

PHYSICAL CHARACTERISTICS, VIABILITY, AND SANITARY ANALYSIS OF *Ilex paraguariensis* SEEDS AND SEEDLING EMERGENCE FROM DIFFERENT MOTHER TREES

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Resumo

Características físicas, viabilidade e análise sanitária de sementes de Ilex paraguariensis e emergência de plântulas de diferentes árvores matrizes. O presente estudo objetivou analisar as características físicas, viabilidade e sanidade de sementes e a emergência de plântulas de nove matrizes de *Ilex paraguariensis*. Para a caracterização física foram determinados o peso de mil sementes, número de sementes por quilo e o grau de umidade. A viabilidade e desenvolvimento embrionário foram determinados a partir do teste do tetrazólio, com utilização de cinco repetições de 20 sementes para cada matriz. Na análise da emergência de plântulas, foram realizados três tratamentos: sementes não estratificadas, sementes armazenadas por 70 dias e sementes estratificadas por 180 dias, com três repetições de 2 g, semeadas em substrato comercial. A análise sanitária foi realizada pelo *Blotter-test*, com cinco repetições de 20 sementes não desinfestadas. Houve diferença significativa entre as matrizes para as variáveis físicas e biométricas. A viabilidade inicial das sementes foi de 52%, observando-se sementes nos estágios de coração e pós-coração. *Trichoderma* sp., *Aspergillus* sp. e *Penicillium* sp. foram os gêneros fúngicos com maior incidência nas sementes de erva-mate.

Palavras-chave: dormência embrionária; patologia de sementes; erva-mate; teste de tetrazólio.

Abstract

The present study aimed to analyze the physical characteristics, seed health, and seedling emergence of nine *Ilex paraguariensis* mother trees. Physical characterization included determining the thousand-seed weight, number of seeds per kilogram, and moisture content. Viability and embryonic development were determined by the tetrazolium test, using five replications of 20 seeds for each mother tree. Seedling emergence was evaluated under three treatments: non-stratified seeds, seeds stored for 70 days, and seeds stratified for 180 days, using three replications of 2 g, being sown in a commercial substrate. Sanitary analysis was conducted by *Blotter-test*, using five repetitions of 20 non-disinfested seeds. Significant results were observed among the mother trees for physical and biometric variables. The initial seed viability was 52%, with embryos in the heart and post-heart stages. The most prevalent fungal genera in yerba mate seeds were *Trichoderma* sp., *Aspergillus* sp., and *Penicillium* sp.

Keywords: embryonic dormancy; seeds pathology; yerba mate; tetrazolium test.

INTRODUCTION

Widely consumed and commercialized in southern Brazil and Mercosul countries, *Ilex paraguariensis* A.St.-Hil. plays a significant role in one of the most important extractive activities of Brazil (IBGE, 2021); especially because of the chemical compounds, its leaves are raw material for the production of a range of tonic and stimulating beverages, garnering growing interest from national and international markets (BARZOTTO; ALVES, 2013). In response to this increasing demand, establishing yerba mate plantations requires seeds sourced from selected plus trees with high genetic, sanitary, and physiological standards.

Laboratory germination and seedling emergence tests in nurseries are the most used methods to determine the viability of seed lots of forest species. On the other hand, the tetrazolium test has been applied as an alternative for determining viability, mainly because it is an easy and quick method of obtaining results (NOGUEIRA *et al.*, 2014), especially for species with seed dormancy, such as yerba mate. In addition to viability, in the tetrazolium test, we can determine the embryos' development stages, and this information may help planning and logistics in the production of seedlings, especially for species whose germination period is too long.

Propagation by seeds is the most used method of implantation or renewal of yerba mate plantations; however, the embryonic immaturity is frequently found in yerba mate seeds. In this case, successful production of yerba mate seedlings requires efforts to elucidate the embryonic immaturity in their seeds. Thus, basic studies, such as selection of mother trees and alternative methods of seed stratification, can provide subsidies for an effective seminal propagation (BITTENCOURT *et al.*, 2022; DUARTE *et al.*, 2023).

