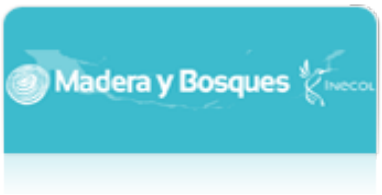

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How to access viability of *Zanthoxylum rhoifolium* seeds? A protocol of tetrazolium test as an alternative to evaluate a dormant seed

¿Cómo acceder a la viabilidad de las semillas de *Zanthoxylum rhoifolium*? Un protocolo de prueba de tetrazolio como alternativa para evaluar una semilla latente

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Abstract

A tetrazolium test method is proposed for evaluating seed quality of *Zanthoxylum rhoifolium* Lam. We used seeds from two lots, which we immersed in distilled water for 8 hours and subjected to two preparations: A - lateral incision into the seed, parallel to the hilum, to expose the endosperm, and B - extraction of the embryos. We then exposed the seeds of both preparations to the tetrazolium solution at two concentrations (0.1% and 0.05%) for two periods (24 hours and 48 hours) at a temperature of 25 ºC, then the seeds were evaluated in a 2 × 8 factorial scheme (lots × treatments) and classified in viable and non-viable. We found that the best preparation for the tetrazolium assay in *Zanthoxylum rhoifolium* was to remove part of the seed coat, this allowed the solution to penetrate the internal tissues without damaging the embryo. There were no significant differences between concentrations of 0.1% at 24 hours and 0.05% for 48 hours. We concluded that immersion of seeds in water for 8 hours, followed by a lateral cut parallel to the hilum exposing the endosperm at a concentration of 0.1% tetrazolium for 24 hours or 0.05% for 48 hours is an efficient method for quality assessment of *Zanthoxylum rhoifolium* seeds.

Keywords: native species, prickly ash, physiological potential, Rutaceae, seed analysis, viability.

Resumen

Este trabajo propone una metodología de prueba de tetrazolio para la evaluación de la calidad de la semilla *de Zanthoxylum rhoifolium* Lam. Se utilizaron semillas de dos lotes, que fueron sumergidas en agua destilada durante 8 horas y sometidas a dos preparaciones: A - corte lateral de la semilla, paralelo al hilio, exponiendo el endospermo; y B - extracción de embriones. Las semillas de ambos tratamientos fueron expuestas a la solución de tetrazolio en dos concentraciones (0,1% y 0,05%), durante dos periodos (24 horas y 48 horas), a una temperatura de 25 ºC. Las semillas fueron evaluadas en un esquema factorial 2 × 8 (lotes × tratamientos) y clasificadas en viables y no viables. Se encontró que la mejor preparación de *Zanthoxylum rhoifolium* para la prueba de tetrazolio es eliminar parte del tegumento, ya que permite que la solución se impregne en los tejidos internos sin dañar el embrión. No hubo diferencias significativas entre las concentraciones de 0,1% en 24 horas y 0,05% en 48 horas. Se concluyó que la inmersión de las semillas en agua durante 8 horas, seguida de un corte lateral, paralelo al hilio, exponiendo el endospermo a concentraciones de 0,1% de tetrazolio durante 24 horas o 0,05% durante 48 horas, son metodologías eficientes para evaluar la calidad de la semilla de *Zanthoxylum rhoifolium*.

Palabras clave: especies autóctonas, aceitilla, potencial fisiológico, Rutaceae, análisis de semillas, viabilidad.

Introduction

Information on the behavior of seeds of native forest species allows support for other studies aimed at producing good quality seedlings (Ribeiro-Oliveira and Ranal, 2014). Among the native species with little available information is *Zanthoxylum rhoifolium* Lam. (Rutaceae), a tree that occurs from Mexico through the Central American lowlands to almost all of South America (Pirani and Groppo, 2020). The wood of this species is used to make tool handles and interior finishes in construction, and it is also planted in rehabilitation areas and landscaping (Costa et al., 2014). *Zanthoxylum rhoifolium* is also an object of study due to its diverse medicinal properties, like antiparasitic, antinociceptive, antioxidant, antitumoral, gastrointestinal and antimalarial activity (Marques et al., 2022).

