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STRATIFICATION OF YERBA MATE SEEDS IN A COMMERCIAL NURSERY AND THE EMERGENCE MONITORING

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ABSTRACT - The propagation of *Ilex paraguariensis* A.St.-Hil. is carried out by seeds that have a low germination rate and morphophysiological dormancy. In order to increase the germination percentage, stratification treatments and emergence monitoring were tested. For this, yerba mate seeds were subjected to stratification treatments: Treatment 1 - seeds between two sand layers; Treatment 2 - seeds between two sand layers and commercial substrate covered with corn straw; Treatment 3 - seeds between two sand layers with the addition of 4.0 g L^{-1} of controlled-release fertilizer; Treatment 4 - seeds between two sand layers covered with corn straw; and a control treatment - seeds without stratification. The stratification period for all treatments was 180 days, and at 120, 150, and 180 days, 100 seeds were removed per repetition from each treatment for emergence tests, with weekly observation for 180-days in a completely randomized design. After that period, the tetrazolium test was performed with the seeds that did not emerge. At 120-days of stratification there was no emergence and at 150-days it was 0.83%. At 180-days, treatment 3 showed the highest percentage - 13.19%. Seeds without stratification do not germinate and after 180-days of emergence the amount of non-viable seeds increased in all treatments. Stratification in sand, combined with controlled-release fertilizer favors yerba mate seedlings emergence. After 180-days of emergence in germinator plants at 25°C, there is an increase in non-viable seeds amount. Seeds that did not undergo the stratification process do not germinate and present a higher dormant seeds percentage after 180-days of emergence.
Key words: *Ilex paraguariensis* A.St.-Hil., Aquifoliaceae, dormancy, tetrazolium test, viability.

ESTRATIFICAÇÃO DE SEMENTES DE ERVA-MATE EM VIVEIRO COMERCIAL E ACOMPANHAMENTO DA EMERGÊNCIA

RESUMO - A propagação da *Ilex paraguariensis* A.St.-Hil. é realizada por sementes que apresentam baixa taxa de germinação e dormência morfofisiológica. Com o intuito de aumentar a porcentagem de germinação foram testados tratamentos de estratificação e acompanhamento da emergência. Para isso, sementes de erva-mate foram submetidas a tratamentos de estratificação: Tratamento 1 - sementes entre duas camadas de areia; Tratamento 2 - sementes entre duas camadas de areia e substrato comercial coberto por de palha de milho; Tratamento 3 - sementes entre duas camadas de areia com adição de 4,0 g L^{-1} de adubo de liberação controlada; Tratamento 4 - sementes entre duas camadas de areia coberta com palha de milho; e um tratamento controle - sementes sem estratificação. O período de estratificação de todos os tratamentos foi de 180 dias, e aos 120, 150 e 180 dias foram retiradas 100 sementes por repetição de cada tratamento para os testes de emergência, com acompanhamento semanal durante 180 dias em delineamento inteiramente casualizado. Após esse período, com as sementes não emergidas foi realizado o teste de tetrazólio. Aos 120 dias de estratificação não houve emergência e aos 150 dias foi de 0,83%. Aos 180 dias, o tratamento 3 apresentou a maior porcentagem - 13,19%. Sementes sem estratificação não germinam e após 180 dias de emergência aumentou a quantidade de sementes não viáveis em todos os tratamentos. A estratificação em areia, aliada ao adubo de liberação controlada favorece a emergência das plântulas de erva-mate. Após 180 dias de emergência em germinador a 25°C ocorre aumento na quantidade de sementes não viáveis. Sementes que não passaram pelo processo de estratificação não germinam e apresentam maior porcentagem de sementes dormentes após 180 dias de emergência.
Palavras-chave: *Ilex paraguariensis* A.St.-Hil., Aquifoliaceae, dormência, teste de tetrazólio, viabilidade.

INTRODUCTION

The Aquifoliaceae family comprises trees, shrubs, and sub-shrubs, represented by the genus *Ilex* with more than 500 species, which occur in tropical and temperate regions. In Brazil, the genus is registered in five geographic regions (FLORA DO BRASIL, 2021), with 58 species (SOUZA; LORENZI, 2012) confirmed in 20 states (FLORA DO BRASIL 2021). The most popular species in the family is *Ilex paraguariensis* A.St.-Hil., known as yerba mate, mate, caá, among other names. In Brazil, its occurrence is registered from Mato Grosso to Rio Grande do Sul, where it predominates in Mixed Rain Forest areas, associated with *Araucaria angustifolia* (Bertol.) Kunzte

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(CARVALHO, 2003). The species also occurs in Argentina, Bolivia, Paraguay, and Uruguay (SOBRAL et al., 2013).