Another important aspect related to obtaining quality seedlings is the seed health. The presence of pathogens can directly influence in germination and seedlings development, causing anomalies, injuries, and even the seedlings' death. In addition, these microorganisms can cause seed deterioration and impair the results of germination and emergence tests, impacting the diagnose of physiological quality of seed lots (PIVETA *et al.*, 2010). In this way, the knowledge of the most frequent pathogens in yerba mate seeds contributes to adopting seed storage strategies and to improving seedling quality in the nursery.

Besides viability and health, the seed morphobiometric characteristics can be an important tool used in the selection of mother trees and the improvement of genetic programs (ARAÚJO *et al.*, 2015). Also, when combined with physical analyses, these studies provide useful information in yerba mate silviculture, allowing classification, standardization of seedling emergence, and standardization of size and vigor in seedling production (CARVALHO; NAKAGAWA, 2012). Hence, it reinforces the idea that to obtain quality seedlings with good development in the field, it is necessary to know the seeds characteristics of the species to be propagated. Thus, the primary objective of the study was to comprehensively assess the physical attributes, viability, and health of seeds, as well as the seedlings emergence from nine mother trees of *Ilex paraguariensis*. This evaluation encompasses their utilization for mother trees selection and seed quality tests.

MATERIAL AND METHODS

Provenance and seed harvest

Seeds from the 2017-crop were obtained from yerba mate producers in the states of Paraná, Santa Catarina and Rio Grande do Sul, and collected in nine mother trees: four from São Mateus do Sul (1PR, 2PR, 3PR and 4PR) (25°52'27" S and 50°22'58" W); three from Itaipópolis (1SC, 2SC and 3SC) (26°20'11" S and 49°54'23" W); and two from Chapada (1RS and 2RS) (28°03'19" S and 53°04'04" W). Each mother tree was considered a seed lot, which was received in April 2017 at Embrapa Florestas, Colombo/PR; the lots were stored in hermetically sealed glass, and placed in a cold chamber for 10 days (10 ± 3 °C and $25 \pm 3\%$ - relative humidity). The period between harvester and testing was approximately 30 days.

Physical analyzes

Physical characterization of each lot was carried out followed the Rules for Seed Analysis (BRASIL, 2009b). The thousand-seeds weight was determined from eight subsamples of 100 seeds, weighed on a precision scale (0.001 g). The number of seeds per kilogram was calculated from thousand-seeds weight; seed moisture content was obtained by the oven method at 105 ± 3 °C for 24 hours, with six replications of 25 seeds. Biometric characterization was performed with 30 seeds (randomly chosen) and we measured with a digital caliper the longitudinal length, width, and thickness, taken in the middle portion of the seed (accuracy of 0.05 mm).

Viability and embryonic development

The initial seed viability was determined by tetrazolium test (2,3,5-Triphenyltetrazolium chloride), conducted following Brasil (2009b - with adaptations) in four stages: 1) *pre-conditioning*: four replications of 25 seeds from each lot were immersed in distilled water and kept at 30 °C for 24h; 2) *exposure of the tissues for staining*: seeds were lengthwise cut, slightly exposing the endosperm; 3) *staining in tetrazolium solution*: excised seeds immersed in tetrazolium solution at 0.1% concentration, and kept at 35 °C for 24 hours, in the dark; 4) *evaluation*: using a stereo microscope and scalpel the seeds were classified as viable (embryo with pink or light red color) or non-viable (embryo with purple or white color and deteriorated seeds). For embryonic development evaluation, embryos were observed in stereoscopic microscope with a 100x magnification; embryo dimensions were measurements and classified into globular (0 to 25 mm²), heart (25 to 45 mm²), post-heart (45 to 60 mm²), torpedo (60 to 80 mm²) or mature (> 80 mm²) (MIRESKI *et al.*, 2019). For embryonic development stages, only viable embryos were analyzed.