Although the species has economic and ecological potential, studies related to its seeds report low germination (5% to 45%) (Silva & Paoli, 2000; Carvalho, 2006; Souza Junior & Brancalion, 2016) caused by the existence of combined dormancy (physical+physiological) and by the presence of damaged seeds, making it difficult to access parameters related to their physiological quality (Corrêa et al., 2022).

An alternative method to access the viability of dormant or long germinating seeds is the use of tetrazolium test, that consists in reaction of dehydrogenase enzymes in living tissues of the seeds by the application of a solution (2, 3, 5 triphenyltetrazolium chloride), thus resulting in a pinkish-red, insoluble and stable color (triphenylformazan), which shows that the embryo is in respiratory activity. This test is not only rapid, but as comproved efficiency for most dormant seeds (Soares et al. (2016).

In forest seeds, the tetrazolium test has proven its effectiveness, as in *Araucaria angustifolia* (Oliveira et al., 2014), *Libidibia ferrea* (Carvalho et al., 2017) *Jatropha curcas* (Araújo et al., 2019) and *Campomanesia phaea* (Silva et al., 2021). However, there is still a need for appropriate methodologies sorted by species, especially for native ones (Oliveira et al., 2018) like *Z. rhoifolium*, which are known especially by the difficulty in promoting their germination.

Objectives

The aim of this article was to determine a methodology for the tetrazolium assay to assess the viability of *Zanthoxylum rhoifolium* seeds.

Materials and methods

The seeds of *Z. rhoifolium* were obtained from two sources, both from natural collection areas. Lot 1 was Penápolis, in the state of São Paulo, with an average altitude of 416 meters, whose predominant climate is classified as high altitude tropical climate – "Cwa" (Alvares et al., 2017). Lot 2 was Dois Vizinhos, in the state of Paraná, with an average elevation of 409 meters (Ipardes, 2019) and humid subtropical climate type – "Cfa" (Alvares et al., 2017).

Lot 1 was obtained through purchase (collected in March 2017 and stored at cold chamber at 10 °C and 55% RH). Lot 2 we acquired in 2018 with the harvest of ripe, reddish fruits at the beginning of spontaneous opening. After harvest, we used a sieve to remove fragments and fruit debris from the seeds and dried them in trays for 24 hours at room temperature.

We subjected the seeds of the two lots to the determination of the water content, germination test and different methods of the tetrazolium test. Water content was determined by the drying oven method at 105 °C ± 3 °C, for 24 hours (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009), with two repetitions of 5g each. We weighed the seeds on a semi-analytical balance, in aluminium capsules and placed them in a drying oven. Based on the weight of the wet seeds, we calculated the water content.

The germination test was performed in a B.O.D. (Biochemical Oxygen Demand) type chamber at a temperature of 25 °C and constant white light. We used four replicates with 25 seeds per lot. Disinfestation was done by immersion in 1% sodium hypochlorite for 30 seconds followed by washing in distilled water. We placed the seeds for germination in Gerbox-type acrylic boxes, on Germitest paper moistened in distilled water to 2.5 times the weight of the paper. We performed the assessments daily from the 4th to the 120th day of the establishment of the test.

For the tetrazolium assay, seeds were soaked in water at 25 °C in B.O.D. for 8 hours to soften the coat, and then subjected to two preparations. 1) Cut the coat (preparation A): a scalpel was used to incise the seeds laterally, parallel to the hilum, so that the endosperm was exposed. 2) Removal of the embryos (preparation B): a scalpel and a needle were used to remove the embryos from the seeds without damaging them (Fig.1).

Seeds and embryos were placed in plastic cups and immersed in a tetrazolium solution at a concentration of 0.1% and 0.05% and kept in a B.O.D. for 24 hours and 48 hours, respectively, excluding light and at a temperature of 25 °C: Eight treatments were analyzed that consisted of combinations of type of cut, concentrations (0.5% and 1%) and immersion duration (24 h and 48 h). Four replicates of 25 seeds were used for each treatment. After each period, the tetrazolium solution was removed and the seeds/ embryos were washed under running water and evaluated.

