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TETRAZOLIUM TEST FOR THE VIABILITY ASSESSMENT OF COCOA SEEDS

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ABSTRACT

Cocoa seeds are recalcitrant, resulting in a faster loss of viability with storage time and the tetrazolium test is an alternative to determine the viability of these seeds as quickly as possible. The objective of this study was to develop a tetrazolium test protocol for cocoa seeds to assess seed viability. Seeds were extracted from fruits and after the processing were subjected to the tetrazolium test considering 7 times in tetrazolium solution (2, 3, 4, 5, 6, 7 and 8 hours) and 2 concentrations of tetrazolium solution (0.05 % and 0.075 %). Before this, seeds were soaking for 6 hours in germination paper and the seed coats were removed. During the immersion on tetrazolium solution, seed were maintained in a germination chamber at 42 °C. After this, seeds were categorized into 4 classes, according to the coloration of embryonic axis, with the living tissues being dyed with light pink color while dead tissues were non-colored. To confirm the results of tetrazolium test, a germination test was carried out to associate the percentage of germination with the percentage of viable seeds obtained by the tetrazolium test. Considering this, the recommended immersion time in the tetrazolium solution is 3 hours with a concentration of 0.05 %.
Additional keywords: Germination test, recalcitrant, *Theobroma cacao*, viability

RESUMEN

Prueba de tetrazolio para evaluar la viabilidad de las semillas de cacao

Las semillas de cacao son recalcitrantes, por lo que su viabilidad disminuye con el tiempo de almacenamiento. La prueba de tetrazolio es una alternativa para determinar su viabilidad lo antes posible. El objetivo de este estudio fue desarrollar un protocolo de prueba de tetrazolio para semillas de cacao y evaluar su viabilidad. Las semillas se extrajeron de frutos y, tras el procesamiento, se sometieron a la prueba de tetrazolio considerando 7 tiempos de exposición a la solución de tetrazolio (2, 3, 4, 5, 6, 7 y 8 horas) y 2 concentraciones de solución de tetrazolio (0,05 % y 0,075 %). Previamente, las semillas se remojaron durante 6 horas en papel de germinación y se les retiró la cubierta. Durante la inmersión en la solución de tetrazolio, las semillas se mantuvieron en una cámara de germinación a 42 °C. A continuación, las semillas se clasificaron en cuatro clases según la coloración del eje embrionario: los tejidos vivos se colorearon de rosa claro y los tejidos muertos no se colorearon. Para confirmar los resultados de la prueba de tetrazolio, se realizó una prueba de germinación para asociar el porcentaje de germinación con el porcentaje de semillas viables obtenido por la prueba de tetrazolio. Teniendo esto en cuenta, el tiempo de inmersión recomendado en la solución de tetrazolio es de 3 horas y la concentración más adecuada es del 0,05 %.
Palabras clave adicionales: Prueba de germinación, recalcitrante, *Theobroma cacao*, viabilidad

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INTRODUCTION

After reaching physiological maturity, with maximum germination, vigor and dry matter mass, seeds are exposed to the conditions of the cultivation or storage environment (such as temperature, rain, insects and microorganisms) and have their physiological potential reduced (Carvalho and Nakagawa, 2000). This problem becomes particularly important for recalcitrant species, such as cocoa (Fajardo *et al*., 2011), which has low tolerance to desiccation after the seeds reach physiological maturity (Li and Sun, 1999). It happens due to the species characteristic

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of germinating soon after releasing the seed into the soil, which means that it does not develop desiccation tolerance mechanisms (Barbedo, 2018). Therefore, it is important to evaluate physiological quality through rapid tests to determine the seeds viability of this species. The production of rootstocks of these species is done by seeds (Sodré, 2013). So, rapid viability assessment by the tetrazolium test is important for maintaining quality seed.