Seedlings emergence

To carry out the seedling emergence tests, seed samples from each mother trees were subjected treatments:

- *Treatment 1* (non-stratified seeds): stored seeds in hermetic packaging, placed in a cold chamber (temperature of 10 ± 3 °C and relative humidity of $25 \pm 3\%$) for 10 days.
- *Treatment 2* (70-days stored seeds): a layer of seeds was placed in an open plastic tray, remaining in a cold chamber (temperature of 10 ± 3 °C and relative humidity of $25 \pm 3\%$) for 70 days.
- *Treatment 3* (stratified seeds): seeds were packed in fabric envelopes and placed between two layers of 4 centimeters of sand, allocated in perforated polyethylene boxes, and kept in a greenhouse with automated irrigation (three daily irrigations of 10 minutes, with an average flow of 56 L/h), without temperature control, for 180 days.

After each treatment, three repetitions of 2 grams were sown at a depth of 1 cm, in polyethylene boxes (perforated bottom) containing commercial substrate. The boxes were kept in a greenhouse with automated irrigation (three daily irrigations of 10 minutes, with an average flow of 56 L/h). From the emergence of the first seedling, evaluations were carried out for 60 days; the results were expressed in normal seedlings percentage (BRASIL, 2009b).

Sanitary quality

The seeds health quality was determined by *Blotter test*, using 100 seeds from each mother tree, freshly harvest and not disinfected (BRASIL, 2009a). Seeds were divided into five replications, placed in gerbox boxes previously disinfected with 1.0% (v/v) sodium hypochlorite solution, on sterilized blotting paper, and moistened with sterilized distilled water. For the control treatment, 100 seeds not disinfected from each mother tree were used; the seeds were disinfected with 1.0% sodium hypochlorite solution (v/v) for three minutes, and after washed in sterilized distilled water, and placed in a Petri dish containing PDA medium (Potato Dextrose Agar). Incubation was carried out in a room with controlled temperature (20 ± 3 °C), a photoperiod of 12 hours, for seven days. The fungal incidence was evaluated using a microscope and the identification of fungi was performed morphologically at the genus level.

Statistical analysis

The experiments involving physical analysis and embryonic characterization were carried out using a completely randomized design. The seedling emergence experiment was designed using a 3 x 9 factorial scheme. The data experiments were subjected to Bartlett's test and to Analysis of Variance (ANOVA). The means were compared using the Tukey test at a 5% significance level. For the sanitary analysis, fungal incidence was observed, and the results were expressed as percentages.

RESULTS

Physical analyzes

There were significant differences in all physical characteristics analyzed for the nine yerba mate mother trees (Table 1). For thousand-seed weight and number of seeds per kilogram, the matrices formed four distinct groups according to Tukey's test. Moisture content was not related to seed weight, exhibiting significant variation among parent trees. The dimension parameters revealed that the length presented the greatest variation, followed by the thickness and width of the seeds.

Table 1. Physical characteristic of seeds from nine *Ilex paraguariensis* A.St.-Hil. mother trees., thousand-seed weight (TSW), number of seeds per kilogram (N/Kg), seed moisture contents (SMC), length, width, and thickness.

Tabela 1. Características físicas de sementes de nove matrizes de *Ilex paraguariensis* A.St.-Hil., peso de mil sementes (TSW), número de sementes por quilograma (N/Kg), grau de umidade (SMC), comprimento, largura e espessura.