For evaluation, the integuments and endosperm were removed from the seeds of Preparation A (Fig. 1), revealing the embryo. The two cotyledons were separated for analysis of internal characteristics. They were adapted for this work according to the standards proposed by França-Neto et al. (1998): light red (healthy tissue); intense red (deteriorating tissue) and discolored tissue (dead tissue). Embryos that exhibited certain colored areas were considered viable, as was the intensity and extent of the color and the appearance of the tissue. Viable seeds were those that showed light red color in tissues with normal appearance and a completely light pink colored embryonic axis and 50% of the cotyledons after the axis. The non-viable seeds were those that presented more than 50% of the discolored cotyledons, tissues with intense red coloring and/or discolored embryonic axis. We made a schematic image with all the categories of seeds found during the evaluation of the tetrazolium test (Fig. 2), which allowed calculating the number of embryos in each of the categories described for the species. The results were expressed as a percentage of viable seeds.

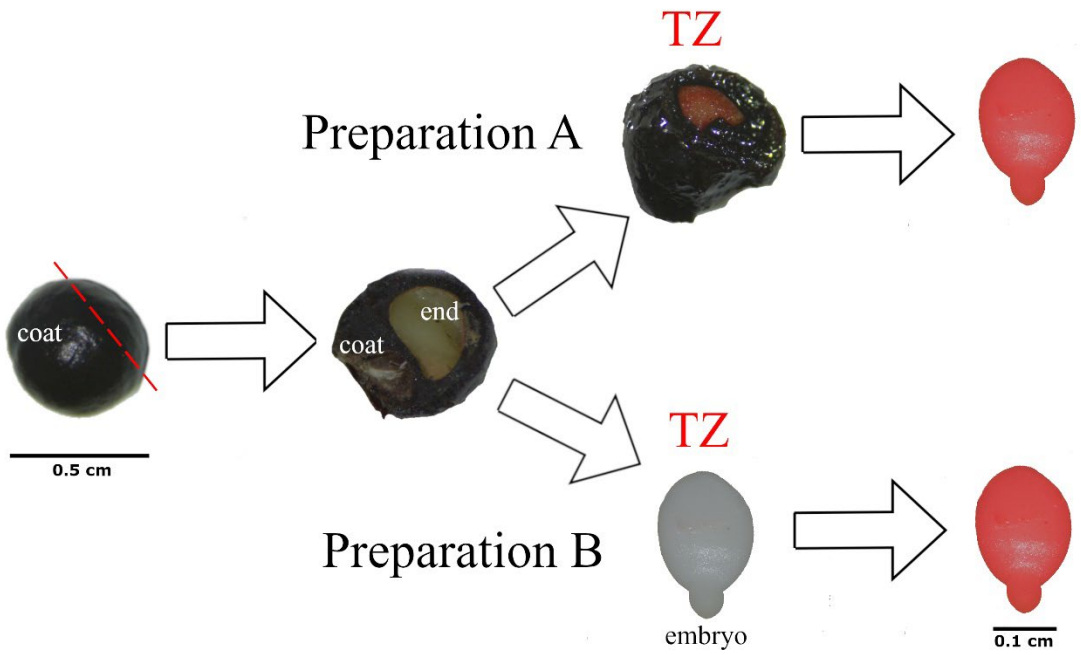

FIGURE 1. Schematic showing the preparations for the seeds exposed to tetrazolium tests.

Preparation A: Cut the coat - With a scalpel was incise the seeds laterally, parallel to the hilum, so that the endosperm was exposed, then put in contact with tetrazolium;

Preparation B: Embryos removed - with a scalpel and a needle the embryos were removed and put in contact with tetrazolium.

TZ=solution of Tetrazolium; end=endosperm; Dotted red line=Coat cut site.

