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Original Paper

Causes of dormancy in *Ilex paraguariensis* pyrenes

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Abstract

Ilex parguariensis pyrenes, popularly known as "yerba mate", were classified as dormant. This study aim to investigated the causes of dormancy in *Ilex paraguariensis*' pyrenes. Hence, the following tests were performed: a) Physical dormancy: rate of absorption in water and methylene blue; detection of lignin and lipophilic compounds in the endocarp and integument; b) Physiological dormancy: bioassays in lettuce seeds, detection, and quantification of phenolic compounds; c) Morphological dormancy: embryo analysis. For the absorption rate, an increase was observed in the mass of the pyrenes, however, when imbibition was performed in methylene blue, absorption only occurred in the endocarp. Lignin was also observed in the form of a sclerenchyma layer next to the seed coat. Similarly, lipophilic compounds were observed in a layer, external to the endosperm. The bioassays with lettuce seeds indicated the presence of chemical inhibitors. In the morphological evaluation of the pyrenes, only 55.5% of the embryos were visualized and they were in the globular or heart stages. *Ilex paraguariensis* pyrenes have combined dormancy: physical (not water absorption), morphological (due to the underdeveloped embryo), and there are shreds of evidence about physiological dormancy (presence of inhibitors); however, it is recommended to investigate the inhibitory agent.

Key words: combined dormancy, *Ilex paraguariensis*, morphological dormancy, physical dormancy, physiological dormancy.

Resumo

Pirênios de *Ilex parguariensis*, popularmente conhecida como erva-mate, foram classificados como dormentes. O objetivo deste estudo foi investigar as causas da dormência dos pirênios de *Ilex paraguariensis*. Para isso, foram realizados os seguintes testes: a) Dormência física: taxa de absorção em água e em azul de metileno; detecção de lignina e compostos lipofílicos no endocarpo e tegumento; b) Dormência fisiológica: bioensaios em sementes de alface, detecção e quantificação de compostos fenólicos; c) Dormência morfológica: análise embrionária. Para a taxa de absorção, observou-se um aumento na massa dos pirênios, porém, quando a embebição foi realizada em azul de metileno, a absorção ocorreu apenas no endocarpo. A lignina também foi observada na forma de uma camada de esclerênquima próxima ao tegumento da semente. Semelhantemente compostos lipofílicos foram observados em uma camada externa ao endosperma. Os bioensaios com sementes de alface indicaram a presença de inibidores químicos. Na avaliação morfológica dos pirênios, apenas 55,5% dos embriões foram visualizados e estavam nos estágios globular ou coração. Pirênios de *Ilex paraguariensis* possuem dormência combinada: física (não há absorção de água), morfológica (devido ao subdesenvolvimento do embrião) e há evidências de dormência fisiológica (presença de inibidores); Contudo, recomenda-se investigar os agentes inibidores.

Palavras-chave: dormência combinada, *Ilex paraguariensis*, dormência morfológica, dormência física, dormência fisiológica.

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Introduction

The yerba mate (*Ilex paraguariensis* A. St. - Hil.) is a tree species from Brazil, Argentina, Uruguay, and Paraguay, belonging to the Aquifoliaceae family (Carvalho 2003). The leaves are dried to prepare a traditional tea (mate tea), which is consumed by millions of people in South America (Oliveira & Waquil 2014; Bergottini *et al.* 2015). In recent years, the use and export of yerba mate by new sectors, such as the pharmaceutical and cosmetics industry, have been intensified; it means that many studies have been carried out on this species, due to its economic importance (Horbach *et al.* 2011; Santin *et al.* 2013). Although it has been used for several decades, some silvicultural problems persist, including low and distributed germination over time (Medeiros *et al.* 1999; Fowler & Sturion 2000).

The dormancy is an adaptation that prevents seed germination even when environmental conditions are favorable (Baskin & Baskin 2004; Finch-Savage & Leubner-Metzger 2006). Although dormancy is considered a survival mechanism of the species (Bewley *et al.* 2013), for plant production, it can be a problem - since it is challenging to obtain seedlings.

