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Biology- Botany

Embryo development in seeds of *Ilex paraguariensis* A.St.-Hil. in stratification treatments

Desenvolvimento embrionário em sementes de *Ilex paraguariensis* A.St.-Hil. em tratamentos de estratificação

Rosani Klein Reinke[I], Mara Cíntia Winhelmann[II], Leo Jaime de Vargas[III], Julia Gastmann[III], Elisete Maria de Freitas[III], Claudimar Sidnei Fior[II], Shirley Silva Martins[IIII]

[I] Universidade Estadual Paulista, São Paulo, SP, Brazil
[II] Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[III] Universidade do Vale do Taquari, Lajeado, RS, Brazil
[IIII] Universidade Estadual do Oeste do Paraná , Cascavel, PR, Brazil

ABSTRACT

The specie *Ilex paraguariensis* A.St.-Hil., known as yerba mate, is propagated by seeds that have morphophysiological dormancy. This study aimed to answer the following questions: (1) What are the changes that occur in the *Ilex paraguariensis* embryos during moist stratification? and (2) May the incorporation of chemical fertilizers in moist stratification medium promote an increase in the germination percentage in yerba mate seeds? For this purpose, yerba mate seeds were subjected to two stratification treatments: between two layers of moist sand and between two layers of moist sand with mineral fertilizer plus a control, for 180 days. At intervals of 30 days, seeds were removed for structural anatomical analysis and, at 150 and 180 days, also for germination tests at 25 °C, which were monitored for 180 days. The treatment with the highest germination percentage during stratification (15.4%) and in the germination tests (15.2 and 20.1%) was the one with mineral fertilizer incorporated in the moist stratification medium. Only in this treatment the embryos in more advanced stages of development (torpedo and mature). It is concluded that embryo development and germination were favored by moist stratification medium with the addition of chemical fertilizers.

Keywords: Anatomy; Aquifoliaceae; Yerba mate; Morphophysiological dormancy

RESUMO

A espécie *Ilex paraguariensis* A. St.- Hil., conhecida como erva-mate é propagada por sementes que tem dormência morfofisiológica. Este estudo teve como objetivo responder às seguintes questões: (1) Quais são as alterações que ocorrem no embrião de Ilex paraguariensis durante a estratificação úmida? e (2)

incorporação de fertilizantes químicos em meios de estratificação úmida pode promover aumento na porcentagem de germinação em sementes de erva-mate? Para tanto, sementes de erva-mate foram submetidas a dois tratamentos de estratificação: entre duas camadas de areia úmida e entre duas camadas de areia úmida mais adubo mineral, além do controle, por 180 dias. Em intervalos de 30 dias, as sementes foram retiradas para análise anatômica estrutural e, aos 150 e 180 dias, também para testes de germinação a 25 °C, que foram monitorados por 180 dias. O tratamento com adubo mineral incorporado na areia de estratificação apresentou a maior porcentagem de germinação durante estratificação (15,4 %) e nos testes de germinação (15,2 e 20,1%). Somente neste tratamento foram observados embriões em estágios mais avançados de desenvolvimento (torpedo e maduro). Conclui-se que o desenvolvimento embrionário e a germinação foram favorecidos pela estratificação em areia com adição de adubo químico.

Palavras-chave: Anatomia; Aquifoliaceae; Erva-mate; Dormência morfofisiológica

1 INTRODUCTION

Ilex paraguariensis A.St.-Hil., popularly known as yerba mate, is an arboreal and evergreen species, originally from Argentina, Paraguay, Uruguay, and Brazil (Sobral et al., 2013). Yerba mate is widely consumed in chimarrão, tererê, and traditional tea form (Coelho et al., 2002); however, there is an increase in the number of studies whose aim is the creation of other items for consumption, such as beverages (beers, soft drinks, and energy drinks), cosmetics, functional foods and animal feed (Croge et al., 2021). In addition, it is also used as medicine (Junior & Morand, 2016; Riachi & De Maria, 2017), with stimulating, anxiolytic and neuroprotective properties (Santos et al., 2015).

The production of yerba mate plants is accomplished mainly from seeds (Wendling & Santin, 2015), thus the occurrence of morphophysiological dormancy is identified as one of the main causes of the low germination rates of yerba mate (Niklas, 1987; Heuser et al., 1993; Wendling & Santin, 2015; Galíndez et al., 2018; Mireski et al., 2019; Souza et al., 2020) and also in other species of the genus *Ilex* (Li et al., 2023).

The morphophysiological dormancy in these species is associated to the underdeveloped embryo (Bewley et al., 2013; Baskin & Baskin, 2014), in addition to inhibitory mechanisms, such as the polyphenols in the seed coat (Mireski et al., 2018), which hamper the embryo development and seed germination (Bewley et al. 2013; Baskin & Baskin, 2014).

Undeveloped embryos may be in the cotyledonary to torpedo stages. In the cotyledonary stage, lateral emergence of the cotyledonary primordium, beginning of procambium differentiation and the presence of the suspensor are observed; in the initial cordiform, differentiation of meristematic tissues and cotyledonary primordium occurs, the digestive cavity has more degraded cells and the endosperm presents cells in division; in the late cordiform there is an increase in cell volume and cotyledons are developed and, finally, in the torpedo the meristematic tissues (protoderm, ground meristem and procambium) and the cap are evident and the endosperm presents vacuoles with amorphous proteins. The mature embryo presents an embryonic axis (hypocotyl-radicle) defined with evident and well-defined meristematic zones and cap, it is more elongated than the torpedo stage and with larger and more vacuolated cells.

To accelerate the embryon development that are in the cordiform to torpedo stages, method of moist stratification are commonly used, a process in which the seeds remain in sand for 180 days (Cuquel et al., 1994; Wendling & Santin, 2015). In light of this, the moist sand stratification combined with other treatments can be an alternative to reduce the time required for the embryo development and contribute to homogeneous germination, consequently, bringing uniformity to seedling production.

Different stratification methods have been tested by researchers to accelerate the germination process, although the germination percentagens remained low (0% to 3.8%), even after six months of stratification moist sand (Cuquel et al., 1994; Meneguet et al., 2004). Dolce et al. (2010) recorded 74.4% germination of embryos kept isolated in in vitro culture for 120 days. There are reports in the literature that the seeds of this species may show a resting period of two years or more (Cuquel et al., 1994). However, the approaches related to embryo development during the stratification process are still unknown.

For the seeds of *Malus domestica* Borb cv. Antonovka (apple), subjected to cold stratification, morphological changes were observed in the embryos over the months in stratification (Dawidowicz-Grzegorzewska & Lewak, 1978), and in seeds of *Sambucus*

chinensis Lindl., there was a 25% increase in embryo size and differences in its shape during its development until overcoming its morphophysiological dormancy (Chen et al., 2014). In other species of the genus *Ilex*, considerable embryo growth and subsequent germination after stratification treatment were observed (Chien et al., 2011; Liu et al., 2023)..

