






Molecular aspects during seed germination of *Erythrina velutina* Willd. under different temperatures (Part 2): isoenzyme activity and DNA integrity

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ABSTRACT: *Erythrina velutina* Willd. (Fabaceae) is a Brazilian native tree with economic, ecological and social potential. The aim of this study was to evaluate isoenzyme activity and changes in DNA integrity during germination of *E. velutina* at different temperatures. The seeds were placed to germinate at 5, 15, 25, 35 and 45 °C, evaluating isoenzyme activity and degradation of the DNA during germination. Isoenzyme expression occurs differently for seed germination under different temperatures, with varied expression between seedlings and cotyledons. The esterase enzyme was more sensitive to express the response of the *E. velutina* germination seeds at different temperatures. DNA repair is more efficient during the germination of *E. velutina* seeds when submitted to a temperature of up to 25 °C, with damage to the genetic apparatus with an increase higher than this temperature.

Index terms: DNA repair, seed biochemical, germination ecophysiology, forest seeds.

Aspectos moleculares durante a germinação de sementes de *Erythrina velutina* Willd. sob diferentes temperaturas (Parte 2): atividade isoenzimática e integridade do DNA

RESUMO: *Erythrina velutina* Willd. (Fabaceae) é uma espécie nativa brasileira com potencial econômico, ecológico e social. O objetivo deste estudo foi avaliar a atividade de isoenzimas e as alterações na integridade do DNA durante a germinação de *E. velutina* em diferentes temperaturas. As sementes foram colocadas para germinar a 5, 15, 25, 35 e 45 °C, avaliando-se atividade isoenzimática e a degradação do DNA durante a germinação. A expressão isoenzimática ocorre de maneira diferente para a germinação das sementes sob diferentes temperaturas, com expressão variada entre plântulas e cotilédones. A enzima esterase foi mais sensível para expressar a resposta de sementes de *E. velutina* sob diferentes temperaturas de germinação. O reparo do DNA é mais eficiente durante a germinação das sementes de *E. velutina* quando submetidas à temperatura de até 25 °C, com prejuízo ao aparato genético em temperaturas superiores.

Termos para indexação: reparo do DNA, bioquímica de sementes, ecofisiologia da germinação, sementes florestais.

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INTRODUCTION

Erythrina velutina Willd. (Fabaceae) is a Brazilian native tree (Rodrigues et al., 2018). Its bark is used as a tranquilizer extract (Candido et al., 2015) and has an anxiolytic hydroalcoholic effect (Sousa et al., 2018). The leaf extracts contain phenols, tannins, alkaloids, flavonoids, sterols, triterpenoids, catechins, saponins and xanthenes (Palumbo et al., 2016, Rodrigues et al., 2017).

Despite the social, ecological and economic importance of *E. velutina* to the Northeast region of Brazil (Caatinga and Cerrado phytogeographic domains), there is still little information on this species, especially related to the physiological and biochemical aspects during germination. In this process, unfavorable environmental conditions have been affecting the initial establishment and development in the field.

The temperature directly affects the biochemical reactions of regulating the seed metabolism, influencing the germination and its speed (Gonçalves et al., 2015). These events are mainly due to the regulation of cellular respiration intensity in mitochondria and the enzymatic activity (Marcos-Filho, 2015). In this sense, the optimal germination temperature is that for which the seed expresses its maximum germination performance, while the minimum and the maximum are characterized by lower germination, at which germination does not occur at below or above it (Bewley et al., 2013).

The effect of temperature on seedling development can be associated with the hydrolysis of reservations and enzymatic expression. The high temperatures reduce seed germination due to deteriorative processes, protein denaturation and inactivation of enzymes (Catão et al., 2016; Dubal et al., 2016). Furthermore, different seedling organs demonstrate different enzyme activities responses to temperature during germination (Henson et al., 1980).

In this context, the determination of the isoenzyme activity has been used to complement physiological seed analyzes, considering more researched isoenzymes that act in the respiratory process, specific enzymes of the lipid metabolism, those involved in the nitrogen-carbon metabolism, in addition to enzymes related to destructuring of the membrane system (Carvalho et al., 2000). Thus, it is recommended to evaluate different isoenzyme systems to better understand the effect of temperature on seed performance.

