Morphological aspects and embryonic development after the dispersion of pirenes of *Ilex paraguariensis* A. St. Hil

Anieli Cioato de Souza[1], Luciana Magda de Oliveira[2], Katiane Paula Bagatini[3], Louise Zago Puchale[4]*

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ABSTRACT

The objective of this work was to identify and describe the morphological aspects of the pyrene and the different stages of developing embryos of *Ilex paraguariensis*. The pyrenes were collected in the year 2016 and 2017, of ripe fruits. The pyrenes, a total of 400 per site and year of collection, remained for 24 h immersed in water for the section and internal observation. The pyrenes, with the visualized embryo, were used for the anatomical description and fixed for the histological evaluation. They were fixed in paraformaldehyde solution and included in Leica™ historesin. The samples were sectioned in Leica microtome with a thickness of 7 ì m, and the sections were stained with Toluidine Blue. The pyrenes are formed by endocarp, integument, endosperm, and embryo. The embryos were classified in the globular, heart (occupying approximately 10% of the length of the seed), post heart (15% of length), torpedo (20% of length), and mature (40% of length) stages; and were located below the micropyle, aligned to the external point. The region surrounding the embryo, called the cavity, consists of a lignified layer.

Keywords: yerba mate; embryo; seed; stages of development.

INTRODUCTION

Ilex paraguariensis A. St. Hil, known as yerba mate, is a tree species native to the Mixed Ombrophilous Forest in South America, whose leaves are widely used in medicine and consumed as tea (mate tea). The species is of great socioeconomic importance for the southern region of Brazil, Paraguay, and Argentina (Fowler & Sturion, 2000). Although this specie has been used for several decades, some silvicultural problems persist, among which, the germination is low (around 5%) and distributed over time (Cuquel *et al.*, 1994; Medeiros *et al.*, 1997; Fowler & Sturion, 2000).

The factors that influence germination, in general, is dormancy, related to physiological and structural aspects (Debeaujon *et al.*, 2007), such as the presence of mechanical, chemical, and physical barriers, as well as differentiation and maturation of the structures of the embryo, known as morphological dormancy (Baskin *et al.*, 2000; Hilhorst, 2007; Cardoso, 2008). Although morphological dormancy is already described in *I. paraguariensis* (Mello, 1980; Ferreira *et al.*, 1991; Heuser, 1999; Fowler & Sturion, 2000; Medeiros, 2001), the details of the stages of embryo development are scarce. Some works classify embryos of the species based on their sizes, such as Niklas (1987); Heuser (1993), Catapan (1998) and Mireski *et al.*, (2019). However, this classification may be subjective, because of the differences in the dimensions of the pyrenes, justifying the need for a classification using the space occupied by the embryo in the endosperm, and also the morphology of the embryo.

The embryo immaturity is one of the reasons for low germination and its distribution over time, only the embryos at the mature stage will be able to germinate, it is believed that research is needed to better describe the pyrenes, the location, and the different stages of development of the embryos, through histological studies and the evaluation of the space that it occupies on the endosperm.

In research carried out by Baskin & Baskin (2005), it was reported that to determine if small embryos (i.e. low

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[1]Universidade do Estado de Santa Catarina, Lages, Santa Catarina, Brazil. anielicioato@hotmail.com; louise_zp@hotmail.com
[2] Universidade do Estado de Santa Catarina, Programa de Pós-Graduação em Engenharia Florestal, Lages, Santa Catarina, Brazil. lucianda.oliveira@udesc.br
[3] Universidade do Oeste de Santa Catarina, Joaçaba, Santa Catarina, Brazil. katianebagatini@unoesc.edu.br
*Corresponding author: anielicioato@hotmail.com

embryo length: seed length ratio) in mature dwarf seeds (0.2–2 mm) are underdeveloped, they would grow (inside the seed) prior to germination, seeds to be considered with morphological or morphophysiological dormancy, as they observed in seeds of *Drosera anglica* (Droseraceae), *Campanula americana*, *Lobelia appendiculata*, L. spicata (Campanulaceae) and *Sabatia angularis* (Gentianaceae) that increased 0, 103, 182, 83 and 57%, respectively. According to the authors, since small embryos in dwarf seeds may or may not be underdeveloped, assignment of seeds to a dormancy class requires that studies be done to determine if embryos grow inside the seed before germination can occur. Such information is important in understanding the evolutionary relationship of the different kinds of seed dormancy.