In the literature, the *I. paraguariensis* pyrenes, were classified as dormant; however, there is still no consensus on the causes of this dormancy. Grigoletti Júnior *et al*. (1999) affirm that the pyrenes have physical dormancy, which is overcome by saprophytic fungi's action, under natural conditions. Physical dormancy is defined as that in which the integument and / or the endocarp are impermeable (Baskin & Baskin 2004). According to Dolce *et al.* (2010), the integument and pyrenes of the *I. paraguariensis* are resistance to the expansion of the tissues (mechanical dormancy), making the germination difficult.

Other authors describe the dormancy of *I. paraguariensis* pyrenes as a morphological dormancy (Niklas 1987; Sansberro *et al.* 1998; Menegueti *et al.* 2004). This classification is due to their embryo immaturity, which requires an additional period to complete their development and germinate. The mature pyrenes are detached from the parent plant before the embryos are morphologically mature, with the maturation of embryos ending after dispersion (Malavasi 1988).

Combined dormancy was also reported for *Ilex* genus by Baskin & Baskin (2014), and specifically for *I. paraguariensis*, as morphological and physiological (morphophysiological) dormancy (Cuquel *et al*. 1994; Galíndez *et al*. 2018). Physiological dormancy occurs when there are inhibitory substances or absence of substances that promote germination, preventing it from occurring (Baskin & Baskin 2004). Thus, the germination only happens after the inhibitory substances have been removed, for example, through the digestive tract of animals (Zaidan & Barbedo 2004). It was also reported morphological and physical dormancy in these pyrenes (Medeiros 1998).

The definition of the cause(s) of dormancy is fundamental for the efficient methods to overcome it. The objective of this study was to investigate the cause(s) of dormancy in *Ilex paraguariensis* pyrenes.

Material and Methods

Mature fruits with a dark purple coloration (color 2,5/1 F: 5Y of the Munsell Color Chart) of *Ilex paraguariensis* were harvested in 2016, under screens installed below the matrices. The harvest was carried out in the altitude of 906 m, where the climate is warm and temperate, with a Cfb classification - according to Köppen and Geiger - and 16.7 °C average temperature. The average annual rainfall is 2045 mm (Climate 2016).

After the harvest, the pyrenes were extracted, using a strainer and running water, benefited by using a blower to remove impurities; later, they were used in the analyses. Initially, the water content of the pyrenes was determined by the greenhouse method at 105 ± 3 °C for 24 h, according to ISTA (2007).

To identify the causes of dormancy, the following tests were performed:

Physical dormancy

Absorption in water and methylene blue, lignin and lipophilic compounds detection

The water absorption was performed in four replicates of 100 pyrenes, which had their mass measured and submitted to imbibition in a transparent plastic germination boxes (11 × 11 × 3,5 cm) with three layers of paper towel, moistened with water 2.5 times the mass of the paper and at a temperature of 25 °C. The pyrenes' mass was measured every hour in the first 24 hours, every twelve hours on the second day, every twenty-four hours from the third to the fifth day. From the sixth day on, the measurement occurred every 48 hours. The absorption was calculated by the difference between the initial mass and the obtained mass.

The absorption also was evaluated in methylene blue solution 1% (Orozco-Segovia *et al*. 2007), in full pyrenes and scarified pyrenes with sandpaper, until the visualization of the endosperm. The pyrenes were immersed in a solution during 48 hours; later, they were longitudinally sectioned and analysed with stereomicroscope - zoomed up to 60x.

For the detection of lignin and lipophilic compounds, histochemical methods were used. The pyrenes were fixed in FAA70 for 72 hours and stored in 70% alcohol. After that, dehydration was carried out in an ethanolic series of 2 hours each (80%, 90% and 100%). The pyrenes were included in Leica® meta-acrylate glycol resin and placed in a pre-infiltration solution for 24 hours and 15 minutes in a vacuum chamber (-20 mbar). Afterwards, the pyrenes were packed in the infiltration solution for 72 hours, at 10 °C, and placed in the inclusion solution. After 48 hours, longitudinal anatomical sections were made with disposable blades in Leica RM 2025 microtome and 7 μm thickness.