Like most species in the family, yerba mate is dioecious, the result of the abortion of one of the sporangia. Flowering occurs from September to December and fruiting from December to April, showing high heterogeneity in maturation (ZANON, 1988; PIRES et al., 2014). The fruit is the drupe type, usually with four pyrenes, with fleshy mesocarp and woody endocarp. Pyrene is the dispersion unit of the species, formed by the woody endocarp and seed, the latter is composed by integument, endosperm, and embryo (HEUSER et al., 1993). Yerba mate is widely consumed in the traditional form of chimarrão, tererê, and tea (COELHO et al., 2002), as medicine (CARDOZO-JUNIOR; MORAND, 2016; RIACHI; DE MARIA, 2017), in cosmetics (DALLABRIDA et al., 2016), dyes, preservatives, food supplements, among other uses (CROGE et al., 2021).

The propagation of *I. paraguariensis* is carried out mainly from seeds (WENDLING; SANTIN, 2015) which, in most cases, show low physiological and genetic quality, negatively affecting the production of quality seedlings, resulting in unequal plants with high mortality (FOWLER et al., 2007; WENDLING; SANTIN, 2015). Seeds also have low germinative power, unequal germination, and morphophysiological dormancy (MEDEIROS, 1998; DOLCE et al., 2010; WENDLING; SANTIN, 2015; GALÍNDEZ et al., 2018; SOUZA et al., 2020).

Morphophysiological dormancy is identified as one of the main causes of yerba mate's low germination rates (NIKLAS, 1987; WENDLING; SANTIN, 2015). The embryo is in an undifferentiated stage or incomplete development (globular to late cordiform stage) and the endosperm shows inhibitory mechanisms (BEWLEY et al., 2013; BASKIN; BASKIN, 2014), in addition to the presence of phenolic compounds in the integument (MIRESKI et al., 2018).

To overcome the two types of dormancy (morphological + physiological), stratification in sand is the most used and widespread technique among nursery workers. It consists in mixing the seeds in sand or in layers interspersed with it, keeping them in boxes or buckets for six months. Such needs demand a long period until the seedlings are formed, corresponding to at least 18-months. Besides, the germination is unequal, starting around 100 days and extending up to 360 days (CUQUEL et al., 1994; WENDLING; SANTIN, 2015).

In addition to stratification in sand, other techniques to try accelerating germination were tested. It is the case of seeds immersion in distilled water and coconut water for 24 h, immersion in hot water at 50 and 100°C for 30 min., storage under 5 and -5°C temperature in light absence for 24 h (SCHAPARINI; VIECELLI, 2011), 9°C pre-cooling for 30 days (MELLO, 1980), usage of different substrates (sand, vermicultite and agar) and temperatures (20, 25 and 30°C constant, alternating temperature 20-30°C) in the presence and absence of light (CATAPAN, 1998), stratification in sand at different temperatures (-12°C, -2°C and 25°C) (MENEGUETI et al., 2004), stratification with wetting of sand with water, gibberellic acid and potassium nitrate solution, with alternation of light and temperature (CUQUEL et al., 1994), chemical scarification with sulfuric acid and integument cut on the micropyle opposite side (MIOTTO, 2014). However, in all works cited, germination was less than 20%. Thus, to increase this germination percentage and accelerate seedling formation, different stratification treatments were tested to overcome seed dormancy in a commercial nursery located in Ilópolis, Rio Grande do Sul, with subsequent emergence monitoring in the laboratory.

MATERIAL AND METHODS

Although the species' dispersion unit is a pyrene, the term seed will be used through the work, representing a diaspore, consisting of endocarp and seed. Yerba mate seeds from a mother-plant located in Ilópolis, Rio Grande do Sul, Brazil (28º 53' 25" S 52º 08' 46" W) were used to carry out the work. In this region, the climate is *Cfb* (humid temperate and temperate summer) according to the Köppen and Geiger classification, with mean temperature of 17.1°C, and mean annual rainfall of 1689 mm (CLIMATE, 2020).

In January 2018, naturally dispersed (abscised) fruits were collected on plastic tarps arranged on the ground, in the tree's canopy projection, before their maturation began. After the fruits' collecting, they were taken to the laboratory and macerated under running water. The mass consisting of macerated pulp and seeds was submerged in a container with water and the supernatant seeds were discarded (WENDLING; SANTIN, 2015), with approximately 10% of the seeds discarded. Then, seeds dried under paper towels on a laboratory bench for five-days, when the water content was determined through the difference in the mass percentage after oven drying at 105± 2ºC for 24 h, using three repetitions of 0.5 g (BRASIL, 2009). The remaining seeds were stored in snap-cap type glasses with plastic lids and kept for four months under 5±2°C controlled temperature.