The objective of this work was to identify and describe the morphological aspects of the pyrene and the different stages of developing embryos of *Ilex paraguariensis* and to propose a classification of the embryos based on the space occupied in the endosperm.

MATERIAL AND METHODS

Mature fruits, with dark purple coloration (color 2.5 / 1 F: 5Y of the Munsell Table) of *I. paraguariensis*, were collected in the years 2016 and 2017, under screens located below the trees, located in the municipalities of Campo Erê, SC, and Urupema, Santa Catarina, Brazil; a total of 15 seed trees. The first presents a warm and temperate climate, classified as Cfb, according to Köppen and Geiger, with an annual average temperature of 16.7 °C, with 2045 mm of annual average rainfall, and an altitude of 903 m (Climate, 2016). The second presents the same climate classification as the previous one but with an annual average temperature of 14.1 °C, with an annual rainfall of 1634 mm and an altitude of 1324 m (Climate, 2016).

After the harvest, the pyrenes were extracted, using a sieve and running water, benefited by the use of a blower to remove impurities and used in the analyzes. The water content was determined by the greenhouse method at 105 ± 3 ºC, for 24 h, according to ISTA (2007).

The pyrenes, a total of 400 per site and year of collection, remained for 24 h immersed in water for the section and internal observation. The pyrenes, with the visualized embryo, were used for the anatomical description and fixed for the histological evaluation. We discarded those in which the embryos were not found, the empty and the predated.

The morphological description was performed with Stereo Microscope Zeiss Stemi-305, with a camera coupled, with zoom up to 60x. The illustrations were done in drawing paper with a weight of 224 g / m² with a 6b graphite pencil.

For the histological evaluation, the pyrenes were fixed in paraformaldehyde solution (2.5%) in phosphate buffer (0.2M) at pH 7.3, in a ratio of 1: 1, for 24 hours at 4° C. After fixation, the material was washed in phosphate buffer three times for thirty minutes (Bouzon, 2006), and dehydrated in a three-minute thirty-minutes wash with aqueous solutions of ethanol in increasing concentrations (30%, 50% 70%, 90%, and 100%). Pre-infiltration of the samples was initially performed with a 1: 1 mixture of 100% ethanol and Leica ™ historesin for 12 hours; subsequently the material was infiltrated in pure historesin for 24 hours. The inclusion was performed with historesin and addition of polymerizer (Leica Historesin, Heidelberg, Germany) at room temperature for 2-3 h (Arnold *et al.*, 1975).

The samples were sectioned in the Leica microtome (RM 2125) with disposable slides. The 7 ìm thick slices were stretched at room temperature and placed directly on the slides on distilled water droplets and dehydrated in a heating plate at 37 °C, until the water slab was dried.

The sections were colored with Toluidine Blue (O'Brien *et al.*, 1965), which is a polychromatic coloration that detects pectic substances and lignin, and because of their coloration allowed better identification of the different stages of development of the embryos. The records of embryo structures were performed under a light microscope with Leica ICC50 HD coupled camera.

RESULTS

The water content of the pyrenes was 12% for the lots of Urupema city 2016 and 2017; and 9% for Campo Ere city 2016 and 2017. The dispersion organ of the species *I. paraguariensis* is the pyrene, which consists of the endocarp, tegument, endosperm, and embryo (Figure 1).

Near the endocarp, in the inner portion, there is the tegument, formed by lignin and pectic substances, being able to offer resistance to water entry (Figure 2).

There is a filled layer of spongy tissue, between the external pore and the micropyle (Figure 1). The cut, for the exposure of the embryo, must be in the longitudinal direction, that is, in the direction of the length of the pyrene, beginning in the external pore, which can be identified with the still intact pyrene. Next to the micropyle, in the lower portion, is the embryo (Figure 3).

In the histological analyzes of the lots used, were not identified globular stage embryos (Figure 4).

The embryo characterized in the heart stage (Figure 5) has a cellular organization, but it is not yet possible to differentiate the procambial elements that are the precursors of the conduction tissues. The protoderm and cotyledon primordia can be identified. The embryos measure 0.29 mm on average, occupying approximately 10% of the longitudinal length of the endosperm.