Germination tests, combined with anatomical analysis during stratification for the monitoring of embryo development, are important to prove the effectiveness of the stratification treatments. In addition, the tetrazolium test can be an alternative to check occurrence of dormant seeds at the end of the experimental period. This is because, for species that present slow germination, longer than 60 days (Brasil, 2009), such as the case of yerba mate, seeds may still be dormant. Consequently, there are reports in the literature that the seeds of this species may show a resting period of two years (Cuquel et al., 1994) or more.

Some studies have shown that the use of mineral elements, such as potassium nitrate, can overcome dormancy in some species (Batak et al., 2002; Alboresi et al., 2005). This compound, associated with low temperatures during the stratification of Acer morrisonense Hayata, favored the overcoming of seed dormancy, with changes in the concentration of the hormones abscisic acid (ABA) and gibberellic acid (GA_3), proteins, lipids, sugars, and amino acids (Chen et al., 2015). While in an apple cultivar Ligol, the potassium nitrate used in the imbibition of the seeds during stratification, assisted in the overcoming of the dormancy, increasing the germination percentage (Grezsik et al., 2017). For yerba mate seeds, treatments with the addition of potassium nitrate and alternation of temperature and light in the stratification reduced dormancy in some seed lots, while in others lots it had no effect (Cuquel et al., 1994).

In this context, anatomical analysis aimed at monitoring embryo development can be important to confirm the influence of stratification methods. The germination test, in addition to the tetrazolium test, may assist in evaluating the effectiveness of the stratification treatments to overcome dormancy. Thus, this study aimed to answer

the following questions: (1) What are the changes that occur in the *Ilex paraguariensis* embryos during seed stratification? And (2) May the incorporation of chemical fertilizers in moist stratification medium to promote an increase in the germination percentage in yerba mate seeds?

2 MATERIALS AND METHODS

2.1 Seed collection and processing

Due to circumstances of physiological description, pyrenes (endocarp plus seed) are referred to as seeds throughout this work. Seeds of *Ilex paraguariensis* were extracted from red to dark red ripe fruits, collected in January 2018 from four mother plants located in the municipality of Ilópolis, Rio Grande do Sul, Brazil (28° 53' 25" S 52° 08' 46" W). In this region, the climate is Cfb (humid temperate climate with temperate summer) according to the Köppen and Geiger classification, with a mean temperature of 17.1 °C, and mean annual rainfall of 1690 mm (Climate, 2020).

In the laboratory, the seeds were extracted from the fruits by washing in running water with the aid of sieves and then placed on absorbent paper, where they remained on a laboratory bench at room temperature for five days. Subsequently, the water content was determined by the humidity percentage difference after oven drying at 105 ± 2 °C for 24 hours, using three repetitions of 0.5 g (Brasil, 2009), whose result indicated 6.85% humidity for the batch. Then, they were stored in a glass snap cap vial and kept under a controlled temperature of 5 °C ± 2 °C for 30 days until the experiment installation.

2.2 Stratification tests

The seeds of the four sources were mixed, constituting a single batch, as they were collected in the same period, from nearby source plants, in places with similar environmental conditions and no statistical differences ($p<0.05$) for the humidity

percentage. Then, they were subjected to two stratification treatments for 180 days: (T1) Treatment 1 - seeds in trays, between two moist sand layers with 60% water retention capacity (WRC), each one 3 cm deep (1.0 L each); (T2) Treatment 2 - seeds arranged between two shading screens and two moist sand layers (60% WRC), each 3 cm deep (1.0 L each), with 4.0 g L^{-1} of Basacote Plus 6M® controlled-release fertilizer (CRF) added (16-8-12) to the upper layer and the treatment; (C) Control - seeds without stratification, kept in paper bags inside Styrofoam boxes, under refrigeration (5 °C ± 2 °C).

The Basacote Plus 6M® controlled-release fertilizer (CRF) contains nitrogen (8.6% in the ammoniacal form and 7.4% in the nitric form), phosphorus (5.6% P_2O_5), potassium (12% K_2O), magnesium (2% total MgO), sulfur (12% total SO_3) and micronutrients (0.02% boron, 0.05% copper, 0.4% iron, 0.06% manganese, 0.015% molybdenum and 0.02% zinc).

The T1 and T2 treatments were established in a greenhouse, in white polyethylene trays (192 × 116 × 64 mm) with a perforated bottom, with twelve holes of 0.3 cm diameter in each tray, for drainage. During the stratification period, irrigation was manual, keeping the substrate always moistened (approximately 60% of the WRC), with a maximum average temperature of 32.5 ºC and a minimum of 8.1 ºC (Table 1) .

Five thousand seeds were used for each treatment. They were distributed in four replications (4 × 1250). Before the installation of the tests, the seeds were disinfected with 70% alcohol for one minute, followed by washing in autoclaved ultra-purified water. Afterward, they were immersed for 20 minutes in sodium hypochlorite (1.5%) containing five drops of *Tween*TM20 and then subjected to triple washing with autoclaved ultra-purified water and drying on absorbent paper to remove excess water.

During the stratification experiment period, the minimum, mean and maximum temperature values for Lajeado City, Rio Grande do Sul, Brazil were obtained from the Meteorological Station of the Universidade do Vale do Taquari (Univates) (Tab. 1).

Table 1 – Maximum (T max), minimum (T min) and mean (mean T) temperature in °C and mean relative humidity (RH) (%) recorded at the Universidade do Vale do Taquari weather station in Lajeado - RS, between March and September 2018

Months	T max (°C)	T min (°C)	Mean T (°C)	Mean RH (%)
March	37.2	13.1	23.4	77
April	35.3	16.3	23.5	80
May	29.7	5.8	18.5	83
June	30.5	2.1	14.1	83
July	29.7	5.3	14.8	86
August	30.3	4.2	14.9	79
September	34.6	9.8	19.8	82
Mean	32.5	8.1	18.4	81.4

Source: Authors (2023)

2.3 Pyrene and seed structural characterization during stratification

For the structural analysis, 10 pyrenes were removed from the control (C) and each of the stratification treatments (T1 and T2) in periods of zero, 30, 60, 90, 120, 150, and 180 days of stratification. The pyrenes were fixed in formaldehyde, acetic acid, and alcohol (FAA50) (Johansen, 1940), and after 24 hours, they were transferred to 70% alcohol storage. To optimize the infiltration process, preserving the embryo structure, the endocarp was removed, and afterward, the seeds were sectioned longitudinally with the aid of a steel blade, dehydrated in an increasing ethyl series, infiltrated, and included in historesin (Leica Historesin Embedding Kit) (Feder & O'brien, 1968, with modifications).

