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Dormancy breaking and biochemical processes associated with germination of *Erythrina falcata* Benth. seeds

Mariana Aline Silva Artur[1]✤, José Marcio Rocha Faria[2], Anderson Cleiton José[2], Jean Marcel de Sousa Lira[3], Kamila Rezende Dázio de Souza[4], Fernanda Carlota Nery[5], Amauri Alves de Alvarenga[2]

[1]Wageningen University, Netherlands
[2]Federal University of Lavras, Brasil
[3]Federal University of Viçosa, Brasil
[4]Federal University of Alfenas, Brasil
[5]Federal University of São João Del Rei, Brasil

SILVICULTURE

ABSTRACT

Background: The aim of this research was to investigate the efficiency of methods to overcome primary dormancy and biochemical processes associated with germination of *Erythrina falcata* Benth seeds. Seeds were submitted to dormancy overcoming treatments and the water uptake pattern was analysed. We then evaluated the activity of the antioxidant enzymes SOD, CAT, and APX, and quantified lipid peroxidation levels, hydrogen peroxide content and total protein content. The experiments were performed in a completely randomized design and the statistical analysis used was Scott-Knott test at 5% probability.

Results: Mechanical scarification with sandpaper leads to approximately 94% of germination in *E. falcata*, while control non-scarified seeds show only approximately 37% of germination. Treatments with hot water led to high mortality in *E. falcata* seeds. Rapid water absorption was observed in the first 18 hours of imbition when the seeds were scarified with sandpaper, and radicle emergence was observed after 36 hours. During imbibition there was an increase of antioxidant enzyme activity and a decrease in lipid peroxidation and H_2O_2 production, suggesting an efficient mechanism for regulating imbibition damage. A decrease in the total protein content was observed during germination of *E. falcata* seeds.

Conclusions: Mechanical scarification is an efficient method for breaking dormancy of *E. falcata* seeds. During germination of *E. falcata* seeds there is a reduction in the production of H_2O_2 and lipid peroxidation, and an increase in the activity of antioxidant enzymes. The total protein content decreased along the germination time.

Key words: Primary dormancy; seed metabolism; antioxidants; enzyme activity.

HIGHLIGHTS

Mechanical scarification is an efficient method to overcome dormancy in *E. falcata* seeds.
Scarified *E. falcata* seeds show rapid and uniform germination.
The activity of antioxidant enzymes increases during the germination of *E. falcata* seeds.
There is a decrease in total protein content during germination of *E. falcata* seeds.

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✤Corresponding author: mariana.silvaartur@wur.nl Received: September, 28/2022 Accepted: June, 1/2023

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INTRODUCTION

Germination begins with the absorption of water by the seed and ends when part of the embryo, usually the radicle, penetrates the structures that surround it. In this way, water is necessary for the reactivation of metabolic activity and stimulation of the growth of the embryonic axis (Steinbrecher; Leubner-Metzger, 2017). The rapid influx of water into the dry seed cells during early imbibition results in temporary structural disturbances, particularly during the transition from rigid gel to liquid crystalline phase membranes and cytoplasm, leading to an immediate release of solutes and metabolites to the surrounding solution (Bewley et al., 2013). During seed imbibition there is production of reactive oxygen species (ROS) that can attack biological membranes and lead to reduced germination (Wojtyla et al., 2006; Nonogaki et al., 2010).

The levels of ROS in germination are kept in dynamic equilibrium due to removal systems, among which the enzymes of the antioxidant system Superoxide Ion Dismutase (SOD), Catalase (CAT) and Ascorbate Peroxidase (APX) have been proposed as agents of fundamental importance for the conclusion of the germination process (Bailly, 2004). The inefficiency of the ROS removal system in the germination process causes damage to the membranes due to, for example, increase in the concentration of hydrogen peroxide (H_2O_2) and through lipid peroxidation. Lipid peroxidation exacerbates oxidative stress through the production of lipid-derived radicals capable of reacting with each other and of damaging proteins and DNA (Gill; Tuteja, 2010; Sharma et al., 2012). The breaking of lipid chemical bonds in the plasma membrane causes damage to the cell structure, eliminating membrane selectivity and, consequently, causing cell death.