The tetrazolium test is one of these rapid tests and its principle is to evaluate the viability and vigor of seeds by exposing them to a 2, 3, 5 triphenyl tetrazolium solution. Alive seeds are breathing and releasing hydrogen ions (H+). Dehydrogenase enzymes catalyze the reduction reaction of 2, 3, 5 triphenyl tetrazolium to triphenylformazan. As this reaction occurs, it is possible to separate the living tissues from the dead tissues of a seed based on the difference in the color of the seed tissues. Living tissues are colored from pink to red, depending on the intensity of metabolic activity, while dead tissues do not color (MAPA-SDA, 2009).

By this biochemical test we can diagnose different types of damage which interfere on the seeds physiological. Some defined damages for different crops are: mechanical damage; weathering damage; insect damage; damage due to drought and high temperatures (França and Krzyanowski, 2019). The tetrazolium test can also be used to determine if seeds are viable and vigorous (Ramirez *et al.*, 2021).

In *Acca sellowiana* (O. Berg) Burret (Myrtaceae), the tetrazolium test was used to evaluate the physiological potential of the seeds by separating them into viability classes based on the difference in color of the seed tissues (Sarmento *et al*., 2013). Similarly, Sukkaew *et al*. (2023), showed the change in tissue color, with a soft or intense pink color being present in the embryonic axes of rice seeds considered viable. This test can also be used to assess the viability of seeds that have been stored for a long time (Maia *et al*., 2024).

Tetrazolium test is interesting specially in forest seeds, as it is a quick test for evaluating seed viability when compared to the germination test Belniaki *et al*. (2020). For the species *Himatanthus sucuuba* (Apocynaceae) germination took 25 days after sowing, while for the tetrazolium test the time required was 90 minutes at a concentration of 0.075 % (at a temperature of 40 °C) (Ramirez *et al*., 2021). The same for commercial crops, such as rice, which takes between 14 days to complete germination, while for the tetrazolium test it takes two hours to assess seed viability (Sukkaew *et al*., 2023).

For recalcitrant seeds, this same advantage of the tetrazolium test is observed. For example, in peach palm seeds (*Bactris gasipaes* Kunth), it took 4 h in a concentration of 1 % tetrazolium solution at 30 °C, while their germination takes an average of 120 days to occur (Belniaki *et al*., 2020). In other recalcitrant seeds, such as *Eugenia brasiliensis*, *Eugenia uniflora* and *Eugenia pyriformis*, the tetrazolium test has also been shown to be a faster assessment test for identifying the loss of viability than compared to the germination test (Lamarca and Barbedo, 2014). The viability of recalcitrant seeds subjected to the drying process can also be assessed using this test as in seeds of *Butia eriospatha* (Arecaceae) (Dambros *et al*., 2024).

Therefore, as cocoa seeds are recalcitrant, which results in a faster loss of viability with storage time, it is necessary to use the tetrazolium test to determine the viability of these seeds as quickly as possible. Therefore, the present study aims to develop a tetrazolium test protocol for cocoa seeds to evaluate the seeds viability.

MATERIALS AND METHODS

The seeds were extracted from 40 ripe orange-colored cocoa fruits. These fruits were 15-20cm length and 7-10 cm width, oval shape, with yellow-orange skin and white mucilaginous pulp containing the seeds. Seed processing was carried out by manual rubbing in sand to remove mucilage. After that, the seeds were washed in running water to remove excess sand. After that, they were exposed to a surface drying at environmental temperature. After drying, the seeds were subjected to the tetrazolium test considering different concentrations of tetrazolium solution and immersion times in the solution. The research was conducted at the Organic Agriculture Laboratory from the Agricultural Sciences Center of the Federal University of São Carlos, Araras campus, Brazil.

The experimental design was completely randomized with two concentrations of tetrazolium solution and seven times of immersion

of seeds in the solution, within a factorial arrangement. The concentrations tested were 0.05 % and 0.075 %, and the immersion times were 2, 3, 4, 5, 6, 7 and 8 hours.