Serial longitudinal sections were made in the seed with the aid of a rotating microtome (model RM 2245 Leica Microsystems Inc.®) with 5.0 µm thickness. The sections were stained with 0.05% toluidine blue (Feder & O'brien, 1968) and mounted on a permanent slides with Entellan®. The images were captured with the aid of a digital camera DP041 coupled to the photomicroscope Olympus BX70 using the DP Controller program.

For Scanning Electron Microscopy (SEM) analysis, two pyrenes of each treatment and period (zero, 30, 60, 90, 120, 150, and 180 days of stratification) were sectioned longitudinally, fixed in 2.5% glutaraldehyde, washed in phosphate buffer (0.1 mol L^{-1} and pH 7.3). Then, they were dehydrated in an ethyl series and metalized in gold. The images were captured using a Zeiss EVO® LS-10 scanning electron microscope and SmartSEM® V05.06 software.

The terminology used to describe the embryo developmental stages followed Heuser et al. (1993) and Heuser (1999). Seed and embryo measurements were performed using the Image Pro Plus program.

2.4 Seed germination during stratification

During stratification, seeds were monitored weekly to verify germination, and those with at least 2 mm of root protrusion were considered germinated (BRASIL, 2009). Germination percentage data was performed based only in the viable seed percentage in the batch. Data of dead or empty seeds and seeds showing evidence of predation were discarded as soon as the experiment started.

2.5 Germination test after stratification

This test consisted of sampling 100 seeds from each of the four replicates of the different treatments, taken from the stratification at 150 (Test 1) and 180 days (Test 2). These seeds were thrice washed in autoclaved ultra-purified water and placed on paper towels. After that, the seeds were subjected to germination in gerbox-type plastic boxes, containing 250 g of sieved and autoclaved sand moistened with ultra-purified autoclaved water at 60% WRC with four replications of 25 seeds (4 X 25).

The seeds were immersed in the sand at a depth of 1 cm. Subsequently, they were stored in Mangelsdorf-type seed germinator chambers, with no photoperiod regulation, temperature of 25 °C ± 1 °C and relative humidity above 90%. The germination percentage was calculated based on the percentage of viable seeds

present in the batch at experiment initiation. For those which did not germinate after 180 days, the tetrazolium test was performed. To count the emerged seeds (Brasil 2009), an emergence assessment was performed weekly for 180 days.

2.6 Tetrazolium test

The seeds were sieved and washed with autoclaved ultra-purified water to remove excess sand, and then a longitudinal cut was performed with tweezers and a scalpel. The seeds were examined using a Leica EZ4 HD stereoscopic microscope, with 20x to 30x magnification. In this preliminary evaluation, they were classified as one of the following: (1) not-full (empty + degraded) or (2) full seeds (with and without visible embryo).

For the viability determination by the tetrazolium analysis, the seeds classified as full were subjected to 0.1% tetrazolium solution for 24 hours at 35 °C. Then, the seeds were washed thrice with ultra-purified water, and subsequently, a visual assessment was performed to determine viability. The seeds were evaluated in response to the solution and those that showed a firm and colored endosperm, visible and colored embryo, no damage, and firm consistency were considered viable. All seeds that did not demonstrate these characteristics were classified as non-viable (Brasil, 2009).

2.7 Statistical analysis

The data of germination percentage during stratification, germination percentage at 25 °C, and percentage of not-full, non-viable, and viable seeds were subjected to analysis of variance (ANOVA) and comparison of means by the LSD-Fisher test at the level of 5% error probability, with the use of Costat 6.4 and SigmaPlot 11.0 software. The data of germination in the stratification (%), germination at 25 °C (%), and non-viable seeds (%) did not satisfy the analysis of variance (ANOVA) assumptions. Consequently, they were transformed to $\sqrt{x}$.

3 RESULTS

3.1 Pyrene and seed structural characterization during stratification

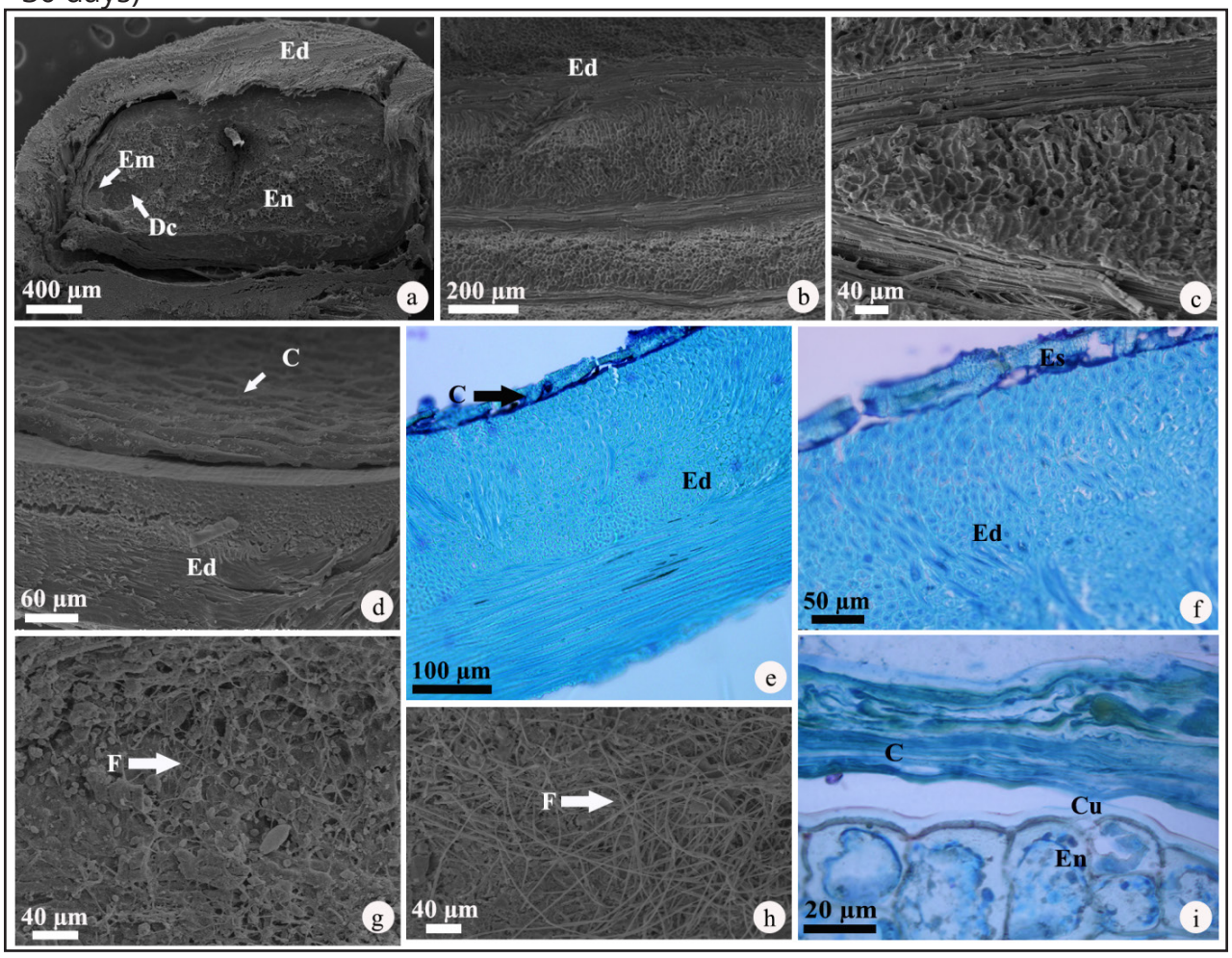
The seeds showed sizes between 1.85 mm and 3.54 mm long. The pyrene has a woody endocarp, followed by the seed coat, endosperm, and embryo, which ia located in the micropylar region (Fig. 1A). The seed involucre shows a woody and multi- layered endocarp, constituted of rows of fibrous cells arranged along the seed's longest axis (Fig. 1 A-F). The seed coat is constituted of parenchymal and lignified cells, followed by the endosperm (Fig. 1E, 1I). For the seeds of the stratification treatments (T1 and T2) fungal hyphae were observed in the endocarp, mainly after 30 days of stratification, visibly increasing over the sample periods (Fig. 1 G-H).