The integrity of the genetic material also plays an important role in the first hours of germination (Sano et al., 2016). This characteristic is involved in the control of several essential cellular processes, and its degeneration causes damage and death of the seeds (Waterworth et al., 2019). Therefore, studies that take this approach into account are necessary.

The scarcity of research on the molecular aspects in seeds of forest species is well-known, especially those linked to the influence of environmental factors such as temperature. In this sense, the aim of this study was to evaluate isoenzyme activity and changes in DNA integrity during the germination of *E. velutina* seeds at different temperatures.

MATERIAL AND METHODS

The study was developed with lots of *E. velutina* seeds coming from the municipality of Ilhéus, Bahia State, Brazil. Seeds were acquired commercially (> 70% viability) in 2017 and kept in a 4 °C refrigerator until the execution of this experiment. Germination tests of *E. velutina* were performed with four replications of 50 seeds, using paper towel substrate (Germitest®), moistened with distilled water in the amount equivalent to three times the dry mass of the paper (Brasil, 2009). The seeds were previously scarified in the region opposite to the micropyle by means of iron sandpaper and sown. Then the paper rolls with the seeds were kept in a Biochemical Oxygen Demand (BOD) type germinator at 5, 15, 25, 35 and 45 °C with a photoperiod of 12 h.

Isoenzyme activity: The seeds were placed to germinate in the same procedure as the germination test at temperatures of 15, 25 and 35 °C. Ten biological replications were collected for each temperature after 20 days to extract isoenzymes from the seedling and cotyledon, in addition to the dry seed. The extraction and electrophoresis methodology was adapted from Scandalios (1969), wherein a sample 200 mg of plant material was weighed, macerated in an ice bath with extraction buffer (0.2 M lithium borate at pH 8.3, Tris citrate and 0.2 M at pH 8.3, plus 0.15% of 2-mercaptoethanol) in the ratio 1:2 (m.v⁻¹), and placed overnight at 10 °C. After, the samples were centrifuged at 12,000 rpm for 30 min; 20 µL

of the supernatant was collected and applied on 8% polyacrylamide gel. Electrophoresis was run in a vertical vial using 0.2 M lithium borate buffer at 80 V for 2 h. Gel development systems were according to Scandalios (1969) for esterase (EST) and glutamate oxalacetate transaminase (GOT) enzymes, and according to Alfenas (1998) for alcohol dehydrogenase (ADH), glutamate dehydrogenase (GTDH) and sorbitol dehydrogenase (SBTDH). The isoenzymatic activity was visually evaluated in according the presence or absence and intensity of bands' expression, by a white light transilluminator. For the visual analysis of the gels there were no statistical analyses.

Integrity of the DNA during the germinative process: Two times (24 and 72 h) were defined to make the samples according to the water absorption pattern by the seeds at temperatures of 5, 15, 25, 35 and 45 °C, in addition to the dry seed. The three-phase water absorption pattern was obtained by weighing 100 seeds in an analytical scale (0.001 g). The seeds were placed under the same conditions as those used in the germination test described above during the period of 78 h.

Ten-seed embryonic axes for each temperature were extracted at 24 and 72 h after sowing. These were stored in tubes containing 2% CTAB (cetyltrimethylammonium bromide) and stored in a freezer at -20 °C. The DNA was extracted according to Doyle and Doyle (1987) protocol, quantified in spectrophotometer (Epoch™) (260/280 purity ratio of 1.26 ± 0.11 between samples) and the results expressed in DNA concentration ($\text{ng} \cdot \mu\text{L}^{-1}$ dry weight⁻¹). Then, 5 μL of the DNA from each treatment was stained with 4 μL of bromophenol and Gelred™ blue, which was then inserted into agarose gel ($1.0 \text{ m} \cdot \text{v}^{-1}$) and run into a horizontal electrophoresis cell, in addition to a standard molecular weight sample of 1Kb (Ladder Kasvi®). The agarose gel was then immersed in 1X TAE buffer (Tris-acetate-EDTA) and fed by a 100 V electric current for 1 h. Finally, the photograph was captured under ultraviolet light in photo-documenting equipment (E-Box VX2).