The post-heart embryo (Figure 6) has evident more organized transfer cells. The embryos measure 0.30 mm on average, occupying 15% of the length of the endosperm.

In the torpedo embryo (Figure 7) the embryonic axis and the cotyledons are well developed, the procambium is organized, with more pronounced differentiation of the procambial elements, and with well visible root apex cells. Embryos measure 0.5 mm on average, occupying 20% of the length of the endosperm.

The mature embryos (Figure 8) measure above 1.0 mm, occupying an average of 40% of the length of the endosperm, the cotyledons are developed, the procambium is distinguished. The initial cells of the root apex are very evident, with the thicker cell wall, covered by cells of more dense content, forming the hood. There is the differentiation of the conduction system, with long and narrow cells in the region of the vascular meristem and differentiation of conducting elements of the xylem.

During the histological analysis, the presence of a lignified layer was observed in the region around the embryo (Figure 9).

DISCUSSION

The water contents of the seeds coincide with those observed in other works (Souza & Silva, 2001; Menegueti *et al.*, 2004). The endocarp is osseous-woody and involves the seed (Kuniyoshi, 1983), similar with others *Ilex* species

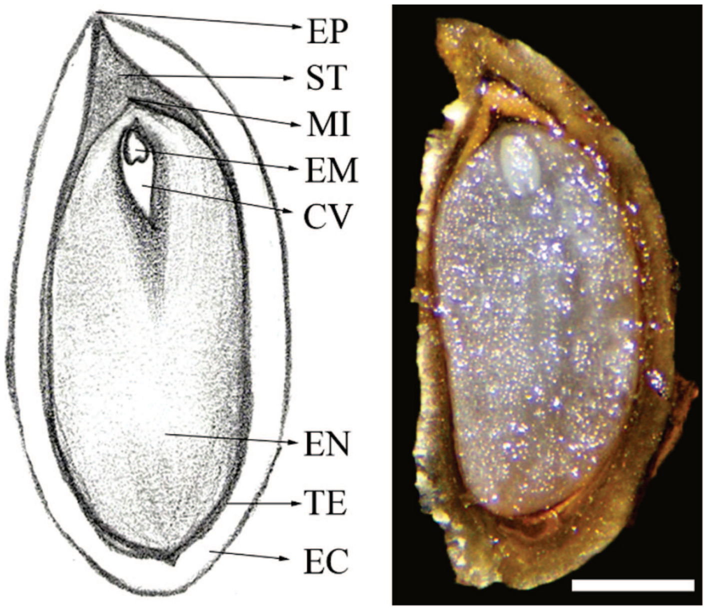

Figure 1: Anatomy of the pyrenes of *Ilex paraguariensis* A. St. Hil. EP - External pore; ST - Spongy tissue; MI - Micropyle; EM - Embryo; CV - Cavity; EN - Endosperm; TE - Tegument; EC – Endocarp.

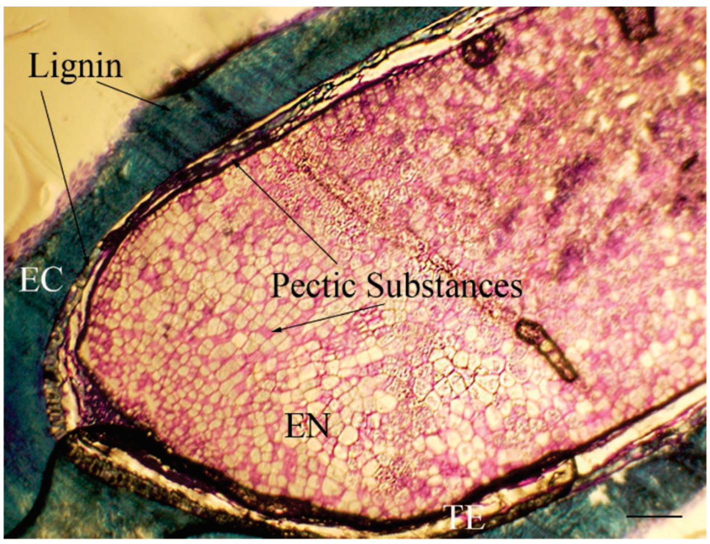

Figure 2: Histological section of *Ilex paraguariensis* A. St. Hil. pyrenes, with pectic substances and lignin. Scale bar: 0.2 mm. EN - Endosperm; TE - Tegument; EC – Endocarp.