For the lignin staining, 2% Phloroglucinol was used and for the reaction of lipophilic compounds, Sudam III; then, semi-permanent blades were made with stained glass. Later, the slides were evaluated to investigate the presence of staining reaction in lignin and lipophilic compounds - under an optical camera microscope.

Physiological dormancy

Bioassay on lettuce seeds, detection and quantification of phenols.

Lettuce seeds were submitted to germination tests in substrates moistened with hydroalcoholic and aqueous extracts of *Ilex paraguariensis* pyrenes. For the extracts preparation (Matos 1988), the pyrenes were separated in two parts: 1) endocarp; and 2) seed (integument, endosperm, and embryo). Then, they were dehydrated in a forced air circulation oven for 48 hours at 40 °C, until they became dry fragments with constant mass. The fragments were triturated in a knife mill, until the generation of powder; hence, from the powder, the extracts were made. In the hydroalcoholic extracts, 70% ethyl alcohol was added and left in the dark for seven days. Later, they were filtered in filter paper and the alcohol was evaporated in a rotavaporator at 100 rpm and 55 °C, until constant volume. Dimethyl sulfoxide (DMSO) was added to the extract and diluted with distilled water, with concentration of 15% for the seeds and 20% for the endocarps.

Water was added at 80 °C, from the powder to the aqueous extracts, and closed in a bottle for 24 hours. The extract was diluted with distilled water until the concentration of 30% for the seeds and 20% for the endocarp.

The germination test and treatments of lettuce seeds were performed, according to recommendations previously described by ISTA (2008). The lettuce germination was conducted in a BOD germination chamber, at 20 °C, with 4 replicates of 50 seeds, in paper filter substrate, which was moistened with 2.5 times the weight of the paper with the extracts. The first germination count was performed 4 days after the beginning of the test and the final count was 7 days after.

Germination tests of lettuce seeds on substrates moistened with distilled water (Witness-water) and DMSO (Witness-DMSO) were also carried out to evaluate the product's influence on seed germination. The germination was evaluated daily, to determine the germination speed index (IVG), until the final count; thus, the value was obtained using the Maguire (1962) equation.

The phenolic compounds detection were performed by histochemical analysis, using the inclusion of the pyrenes in resin (as described in the previous item for lignin and lipophilic compounds) and reaction with ferric chloride (Johansen 1940).

The phenolics compounds quantification was made from the seed (integument, endosperm, and embryo) and endocarp. The samples were macerated in a porcelain mortar using methanol and water (1:1). The solution was filtered, and the total phenol compound content was determined in a spectrophotometer with absorbance of 760 nm. Folin-Ciocalteu reagent was used for the staining reaction. Data were expressed as mg of Gallic acid, equivalent per gram. A calibration curve of Gallic acid (standard curve) was constructed to determine the total phenolic compounds content (Fig. 1).

Morphological dormancy

Morphological analysis of embryos.

Four replicates with 100 pyrenes were used, which were immersed in water for 24 hours, and later longitudinally sectioned, to evaluate the embryos' development. The evaluations were performed in Stemi-305 stereomicroscope with zoom up to 60x.

The embryos were classified considering the stages:

1. Globular: when the embryo is composed of a mass of cells; it is not possible to identify cotyledonary primordial.

2. Heart: it is possible identify cotyledonary primordial, where the shape resembles a heart (occupies approximately 10% of the length of the seed).

3. Post-heart: the cotyledons are already larger (occupies approximately 15% of the length of the seed).

4. Torpedo: cotyledons are differentiated (occupies approximately 20% of the total length).

5. Mature: the embryo is developed and completely differentiated (occupying approximately 40% of the length of the seed).

The experiments were conducted in a completely randomized design. To compare the means between treatments, the Tukey 5% probability test was used, using the software Assistat (Silva & Azevedo 2016).

Results

The water content of the pyrenes was 8.7%. During the experiment related to physical dormancy, an increase in the mass of the pyrenes exposed to the moistened substrate was observed, probably due to the absorption of water (Fig. 2).

Although the absorption in water was verified, it was observed, in the integral pyrenes immersed in methylene blue, that it is related only to the entrance of water in the endocarp (Fig. 3a). In the pyrenes that were scarified with sandpaper (Fig. 3b), the pigment entry in the endosperm and embryo were observed during the 48 hours of imbibition.