In several Brazilian forest species, the physiological and biochemical mechanisms of germination have not yet been studied partly due to seed dormancy. This lack of information makes it difficult to understand the factors that promote the success in the germination process. Developing an efficient method to overcome dormancy can be useful for a more homogeneous seed germination and seedling growth.

E. falcata (*a.k.a.* as corticeira-da-serra, mulungu, beak-parrot, swine and corticeira) is a native and not endemic forest species of Brazil (Lima, 2013) that has several classes of active ingredients (Almeida, 2010) and medicinal properties (Rambo et al., 2019). It is a plant adapted to open areas in very wet and swampy soils, and it is recommended for planting in degraded riparian areas (Duarte; Krentkowski, 2015). Annually, *E. falcata* trees produce a moderate number of seeds and present primary physical (tegumentary) dormancy (Matheus et al., 2010; Carvalho et al., 2006), requiring specific methods to overcome it. In view of this, the main goal of this research was to evaluate the efficiency of different methods in breaking dormancy and to characterize biochemical processes associated with germination of *E. falcata* seeds.

MATERIAL AND METHODS

Plant material

Ripe fruits of ***E. falcata*** were collected from six mature trees in the municipality of Lavras, Minas Gerais, Brazil, 21°14'43"S, 44°59'59"W and altitude of 919 meters. The seed processing was carried out manually, the fruits were broken to extract the seeds and those that were damaged and attacked by pathogens or insects were discarded. Subsequently, the seed batches were mixed and stored in a dry, cold chamber at 10ºC until the experiments were carried out.

Determination of water content and weight of a thousand seeds

The water content before storage was determined by the oven method at 103 ± 3 ºC for 17 hours, with three replications of 10 seeds, and the calculation was performed on the wet basis and expressed in percentage. The weight of a thousand seeds was determined using eight samples with 100 seeds each (Brasil, 2009).

Dormancy breaking

The treatments to break physical dormancy consisted of mechanical scarification using sandpaper n° 120 on the side opposite the embryo (Matheus et al., 2010); immersion of seeds in water at room temperature (25 ºC) for 24 hours (Davide and Chaves, 1996); immersion of the seeds in hot water at a temperature of 80 ºC, followed by resting in the same water for 24 hours and immersion of the seeds in hot water at a temperature of 100 ºC for 30 seconds (modified from Davide et al., 1995), followed by resting in the same water for 10 minutes. The control treatment consisted of seeds without treatment to overcome dormancy. Four replications of 20 seeds were used, arranged in rolls of paper for germination that were changed every 7 days. The data obtained were used to calculate the percentage of germination, and the germination speed index (GSI) was calculated using the formula proposed by Maguire (1962). The tests were carried out in BOD germination chambers under constant white light at 25 ºC. The evaluations were carried out daily until 35 days, and we adopted as germination parameter the root protrusion with approximately 1.0 mm and subsequent formation of normal seedlings. Seeds that did not germinate and remained intact at the end of the test were considered dormant, and those that did not germinate and deteriorated or those infected by microorganisms were considered dead.

Imbibition curve

The seeds were scarified with sandpaper n° 120 and placed in rolls of germination paper moistened with 2.5 times of distilled water in relation to the weight of the paper and placed in germination chambers of the BOD type under constant white light at 25 ºC. Three replications of 10 seeds were used, and the imbibition process was analysed by weighing the seeds on an analytical balance with a precision of 0.001 g, at regular intervals of one hour during the first 12 hours and every 6 hours until 78 hours. The increase in fresh mass was calculated using the equation [1]. Where: MFI (%) - % increment over initial fresh mass, MFU = wet seed mass and MFI = dry seed mass.

$$MFI(\%) = \frac{MFU - MFI}{MFU} \times 100 \quad (1)$$

Quantification of hydrogen peroxide (H_2O_2)

Three replications of 5 seeds without the tegument submitted to imbibition (0 [dry seeds], 6, 12, 18, 27 and 36 hours and seeds with primary root of 1 mm and 3 mm) were used. H_2O_2 was determined by measuring the absorbance at 390 nm in an ELISA spectrophotometer (Velikova et al., 2000).