Mother tree	TSW (g)	N/Kg	SMC (%)	Length	Width (cm)	Thickness
1PR	8.56 a	116,795 e	9.11 d	4.12 ab	2.48 b	1.85 bc
2PR	7.22 b	138,579 d	11.62 bc	3.91 bc	2.33 b	1.82 bc
3PR	4.60 d	217,185 ab	11.42 bc	3.36 e	2.07 c	1.55 d
4PR	6.25 c	160,029 c	10.74 c	3.77 c	2.44 b	1.73 c
1SC	8.29 a	120,698 e	11.85 b	3.71 cd	2.46 b	1.75 c
2SC	4.98 d	200,622 b	11.91 b	3.48 de	1.91 c	1.50 d
3SC	4.91 d	203,770 ab	14.43 a	4.15 a	2.47 b	1.92 ab
1RS	4.57 d	218,902 a	11.02 bc	4.13 ab	2.74 a	1.97 a
2RS	7.21 b	138,749 d	10.66 c	4.19 a	2.83 a	2.00 a

Means followed by the same letters between the mother trees (columns) do not differ statistically by Tukey's test at a 5% probability level.

Viability and embryonic development

There were no significant differences in seedling emergence between non-stratified seeds and seeds stored for 70 days (Figure 1). The initial viability mean was 52%, and consequently, 48% were unviable and/or

deteriorated. Regarding embryonic development, we observed heart and post-heart stages, with no significant difference for both variables (Figure 1), with an overall mean of 46% and 54%, respectively. Moreover, no embryos in globular, torpedo, and mature stages were observed in any mother trees evaluated.

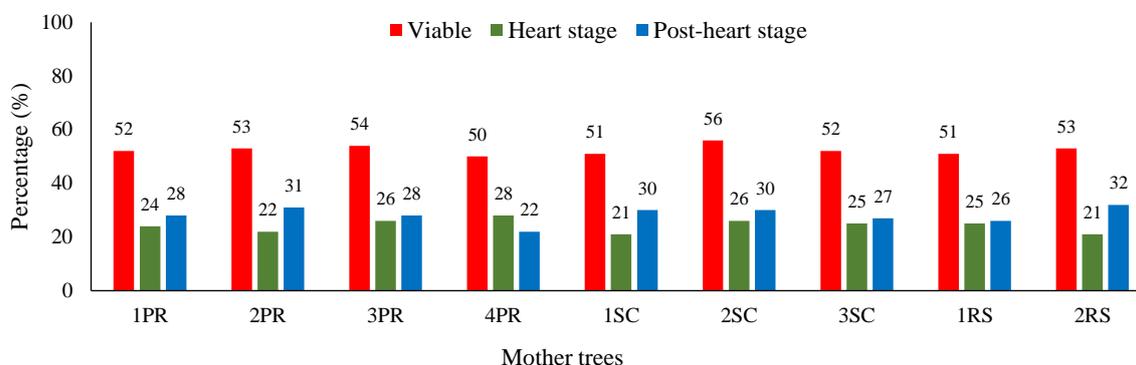


Figure 1. Viability and embryonic stage of seeds of nine *Ilex paraguariensis* A.St.-Hil. mother trees, analyzed by tetrazolium test.

Figura 1. Viabilidade e estágio embrionário de sementes de nove matrizes de *Ilex paraguariensis* A.St.-Hil., analisadas pelo teste de tetrazólio.

Seedling emergence

For non-stratified seeds, there was no emergence after two months of evaluation. Significant differences in seedling emergence were observed between non-stratified seeds and seeds stored for 70 days. The 70-day stored seeds exhibited seedling emergence percentages ranging from 1.96% to 3.99%, with no significant differences among mother trees. Regarding to stratified seeds, six mother trees demonstrated superior seedling emergence with values between 7.98% and 8.79% (Table 2).

Table 2. Seedling emergence percentage of nine *Ilex paraguariensis* A.St.-Hil. mother trees, at 60 days after seed pre-germination treatments.

Tabela 2. Porcentagem de emergência de plântulas de nove matrizes de *Ilex paraguariensis* A.St.-Hil. aos 60 dias após tratamentos pré-germinativos das sementes.