Anatomical sections: we can observe lignin's presence in the sclerenchyma layer next to the *Ilex paraguariensis* seed wrappers (Fig. 4).

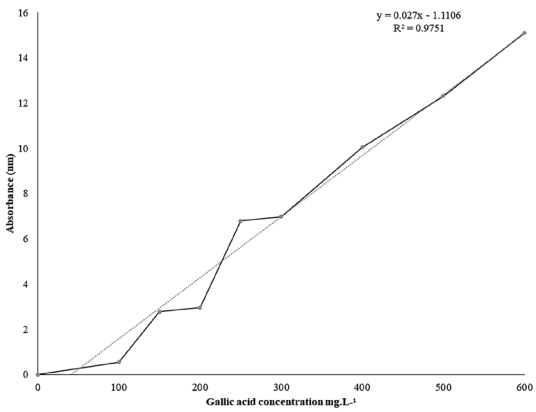

Figure 1 – Standard curve of Gallic acid.

Using the reagent Sudam III, in the histochemical tests, it was possible to identify a layer - external to the endosperm - of lipophilic compounds (Fig. 5).

Test of lettuce seeds germination with the aqueous extract: there was germination; however, it was slow compared to the control, differing statistically both from the IVG and from the first germination count. With the hydroalcoholic extract from the seeds and the endocarp, germination did not occur; hence, it may indicate the presence of chemical inhibitors (Tab. 1).

The control, with water or DMSO, in the first count, during four days, showed high germination (99% and 98%). This germination was statistically different from the aqueous extract of endocarp (90%), and different from the aqueous extract of seeds (integument, endosperm and embryo), that was 69% in the final count - seven days after the beginning

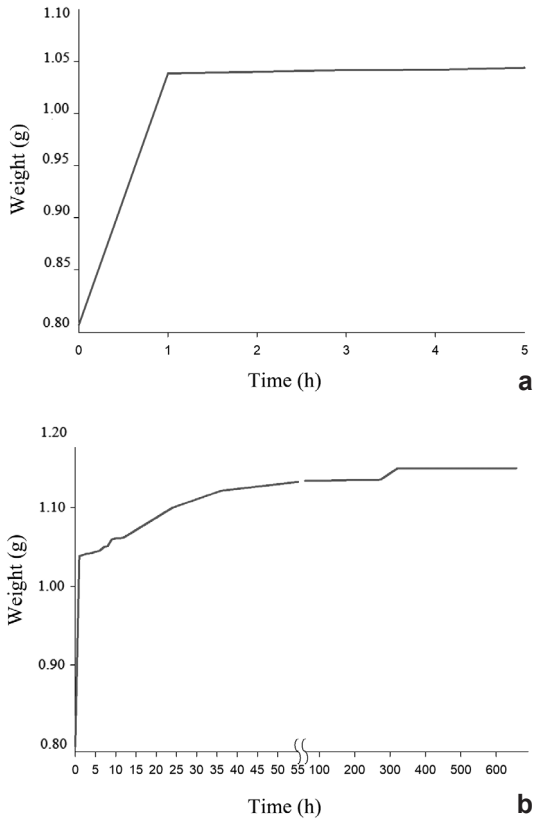

Figure 2 – a-b. Water absorption of the *Ilex paraguariensis* pyrenees based on mass increase (g) over time (h) – a. absorption in the first 5 hours; b. absorption in 600 hours.

of the test - the germination of the control with water or DMSO did not change. Seed germination with aqueous seed extract increased to 91% and endocarp extract to 98%. Both hydroalcoholic extracts did not encourage germination. The best number of IVG was the two controls, after aqueous extract of endocarp (8.87) and aqueous extract of seeds (6.25).

The presence of phenols in the endocarp and in the seed (integument, endosperm and embryo) was verified in the *Ilex paraguariensis* pyrenes, with the highest amount present in the endocarp (Fig. 6).

In the morphological evaluation was verified in that, in 55.5 % of the pyrenes, the embryos were in the globular or heart stage (Fig. 7); furthermore, the embryos were not visualized in the other stages (44.5 %).