Quantification of lipid peroxidation

The same treatments and the same extract used for the quantification of hydrogen peroxide were used, and the analysis was performed according to the protocol proposed by Buege and Aust (1978) in which the concentration of malondialdehyde (MDA) is quantified, a secondary product of the peroxidation process. The calculation was performed using the equation [2]. Where: MDA - concentration of malondialdehyde, ξ - molar extinction coefficient = 1.56 x 10^{-5}, and b - optical length = 1.

$$MDA = \frac{A535 - A600}{\xi b} \quad (2)$$

Antioxidant system enzyme activity

Seeds without the seed coat were sampled after different imbibition times and primary root lengths (0 h [dry seeds], 6 h, 12 h, 18 h, 27 h, 36 h, 1 mm and 3 mm) and then macerated in liquid nitrogen and stored at -80 ºC, using three replications of 5 seeds for each treatment. SOD activity was quantified by its ability to inhibit nitrotetrazolium blue (NBT) photoreduction, as proposed by Giannopolitis and Ries (1977). CAT enzyme activity was analysed according to Havir and McHale (1987), and APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm, according to Nakano and Asada (1981). From the same extract, the total proteins were quantified by the method of Bradford (1976) to determine the specific activity of antioxidant enzymes.

Quantification of total proteins

Three replications of 5 seeds without the tegument were used, submitted to different imbibition times and primary root lengths (0 h [dry seeds], 6 h, 12 h, 18 h, 27 h, 36 h, 1 mm and 3 mm) macerated in liquid nitrogen and stored at -80 ºC. The quantification of total proteins was performed according to the method of Bradford (1976).

Statistical analysis

The experimental design used was completely randomized for all experiments, and the variables were analyzed using the Shapiro Wilker normality test, analysis of variance and Scott-Knott test with $p < 0.05$. The variables that did not follow a normal distribution (dormancy and mortality percentage, shoot and root dry mass and lipid peroxidation) were transformed (square root). All analyzes were performed using the Analysis of Variance System for Balanced Data - SISVAR® (Ferreira, 2000) and graphs were made using the Microsoft Excel® program.

RESULTS

Under control conditions (no dormancy overcoming treatment) *E. falcata* seeds presented 55 % dormancy, 7.5 % mortality, and 37.5 % of germination at the end of the experiment (Figure 1). The highest germination percentage (93.75 %) was observed when the seeds were scarified with sandpaper. Immersion of seeds in water at 25 ºC led to no differences in germination, dormancy or mortality when compared to the control. However, immersion of the seeds in water at higher temperatures (80 ºC and 100 ºC) led to an increase in mortality. Treatment with water at 100 ºC was the most detrimental to *E. falcata* seeds, leading to a significant reduction in germination percentage (6.26 %) and a significant increase mortality (91.25 %).

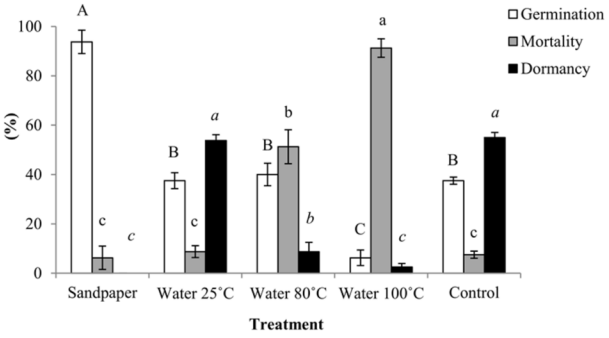

Figure 1. Percentage of germination, mortality and dormancy of *E. falcata* seeds after treatments to overcome dormancy. Means followed by equal letters (uppercase for germination, lowercase for mortality and lowercase in italics for dormancy) do not differ from each other by the Scott-Knott test at 5 % probability. Bars indicate standard error.

The initial moisture content and weight of one thousand *E. falcata* seeds were 9.89 % and 275.80 g, respectively. Higher GSI was observed in seeds scarified with sandpaper when compared to the other treatments (Table 1).

To investigate the physiological and biochemical processes associated with germination of *E. falcata* seeds we used the scarification with sandpaper treatment as it was the most efficient method to overcome dormancy (Figure 1). A more rapid water absorption was observed in seeds scarified with sandpaper when compared to the control treatment (not scarified) after 78 hours of analysis (Figure 2). This fact explains the highest germination speed index (GSI) found in seeds scarified with sandpaper (Table 1). Based on the increase in fresh weight during imbitition,

Table 1. Mean values of germination speed index in *E. falcata* seeds submitted to different treatments to overcome dormancy after 35 days.