Treatment	Seedling emergence (%)								
	1PR	2PR	3PR	4PR	1SC	2SC	3SC	1RS	2RS
Non-stratified	0	0	0	0	0	0	0	0	0
70-days stored	2.0 Aa	2.50 Aa	1.96 Aa	3.99 Aa	3.31 Aa	3.12 Aa	3.66 Aa	3.47 Aa	3.36 Aa
Stratificated	8.79 Ab	8.27 Ab	5.95 Bb	7.98 Ab	8.39 Ab	4.84 Ba	5.49 Ba	6.39 Bb	8.16 Ab

Mean followed by the same uppercase letter between mother trees (rows) and lowercase letter between pre-germination treatments (column) do not differ statistically by Tukey's test at a 5% probability level.

Sanitary quality

Microorganisms' presence on yerba mate seeds was varied with 13 fungal genera observed in the nine mother trees evaluated (Table 3). Among these, *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp. was found in all mother trees. *Alternaria* sp., *Colletotrichum* sp., *Curvularia* sp., *Fusarium* sp., *Mucor* sp., *Nigrospora* sp., *Pestalotiopsis* sp., *Phomopsis* sp., and *Rhizopus* sp. were detected in varying of mother trees, each with different incidence levels. *Alternaria* sp. and *Cladosporium* sp. showed lower incidences of fungi in the contaminated matrices.

Table 3. Seed fungi incidence percentage in nine *Ilex paraguariensis* A.St.-Hil. mother trees.

Tabela 3. Porcentagem de incidência de fungos em sementes de nove matrizes de *Ilex paraguariensis* A.St.-Hil.

Genus	1PR	2PR	3PR	4PR	1SC	2SC	3SC	1RS	2RS
<i>Alternaria</i> sp.	2	0	0	0	4	1	2	1	1
<i>Aspergillus</i> sp.	28	24	45	12	27	15	14	18	22
<i>Cladosporium</i> sp.	0	0	0	0	0	0	0	1	1
<i>Colletotrichum</i> sp.	0	1	1	2	9	14	13	0	1
<i>Curvularia</i> sp.	1	1	0	0	0	2	2	2	1
<i>Fusarium</i> sp.	0	2	1	2	10	2	3	0	1
<i>Mucor</i> sp.	3	1	0	0	0	0	0	8	4
<i>Nigrospora</i> sp.	1	4	0	0	0	0	0	5	2
<i>Penicillium</i> sp.	20	18	27	16	19	18	19	15	14
<i>Pestalotiopsis</i> sp.	3	4	0	0	0	0	0	9	2
<i>Phomopsis</i> sp.	6	1	0	0	0	0	0	8	0
<i>Rhizopus</i> sp.	5	16	3	7	2	6	4	10	5
<i>Trichoderma</i> sp.	20	14	13	17	20	25	27	13	18
Control	11	14	10	44	9	17	16	10	28

Fungal potentially pathogenic, such as *Colletotrichum* sp., occurred in higher percentages in Itaiópolis/SC mother trees (1SC, 2SC and 3SC), ranging from 9 to 14%. Likewise, *Fusarium* sp. occurred in 10% of 1SC seeds. The genera *Cladosporium* sp., *Curvularia* sp., *Mucor* sp., *Nigrospora* sp., *Pestalotiopsis* sp., and *Phomopsis* sp., were observed, but those with lower incidence. For disinfected seeds (control treatment) was observed fungi presence in all mother trees (Table 3).

DISCUSSION

The results of physical analysis demonstrate significant divergences among parent trees, both within and between the three provenances, all originate from natural ecosystems. The seed size variables did not correlate with the thousand-seed weight, the number of seeds per kilogram, and moisture, indicating different seed densities among the matrices. These results confirm the higher phenotypic variation of seeds originating from wild areas due to environmental and genetic factors (BRÜNING *et al.*, 2011; WINHELMANN *et al.*, 2022). These aspects underscore the importance of seed analysis for commercialization, as such knowledge regarding variations in each mother tree directly influences the planning and execution of seedling production activities.