Discussion

The water content of the pyrenes was 8.7%, similar to what was observed in other studies with this species (Souza & Silva 2001; Menegueti *et al.* 2004).

The increase in the mass of the pyrenes was 0.3 g in relation to the initial weight, exposed to the moistened substrate; in the first 24 hours, the mass increased 78%. In the integral pyrenes, immersed in methylene blue, the water entrance is related only in the endocarp. The absorption of the methylene blue by the pyrenes was followed during 48 hours, due to the observed results in the absorption of water (mass increase before that period). In the scarified pyrenes, the entrance of water occurred in the endocarp, tegument, and endosperm, in the first hours.

In studies with *I. latifolia* and *I. ronduta,* the pyrenes were immersed in distilled water and methylene blue (1%) for 48 hours. Staining was verified only in the endocarp and in the integument; however, the endosperm and embryo did not color (Tezuka *et al.* 2013). These results are similar to those observed in this study.

Physical dormancy in *Ilex paraguariensis* pyrenes was also observed by Grigoletti Júnior *et al.* (1999). The seeds integuments can cause dormancy, what is called physical dormancy (Baskin & Baskin 2004). The integument protects

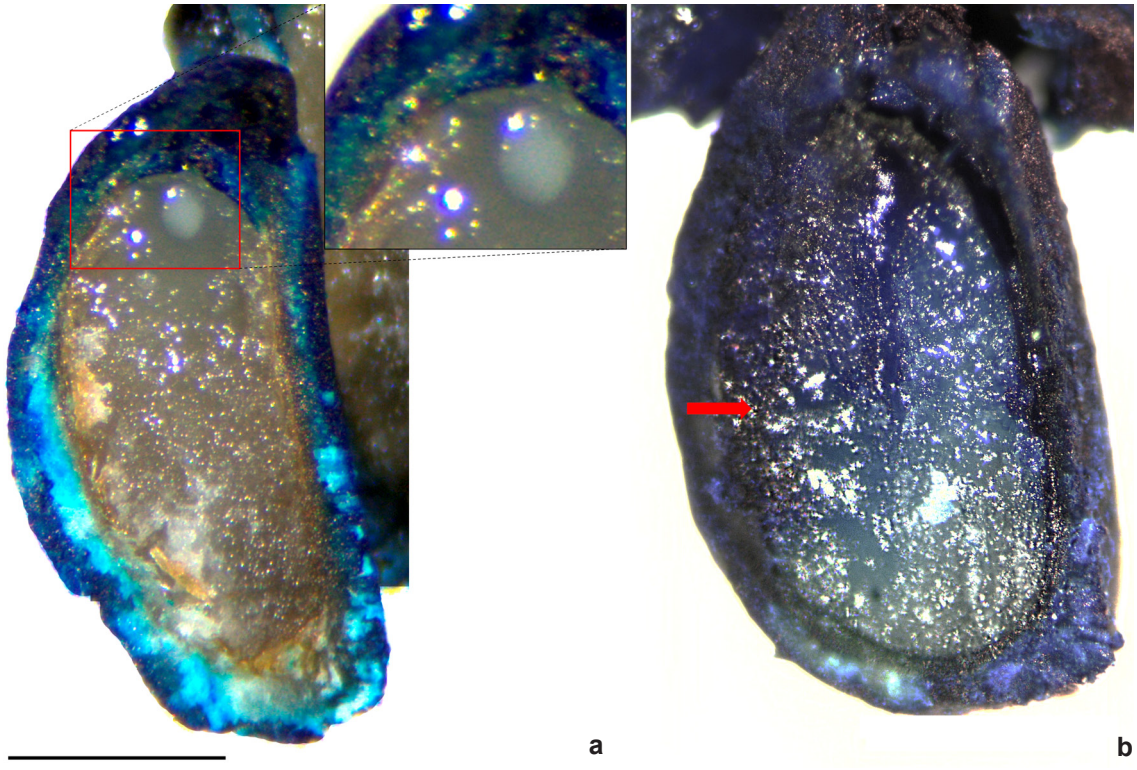

Figure 3 – a-b. *Ilex paraguariensis* pyrenees after immersion in 1% methylene blue solution for 48 hours – a. intact pyrene; b. sanded pyrene, the red arrow indicates the scarified location. Scale bar: 1 mm.

the embryo, besides regulating the absorption of water and oxygen (Zeng *et al.* 2004); however, the coating can infer dormancy by preventing the passage of water (Debeaujon *et al.* 2000) and gas exchanges or mechanically restrict the output of the radicle (Bewley 1997). Studies carried out by Dolce *et al.* (2010), about *I. paraguariensis* pyrenes, demonstrated that germination was only possible after the pyrenes were sectioned.