Statistical analysis: The experiment was conducted in a completely randomized design with analysis of variances (ANOVA) performed using the BioEstat® 5.0 statistical program, as well as mean comparisons by the Tukey test at the level of 5% of probability.

RESULTS AND DISCUSSION

The enzyme alcohol dehydrogenase (ADH) only showed activity in the cotyledons of *E. velutina* when the seeds germinated at 15 and 25 °C (Table 1). This activity was of low intensity and can also be verified in the dry seed, where the expression of 3 alleles was observed. The seedling did not express this isoenzyme at any of the evaluated temperatures, similarly to the work of Tunes et al. (2011), in which the ADH activity was quite pronounced in the seeds, whereas it was practically null in the seedlings.

The ADH enzyme is involved in anaerobic metabolism, catalyzing the reduction of aldehyde to ethanol, a compound less toxic to the plant (Kato Noguchi and Yasuda, 2007). This enzyme is stored in the seed during the maturation process, and remains stable until germination begins, and its metabolism is reactivated in phases I and II of imbibition. Their levels decrease drastically after imbibition (Weitbrecht et al., 2011), indicating the predominance of the aerobic process of energy generation. This is consistent with the low intensity of ADH expression observed in the electrophoretic profile from *E. velutina* cotyledons after 20 days of seed imbibition. It is also consistent with the lack of activity of this enzyme when germinated seeds were evaluated at 35 °C, with their development anticipated by the temperature increase as morphologically observed in Figure 1 in the part 1 (stage II), accelerating ADH consumption and establishing the totally aerobic respiratory process, where this enzyme is no longer necessary. It can also be considered that the maintenance of the ADH activity at 25 and 30 °C was favorable to the germinative processes, because it reduces the exposure of the seeds to the deleterious action of acetaldehyde.

Studies demonstrate that de novo synthesis of ADH can be induced under cold stress conditions, and then this enzyme is quantitated expressively in seedling tissues (Kato Noguchi and Yasuda, 2007; Mertz et al., 2009; Weitbrecht et al., 2011). However, this was not verified in the present research when *E. velutina* seeds germinated at 15 °C, probably because this native species did not present evolutionary adaptation of tolerance to cold stress since its occurrence is in regions with higher temperatures.

Table 1. Isoenzymes alcohol dehydrogenase (ADH), esterase (EST), glutamate oxalacetate transaminase (GOT), glutamate dehydrogenase (GTDH) and sorbitol dehydrogenase (SBTDH) expressed in *Erythrina velutina* seeds germinated at 15, 25 and 35 °C in the cotyledons and seedlings, in addition to the dried seed.

Isoenzymes	seedling			cotyledons			dry seed
	15 °C	25 °C	35 °C	15 °C	25 °C	35 °C	
ADH	Allele 1						
	Allele 2						
	Allele 3						
EST	Allele 1						
	Allele 2						
	Allele 3						
	Allele 4						
GOT							
GTDH	Allele 1						
	Allele 2						
	Allele 3						
	Allele 4						
SBTDH							
	Allele 1						
	extremely weak						
	weak						
	moderately intense						
	intense						

In analyzing the isoenzyme patterns of the esterase enzyme (EST), it was possible to observe the presence of 4 low-intensity alleles in tissues, seedlings and cotyledons: allele 1 in cotyledons that developed at 25 °C; allele 2 in seedlings at 25 °C; allele 3 in seedlings and cotyledons at 25 °C; and allele 4 in seedlings at 15 and 25 °C, and cotyledons at 15 °C. Its expression was not observed at 35 °C, nor in dry seed (Table 1).