For the stratification test, four treatments were established: treatment 1 (T1) - seeds between two sand layers; treatment 2 (T2) - seeds arranged between two sand layers, with 1.0 L of sand at the tray bottom as the bottom layer and 500 mL of sand plus 500 mL of commercial substrate (composed of sphagnum peat, expanded vermiculite, dolomitic limestone, agricultural plaster, NPK fertilizer, and micronutrients) covered by 20 g of shredded corn straw as the second layer; treatment 3 (T3) - seeds between two sand layers with the addition of 4.0 g L^{-1} of controlled-release fertilizer Basacote Plus 6M® (16-8-12 + 2) to the top sand layer. The fertilizer used consists of nitrogen (8.6% in ammoniacal form and 7.4% in nitric form), phosphorus (5.6% P_2O_5), potassium (12% K_2O), magnesium (2% of 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); treatment 4 (T4) - seeds between two sand layers with the addition of 20 g of shredded corn straw on the top sand layer. To these treatments, a control (CT) was added - seeds with no stratification, stored a snap-cap type glasses with plastic lids, and kept under 5±2°C controlled temperature.

To establish the stratification treatments, white polyethylene trays (one per repetition, 192 x 116 x 64 mm) with perforated bottoms (9 holes of 0.5 cm diameter in each tray) were used for drainage. Sand was sieved and each layer thickness was approximately 3.0 cm (1.0 L each) and moistened at 60% of the water holding capacity (WHC). Trays were kept under the local nursery environmental conditions.

The design was completely randomized with four repetitions, each one with around 1,000 seeds. The stratification period of all treatments was 180-days, however, at 120, 150 and, 180 days, a 100 seeds sample was taken from each repetition of each treatment. Seeds of each time were transported to the laboratory, where they were washed with ultra-purified water (reverse osmosis apparatus) and autoclaved. Then they were submitted to germination in gerboxes containing 250 g of sieved and autoclaved sand, moistened with ultra-purified water, and autoclaved at 60% of the WHC, in a completely randomized design. The tests were carried out in Mangelsdorf type germinator plants at 25±1°C temperature, with no photoperiod control and at >90% relative humidity for 180 days.

The evaluations were carried out weekly, and seeds were considered emerged when they showed a visible vegetative structure above the sand surface. Emergence percentage calculation was performed based on viable seeds percentage in the lot, obtained through a previous study by tetrazolium test with a 100 seeds sample from the same lot (4 repetitions of 25 seeds). For the definition of viable seeds, the dead percentage, empty and predated seeds were discarded as soon as the experiment started. The lot's initial viability was 36.4%.

After 180-days of emergence evaluation for the three stratification times, a tetrazolium test was performed with seeds that did not germinate. For that, seeds were sieved and washed with ultra-purified water and autoclaved to remove the sand. Then, a longitudinal cut was made in each one with tweezers and a scalpel blade, followed by observation in a Leica EZ4 HD stereoscopic, with an increase of 20 to 30 times - in this stage empty and deteriorated seeds were discarded. The remaining seeds were submitted to 0.1% tetrazolium solution for 24 h at 35°C (CATAPAN, 1998) to determine viability by tetrazolium salt analysis. Seeds were evaluated for reaction to the solution and were considered viable those that showed endosperm stained with a visible embryo, with no damage and firm consistency. Seeds that did not have these characteristics were classified as non-viable (BRASIL, 2009).

The data on emergence percentage, viable and non-viable seeds percentage at 120, 150, and 180-days of stratification followed by 180-days of emergence were subjected to analysis of variance (ANOVA) and the means compared by LSD-Fisher test at the level of 5% probability of error by the statistical programs Costat 6.4 and Sigmaplot 11.0. The viable and non-viable seeds percentage at 180-days of stratification did not attend the ANOVA assumptions, so they were transformed to $\sqrt{x}$ e $\frac{\text{Asen}}{\sqrt{100}}$, respectively.

RESULTS AND DISCUSSION

Seeds showed 10.4% of water content and the analysis of variance showed that there was an interaction (p<0.001%) between treatments and stratification times for the emergence percentage (Table 1). After 120-days of stratification, there was no emergence, while at 150-days the emergence mean percentage was 0.83%. The stratification treatments in sand (T1), sand with fertilizer (T3), and sand covered with corn straw (T4) did not differ between themselves. After 180-days of stratification, the control treatment (CT) and stratification in sand and sand + commercial substrate + corn straw (T2) did not show emergence. The emergence for stratification in sand covered with corn straw (T4) was 2.08% and stratification in sand with fertilizer differed statistically from the others and presented the highest emergence percentage (13.19%).

