the study shows the potential risk of climate change for the survival of forest species.