Treatment	Germination Speed Index (GSI)
Scarification with sandpaper	8.65 a
Water immersion 25 ºC	1.13 c
Water immersion 80 ºC	2.54 b
Water immersion 100 ºC	0.26 c
Control	1.12 c

we physiologically characterized the different germination phases of *E. falcata* seeds (Figure 2). We observed a rapid increase of water uptake in the first 18 hours of imbibition, characterizing the phase I of germination. Between 18 hours and 36 hours there was no substantial increase in water uptake, what is a characteristic of the phase II of germination. At 36 hours we observed radicle protrusion of *E. falcata* seeds, what characterizes the phase III of germination (Figure 2).

During the first 12 hours of imbibition there was no significant difference in SOD activity (Figure 3A). However, after 18 hours of imbibition there was an increase in SOD activity coinciding with the transition from phase I to phase II of germination (Figure 2). After that, SOD activity remained high until the primary root reached 3 mm in length.

Similar to the activity of SOD, the activity of CAT remained low during the first 12 hours of imbibition, increasing from 18 hours onwards and remaining high until the primary root reached 3 mm in length (Figure 3B). On the other hand, APX showed an earlier significant increase in activity at 12 hours after imbibition and did not change at later time points (Figure 3C). We found that concentration of hydrogen peroxide was higher in dry seeds (0 hours of imbibition), significantly decreased after 6 hours of imbibition, and remained unchanged at later timepoints (Figure 4A). We also found that during imbibition there was a significant reduction in the formation of malondialdehyde (MDA) and, therefore, less lipid peroxidation (Figure 4B).

To finally characterize the biochemical processes associated with the germination of *E. falcata* seeds, we analysed the total protein content. We observed a reduction of the total protein content during the first 12 hours of imbibition, and it remained constant until the primary root reached 3 mm in length (Figure 5).

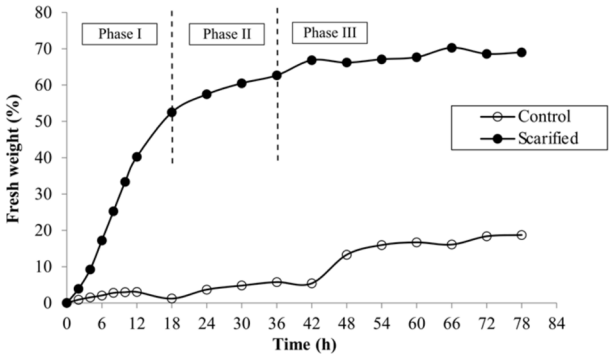

Figure 2. Imbibition curve and germination phases of *E. falcata* seeds without scarification and mechanically scarified with sandpaper. The seeds were germinated in water at 25 ºC under constant light. Each point is the average of three replicates of 10 seeds.