TABLE 1 - Germination percentage of *Ilex paraguariensis* A.St.-Hil. in different treatments and stratification times.

Stratification times	120 days	150 days	180 days
Stratification treatments	----------% emergence----------		
CT - seeds stored at 5 °C	0 aA*	0 bA	0 bA
T1 - seeds between two sand layers	0 aA	0.69 abA	0 bA
T2 - seeds between two sand layers + commercial substrate + corn straw	0 aA	0 bA	0 bA
T3 - seeds between two sand layers with fertilizer	0 aC	2.78 aB	13.19 aA
T4 - seeds between two sand layers + corn straw	0 aA	0.69 abA	2.08 bA
Mean	0	0.83	3.06

*Means followed by different letters in the column and distinct upper-case letters in the row differ by the LSD-Fisher test, at 5% error probability.

In the different stratification times, treatments with no stratification (CT), stratification in sand and sand + commercial substrate + corn straw did not differ. Treatment with stratification in sand covered with corn straw showed a higher emergence percentage at 180-days (2.08%) and stratification in sand with fertilizer differed in the three periods, with no emergence at 120-days, with 2.78% at 150-days and a higher percentage at 180-days (13.19%).

Stratification in sand has been indicated as the most usual and widespread process among nursery workers (CUQUEL et al., 1994; CATAPAN, 1998; WENDLING; SANTIN, 2015) since the 90's when the first studies on yerba mate seeds dormancy began in southern Brazil. Sand has the function of keeping seed moist and, due to their physical characteristics, contributes to the leaching of inhibitory compounds that give physiological dormancy to seeds (BEWLEY et al., 2013; BASKIN; BASKIN, 2014). However, in this work, it was possible to verify that this methodology is not so efficient, since the present study showed that seeds, when placed only in sand, showed a low germination percentage, besides this occurred only after 11-months (150-days of stratification and 180 more days of emergence).

The use of an alternative stratification treatment, composed of a commercial substrate and corn straw, was intended to simulate the stratification performed by some nursery workers, which present a satisfactory germination percentage and quality seedlings. This treatment is differentiated for presenting, in the composition of the commercial substrate, some nutrients that could assist in the germination process, not totally inert like sand. However, in this study, there was no distinction in the results in relation to treatment with no stratification (CT), with no observation of emergence.

The use of controlled-release fertilizer in the composition of the upper layer (T3) aimed to provide nutrients, mainly nitrate. According to the producer, the fertilizer used in the T3 treatment may gradually provide nutrients for five to six months, following the plant's nutritional needs. Possibly, fertilizer provided nutrients, mainly nitrate through the stratification period, interfering positively in the germination, mainly after 180-days of stratification followed by 180-days of emergence, which emergence percentage was 13.19%.

In the literature, the importance of substances such as potassium nitrate in overcoming seed dormancy is emphasized. The Seed Analysis Rules (SAR) suggest some species seeds imbibition in KNO_3 (BRASIL, 2009). This substance use is based on the hypothesis that the release of nitrate (NO^3) or nitrite (NO^{2-}) occurs and that these chemicals act as electron receptors via pentose-phosphate. Consequently, they reduce the dormancy state which culminates in germination (CARVALHO; NAKAGAWA, 2012). Also, nitrate is likely to influence abscisic acid (ABA) metabolism (YAN; CHEN, 2020), reducing the levels of this hormone and increasing the gibberellins (GA) synthesis (BETHKE et al., 2007). This is a key process in reducing dormancy and inducing germination, as these two hormones (ABA and GA) are antagonists in this cycle (YAN et al., 2014).

Another factor that may have influenced the results was the outdoor trays maintenance process, even in the rainiest periods. This may have contributed to excessive fertilizer leaching in treatment T3 and the substrate nutrients leaching in treatment T2, reducing nutrients availability. Stratification requires strict irrigation management, as rainy periods or even excess watering can contribute to the excessive nutrients leaching, as in the T2 and T3 cases.

Regarding seed viability, data obtained from tetrazolium tests after stratification periods, there was no interaction between treatments and stratification times, showing significant differences only between the treatments themselves (Table 2). The control treatment (CT) (with no stratification) showed the highest viable seeds percentage for the three analyzed periods, and consequently the lowest non-viable seeds percentage. In general, the stratification treatment in sand + commercial substrate + corn straw (T2) presented the lowest viable seeds percentage at 120 and 150-days of stratification. At 180-days of stratification, seeds stratified in sand covered with corn straw (T4) showed similar behavior to sand with fertilizer (T3), with the highest non-viable seeds rates. Other treatments showed an intermediate behavior between the CT and T3.

