# Enzymatic activity in braúna seeds subjected to thermal stress

## Atividade enzimática em sementes de braúna submetidas ao estresse térmico

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### ABSTRACT

*To occur seed germination distinct hydrolytic enzymes work together, enabling primary root protrusion. The objective of this study was to investigate changes in the activities of α-amylase, β-amylase, and glucose-6-phosphate dehydrogenase (G6PdH) during germination of **Melanoxylon brauna** Schott. seeds under thermal stress. To this end, seeds of this species were germinated under constant temperature (10, 25, 30 or 40°C) and samples were collected every 24h during a 96-h period, in which the activity of the enzymes were evaluated. At 25 and 30°C, optimal temperatures for the germination of the species, the activities of α-amylase, β-amylase, and G6PdH increased with seed imbibitions. At 10 and 40°C, enzyme activities decreased, impairing the germination process.*

**Key words**: *α-amylase, β-amylase, glucose-6-phosphate dehydrogenase, **Melanoxylon brauna**.*

### RESUMO

*Para que ocorra a germinação das sementes, enzimas hidrolíticas distintas atuam em conjunto, permitindo a protrusão da raiz primária. O objetivo neste trabalho foi investigar as alterações nas atividades das enzimas α-amilase, β-amilase e glicose-6-fosfato-desidrogenase durante a germinação das sementes de **Melanoxylon brauna** Schott, submetidas ao estresse térmico. Para tanto, sementes da espécie foram submetidas ao teste de germinação em temperaturas constantes de 10, 25, 30 e 40°C, sendo retiradas amostras de sementes a cada 24 horas, durante o período de 96 horas, nas quais foi avaliada a atividade das enzimas. Nas temperaturas 25 e 30°C, consideradas ótimas para a germinação da espécie, as atividades das enzimas α-amilase, β-amilase e glicose-6-fosfato-desidrogenase aumentam com a embebição das sementes, enquanto, nas temperaturas 10 e 40°C, as atividades das enzimas são reduzidas, prejudicando o desenvolvimento adequado do processo germinativo.*

**Palavras-chave**: *α-amilase, β-amilase, glicose-6-fosfato desidrogenase, **Melanoxylon braúna**.*

## INTRODUCTION

Interest in the propagation of native forest species has been intensified in recent years. It is as a result of the need to recover degraded areas and restore the landscape, in addition to new environmental legislation requirements.

Among the species of ecological and economic importance is **Melanoxylon brauna** Schott. (**Fabaceae - Caesalpinioideae**), commonly known as brauna. This species grows in the Northeast and Southeast regions of the Atlantic Forest in Brazil (LORENZI, 2009), and is well known for the quality and durability of its wood (GONZAGA, 2006). It is currently on the "Official List of Endangered Flora of Brazil" established by Ministério do Meio Ambiente (Ministry of Environment) Normative Instruction No. 06 (MMA, 2008). Therefore, it is crucial to further investigate the propagation strategies of this species, with particular emphasis on the germination of its seeds, which do not display dormancy and germinate easily (FLORES et al., 2014).

Seed germination is a fundamental stage in the plant life cycle (MEI & SONG, 2010) and consists of a complex and orderly set of physiological and biochemical events that result in the rupture of the integument by the primary root, which then continues to develop until a new plant is formed (BEWLEY et al., 2013).

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Among the factors affecting germination, temperature plays a key role, as it determines the capacity and rate of seed germination (ZAMITH et al., 2013), assists in overcoming primary and/ or secondary dormancy (MONDONI et al., 2012), affects the speed of water absorption by the seeds (ATAÍDE et al., 2014), and controls the rate of the biochemical reactions that determine the entire process (MATOS et al., 2014).

For primary root protrusion to occur, various hydrolytic enzymes must participate, many of which are *de novo* transcribed and synthesized (BEWLEY et al., 2013). Among these enzymes are the amylases, which are activated in the embryo at the start of hydration (LEE & KIM, 2000) and constitute a group of enzymes responsible for breaking down starch molecules into various products, including dextrins and progressively smaller glucose polymers (SINGH & KAYASTHA, 2014). The α- and β-amylase enzymes are involved in the main breakdown of carbohydrates used during respiration, specifically starch; this activity is critical to provide the embryo with energy and a carbon skeleton (SFAXI-BOUSBIH et al., 2010).

Another key enzyme, glucose-6-phosphate dehydrogenase (G6PdH), acts on the alternative pentose monophosphate pathway and is responsible for maintaining appropriate levels of NADPH in the cells (ASAI et al., 2011). Due to its involvement in the seeds' respiratory process, it may act as an indicator of declining physiological quality in stressful environments, and in particular, be associated with damage to cell membrane structure (KEVERS et al., 2004).