Embryo development in some seeds were not avaliated due to the absence of embryo. In the control treatment, only 21% of seeds showed visible embryos, while in other treatments the percentages were higher: 31% in T1 and 38% for T2 treatments. Embryos at different stages of development were observed, both in the control and in the moist stratification treatments (Tab. 2).

The cotyledonary stage was observed in the control treatment and T1 (0.23 to 0.30 mm long) (Tab. 2). Essentially, for the control cordiform embryos (0.31 to 0.42 mm long) were observed. In the T1 and T2 treatments, embryos predominated in the cordiform and late cordiform stages (0.43 to 0.60 mm long), however, in T2, the verification of torpedo stage embryos (0.61 to 0.90 mm long) and mature (0.91 to 2.04 mm long) (Tab. 2), considered suitable for germination, are highlighted.

In all stratification periods, the predominance of embryos in the cordiform or late cordiform stages was noted, however, we emphasize the occurrence of more advanced stages of embryonic development (torpedo and mature) in the T2 from the period of 150 days (Tab. 2).

Figure 1 – Electron microscopy images (A-C, D, G-H) and photomicrographs (E-F, I) of longitudinal sections of *Ilex paraguariensis* A.St.-Hil seeds during stratification. A: Overview of the seed (C - 150 days). B-C: Endocarp, overview and detail, respectively (C - 0 day). D: Overview of the endocarp and coat (T2 - 120 days). E: Overview of the endocarp (C - 0 days). F: Detail of the endocarp (T1 - 180 days). G-H: Detail of the endocarp with fungal hyphae. G: T1 - 60 days. H: T2 - 180 days. I: Detail of the coat (T2 - 30 days)



C= coat; Cu = cuticle; Dc = digestive cavity; Ed = endocarp; Em = embryo; En = endosperm; Es = sclerenchyma; F = fungal hyphae.
Source: Authors (2023)

Table 2 - Embryonic stages of *Ilex paraguariensis* A.St.-Hil. seeds submitted to different stratification and control treatments over 180 days

Period (days)	Cotyledonary stage (0.23-0.30mm)	Cordiform stage (0.31-0.42mm)	Late cordiform stage (0.43-0.60 mm)	Torpedo stage (0.61-0.90 mm)	Mature (0,91-2.04mm)
Control (C)					
0	-	+	-	-	-
30	-	+	+	-	-
60	+	-	-	-	-
90	-	+	-	-	-
120	-	+	-	-	-
150	-	+	-	-	-
180	-	+	-	-	-
Treatment 1 (T1)					
30	-	+	+	-	-
60	-	+	-	-	-
90	-	-	+	-	-
120	-	+	-	-	-
150	+	-	+	-	-
180	-	+	+	-	-
Treatment 2 (T2)					
30	-	+	-	-	-
60	-	+	+	-	-
90	-	+	+	-	-
120	-	+	+	-	-
150	-	+	+	+	+
180	-	+	+	-	+

-absent; +present;
Source: Authors (2023)

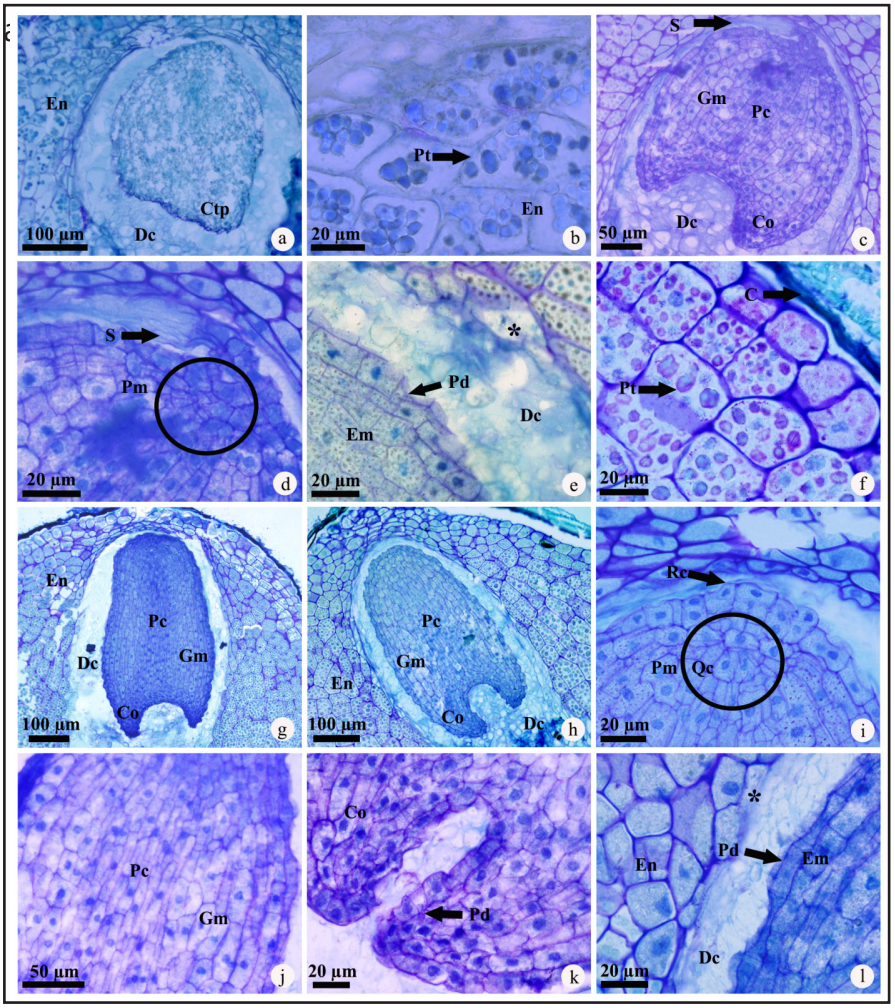
In the embryo cotyledonary stage (Fig. 2A-B), observed in the control treatment and T1, the embryos showed initial differentiation of the cotyledon primordia (Ctp) and the meristematic tissues formed by the protoderm and fundamental meristem presented no clear visualization of the procambium (Fig. 2A). In this phase, the digestive cavity cells (Dc) involve the entire embryo (Fig. 2A) and they are more degraded close

to the cotyledon primordia. The cells of the endosperm have numerous corpuscles (Fig. 2B).