(Ives, 1923; Hu, 1975), consisting of fibers that are arranged in a disorderly manner, presenting crystals between them, compound by lignin. The integument is a layer of sclereids with internal periclinal wall thickening and anticlinal periclinal; in the lower part, there is a layer of parenchyma formed by thin-walled flat cells (Heuser, 1993).

The fleshy, cream-colored endosperm surrounds the embryo (Kuniyoshi, 1983; Brasil, 2009). The species has a relatively small basal embryo, and when mature it occupies 1/3 to 1/2 of the lower portion of the seed; being rudimentary, not developed but differentiated (Martin, 1946; Baskin & Baskin, 2004) as described for the family Aquifoliaceae (Brasil, 2009). The slow development of the embryos and the low rate of germination are not directly related to the permeability of pyrenees, but pyrene as a mechanical barrier, interfering in the expansion of tissues (Dolce *et al.*, 2010). The spongy tissue between the outer pore and the micropyle was also verified by Kuniyoshi (1983).

The embryos of the mature pyrenes of *I. paraguariensis* may be at different stages of development, characterizing the morphological dormancy, as mentioned previously. The globular embryo (Figure 4) is characterized as a mass of disorganized cells, as described by Heuser (1993). The globular embryo, by size, was classified as less than 0.19 mm (Niklas, 1987; Heuser, 1993; Catapan, 1998); and 0.20 by Mireski *et al.* (2019).

The embryo characterized in the heart stage coincides with the description made by Bryant (1985), who reports that certain areas are already designed to develop into specific organs. The rudimentary forms of the organs themselves, particularly the cotyledons and the radicle, become distinguishable, this stage is known as the heart because it has two distinct lobes, having approximately 50 cells (Bryant, 1985). The embryo heart was classified according to size, with dimensions between 0.20 to 0.29 mm (Niklas, 1987); 0.20 to 0.45 mm (Heuser, 1993); 0.40 mm

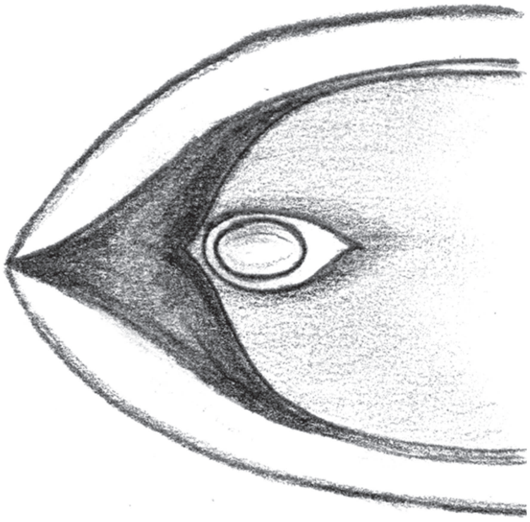

Figure 4: Pyrenes of *Ilex paraguariensis* A. St. Hil. with the globular stage of development.