Considering that little is known about the enzymatic activity of native forest species' seeds during germination, and that this process is influenced by environmental factors, the objective of this study was to investigate the changes in α-amylase, β-amylase, and G6PdH activities in *M. brauna* seeds subjected to temperature stress during germination.

## MATERIALS AND METHODS

This study was conducted at the Forest Seed Laboratory of the Department of Forestry at the Federal University of Viçosa (FUV), from October 2012 to February 2013. The *M. brauna* seeds were collected from the Leopoldina region in Minas Gerais in September 2012, and were then processed, selected, packed into 2-L impermeable fiber drums, and stored in a cold chamber at 5°C and 60% RH for a month.

The *M. brauna* seeds were germinated in Petri dishes containing two sheets of Germi

test paper moistened with distilled water, and provided continuous lighting by four 40 W daylight fluorescent lamps at 10, 25, 30 or 40°C constant temperature for 10 days (FLORES et al., 2014). Seeds were considered to have germinated when primary root protrusion was observed; the average germination percentage was then calculated. The germination rate (GR) was calculated according to the formula provided by MAGUIRE (1962). For the germination test, five replicates of 20 seeds each were used for each temperature.
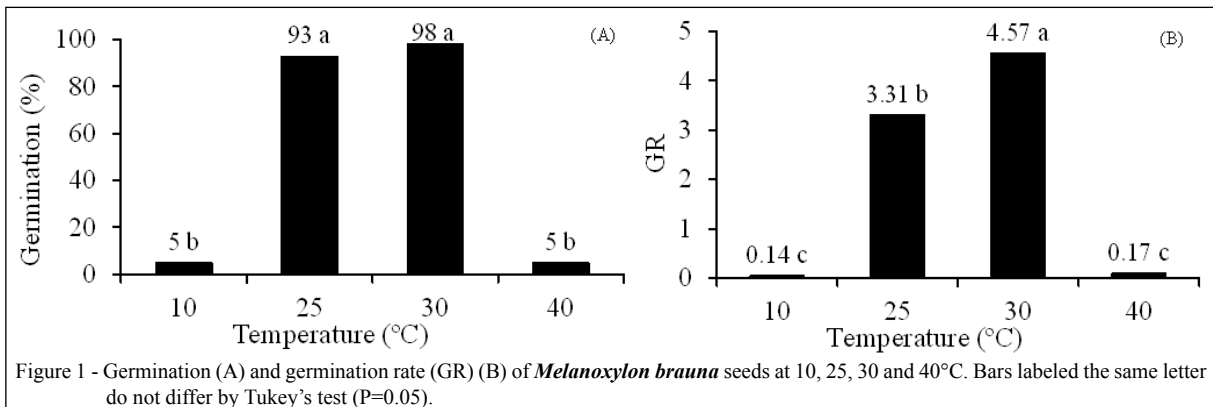
At each interval and aforementioned temperature, the α-amylase, β-amylase and G6PdH activities were evaluated. Enzymes α- and β-amylase were quantified by the method described by BERNFELD (1955), using starch as the substrate. The determination of G6PdH activity was performed according to the methodology described by DUKE et al. (1977). The protein concentration was measured according to Bradford (1976), using a standard curve constructed with bovine serum albumin (BSA). Enzymatic assays were performed in five replicates of 1.0g of cotyledons each for α- and β-amylase, and 0.1g of cotyledons and embryonic axis for G6PdH.

The experiment was conducted in a completely randomized design, subjected to an analysis of variance (ANOVA), and the germination and GR average values compared by Tukey's test at 5% probability level. The activities of the enzymes were evaluated by up to third degree polynomial regression, selected according to the significance of the equation by F-test ($p < 0.05$) and coefficient of determination ($R^2$). The statistical program used for all analyses was Statistica 8.0 (StatSoft, Inc.).

## RESULTS AND DISCUSSION

The highest germination percentages of *M. brauna* seeds (93 and 98%) were observed at 25 and 30°C, respectively. These percentages showed no significant difference from each other and were statistically higher than germination at 10 and 40°C, at which both average germination percentages were 5% (Figure 1A). However, at 30°C the germination rate was greater than at 25°C, displaying statistically different averages (Figure 1B).