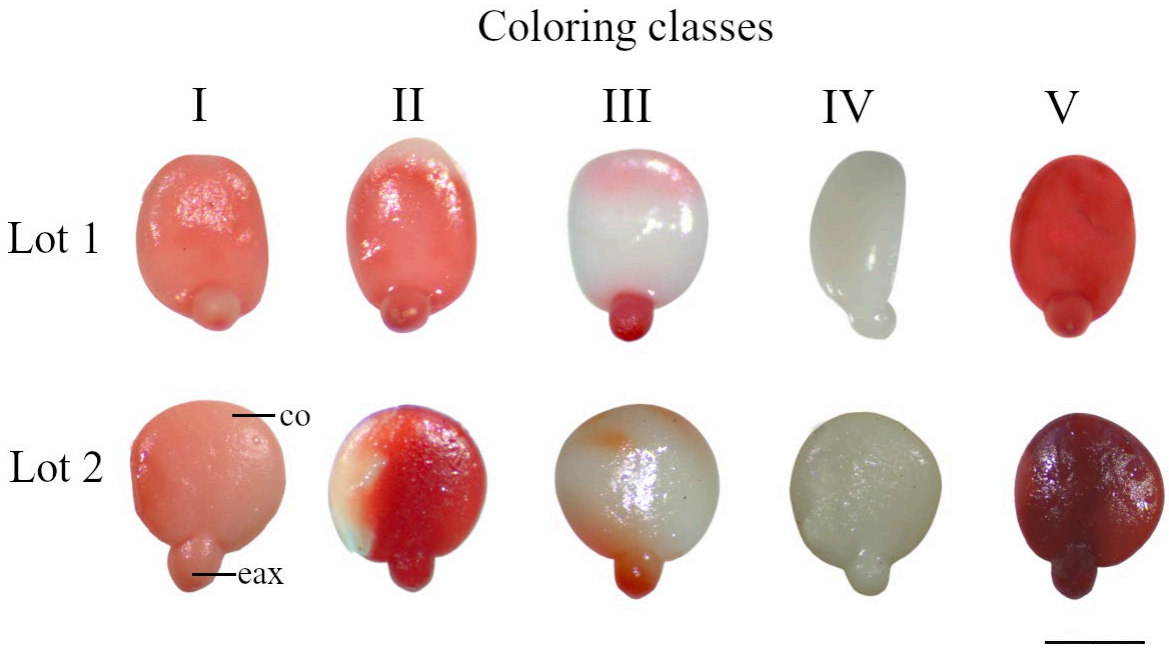

FIGURE 2. Categories of *Zanthoxylum rhoifolium* seed embryos stains exposed to the tetrazolium test.

I (viable): embryo with pink/red color and firm-looking tissue

II (viable): colored embryonic axis and cotyledons with less than 50% discoloration

III (non-viable): colored embryonic axis and less than 50% of colored cotyledons; IV (non-viable): embryo completely discolored; V (non-viable): embryo with intense red/dark coloring in its entire area.

co=cotyledon; eax= embryonic axis; Bar= 1 mm.

The tetrazolium and the germination experiments were laid out in a completely randomized design (CRD) in a 2 × 8 factorial scheme (lots × treatments). The data do not comply the statistics assumptions ($p = 0.0005$), in this way, we perfomed a modeling with generalized linear model (GLM) (binomial distribution with *logit* function in the *stats* package). After adjusting the model, an analysis of variance was performed and a test of means (Scott Knott at 5% of error probability) (Santana et al., 2018), using R Studio statistical program.

RESULTS AND DISCUSSION

The seeds of *Zanthoxylum rhoifolium* from lot 1 had high moisture content (20%) compared to lot 2 (11%). Uncontrolled environments with humidity between 14% and 20% induce metabolic activities that cause stress by increasing respiration rate, which reduces viability (Schmidt, 2007), which was observed in lot 1 (maximum 15% viability). Controlled drying and storage reduces seed moisture and metabolism (Marcos-Filho, 2005), which allowed the viability of lot 2 to be maintained (Table 1).

As for the preparation, it was found that the removal of the embryo for the test (Preparation B) not only requires more labor, but can also damage the embryonic axis and cotyledons, making the embryos unsuitable for the test. These results were confirmed during seed processing when some seeds were discarded due to damage caused by embryo removal. If we cut open the envelope and expose the endosperm (Preparation A), we can immerse the inner tissue in the solution without damaging the structures. This method was also efficient for seeds of *Euterpe edulis* (Oliveira et al., 2017), *Libidibia ferrea* (Carvalho et al., 2017) and *Poincianella pyramidalis* (Sousa et al., 2017).