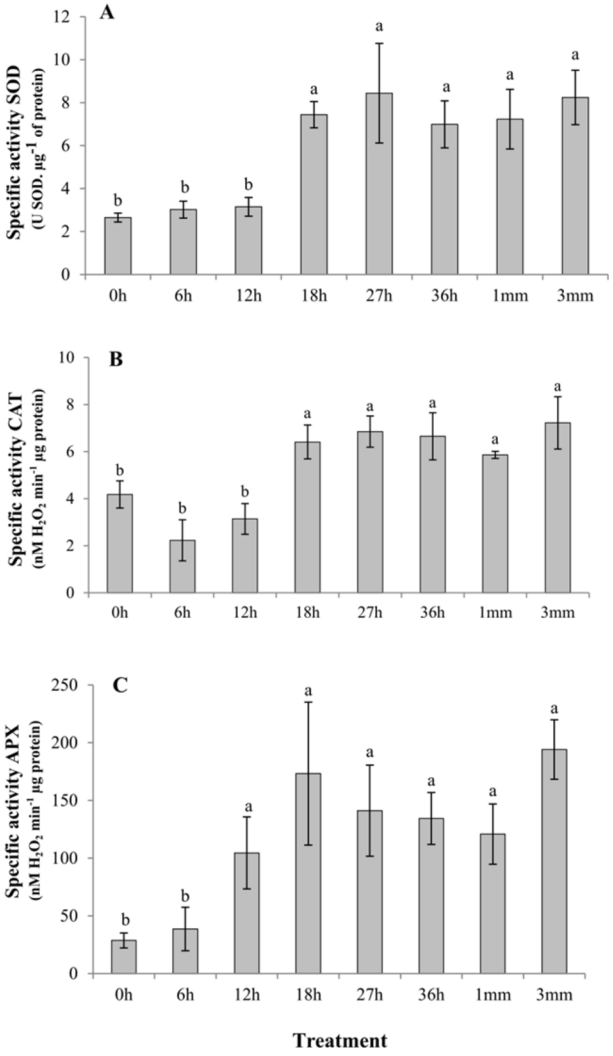

Figure 3. Activity of superoxide dismutase (SOD) - A, catalase (CAT) - B, and ascorbate peroxidase (APX) - C during imbibition of *E. falcata* seeds scarified with sandpaper. Each bar represents the average of three replicates of 5 seeds and the vertical bars represent the standard error. Means followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability.

DISCUSSION

According to Moise et al. (2005), the outermost layer of the seed coat of Fabaceae is a waxy cuticle, which represents the first barrier to imbibition and varies in thickness.

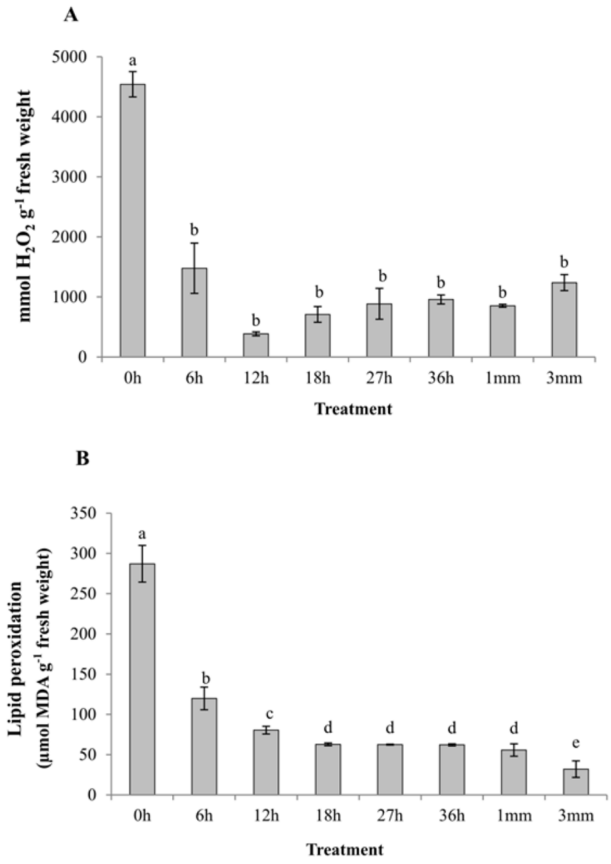

Figure 4. Quantification of hydrogen peroxide (A) and lipid peroxidation (B) through the quantification of MDA during the imbibition in *E. falcata* seeds. Each bar represents the average of three replicates of 5 seeds and the vertical bars represent the standard error. Means followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability.

According to Baskin and Baskin (2005), physical dormancy is caused by one or more palisade layers in the seed coat and overcoming dormancy involves the disruption of a specialized area in the seed, thus creating an opening through which water uptake occurs. We tested different methods to disrupt the tegument and overcome physical dormancy in *E. falcata* seeds (Figure 1). We found that treating the seeds with water at high temperatures was detrimental to germination and led to high mortality percentage (Figure 1). Despite being an effective treatment to overcome dormancy in some species, overall, immersion of seeds in hot water (80˚C and 100˚C) can cause damage to embryos and compromise viability in species of the genus Erythrina (Santos et al., 2013). Similar to our findings, *Erythrina crista-galli* seeds immersed in water at high temperature (96˚C) also showed a low percentage of germination (Silva et al., 2006).