Four replications were carried out for tetrazolium test with 25 seeds for each repetition per treatment. To facilitate the removal of the seed coat, the seeds were wrapped in a sheet of germination paper moistened with 2.5 times the weight of the dry paper for 6 hours. The seed coat was removed using a blade, leaving the seeds with the embryonic axis and cotyledons intact. According to Marcos (2015), pre-conditioning, on wet paper, is a recommended procedure to facilitate the removal of the seed coat, as well as assist in the activation of enzymes and tissue coloring.

Then, the seeds were placed in plastic cups with the tetrazolium solution in each of the treatments, until complete immersion and kept without the presence of light in a germination chamber at 42 °C, an appropriate temperature according to Paiva *et al.* (2017).

After each coloring time, the seeds were washed in running water and kept immersed in water until evaluation (MAPA-SDA, 2009). To assess viability, a longitudinal cut was made in the seed, separating the embryonic axis from the middle. The color evaluation took place exclusively in the region of the hypocotyl-radicle axis and the seeds were classified into 4 classes (Table 1). The approach of Sales *et al*. (2022) was considered to define the relationship between tissue color and the tissue's physiological condition this study. Cotyledons were not evaluated, as in cocoa seeds they have a brown color, which makes it difficult to visualization of tetrazolium staining in these tissues.

Table 1. Classification of cocoa seeds (*Theobroma cacao* L.) using the tetrazolium test.

Classes	Description
Class 1	**Viable** – Hypocotyl-radicle axis with light pink color
Class 2	**Viable** - Hypocotyl-radicle axis with light pink color and some dark pink spots, without compromising essential tissues such as plumule and radicle
Class 3	**Non-viable -** Hypocotyl-radicle axis with 50 % or more white color
Class 4	**Non-viable -** Hypocotyl-radicle axis with 100 % white color or deformed cotyledons or empty seeds with non-existent cotyledons

To confirm the tetrazolium test, a germination test was carried out in order to associate the percentage of germination with the percentage of viable seeds obtained by the tetrazolium test.

For the germination test, the seeds were sown in 4 replications of 25 seeds, in the vermiculite substrate (Voigt *et al.*, 1995) in gerbox-type boxes (11x11x3,5 cm). The substrate was moistened once, at the beginning of the experiment. Subsequently, the boxes were kept in a germination chamber at 25°C. Three evaluations were carried out, with the last evaluation taking place on the 23rd day after the germination test installation, when germination stabilized. Evaluations of normal seedlings were carried out considering the seedlings that had emitted the primary root and secondary roots.

Considering the classification results using the tetrazolium test at different concentrations and immersion times, these were initially analyzed using an analysis of variance and, when there was a significant effect, the means were compared using the Tukey test at 5 % probability.

RESULTS AND DISCUSSION

No important effect of interaction of treatments was observed. On 2 hours of immersion time, for both concentrations (0.05 % and 0.075 %), the viability by the tetrazolium test was underestimated in relation to the germination test (73.14 %) (Figure 1). This may be related to the fact that, in shorter times in tetrazolium solution, the dye was still unable to color the seed the tissue, which resulted in viability values lower than germination (Carvalho-I *et al*., 2017; Mercado *et al*., 2020). It should also consider that concentrations of dye when were low, they

couldn't stain the hypocotyl-radicle axis; Ursulino *et al*. (2025) found that when the concentration was more than a 0,075%, only 38% of the seeds of *Dimorphandra gardneriaria* were dyed in thirty minutes. At 3 hours, the viability values were 75 % for the 0.05 % concentration and 76 % for the 0.075 % concentration (Figure 1). These values were close to the value obtained by the germination test, demonstrating the potential for this coloring time to be ideal for cocoa seeds. Correlating the results of the tetrazolium test with the germination test has been shown to be suitable for studies related to determining protocols for the tetrazolium test (Kusumawardana, 2018).