The variance observed in this study is like the results found by Winhelmann *et al.* (2022). Additionally, the physical characteristics of *I. paraguariensis* are similar to those of the species *I. venusta* and *I. pubiflora* (JIANG *et al.*, 2017; PRUESAPAN *et al.*, 2017). Among the physical variables, only the weight of a thousand seeds and the number of seeds per kilogram showed a correlation with seedling emergence in the stratification treatment; this response is because this variable indicates quality, reflecting a greater accumulation of reserves.

The average viability of the seeds of 52% corroborates with other studies carried out for wild seeds. Literature shows yerba mate seed viability values ranging from 40% (GALÍNDEZ *et al.*, 2018) to 66% (MIRESKI *et al.*, 2019). Duarte *et al.* (2023) observed 75% of viable seeds collected from a provenance and progeny tests. In relation to the embryonic stages, the results showed that mother trees presented only immature seeds, incapable of germinating immediately after the dispersion of the fruits, requiring adequate time and environmental conditions to complete the embryonic development; for this purpose, the stratification method is used in the production of yerba mate seedlings (DUARTE *et al.*, 2023).

The similarity among mother trees related to embryonic stages can be attributed to the low number of individuals evaluated. Regarding the embryonic stages, it is common to find embryos in globular, heart, and post-heart stages (FOWLER *et al.*, 2007; MIRESKI *et al.*, 2019). However, seeds with torpedo stage and mature embryos were found in the studies carried out by Niklas (1987) and Duarte *et al.* (2023). It is worth emphasizing the similarity between the embryonic stages, which can lead to classification and differentiation errors in the literature.

The results for seedling emergence are considered unsatisfactory from a silvicultural point of view. There are a few studies that report germination or seedlings emission, and those demonstrate low, irregular, and slow germination (CUQUEL *et al.*, 1994; FOWLER *et al.*, 2007; MIRESKI *et al.*, 2019; SOUSA *et al.*, 2020; WINHELMANN *et al.*, 2022); that corroborates the observed results in this study. Better results were obtained by

Cuquel *et al.* (1994) and Winhelmann *et al.* (2022), with 23.9 and 19.28% of emergence, both for seeds from mother trees phenotypically selected. This emphasizes the need to install seed production orchards, established with selected mother trees.

The present results for seedling emergence and those observed in the literature are attributed to seed embryonic dormancy. Two months after the stratification period, the maximum emergence observed was 8.79%. In initial embryonic development, there were 52% viable seeds, of which 54% had embryos in the post-heart stage, this would be indicative of a higher percentage of germination/emergence. In addition to embryo heterogeneity and slow germination, these results may be related to seed degradation by biotic or abiotic action during stratification (SOUZA *et al.*, 2020). Moreover, it can be assumed that stratification time and germination were not enough for seedling emergence. Given these results, it is evident the need to investigate dormancy overcoming methods and stratification time, and to select mother trees with greater seed viability.

Fusarium and *Rhizoctonia* genera are the main contaminating pathogens of yerba mate seeds, mainly in the process of fruit pulping and along the stratification time (POLETTI *et al.*, 2015). Furthermore, other studies also report incidence of *Penicillium* sp. in yerba mate seeds; they also showed incidence of *Trichoderma* genus (OLIVEIRA *et al.*, 2015). The pathogenic fungi incidence in the nine mother trees seeds studied can become a source of inoculum of these pathogens, especially for seedlings in the nursery, causing germination inhibition, fungus growth on cotyledons and primary leaves, in addition to radicle necrosis (RODRIGUES; MENEZES, 2002) which can reduce the number of normal seedlings able to become high quality and vigor plants. The presence of pathogenic fungi in the seeds highlights the importance of sanitary control in the production of seedlings.

CONCLUSION

- The seeds show variations in physical characteristics among the mother trees, but do not show distinctions in viability and embryonic stage; the heart and post-heart embryo stages were observed. In addition, seedling emergence varied subtly among mother trees.
- 13 fungal genera were observed in the yerba mate seeds, with *Trichoderma* sp., *Aspergillus* sp., and *Penicillium* sp. exhibiting the highest incidence.

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