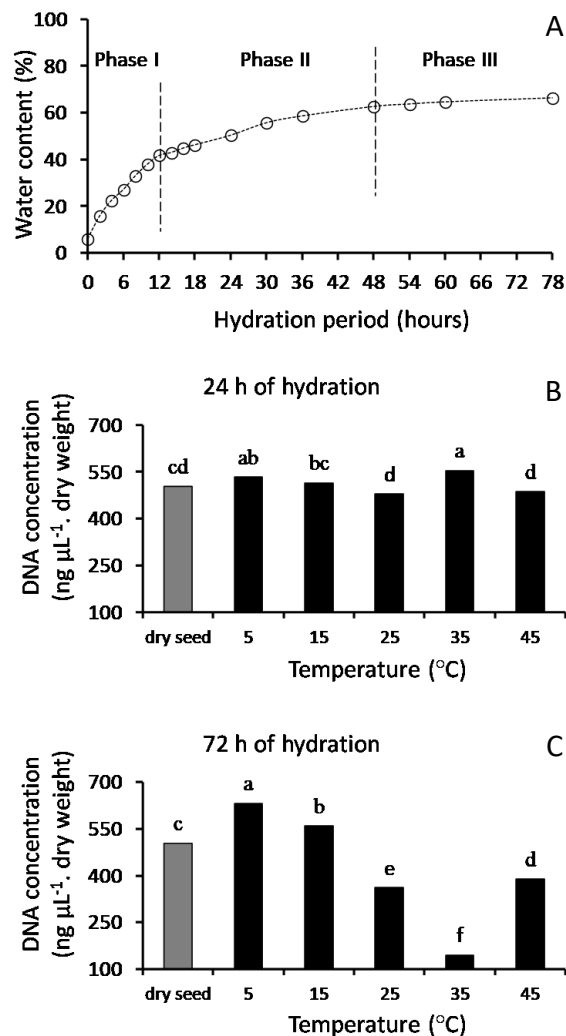
Esterase enzymes have been isolated and purified from various sources, including plants, animals and microorganisms. Esterase enzymes present many isoforms, representing a complex group of hydrolytic enzymes that have the overlapping property of specific substrates, hydrolyzing both endogenous and exogenous esters (Bhavitk et al., 2014). Regarding the isoenzymes evaluated in this study, the esterase enzymes were those that presented the highest number of alleles, and consequently express more isoforms of this enzyme. Furthermore, the esterase enzyme was more sensitive test, resulting in different electrophoretic profile for each treatment evaluated. According to Gillespie and Langley (1974), this is one of the most polymorphic isoenzymatic systems observed in plants.

As the enzymes involved in the ester hydrolysis reactions, they are directly linked to lipid metabolism during the germination process, as well as total cell membrane phospholipids (Santos et al., 2005). No esterase expression was observed in the dried seeds of *E. velutina* in this study, probably due to a minimal amount of this enzyme and the methodology used was not sensitive enough to detect it in medium to dry seed substrate. The low concentration of esterase enzymes reflected in the low intensity of the bands in the polyacrylamide gel and in the absence thereof, is probably decreasing the mobilization of lipids during seed germination, resulting in the accumulation of these compounds, as observed in Figure 2E in the part 1.

The decrease in the number and intensity of bands in the development of esterase enzyme in seeds stored for more than 6 months at 10 and 25 °C was reported by Vieira et al. (2013). Therefore, the storage period of the seeds prior to

carrying out the present study with *E. velutina* may also have influenced the absence of esterase expression in the dry seed, since these enzymes act on the metabolism of phospholipids present in the membranes, their consumption is associated with the first events responsible for the aging of seeds.

As verified in Table 1, there was no esterase expression when the seeds germinated at 35 °C. Parasa et al., (2018) verified that esterase enzymes are stable in several species at temperatures above this, for example: in tomato, zucchini and tamarind at 40 °C, and in *Sesbania grandiflora* (L.) Poiret and *Jatropha curcas* (L.) at 50 °C. These authors state that plant enzymes generally have optimum temperature in the range of 30 to 40 °C. Thus, it is assumed that the absence of ADH and EST activity, as well as glutamate oxalate transaminase (GOT) and sorbitol dehydrogenase (SBTDH) is not due to an instability caused by the high temperature, but to an acceleration of the metabolism which led to the consumption of these enzymes in the early stages of the germination process of *E. velutina*, and their presence was not observed at the time the samples were analyzed (20 days after imbibition). An indicative of this metabolic acceleration is the high germination speed at 35 °C associated with the shorter length of seedlings developed at this temperature.