In embryos in the cordiform stage (Fig. 2C-F), observed in the control and the two stratification treatments, the cotyledons are already more differentiated compared to the cotyledonary stage (Fig. 2C-F). At this stage, the radicle pro-meristem (quiescent center) and meristematic tissues (protoderm, fundamental meristem, and procambium) were observed (Fig. 2C-D), and the procambial cells are long and narrow and extend up to the embryo's central portion (Fig. 2C-D). In this phase, the presence of the suspensor (Fig. 2C-D) was also observed, still connected to the embryo and the digestive cavity involving the whole embryo and with some degraded cells (asterisk) (Fig. 2E). The endosperm cells, which are located close to the embryo, have numerous corpuscles (Fig. 2F).

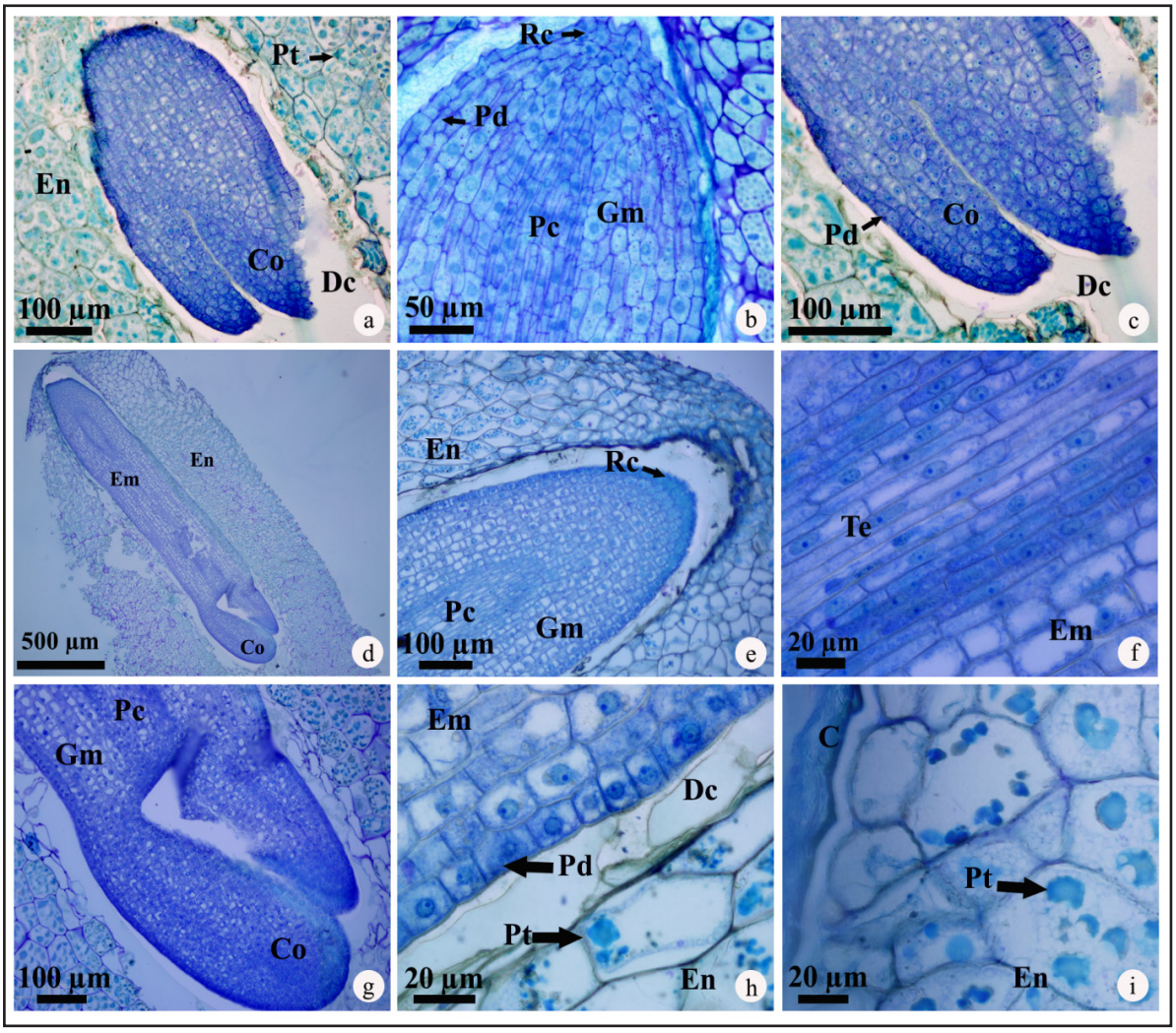
The late cordiform stage (Fig. 2G-L), also found in the control and stratification treatments, was characterized by size (0.42 to 0.60 mm long), greater number of cells, and cell volume (Fig. 2G-H) compared to the cordiform stage. In this phase, a greater delimitation of meristematic tissues was observed, with protoderm covering the embryo, ground meristem in the cortical portion with cells with protein granules and the procambium located in the median region, with long and narrow cells that extend up to the cotyledons (Fig. 2G-J, L). In the cotyledon region, meristematic cells in division are noted, with no differentiation from the stem apical meristem (Fig. 2K). The suspensor was observed in some embryos. In the root subapical portion, the pro-meristem region (Pm) and the beginning of root cap differentiation (Fig. 2I) were identified. The digestive cavity cells surround the embryo and degrading and degraded cells are observed (Fig. 2G-H, 2L). In the endosperm, the presence of many corpuscles was noted inside the cells (Fig. 2L).