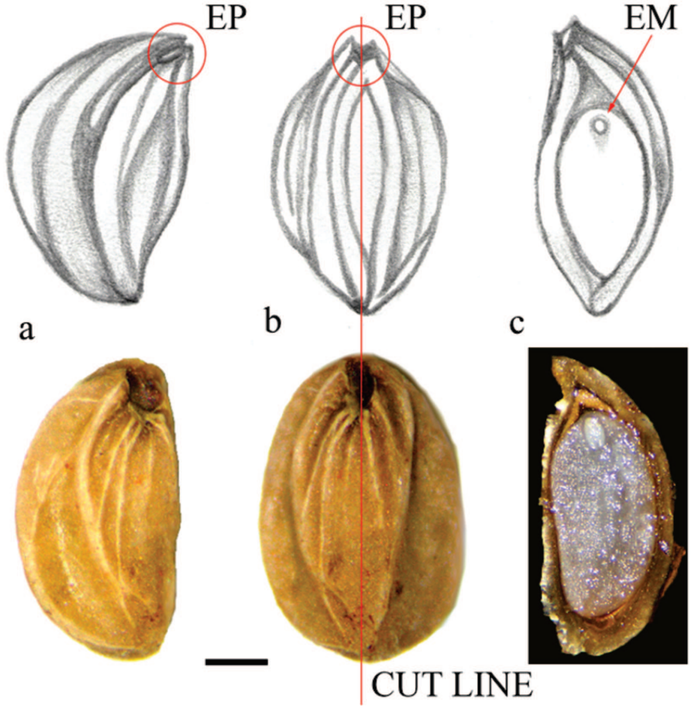

Figure 3: Location of the embryo in the *Ilex paraguariensis* A. St. Hil. pyrene A: Side view 1; B: Side view 2; C: Pyrene cut lengthwise. Scale bar : 0,5 mm. EP – External pore, EM - embryo.

(Mireski *et al.,* 2019); and 0.20 to 0.30 mm (Catapan, 1998), coinciding with the results obtained in this work.

The post-heart embryo (Figure 6) has evident, more organized transfer cells as described by Heuser (1993). The changes that occur represent an increase and maturation of the embryo (Bryant, 1985). Other authors classified post-heart embryo between 0.30 and 0.40 mm (Niklas, 1987); 0.30 to 0.50 mm (Catapan, 1998), coinciding with the results presented in this study; Heuser (1993) sizes of 0.45 to 0.70 mm; and 0.60 mm classified by Mireski

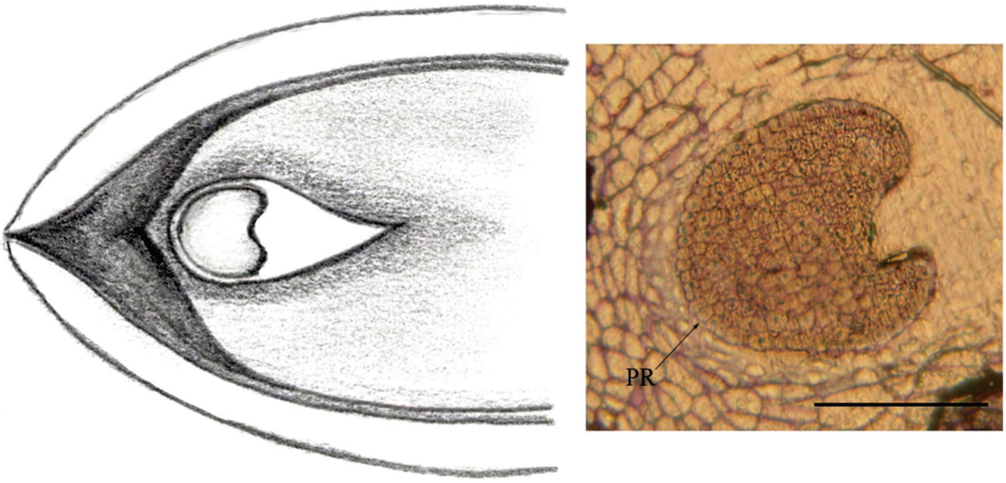

Figure 5: Pyrenes of *Ilex paraguariensis* A. St. Hil. with an embryo in the heart stage of development. Scale bar: 0.2 mm. PR- Protoderm.

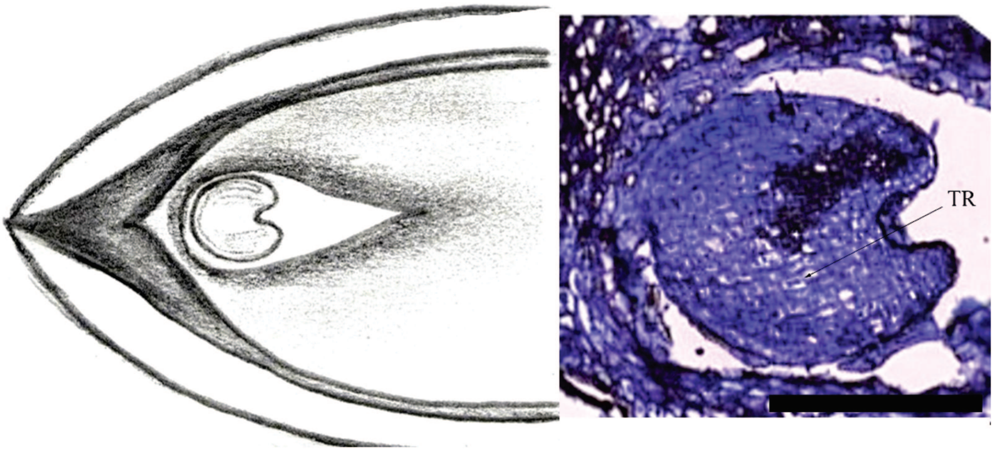

Figure 6: Pyrenes of *Ilex paraguariensis* A. St. Hil. with post-heart stage embryo development. Scale bar: 0.2 mm. TR- Transfer cells.