The specific activity of the α-amylase enzyme tended to increase during the first 24 hours of germination at 10°C, and then remained constant until the end of the evaluation period (96h), as shown in figure 2A. At 25 and 30°C, the enzyme activity continued to increase throughout the germination process, peaking at 96h of imbibitions (Figures 2B
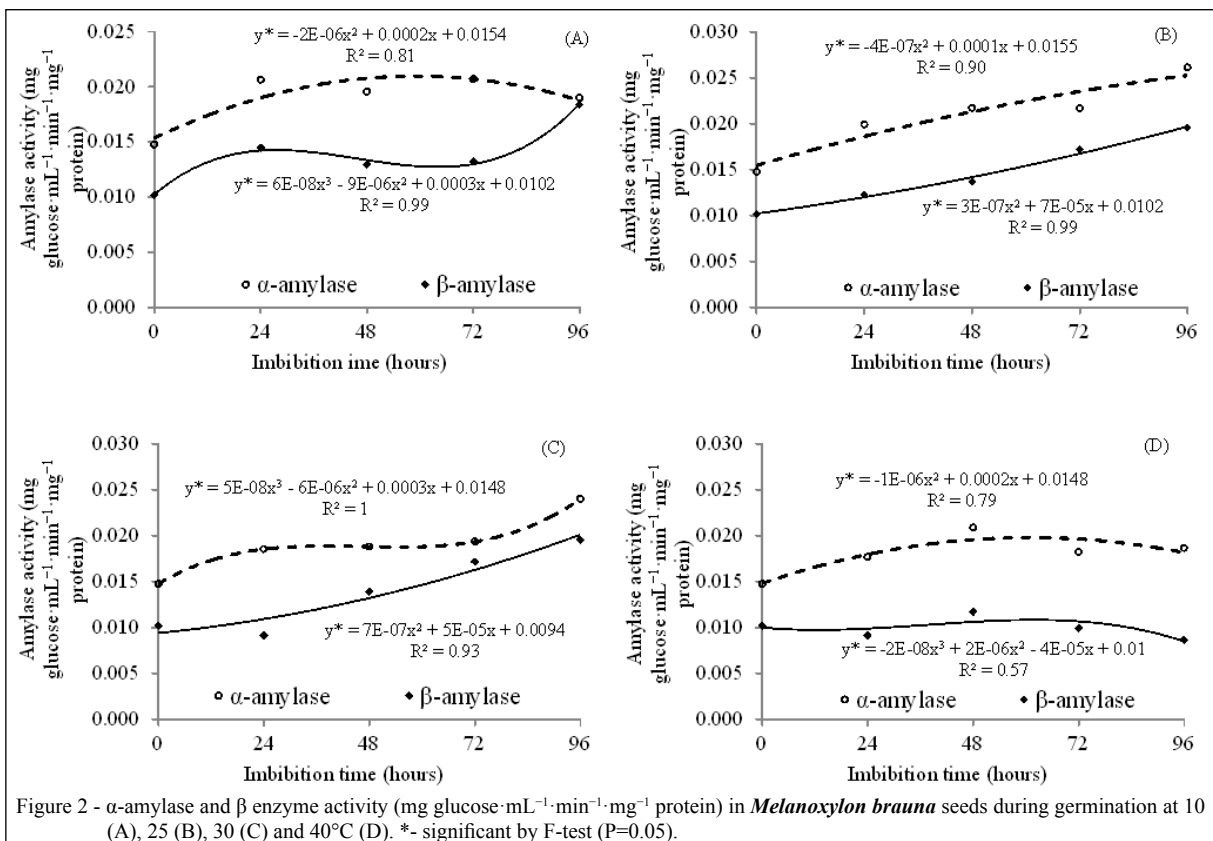
Figure 1 - Germination (A) and germination rate (GR) (B) of *Melanoxylon brauna* seeds at 10, 25, 30 and 40°C. Bars labeled the same letter do not differ by Tukey's test (P=0.05).

and 2C). These values represent a 77.1 and 62.7% increase when compared to the total initial value of the enzyme (0.01475mg glucose·mL$^{-1}$·mg$^{-1}$ protein). During seed germination at 40°C, the specific activity of α-amylase increased in the first 48 h, and had stabilized by 72h, with an average of 0.0186 mg glucose·mL$^{-1}$·mg$^{-1}$ protein at 72 and 96h of imbibitions (Figure 2D).

Conversely, the β-amylase enzyme activity measured at time zero was 0.01018mg glucose·mL$^{-1}$·mg$^{-1}$ protein, which increased

during seed germination at 10, 25 and 30°C, reaching 0.01899, 0.01956, and 0.01955mg glucose·mL$^{-1}$·mg$^{-1}$ protein after 96 h of imbibitions (Figure 2A-C). At 40°C, the enzyme activity remained stable throughout the observation period, such that at 10 and 40°C, which are above and below the optimum range for germination of *M. brauna* seeds, respectively, the enzyme activity was negatively influenced (Figure 2D).