Figure 2 – Photomicrographs of longitudinal sections of *Ilex paraguariensis* A.St.-Hil. seeds during stratification, illustrating embryos in cotyledonary stages, in the cordiform stage and in the late cordiform stage. A: General view of the embryo in cotyledonary stage (T1 - 150 days). B: Detail of the endosperm cells (T1 - 150 days). C-D: Overview and detail of the embryo in cordiform stage respectively, with emphasis on the suspensor (T2 - 150 days) E: Detail of the digestive cavity and protoderm cells (Control - 300 days). F: Detail of the endosperm cells (Control- 0 day) G-H: General view of the embryo in late cordiform stage (T2 - 120 and 60 days) I: Detail of the root cap and pro-meristem (T2 - 60 days). J: Detail of the cells of the procambium and of the ground meristem (T2 - 150 days). K: Detail of the cotyledons (T2 - 150 days). L: Detail of the digestive cavity



C= coat; Co = cotyledon; Ctp = cotyledonary primordium; Dc = digestive cavity; Em = embryo; En = endosperm; Gm= ground meristem; Pc = procambrium; Pd = protoderm; Pm = promeristem; Pt = protein; S = suspensor; * degrading cells.
Source: Authors (2023)

Figure 3 – Photomicrographs of longitudinal sections of *Ilex paraguariensis* A.St.-Hil. seeds during the stratification period, showing embryos in the torpedo stage at 150 days and in the mature stage at 180 days of stratification for the T2 treatment. A: General view of the torpedo stage embryo. B: Detail of the embryo root axis region. C: Detail of the cotyledons. D: General view of the mature embryo. E: Detail of the embryo root axis region. F: Detail of the procambial region, with differentiating tracheal elements. G: Detail of the cotyledons. H: Details of the protoderm cells and endosperm cells. I: Detail of endosperm cells



C= coat; Co = cotyledon; Dc = digestive cavity; Em = embryo; En = endosperm; Gm= ground meristem; Pc = procambium; Pd = protoderm; Pt = protein; Rc = root cap coif; Te = tracheal elements.
Source: Authors (2023)

Embryos in the torpedo stage range between 0.60 and 0.94 mm long, are more elongated than the late cordiform stage and show a well-developed embryonic axis and

cotyledons. The procambium shows a more clear-cut differentiation of meristematic tissues, the root cap is evident without the presence of the suspensor, there is also cell degradation in the digestive cavity, and in the endosperm, there is the presence of corpuscles (Fig. 3A-C).

In mature embryos (Fig. 3D-I) (0.94 to 2.04 mm long), the embryonic axis (hypocotyl-radicle) is defined (Fig. 3A) with clear and well-delimited meristematic zones and root cap (Fig. 3E). It is more elongated than in the torpedo stage and with larger and more vacuolated cells than the previous stages (Fig. 3E-H). In the procambium region, it is possible to see the differentiation of the conductive elements (Fig. 3F). The digestive cavity shows many degraded cells (Fig. 3H) and in the endosperm, the corpuscles are in smaller quantity when compared to the previous embryonic stages, with some of them agglutinated (Fig. 3H-I).

3.2 Seed germination during stratification

There was no statistical difference for the germination percentage among stratification periods, only between stratification treatments (Tab. 3). Germination started at 120 days of stratification, with no presence of germinated seeds before this period for any of the treatments. The T2 treatment (15.4%) differed from the treatment 1 (0.13%) and control (0%) (Tab. 4).

Table 3 shows that there was a statistical difference between the stratification treatments, but there was no statistical difference in the germination percentage between the stratification periods. There was no interaction between the treatments and the stratification period for germination at 25 °C.

Table 3 – Germination percentage during stratification, germination percentage at 25 °C, percentage of not-full, viable and non-viable *Ilex paraguariensis* seeds at 180 days of germination

(Continue)

Analyzed variables (%)	Stratification treatment	Stratification time	Interaction
	p value	p value	p value
Germination during stratification	<0.0001*	0.0899 ns	0.1661 ns

Table 3 – Germination percentage during stratification, germination percentage at 25 °C, percentage of not-full, viable and non-viable *Ilex paraguariensis* seeds at 180 days of germination

(Conclusion)

Analyzed variables (%)	Stratification treatment	Stratification time	Interaction
	p value	p value	p value
Germination at 25 °C	<0.0001*	0.43^{ns}	0.44^{ns}
Not-full seeds	<0.0001*	0.0003*	0.0008*
Viable seeds	<0.0001*	0.0002*	0.0017*
Non-viable seeds	<0.0001*	0.051^{ns}	0.0014*

Germination during stratification = germination during six months of stratification in the greenhouse. Germination at 25 °C = germination during six months at 25 °C in the laboratory in a Mangelsdorf-type seed germinator chambers. ns = not significant; *5% significance.
Source: Authors (2023)

Table 4 – Mean germination percentage of *Ilex paraguariensis* A.St.-Hil. seeds during stratification, in different treatments at 120, 150, and 180 days of stratification

Stratification treatment	Stratification time			
	120 days	150 days	180 days	Total
Control (C)	0	0	0	0 b
Treatment 1 (T1)	0	0	0.13	0.13 b
Treatment 2 (T2)	6.2	3.2	6.0	15.4 a
Average germination percentage	2.07^{ns}	1.07^{ns}	2.04^{ns}	5.18

ns = not significant, means followed by different letters in the column differ by the LSD-Fisher test at 5% error probability.
Source: Authors (2023)

3.3 Germination test after stratification

The statistical analysis showed that germination percentage in the T2 treatment was higher in the two germination tests (Test 1 and Test 2), when compared to the other treatments (Tab. 5). For the control no germinated seeds were counted. For T1, germination was verified in both periods (150 and 180 days of stratification), however, in both the germination was less than 1%. Our results showed that T2 had a mean germination of 17.7%.

Table 5 – Germination percentage of *Ilex paraguariensis* A.St.-Hil. in Test 1 (150 days of stratification + 180 days of germination) and Test 2 (180 days of stratification + 180 days of germination) in the different stratification treatments

Stratification treatment	% Germination Test 1	% Germination Test 2
Control (C)	0 b	0 b
Treatment 1 (T1)	0.12 b	0.7 b
Treatment 2 (T2)	15.2 a	20.1 a
Average germination percentage	3.1	4.4

Means followed by different letters in the column differ by the LSD-Fisher test at 5% error probability.
Source: Authors (2023)

3.4 Tetrazolium test

For the three variables analyzed in the tetrazolium test, there was an interaction between the treatment and the stratification period (Tab. 3). The seeds showed initial viability of 38%.

Table 6 shows that the amount of unfilled seeds at the end of Test 1 was higher for treatment T2 (100%), differing significantly from T1 and control. The percentage of viable and non-viable seeds was higher in the control, differing from the other treatments in both times.

For the variable percentage of viable seeds, in Test 1, the control treatment showed the highest percentage (22%), where as T2 treatment revealed the lowest value (0%). The mean percentage of viable seeds at the end of the experimental period was 10.75% for Test 1 and 4.0% for Test 2 (Tab. 6). Between stratification periods (150 and 180 days), the control and the T1 treatment differed statistically with a higher percentage of viable seeds in Test 1 (Tab. 6).