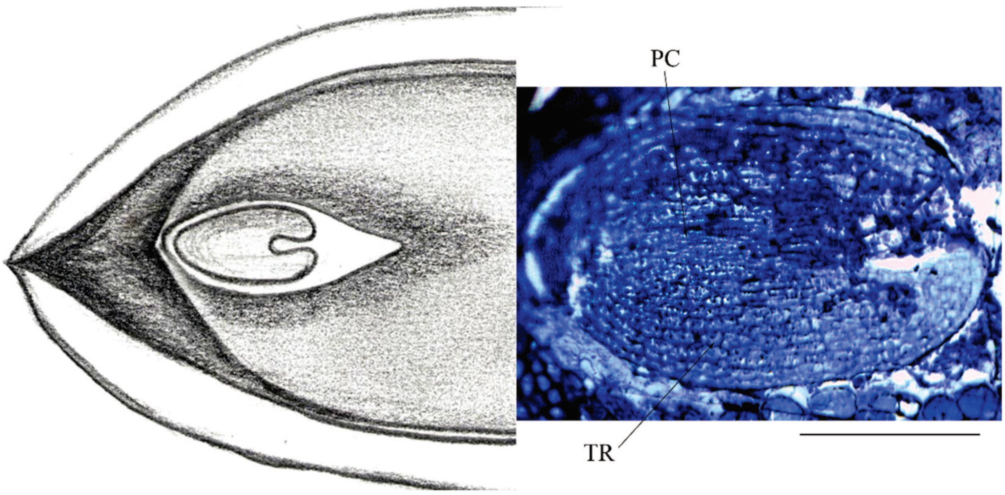

Figure 7: Pyrenes of *Ilex paraguariensis* A. St. Hil. with an embryo in the torpedo stage of development. Scale bar: 0.2 mm. TR- Transfer cells; PC- Procambium.

et al. (2019). The post-heart stage is a middle term between heart and torpedo, as there are some striking heart characteristics, such as cotyledonary primordia, as well as torpedo characteristics, such as transfer cells, with only small size, making it difficult their identification.

In the embryo torpedo (Figure 7), the cells that are in the division are organized in distinct meristematic regions, evidencing the meristematic tissue protoderm, fundamental meristem, and procambium (Bryant, 1985). Embryos measure 0.50 mm on average, according to the results presented in this study. In other studies, embryos in this stage measure between 0.40 and 0.80 mm (Niklas, 1987); 0.70 and 1.00 mm (Heuser, 1993); 0.50 and 0.70 mm (Catapan, 1998); 0.80 mm (Mireski *et al.,* 2019). Mature embryos (Figure 8) were classified as bigger than 1 mm (Niklas, 1987; Heuser, 1993); bigger than 0.9 mm (Catapan, 1998); bigger than 0.81 mm (Mireski *et al.* 2019), confirming the results obtained in this work.

The size of the embryos coincided, at all stages, with the classification by Niklas (1987) and Catapan (1998), but did not coincide with Heuser (1993) and Mireski *et al.* (2019) classifications. The classification by embryo size may be influenced by other factors, such as the seed size itself; in this way, it is believed that the use of the percentage of occupation of the embryo in the endosperm can be an alternative.

The descriptions of morphological characteristics of the stages of development of the embryos, confirm and corroborate those described by Heuser (1993). Embryos increase approximately 4 times the size, from the heart stage to the mature stage, as also verified by Galíndez *et al.* (2018).

The lignified layer, observed in the region around the embryo, was also observed in *I. maximowicziana* and called the cavity by Chien *et al.* (2011). This region, around the embryo, is originated by the lysis of cells of the endosperm, resulting from the action of enzymes, whose function is the opening of the space for the development of the embryo. The size of this cavity is not related to the stage of development of the embryo (Heuser, 1993).