Averages followed by the same letter do not differ by the Tukey test at the 5% probability level.

Figure 1. (A) Three-phase water absorption and DNA concentration in embryonic axes of *Erythrina velutina* seeds submitted to germination at different exposure temperatures (5, 15, 25, 35 and 45 °C and seed without hydration) for (B) 24 h and (C) 72 h.

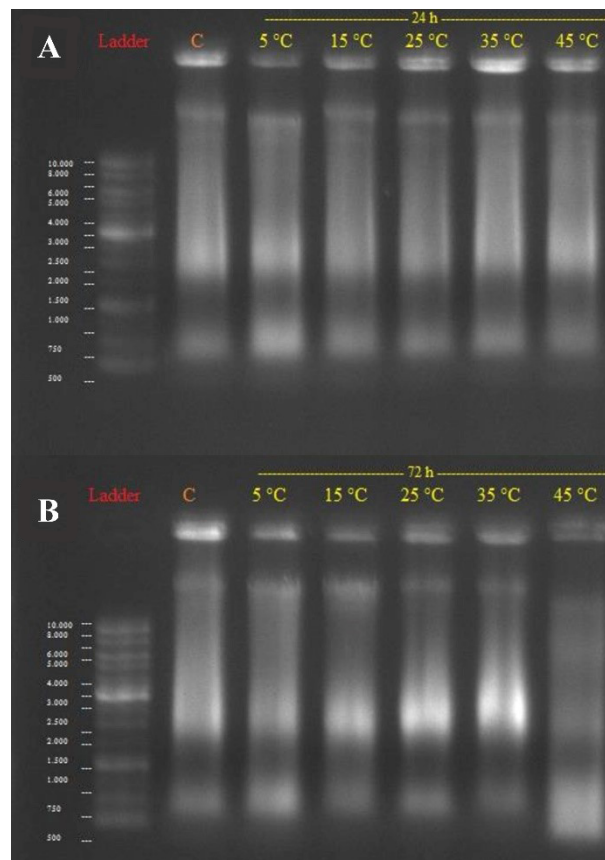


Figure 2. Electrophoresis of embryonic axes of *Erythrina velutina* seeds submitted to germination at different exposure temperatures (5, 15, 25, 35 and 45 °C) for (A) 24 h and (B) 72 h. C: seed without hydration.

In the study by Malone et al. (2007), the decrease and absence of bands were related to the faster catabolism in rice varieties, suggesting that most of the reserve compounds had been metabolized at seven days of germination. The enzyme glutamate oxalacetate transaminase (GOT) showed intense activity in seedlings and cotyledons, both in seeds germinated at 15 °C and at 25 °C. This activity was represented by a single allele, expressed in both seedlings and cotyledons. It was also observed that its expression was extremely pronounced in the dried seeds.

GOT is directly involved in the metabolism of nitrogen, acting in a specific transfer reaction of the aminogroup of an amino acid to the α -ketoglutaric acid to form the glutamic acid and to produce the keto acid, in a reversible reaction (Conn and Stumpf, 1990). Thus, this enzyme is expressed in both the oxidation of amino acids involved in generating energy via the Krebs cycle and in the reduction of α -ketoglutarate, destined to the biosynthesis of new amino acids (Malone et al., 2007). In the present study, intense GOT expression can be observed at temperatures of 15 and 25 °C, indicating that the metabolism processes of reserve proteins and synthesis of amino acids destined to resume embryo growth are active, whereas at 35 °C these processes involving GOT had ceased at 20 days of germination.

The isoenzymatic profile of glutamate dehydrogenase (GTDH) represents activity of this enzyme in the three evaluated germination temperatures for *E. velutina* in the seedling, as well as the cotyledons and dry seeds. The number of alleles and the band intensity presented a variation, observing 1 allele in the seedlings, whereas 4 alleles are identified in the cotyledons and dry seeds (Table 1).