Physical dormancy may be caused by lipophilic compounds, such as: serous cuticle, suberin, lignin, paliçadic tissue, cutin and mucilages in the integument and/or pericarp (Perez 2004). In the histochemical tests, using the reagent Sudam III, it was possible to identify a layer of lipophilic compounds - external to the endosperm; this layer has not been described in the literature yet, in relation to *Ilex paraguariensis*. In *I. opaca*, Ives (1923) described a layer external to the endosperm, composed of suberin, followed by integument and endocarp; thus, this layer was formed by lignin and pectic substances.

The accumulation of suberin forms a coating, a barrier that prevents the entrance of water (Nawrath 2002). Suberin is composed of two distinct types of insoluble polyesters: fatty acid and glycerol (Spurný 1964). Genetic evidence show that suberin deposition controls seed permeability (Beisson *et al.* 2007).

However, the physical barrier is not the only obstacle to the germination process of *Ilex paraguariensis* seeds, since in germination tests of lettuce seeds with the hydroalcoholic extract with seeds and endocarp, germination did not occur; thus, it may indicate the presence of chemical inhibitors. In the aqueous extract, there was germination; nevertheless, it was slow compared to the control, differing statistically both from the IVG and from the first germination count. A factor to be considered is the solubility of the inhibitor(s) in alcohol, which may be used for the future identification of such inhibitor(s). It was observed that there was 98% germination of the lettuce seeds in the substrate moistened with the extractor (DMSO), indicating that it did not influence the results.

Lettuce seeds are used to examine allelopathy, since their sensitivity to secondary metabolites functions as allelochemicals (Ferreira & Aquila 2000). Allelopathic compounds may affect processes, such as: seed germination and seedling growth, nutrient assimilation, photosynthesis, respiration, protein synthesis, enzyme activity, and nutrient loss - through the effects on cell membrane permeability (Durigan & Almeida 1993).

In the same genus *Ilex*, Tezuka *et al.* (2013) indicated the presence of inhibitors in the endosperm, integument and/or endocarp, because isolated embryos germinated 100% in 8 weeks of incubation, confirming the results obtained in this work.

The presence of inhibitors in the germination process conditions promotes the occurrence of physiological type dormancy. Physiological

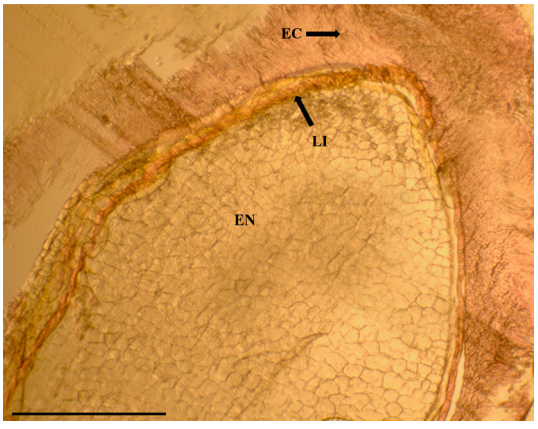

Figure 4 – Presence of lignin in *Ilex paraguariensis* pyrenees. Scale bar: 0.5 mm. EN = endosperm; LI = lignin; EC = endocarp.