During the evaluation of the embryos, it was found that 100% were immature, but at different stages of development. A hypothesis is that this difference in embryo maturation may be related to factors, such as seed moisture and environmental conditions during development. According to Mireski *et al* (2019), to explain the

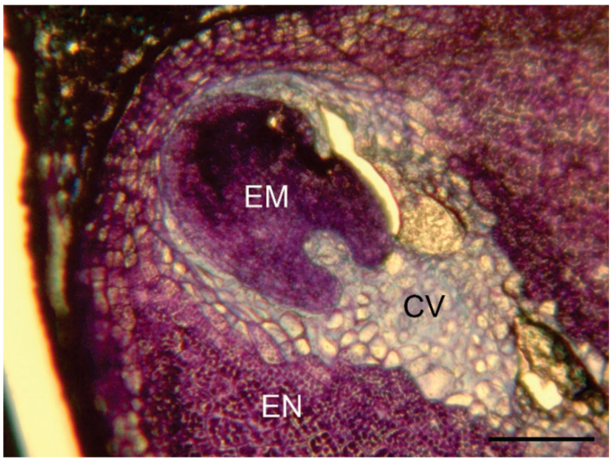

Figure 9: Pyrene of *Ilex paraguariensis* A. St. Hil., around the embryo there is the cavity containing cells with lignified walls. Scale bar: 0.2 mm. EM - Embryo; CV - Cavity; EN - Endosperm.

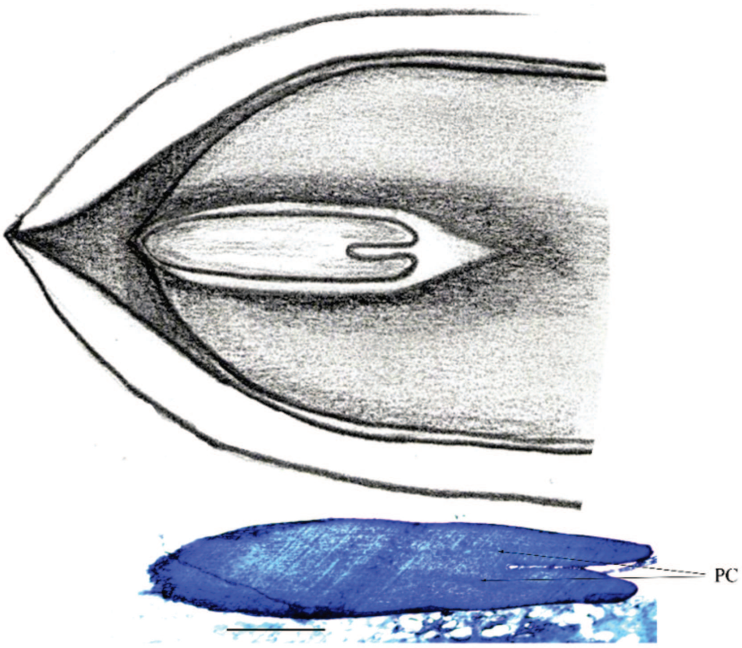

Figure 8: Pyrenes of *Ilex paraguariensis* A. St. Hil. with a mature embryo development stage. Scale bar: 0.2 mm. PC- Procambium.

dormancy problems in yerba mate seeds, there are numerous hypotheses, without, however, there being consistent data that lead to definitive conclusions. These authors found that the development of yerba mate embryos, represented by changes in shape, evolved as the time of exposure to artificial drying extended.

The differences in embryonic development in the seeds of I. paraguariensis, according to Fowler *et al* (2007), may also be due to environmental differences, especially due to the higher average annual temperatures and the greater number of hours of insolation.

CONCLUSIONS

The pyrenes are formed by endocarp, integument, endosperm, and embryo.

The stages of development of the *I. paraguariensis* embryo for definition is indicated beyond the anatomical analysis, the space that the embryo occupies in the endosperm. Embryos can be classified into the stages: globular, heart (occupying approximately 10% of the length of the seed), post heart (15% of length), torpedo (20% of length), and mature (40% of length) and are located below the micropyle, aligned with the external point of the pyrene.

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