TABLE 2 - Percentage of viable and non-viable seeds of *Ilex paraguariensis* A.St.-Hil. after 120, 150 and 180 days of stratification followed by emergency for 180 days.

Stratification times	120 days		150 days		180 days	
Stratification treatments	Viable	Non-viable	Viable	Non-viable	Viable	Non-viable
	%					
CT - seeds stored at 5°C	11.5 a*	88.5 c	8.0 a	92.0 c	8.0 a	92.0 c
T1 - seeds between two sand layers	5.25 b	94.75 b	5.25 ab	94.75 bc	5.25 ab	94.75 bc
T2 - seeds between two sand layers + commercial substrate + corn straw	1.0 c	99.0 a	1.0 c	99.0 a	1.0 cd	99.0 a
T3 - seeds between two sand layers with fertilizer	3.75 bc	96.25 ab	3.75 bc	96.25 ab	3.0 bc	97.0 ab
T4 - seeds between two sand layers + corn straw	2.25 bc	97.75 ab	2.25 bc	97.75 ab	0.75 d	99.25 a
Mean	4.75	95.25	4.05	95.95	3.6	96.4
p value	<0.001	<0.001	0.0026	0.0026	0.0019	0.0035

*Means followed by different letters in the column differ by the LSD-Fisher test, at 5% error probability.

The treatment with no stratification (CT) showed the lowest non-viable seeds percentage and, consequently, the highest viable seeds percentage, due to the fact this treatment did not undergo the stratification process, presenting a greater dormant seed amount at the end of the process, that is, after 360-days.

It is a fact that the stratification followed by germination at 25°C favored seed degradation, as it significantly increased non-viable seeds percentage after 360-days in all treatments. Menegueti et al. (2004) also found greater seeds deterioration in those that were kept in germinator at 25°C compared to ones kept in the refrigerator at -2°C. Some authors verified the seeds total degradation after 240-days of storage under refrigerator temperature, however, the seeds were wrapped around the fruit pulps, a fact that favored deterioration when compared to seeds that were in the same conditions but stratified in sand (CUNHA; FERREIRA, 1987). However, the association of some fungi with yerba mate seeds can be beneficial to germination through endocarp decomposition (GRIGOLETTI-JÚNIOR et al., 1999).

At different stratification times (120, 150, and 180-days), after the emergence period ends (180 days), the endocarp darkening was observed, which may be due to the fungi presence that act in this lignified structure degradation (SOUZA et al., 2019), which possibly contributes to significantly increase to the percentage of non-viable seeds, since many of them showed a softened endocarp and endosperm with dark color, indicating signs of clear degradation.

In addition to that, the low initial seeds viability (36.4%) is an issue that is not taken into account in most studies. Souza et al. (2020) investigated the causes of the low quality of yerba mate seeds and concluded it is due to an empty and deteriorated seeds high percentage, varying between 54 and 93% depending on lot origin. The lot of seeds used in this work, the majority were composed of deteriorated seeds (62%) and, to a lesser extent, of empty seeds (only 2%). This may be a common characteristic of this species and one of the possible causes of low germination percentage, which, in most studies, is only attributed to morphophysiological dormancy.

To favor greater seedlings production, it is necessary to take action with nurseries' worker that increases viable seed quantity in lots they use. This can be guaranteed with the adoption of seed cleaning methods, which will increase germination rates and reduce costs. More specific criteria are also needed for the selection of mother-plants with lower empty and deteriorated seed rates.

Although germination is still low (less than 20%), it can be said that the treatment with controlled-release fertilizer was distinguished from the others. However, further studies are needed, mainly concerning fertilizer dose adjustment and its formulation, in addition to investigating the contribution of each nutrient in embryo development during stratification. Research studies are also required regarding the joint action of more nutrients, even though the current literature highlights the action of nitrate on embryo development, especially in *Arabidopsis* (ALBORESI et al., 2005). Besides that, the factors of impact, such as alternating temperatures during stratification, still require investigation once they may be simultaneously contributing to dormancy overcoming and consequent increase in yerba mate seeds germination rates.

CONCLUSIONS

Stratification in sand, combined with controlled-release fertilizer favors yerba mate seedlings emergence. After 180-days of emergence in germinator plants at 25°C, there is an increase in non-viable seeds amount.

Seeds that did not undergo the stratification process do not germinate and present a higher dormant seeds percentage after 180-days of emergence.

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