For lot 1, there was significant difference between treatments. A maximum of 15% and 10% of viability was observed in this lot at T2 and T4 treatment by the tetrazolium test. In lot 2, the results of T2, T3, T5, T6 and T7 treatments were statistically better than the other

treatments with no statistical difference between them (Table 1). Thus, the treatments with the best results using Preparation A were T2 (concentration of 0.1% over 24 hours) and T3 (0.05% over 48 hours). It is worth noting that despite comparing the results of the tetrazolium test with those of germination, only 1% of the seeds of *Zanthoxylum rhoifolium* germinated, which is due to dormancy. Moreover, the tetrazolium test for seeds with dormancy was indicated in the rules for seed analysis as a substitute for the germination test (MAPA, 2009).

Although one of the advantages of the tetrazolium test is the rapidity of its conduction and evaluation, the tetrazolium salt is expensive and there is an increasing need for methods that reduce the cost of laboratory tests on seeds (Marcos-Filho, 2015). In this regard, the concentration of 0.05% during 48 hours has shown satisfactory results, since it allows the analyst to reduce the amount of salt used, thus making the test cheaper. Furthermore, the embryos studied have a clearer color for visualization (light red). This concentration is also recommended for other forest species, such as *Libidibia ferrea* (Carvalho et al., 2017) and *Parkia multijuga* (Costa et al., 2018).

Most of the viable seeds of lot 1 belonged to class I and the non-viable ones to class IV, indicating complete metabolic inactivity (Fig. 2). Class I colored embryos also predominated in lot 2. Most of the non-viable embryos were assigned to class V and were intensely red in color throughout their extension (Fig. 2).

The results of the tetrazolium test showed that *Zanthoxylum rhoifolium* responded positively to the application of the solution. The tissues showed visible color differences (Fig. 2), which allowed the classification of embryos into viable and non-viable.

Another important factor in the analysis is the distinction between the lots in terms of physiological quality. While lot 2 had viability up to 89%, lot 1 had a maximum of 15% viable seeds (Table 1). This difference was also evident by the tetrazolium test in the lots of *Poincianella pyramidalis* (Sousa et al., 2017) and *Enterolobium contortisiliquum* (Nogueira et al., 2014).

TABLE 1. Viability (%) of seeds of *Zanthoxylum rhoifolium*, assessed using different treatments of the tetrazolium test.

Treatments	*Preparation*	*Tetrazolium concentration*	*Immersion time*	*Viability*	
				Lot 1	Lot 2
		(%)	(h)	(%)	(%)
T1	A	0,05	24	3 bB	41 bA
T2	A	0,05	48	15 aB	88 aA
T3	A	0,1	24	4 bB	83 aA
T4	A	0,1	48	10 aB	34 bA
T5	B	0,05	24	0 bB	89 aA
T6	B	0,05	48	0 bB	85 aA
T7	B	0,1	24	0 bB	83 aA
T8	B	0,1	48	4 bA	4 cA

Averages following the same letter are not statistically different, lowercase letters indicate the column and uppercase letters indicate the row, by Skott-Knott test at 5% probability.

Considering the results of viability for lots 1 and 2, germination was found to be only 1% for both lots after the period of 120 days. The germinated seed of lot 1 exhibited a 2 mm radicle but was attacked by a pathogen (fungus) that rotted the structure and prevented the subsequent development of a seedling. The presence of dormancy, among other factors, influenced the germination result, as dormant seed that has not been subjected to any treatment to overcome dormancy usually has no or very low germination (Baskin & Baskin, 2014). Although the presence of combined dormancy in these seeds has been reported (Corrêa et al., 2022), no effective method to overcome dormancy has been found in the literature, making it impossible to compare the results of the tetrazolium and germination tests.

We emphasize the importance of the tetrazolium test in assessing the quality of these seeds, since in this test the presence of dormancy does not affect the determination of viability (Oliveira et al., 2018; França-Neto & Krzyzanowski, 2019).

Conclusions

The tetrazolium test showed to be adequate to access the viability of the seed lots. The immersion of seeds in water for 8 hours, followed by a lateral cut parallel to the hilum exposing the endosperm, and concentrations of 0.1% tetrazolium for 24 hours or 0.05% for 48 hours are efficient methods for quality assessment of seeds of *Zanthoxylum rhoifolium*.

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