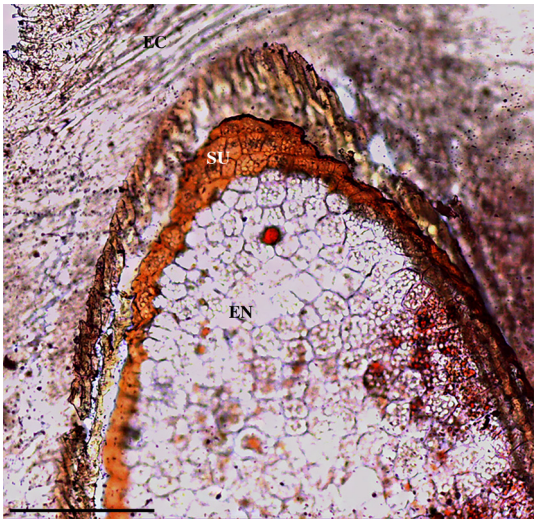

Figure 5 – Presence of lipophilic compounds in *Ilex paraguariensis* seeds. Scale bar: 0.5 mm. EN = endosperm; CL = lipophilic compounds; EC = endocarp.

Table 1 – Germination (%) and Germination Speed Index (IVG) of Lactuca sativa (lettuce), with extracts: aqueous 30% seeds (integument, endosperm, and embryo) and 20% endocarp; hydroalcoholic 15% seeds (integument, endosperm, and embryo) and 20% endocarp of the Ilex paraguariensis A. St. Hil.

Treatment	1st count (4 days) (%)	Final count (7 days) (%)	IVG
Witness - DMSO	98 ± 4.08 a	98 ± 4.08 a	11.19 ± 4.66 a
Ext. hydroalcoholic - seed (15%)	0 d	0 c	-
Ext. hydroalcoholic - endocarp (20%)	0 d	0 c	-
Witness - water	99 ± 2.02 a	99 ± 2.02 a	12.17 ± 2.94 a
Ext. aqueous - seed (30%)	69 ± 26.93 c	91 ± 2.20 b	6.25 ± 11.12 c
Ext. aqueous - endocarp (20%)	90 ± 5.74 b	98 ± 2.36 a	8.87 ± 10.20 b

Means followed by distinct letters in the columns differ from each other at a significance level of 5% in the Tukey test.

dormancy has been related to phytohormones, especially abscisic acid (ABA), or to secondary metabolism products, such as terpenes and phenolic compounds (Taiz & Zeiger 2002). The phenolic compounds can influence the germinative process, because they represent an obstacle to the diffusion of gases, whose function is to protect against leakage of solutes, imbibition damage and oxidative stress (Debeaujon *et al.* 2000; Marcos Filho 2015). These compounds are secondary and chemically heterogeneous - from the shikimic acid and malonic acid routes - with varying solubility, according to the compound (Taiz & Zeiger 2002).

The presence of phenols was verified in the *Ilex paraguariensis* pyrenes, with the highest amount present in the endocarp. In forest seeds of *Piptadenia macrocarpa* Benth, *Joannesia princeps* Vell. and *Dalbergia nigra* Vell., there was a variation of 0.329 to 1.342 $mg.g^{-1}$ of phenols in the integuments, and 0.016 to 0.495 $mg.g^{-1}$ in the seed embryos, indicating a lower concentration of these compounds in the internal tissues of the seeds (Maciel *et al.* 1992). With the bioassays, the presence of germination inhibitors was evident; however, other studies are necessary to identify them.

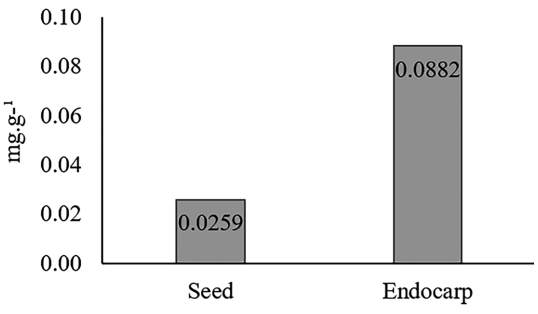

Figure 6 – Quantification of phenolic compounds in the endocarp and seed (integument, endosperm and embryo) of *Ilex paraguariensis*.

In addition to the physical barrier and inhibitors of germination present in the endocarp and seed, it was verified that, during the morphological evaluation and in 55.5% of the pyrenes, the embryos were in the globular or heart stage; nevertheless, embryos were not visualized in the other stages (44.5%). The predominant stages of *Ilex paraguariensis* embryos development - described in the literature - range from globular to post-heart (Niklas 1987; Fowler *et al.* 2007).