In suitable physiological conditions, the enzymes involved in germination are synthesized



Figure 2 - α-amylase and β enzyme activity (mg glucose·mL$^{-1}$·min$^{-1}$·mg$^{-1}$ protein) in *Melanoxylon brauna* seeds during germination at 10 (A), 25 (B), 30 (C) and 40°C (D). *- significant by F-test (P=0.05).

and activated as the hydration of the seeds progresses; during this process, the amylases hydrolytically cleave starch molecules. The enzyme α-amylase begins the mobilization of reserves, hydrolyzing the internal α-1,4 bonds of linear or branched glycans of the amylopectin component of starch molecules (SMITH et al., 2005). The β-amylase acts after the α-amylase, breaking the alternate 1,4-α-D-glucosidiclinkages from the non-reducing end of starchinto maltose, which will be then transported into the cytosol and hydrolyzed by α-glucosidase into glucose (VALERIO et al., 2011).
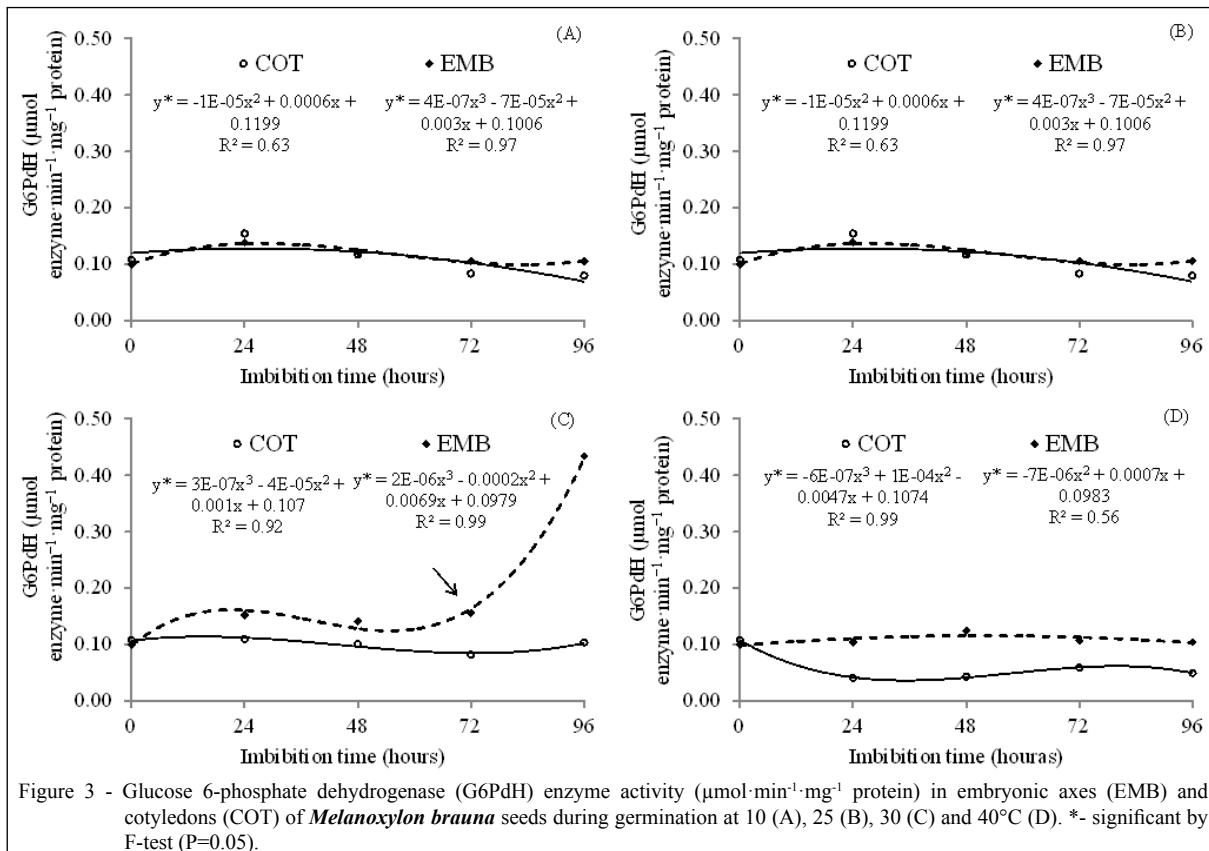
Increases in amylase activity during germination have been observed in *Aniba rosaeodora* (LIMA et al., 2008), *Hordeum vulgare* (SANTOS et al., 2010), *Sorghum vulgare* (RAIMI et al., 2012), and *Cajanus cajan* (LAURA et al., 2013). In *Triticum aestivum* seeds, the production of α-amylase was reduced during germination at temperatures lower than the optimum species (FLEMING et al., 1960); such behavior is related to plant metabolic response to low temperatures.