Table 6 – Percentage of not-full, viable and non-viable *Ilex paraguariensis* A.St.-Hil. seeds for Test 1 (150 days of stratification + 180 days of germination) and Test 2 (180 days of stratification + 180 days of germination) under different stratification methods

Stratification treatment	% Not-full		% Viable		% Non-viable	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Control (C)	69.25 c B	90.50 b A	22.00 a A	7.75 a B	8.75 a A	1.75 a B
Treatment 1 (T1)	89.75 b A	96.25 ab A	10.25 b A	3.50 ab B	0.00 b A	0.25 a A
Treatment 2 (T2)	100.00 a A	99.00 a A	0.00 c A	0.75 b A	0.00 b A	0.25 a A
Average	86.3	95.25	10.75	4.0	2.92	0.75

Means followed by distinct lowercase letters in the column and distinct uppercase letters in the row differ by the LSD-Fisher test, at the level of 5% error probability.
Source: Authors (2023)

Regarding the percentage of non-viable seeds, after performing the tetrazolium test, in Test 1 the control showed the highest percentage (8.75%) and the other treatments did not differ statistically with the time of stratification.

4 DISCUSSION

The statistical analysis showed that the T2 expressed higher germination values both in stratification and in germination tests (Test 1 and Test 2). These results indicate that the controlled-release fertilizer positively influenced the overcoming of dormancy in *I. paraguariensis* seeds through the embryo development induction and, thus,

overcoming the morphological dormancy, since only in this treatment the embryos were in the mature stage after 150 days of stratification.

The fertilizer, used only in T2, has the advantage of making nutrients available progressively over five-six months. Thus, nutrients, mostly nitrate, become available throughout the stratification period, a mechanism that possibly helped in the embryonic development process, since it has 8.6% of the nitrogen in ammonia form and 7.4% in nitric form.

The action of nitrate on overcoming seed dormancy is probably because it affects abscisic acid (ABA) metabolism (Alboresi et al., 2005; Yan & Chen, 2020). This process occurs when nitrate is converted to nitrite by the enzyme nitrate reductase and, through other enzymes, can be transformed into nitric oxide (NO), amino acids, and other nitrogenous compounds (Bewley et al., 2013). NO is considered an important indicator in the germinative process through the transcription of the key enzymes, essential for gibberellin (GA) synthesis, increasing the levels of GAs, which consequently act on the reduction of ABA levels (Bethke et al., 2007). Therefore, it is a key element in reducing dormancy and inducing germination, since in most species, germination is regulated by two antagonistic hormones, ABA and GA (Yan et al., 2014; Duermeyer et al., 2018).

Nitrate is considered capable of stimulating germination, even if it is present in low concentrations, as verified in *Arabidopsis*, in which 0.1 mM was sufficient to overcome dormancy (Alboresi et al., 2005). However, the required concentration may vary among species, with some requiring higher concentrations than others. This nutrient is easily found in the majority of soils, and together with light and alternating temperatures, it is an indicator of depth in the seed bank, because the closer the seeds are to the soil surface, the greater the light, nitrate and ethylene availability, and alternating temperatures (Yan & Chen, 2020).

The stratification of dormant seeds is recommended for overcoming morphophysiological dormancy because the contact of the seed with a moistened

substrate reduces the influence of germination inhibitors due to their leaching (Bewley et al., 2013), this is what occurs, for example, in soil seed banks (Baskin & Baskin, 2014). Thus, it is assumed that during the stratification process of yerba mate seeds, leaching of germination inhibitors occurs (Mireski et al., 2019), allowing the seed to germinate. In the present study, although the occurrence of germination was verified in T1 and germination tests (Test 1 and Test 2), its percentage was low.

According to the data from the structural analyses carried out here, this low germination can be caused by the absence of the embryo in more advanced stages of development, such as torpedo and mature. Thus, even though stratification helps to remove the inhibitors, without the embryo developing to the mature stage, the germination process does not occur. And this fact may have contributed to an increase in the not full and non-viable seed percentage observed in the tetrazolium test (Tab. 6).

For many species, the exposure of seeds to high temperatures followed by low temperatures, simulating summer and winter temperatures, helps to overcome dormancy, but each species has specific condition requirements (Baskin & Baskin, 2014). Yerba mate seeds are dispersed during the summer-autumn and germinate, naturally, only in the following spring-summer (Galíndez et al., 2018), when the environmental conditions are adequate (Bewley et al. 2013; Finch-Savage & Footitt, 2017). Thus, in this species, the immature embryos have part of the autumn and all winter period to develop until the mature stage to germinate during the spring. For species that disperse seeds in the fall and show deep dormancy, ABA signaling and sensitivity are more important than the ABA amount (Finch-Savage & Footitt, 2017).

In this work, the stratification period was from March to September, so the seeds were exposed to both low (minimum mean temperature of 8.2°) and high (maximum mean temperature of 32.5 °C) temperatures. The stratification treatment concluded in early spring. For temperate or subtropical climate species, as in the case of yerba mate, overcoming dormancy is slow, during the winter period, to avoid germination during short periods of slightly higher temperatures, leaving the seeds only able to germinate

in the spring (Bewley et al., 2013). This was identified in this work, as embryos in mature stages were only verified after 150 days (five months) of stratification.

Exposure of seeds to low temperatures can help to reduce the endogenous content of ABA, promoting an increase in gibberellin and cytokinin levels. These two interact sequentially to overcome dormancy (Bewley et al., 2013). This fact contributes to overcoming physiological dormancy, while the morphological dormancy is overcome during higher temperature cycles, favoring embryonic development (Geneve, 2003).

A common feature of environmental signals related to the depth of dormancy and its overcoming is the transcriptional effect of the CYTOCHROME P450 707A (CYP707A) gene family, whose members catabolize ABA, with the increase in these genes' transcription promoted by NO (Penfield, 2017; Yan & Chen, 2020). The corresponding decline in ABA levels allows for GA synthesis, and the following events lead to germination. Nitrate acts on the hormonal metabolism of the seed similarly to other environmental signals (Penfield, 2017). In addition, for the species *Sisymbrium officinale*, nitrate stimulated cell expansion in the embryo and induced testa rupture, accelerating water absorption (Toorop, 2015). For the yerba mate seeds studied here, this was noted in the larger T2 embryos, with cells showing greater cell expansion and differentiated tissues, which consequently culminates in germination, combined with higher mobilization of reserves, mainly proteins.

The yerba mate seeds undergo dormancy even when the seed is connected to the mother plant, and the embryo is in the cordiform stage, considered immature (Heuser, 1999). In newly collected yerba mate seeds, the majority show cordiform stage embryos (70.24%), followed by late cordiform stage (23.6%) and a lower percentage in torpedo stage (2.6%) and mature (0.96%) (Niklas, 1987). For other Ilex species, a low mature embryo percentage (<10%) was also observed (Tsang & Corlett, 2005), which is a characteristic of species of the Aquifoliaceae family (Baskin & Baskin, 2014).