Immature embryos were also reported in other species of *Ilex* (Hu *et al.* 1979; Tsang & Corlett 2005; Chien *et al.* 2011), and it may be a common phenomenon for the genus. Studying the germination of immature embryos of *I. paraguariensis* in vitro, of fruits of different maturation stages, Ferreira *et al*. (1991) reported that there was interruption of embryo growth at the stage known as heart, when the fruits were ripe and could be a consequence of the presence of inhibitors in the endosperm, and possibly in the embryos themselves. On the other hand, Dolce *et al.* (2010) reported that there was no significant difference between percentages of germination of excised embryos and sectioned pyrenes, suggesting that the endosperm does not inhibit the development or germination of embryos. It is believed that the different causes of dormancy, in the same pyrene, are complementary, since the physics makes gas exchanges difficult and prevents the release and assimilation of the inhibitors, which hinder the development of the embryo.

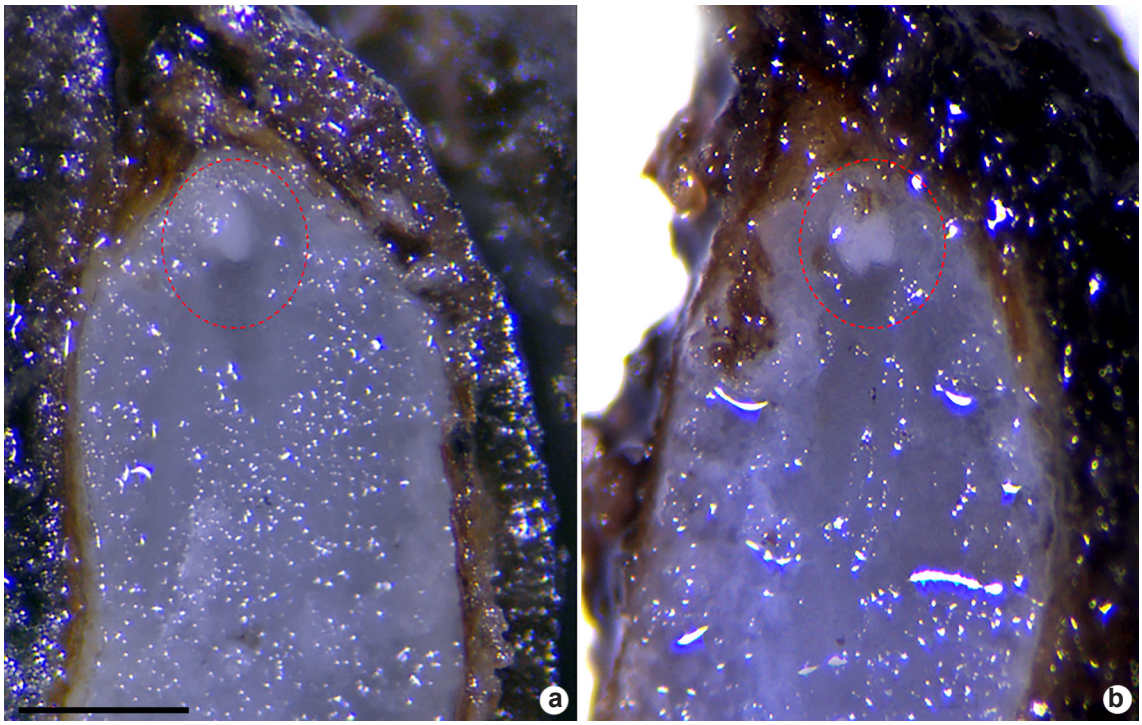

Figure 7 – a-b. *Ilex paraguariensis* pyrenees sectioned longitudinally with embryos – a. at the globular stage; b. at the heart stage. Scale bar: 0.5 mm.

Ilex paraguariensis pyrenes have combined dormancy: physical (not water absorption), morphological (due to the underdeveloped embryo), and there are shreds of evidence about physiological dormancy (presence of inhibitors); however, it is recommended to investigate the inhibitory agent.

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