It was reported, during the germination of *Sorghum bicolor* seeds, (TAWABA et al., 2013) that from 24°C, a 4-8°C rise in the temperature

was sufficient to reduce the synthesis of β-amylase. Diminishing enzyme synthesis is consistent with lower maltose and maltotriose release at high temperatures. Furthermore, KAPLAN & GUY (2004) reported lower amylase activity to be associated with increased germination temperature, when it exceeded the optimum temperature range for seed germination.

In this study, the average activity of G6PdH at time zero in *M. brauna* seeds was 0.10000 and 0.10770 μmol·min$^{-1}$·mg$^{-1}$ in the embryonic axis and cotyledons respectively (Figure 3). At 25 and 30°C, the enzyme activity tended to stabilize in the cotyledons and to increase in the embryonic axis. The increase was most accentuated after 84 h of imbibitions at 30°C, a period in which root protrusion was also observed at this temperature, displaying activity equivalent to 333.36% of initial levels.

At 25°C, G6PdH activity during the 96h imbibitions period increased 79.99% compared to the initial activity, reaching values of 0.18622 μmol·min$^{-1}$·mg$^{-1}$. This contrasts with the enzyme activity at 10°C, which was steady in the embryonic axis and increased in the first 24h of germination, followed by slow and continuous decrease in the cotyledons, where it reached 0.07979μmol·min$^{-}$



Figure 3 - Glucose 6-phosphate dehydrogenase (G6PdH) enzyme activity (μmol·min$^{-1}$·mg$^{-1}$ protein) in embryonic axes (EMB) and cotyledons (COT) of *Melanoxylon brauna* seeds during germination at 10 (A), 25 (B), 30 (C) and 40°C (D). *- significant by F-test (P=0.05).

$^1 \cdot mg^{-1}$ at the end of imbibitions (Figure 3A). At 40°C, G6PdH activity was also stable in the embryonic axis, whereas it decreased in the cotyledons from the beginning of the evaluation period, being observed at $0.04412 \mu mol \cdot min^{-1} \cdot mg^{-1}$ activity after 12h. Then it remained practically the same during the imbibitions of the seeds, and it was $0.04495 \mu mol \cdot min^{-1} \cdot mg^{-1}$ at 96h of imbibitions (Figure 3D).

The G6PdH enzyme is involved in the cellular respiration process, specifically in the alternative pentose monophosphate pathway, and is responsible for maintaining proper levels of NADPH in the cells (SHAN-ZHI et al., 2005). In response to some kind of stress, for instance, low or high temperature (10 and 40°C), the reduction in seed germination may be associated with lower enzyme activity (Figure 1B), indicating a possible decline in internal metabolic functions. A similar decline in physiological quality of *Oryza sativa* seeds was associated with low respiratory rate a and reduction in G6PdH activity; this reduction corresponded to the exposure of the seeds to temperatures above 25°C (MARINI et al., 2013).

The association of G6PdH activity with the loss of quality was also found in seeds of *Glycine max* (MUNIZ et al., 2007). Because it is the first NADPH-generating enzyme in the pentose phosphate pathway (WAKAO et al., 2008), G6PdH-deficient cells are highly sensitive to oxidative stress, in contrast to those expressing appropriate levels of enzyme activity (LIU et al., 2007). The fact that G6PdH is a regulatory enzyme in this oxidative pathway (TAIZ & ZEIGER, 2013) suggests that the stress caused by the temperatures during seed germination of *M. brauna* likely resulted in less available energy for biosynthetic processes during germination, owing to lower enzyme activity.

In contrast, the increase in G6PdH activity during the post-germination period at 30°C expressed the efficiency of the respiratory activity during this period, when the new seedlings presented higher respiratory rates than seeds submitted to imbibitions. According to BEWLEY et al. (2013), respiration increases linearly as more substrates are available for seeds, justifying the large oxygen consumption rates and mobilization of reserves in the post-germination period.

## CONCLUSION

At 25 and 30°C, the α-amylase, β-amylase, and G6PdHactivities increase with the imbibitions time of *Melanoxylon brauna* seeds. The α-amylase, β-amylase, and G6PdH activities at 10 and 40°C are lower compared to 25 and 30°C.

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