Glutamate dehydrogenase is an enzyme that links both of those metabolic pools within a cell, as it can carry out the reaction of reductive amination of 2-oxoglutarate to glutamate (the anabolic activity; NADH–GDH), and the reverse reaction of oxidative deamination of glutamic acid (the catabolic activity; NAD⁺–GDH) (Grabowska et al., 2011).

It is noteworthy that among the enzymes under study, GTDH was the only one that was expressed at 35 °C at

20 days of germination, despite the low viability of seeds at this germination temperature (Table 1 in the part 1). High temperatures and ageing promote nitrogen remobilization, a typical feature of the senescence process, and glutamate dehydrogenase may be of particular importance in the catabolism of amino acids to release ammonia for their incorporation into amides during nitrogen remobilization (Haba et al., 2014).

The sorbitol dehydrogenase enzyme was only expressed in seed cotyledons germinated at 15 and 25 °C and in the dry seed, with 1 low intensity allele. Considering the electrophoretic profiles analyzed, it is possible to verify that the expression of the same enzyme is differentiated between the germinated seed and the dry one, since different cell differentiation and gene expression processes are occurring. Intense metabolic activity is observed during the germination process corresponding to ATP production, the degradation of proteins and reserve polysaccharides, the synthesis of new mRNAs, and the restructuring and repair of damaged membranes and organelles which considerably modifies the isoenzymatic expression, while the dry seed maintains low metabolic activity, referring to the necessary biosynthetic reactions for cellular respiration (Malone et al., 2007).

Regarding the DNA integrity during the germination process, the *E. velutina* seeds present a three-phase water absorption pattern. Phase I lasted 12 hours, followed by Phase II with 36 hours, at which point root protrusion (48 hours after sowing) was observed, indicating the beginning of Phase III (Figure 1A). The evaluation of the three-phase water absorption pattern predicts which biochemical and physiological processes are occurring during seed rehydration, initially governed by physical force (Phase I), followed by metabolic activity intensification (Phase II) and embryo growth resumption (Phase III) (Bewley et al., 2013).

E. velutina embryos showed little change in DNA concentration (Figure 1B) and integrity (electrophoresis of embryonic axes of seeds) of the genetic material in the first 24 hours of hydration (Phase II) (Figure 1A), regardless of incubation temperature, although decreases in DNA concentration were checked at 25 and 45 °C (Figure 1B and 1C). High temperatures after 72 hours of hydration (Phase III) caused damage to the genetic material until the total collapse of the seed at 45 °C, while temperatures of 5 and 15 °C maintained the superior DNA integrity compared to the dried seeds (Figure 1C and Figure 2B). Temperatures near 50 °C is in the ideal range of activity for numerous enzymes and chemical reactions related to DNA degradation processes (Kranter et al., 2011). Therefore, the seeds kept at 45 °C in this study completely lost viability.

Therefore, there is a limitation in DNA repair activity at elevated temperatures (35 and 45 °C). These conditions result in the loss of capacity to produce normal seedlings and to allocate energy reserves. Although DNA damage occurs at 25 °C, embryos are able to produce normal seedlings and efficiently allocate their reserves, which means they repair their genetic material for numerous cellular processes. In this sense, research conducted to evaluate DNA integrity in forest seeds was carried out in *Campomanesia adamantium* (Cambess.) O. Berg (Dresch et al., 2015) and during the desiccation process, where there were alterations in the integrity of the genetic material. However, these studies are still incipient, not expressive and need to be applied using other species.

Given this outline, it is possible to affirm that the molecular analyzes presented herein contribute to understand the complexity of events that occur during seed germination and seedling establishment, as well as to help in interpreting the physiological and biochemical mechanisms associated with the germination of *E. velutina*.

CONCLUSIONS

Isoenzyme expression occurs differently for the seed germination under different temperatures, with varied expression between seedlings and cotyledons. The esterase was more sensitive isoenzyme analyzed; the results expressed more isoforms and different electrophoretic profile for each temperature and part of the plant evaluated.

DNA repair is more efficient during the germination of *E. velutina* seeds when submitted to a temperature of up to 25 °C, with damage to the genetic apparatus with an increase higher than this temperature.

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