In many cases, the mature seed endosperm is also involved in germination control, serving mainly as a barrier to root development. When germination is

stimulated, several enzymes are activated, the micropylar endosperm weakens, allowing the radicle to expand to adjacent tissues (Linkies et al., 2010). In the seeds studied here, two aspects were verified. One of them is a decrease in the width of the micropylar endosperm cells from the torpedo stage on. The other is an increase in the digestive cavity. According to Heuser et al. (1993) the corpuscles presente in the endosperm tissue are constituted of lipids and proteins.

The primary root protrusion only occurs after two events. The first one is when the embryo reaches the pre-determined length for the species. The second is when the micropylar endosperm has been sufficiently degraded (Bewley et al., 2013). These characteristics were observed in embryos classified as mature, with a defined hypocotyl-radicle axis, digestive cavity with degraded cells, and few protein corpuscles in the endosperm cells. These variations were observed in the torpedo and mature embryos after 150 days of stratification for the T2 treatment. For seeds that presented embryos in the mature stage, the endosperm proteins are in less quantity and more agglutinated since they were mobilized to be used in embryo development.

The presence of a narrow integument and a woody endocarp, as observed in electron microscopy and photomicroscopic images, is treated as a common characteristic in drupoid fruits, in which the seed protection function is performed by the endocarp (Souto & Oliveira, 2005). In this work, it was possible to observe the presence of several layers of lignified cells in the pyrene endocarp. This aspect is highlighted by some authors as responsible for preventing water from getting inside the yerba mate seeds (Grigoletti-Júnior et al., 1999), hindering tissue expansion and, consequently, embryo growth (Dolce et al., 2010).

The participation of saprophytic fungi, under natural conditions, acts on the endocarp decomposition of the yerba mate pyrenes, favoring germination (Grigoletti-Júnior et al., 1999). For the pyrenes evaluated in this work, the presence of fungi in the endocarp was verified in all stratification treatments, however, germination was not observed in all treatments. The fungi acting on the disruption of the endocarp

cell walls can favor the hydration of the seeds and facilitate the entrance of chemical elements, such as the nutrients present in the T2. However, the presence of fungal hyphae alone did not directly influence embryonic development and consequently did not favor germination.

The presence of fungi can lead to seed rot and death (Souza et al., 2019), a fact evidenced at the end of the experimental period for both germination tests (Test 1 and 2), as the percentage of not-full seeds (empty + deteriorated) considerably increased. Before beginning stratification, the batch showed 50% of not-full seeds, of which only 2% were empty. Thus, the increase was of deteriorated seeds, as during all treatments in the tetrazolium test, the presence of seeds with gelatinous endosperm, without firm consistency, with color changes, often yellow or darkened, were verified. There was also an increase in the number of necrotic embryos, with blackened color.

As previously mentioned, stratification helps remove germination inhibitors, however, embryo development takes a certain period. Such embryo development is also reported for three other *Ilex* species from Taiwan (LIU et al., 2023). Faced with the increase in the amount of deteriorated seeds, the overcoming of physiological dormancy, probably by removing substances that inhibit germination, may have occurred before morphological (mature embryos) overcoming, which may have contributed to the seeds' degradation and death.

Such embryo development is also reported for three other Ilex species from Taiwan (Liu et al., 2023). The fact that T2 showed a higher percentage of unhealthy seeds at the end of the experimental period is because most of the seeds that were able to germinate (38% of initial viability by tetrazolium test) germinated during the experiment (germination in stratification, Test 1 and 2) and the others died, increasing the percentage of not-full seeds.

The use of KNO_3 is widely recommended by the Rules for Seed Analysis (RSA) to be used to overcome dormancy in a wide range of species (BRASIL, 2009). Although

studies performed using KNO_3 to overcome dormancy in I. paraguariensis seeds during stratification, the association of alternating temperature and light obtained a low germination rate (4.2%) (Cuquel et al., 1994). In the present work, the use of controlled-release fertilizer favored nutrient supply throughout the experimental period, mainly nitrate. This component contributed positively to the development of embryos and culminated in germination. However, there is a possibility that other nutrients present in the fertilizer are involved in the processes of overcoming dormancy and germination, especially because scientific investigations of this theme are rare.

The data obtained here show a higher occurrence of late cordiform stage embryos in T1 and T2, confirming the positive effect of stratification in sand. In addition, the observation of torpedo stage and mature embryos only in T2, with the effective influence of the sand substrate, shows the efficacy of the use of the controlled-release fertilizer to stimulate embryonic development in *Ilex paraguariensis* seeds. This fact is also corroborated by the germination percentage verified in T2.

Given the results obtained in the present study, it is possible to combine chemical substances to encourage germination of *Ilex paraguariensis* seeds, but more studies are needed regarding seed conditioning for three reasons: firstly, to increase the viability of seeds in the batch, so more fertilizer concentrations can be tested later on, secondly, to verify which dose favors greater embryonic development in a shorter period and lastly, to investigate the effect of isolated nutrients to explore the contribution of each one to this process.

Considering the data collected in this work, it is concluded that there is heterogeneity in embryonic development in seeds under the same variables of maturation. Also, the reduction in time for embryo development and the consequent germination seems to depend on specific environmental conditions, including a source of nutrients over the period.

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Authorship contributions

1 – Rosani Klein Reinke

Master in Conservation and Management of Natural Resources from the State University of Western Paraná UNIOESTE
https://orcid.org/0000-0001-8637-4019 • rosanikr@gmail.com
Contribution: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

2 – Mara Cíntia Winhelmann

PhD in Phytotechnics, Federal University of Rio Grande do Sul - UFRGS.
https://orcid.org/0000-0003-3431-9442 • mcwinhelmann@universo.univates.br
Contribution: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

3 – Léo Jaime de Vargas

Graduated in Biological Sciences - Degree (Univates)
https://orcid.org/0000-0001-7764-5008 • leo.vargas@universo.univates.br
Contribution: Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

4 – Júlia Gastmann

Master in Biotechnology from the Postgraduate Program in Biotechnology at the University of Vale do Taquari - Univates
https://orcid.org/0000-0003-3941-9493 • julia.gastmann@universo.univates.br
Contribution: Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

5 – Elisete Maria de Freitas

PhD in Botany Federal University of Rio Grande do Sul, UFRGS
https://orcid.org/0000-0002-9292-1557 • elicauf@univates.br
Contribution: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

6 – Claudimar Sidnei Fior

PhD in Phytotechnics, Federal University of Rio Grande do Sul, UFRGS
https://orcid.org/0000-0001-9893-081X • csfior@ufrgs.br
Contribution: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

7 – Shirley Silva Martins

PhD in Biological Sciences Universidade Estadual Paulista Júlio de Mesquita Filho, UNESP.
https://orcid.org/0000-0002-7696-8865 • shirley_botany@yahoo.com.br
Contribution: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization Writing – original draft, Writing – review & editing.

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