We found that mechanical scarification with sandpaper was the most effective treatment to overcome physical dormancy in *E. falcata* seeds. The increase in fresh mass over time and the beginning of root protrusion were then used to determine germination stages as described by Bewley et al. (2013) for scarified seeds (Figure 2). At 18 hours an increase of 52 % was observed on the fresh mass, what determined the phase I of germination. This phase is characterized by a relatively rapid increase in water absorption (Bewley et al., 2013). Phase II was determined considering the times after 18 hours of imbibition until the beginning of radicle protrusion, in this period there was no significant increase in fresh mass until the protrusion observed after 36 hours of imbibition. In phase III there was an increase of 63 % in the fresh mass and at the end of the analysis we observed an increase of 69 % on the fresh mass. Despite the clear triphasic imbition pattern of *E. falcata* seeds, a recent study has shown that this is the rarest pattern of imbibition for native brazilian forest species (Pereira et al., 2022).

Seeds without mechanical scarification had slow water absorption, not allowing to differentiate between phases I and II (Figure 2), with phase III observed after 60 hours of imbibition. This quicker radicle protrusion in scarified seeds was also observed in seeds of *Amburana cearensis* and *Ormosia grossa* belonging to the same subfamily as *E. falcata* (Faboideae a.k.a. Papilionoideae), indicating that the impermeability of the seed coat is a common feature in species of this subfamily (Loureiro et al., 2013; Pinheiro et al., 2021). We then investigated how the activity of the antioxidant enzymes SOD, CAT and APX changed during the different phases of *E. falcata* seed germination. For SOD and CAT, we found an increase in the activity only after 18 hours of imbibition, correlating with the begining of phase II of seed germination (Figure 3). SOD activity has been found to vary between different species and seed tissues during imbibition. In the seeds of the tree species *Butia capitata* (Bicalho et al., 2018) and *Delonix regia* (de Souza Barbosa et al., 2022), an increase in SOD activity was observed after 24 hours of imbibition. In the seeds of *Lupinus luteus* L. there was an increase in SOD activity in the embryonic axes until the emergence of the radicle, while in the cotyledons the activity remained constant (Garnczarska; Wojtyla, 2008). According to these authors, the increase in the activity of the enzyme may have been stimulated by the increasing formation of the superoxide anion during the imbibition.

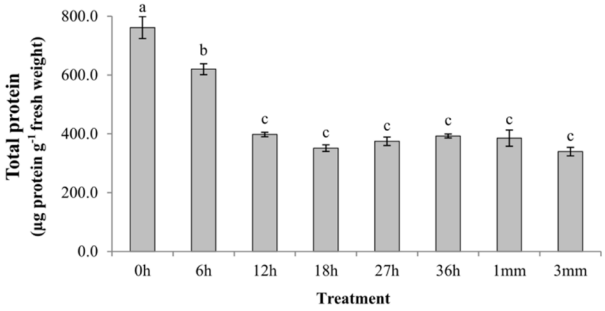

Figure 5. Quantification of total proteins during germination of *E. falcata* seeds. Each bar represents the average of three replicates of 5 seeds and the vertical bars represent the standard error. Means followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability.

In pea seeds, an increase in CAT activity was observed both in the embryonic axes and in the cotyledons (Wojtyla et al., 2006), the same occurring in axes and cotyledons of *Lupinus luteus* L. throughout the imbibition (Lima et al., 2021). Karuppanapandian et al. (2011) suggested that the increase in CAT activity in plants is an adaptive trait that helps to overcome tissue metabolism damage by decreasing toxic levels of H_2O_2. For APX we observed a significant increase in activity after 12 hours of imbibition (Figure 3C). APX has a higher affinity for H_2O_2 than CAT (Sharma et al., 2012), what might explain the fact that in a shorter imbibition time (12 hours) an increased activity was observed when compared to CAT (18 hours) (Figure 3B).

An early event of phase I of germination is the resumption of energy metabolism (Bewley et al., 2013; Wojtyla et al., 2006; Nonogaki et al., 2010). After the initial phase of increased oxygen consumption by respiration, the rate declines until the radicle protrudes from adjacent tissues (Bewley et al. 2013). It is known that respiratory metabolism is one of the potential sources to produce ROS (Mittler, 2002; Bailly, 2019). With increased metabolism, it is possible that there is an increase in the production of ROS that can cause lipid peroxidation and cellular damage (Gill et al.; Tuteja, 2010; Li et al., 2022). In this way, activation of the antioxidant machinery is needed to avoid the damage caused by the reactive species (Li et al., 2022). The decrease in lipid peroxidation during imbibition of *E. falcata* seeds (Figure 4B) may have been caused by decreased production of ROS such as H_2O_2 (Figure 4A), what might have been achieved through increased activity of the antioxidant enzymes SOD, CAT and APX (Figures 3A, 3B and 3C).

For all enzymes analyzed in this work, specific activity was observed at time zero of imbibition (dry seed). According to Garnczarka and Wojtyla (2008), it is assumed that the structures and enzymes necessary for the initial resumption of metabolic activity are generally present in dry seeds, having survived, at least partially intact, the desiccation phase that ends seed maturation. After 6 hours of imbibition, hydrogen peroxide concentration was reduced. Hydrogen peroxide is a byproduct of SOD activity to prevent cell damage and must be eliminated through conversion to water in subsequent reactions involving enzymes such as APX and CAT. Thus, these enzymes regulate the levels of hydrogen peroxide in plant cells. Hydrogen peroxide can be beneficial for germination in the case of overcoming dormancy, or harmful, in the case of aging, depending on the level of accumulation in the embryo's cells (Bailly, 2019).

Although membranes are protected in mature and dry seeds, damage can occur during imbibition (Nonogaki et al., 2010). The role of the integument, lens, micropyle and hilum composition in controlling water absorption in Fabaceae seeds is described in the literature (Rodrigues-Junior et al., 2014; Wojtila et al., 2006; Hu et al., 2009). Since the seeds used in this work underwent mechanical scarification with sandpaper until the cotyledon was exposed, the water absorption by the seeds may have been rapid, causing high lipid peroxidation in the early stages of imbibition. According to Gill and Tuteja (2010), lipid peroxidation, as well as damage to the DNA molecule, individually, can be considered as the major determinants of seed viability loss. Malondialdehyde (MDA) is the end product of peroxidation of unsaturated fatty acids from phospholipids and is one of those responsible for damage to cell membranes (Sharma et al., 2012). The breakdown products of aldehydes such as MDA can form conjugates with DNA and proteins (Gill; Tuteja, 2010). In *E. falcata* seeds the concentration of MDA and, thus, lipid peroxidation, decreased during imbibition (Figure 4B).

The process of protein degradation during germination is common for different species, genera, and families of plants. In Fabaceae, germination initiates hydrolysis of the storage proteins and increases free aminoacids which are important for human digestibility and seed nutritional value (Ohanenye et al., 2020). We observed a decrease in total proteins in the first 12 hours of *E. falcata* seed germination (Figure 5). In seeds of *Erythrina velutina form aurantiaca*, a decrease in protein content was also observed during germination and seedling formation (Oliveira et al., 1998). Kirmizi and Güleryüz (2006) observed a decrease in total protein values during 7 days of germination of *Vicia faba* seeds, coinciding with an increase in the total free amino acid content. In seeds of *Caesalpinia peltophoroides* Benth. a drop in total protein content during germination was also observed (Corte et al., 2006). Protein mobilization during seed imbibition is a crucial process for seedling establishment, since proteins that were synthesized and stored in abundance in the seeds are metabolized into free amino acids for biosynthesis and energy generation (Wilson; Wilson, 2012).

CONCLUSIONS

Mechanical scarification with sandpaper is an efficient method for breaking physical dormancy in *E. falcata* seeds. Such seeds present rapid water absorption in the first 18 hours when scarified with sandpaper and the radicle emergence occurs after 36 hours of imbibition. During imbibition of *E. falcata* seeds, a reduction in the production of H_2O_2 and lipid peroxidation, and an increase in the activity of antioxidant enzymes were observed. The total protein content decreased during imbibition.

AUTHORSHIP CONTRIBUTION

Project Idea: MASA, JMRF, AAA, FCN

Funding: AAA, JMRF

Database: MASA

Processing: MASA

Analysis: MASA, JMSL, KRD

Writing: MASA, AAA, JMRF

Review: MASA, ACJ, JMRF, JMSL, KRD, FCN, AAA

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