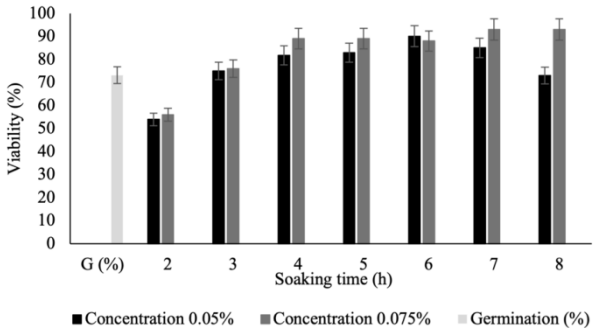

Figure 1. Germination percentage and viability of *Theobroma cacao* L. seeds percentage by the tetrazolium test at different solution times and tetrazolium salt concentrations.

When it was tested 4 hours of immersion time, the viability values were higher than those determined by germination. Virgens e*t al*. (2019) found that longer times and higher concentrations indicated higher viability values. The same was observed by Carvalho-S *et al.* (2017) in which longer times promoted a more intense coloration in the tissues of *Libidibia ferrea* seeds, and the best evaluation occurred at concentration of 0.05 % and time of 3 hours similarly to the present work.

Therefore, the 3 h immersion time in the tetrazolium solution at a concentration of 0.05 % was more suitable for evaluating the viability of cocoa seeds. This concentration was chosen due to the lower need for tetrazolium salt to prepare the immersion solution, which reduces the cost of carrying out the test.

With the increasing time on tetrazolium solution, from four hours onwards, the percentage of seeds in class 2 increased (Table 2). This indicates that the tetrazolium salt colored the seeds more intensely and, consequently, resulted in an overestimation of viability. The non-coloration of the tissues indicates that the seeds were non-viable, as in class 3 and class 4, in which, seeds show 50 % or more of white color, and 100 % of white color, respectively. According to Silva *et al.* (2016), a deep red color indicates deteriorations, while a milky white color characterizes mortality.

During the evaluation process, it was identified that after cutting the embryonic axis, an intense oxidation of the plant tissue was observed. This is a typical characteristic of recalcitrant seeds subjected to desiccation, since changes in metabolism occur in aqueous solution, leading to oxidative degradation. The oxidation of seed structures depends on their metabolic activity and drying rate (Marcos, 2015). The darkening of the cotyledons caused by oxidation was observed by Andrade and Ferreira (2000) shortly after extraction of the seed from the uvaia fruit (*Eugenia pyriformis*), concluding that the oxidation of seed structures associated with the reduction in the humidity rate can cause rapid loss of seed viability. This characteristic makes it difficult to evaluate this part if it were not carried out immediately after cutting, making it necessary to also observe the back of the axis that was protected by the cotyledons (where the color resulting from tetrazolium remained clearer) (Figure 2).

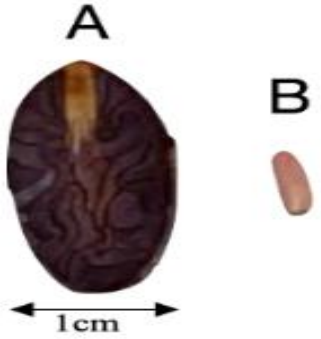

Figure 2.Viable cocoa seeds (*Theobroma cacao* L.), in class 1, after 3 hours in a tetrazolium salt solution, at a concentration of 0.075 % (with A referring to the place where the cut was made and already oxidized and B being the back of the hypocotyl-radicle axis with more visible color).

Table 2. Percentage of cocoa seeds (*Theobroma cacao* L.) classification at different times in tetrazolium solution and different concentrations, considering each evaluation class [(1)].

CLASS 1							
	Time (hours)						
Concentration	2	3	4	5	6	7	8
0.05 %	54Ba*	75Aa	82Aa	81Aa	82Aa	81Aa	73ABa
0.075 %	56Ba	76Aa	72ABa	82Aa	85Aa	85Aa	84Aa
CLASS 2							
	Time (hours)						
Concentration	2	3	4	5	6	7	8
0.05 %	0Aa	0Aa	0Aa	2Aa	8Aa	4Aa	0Ab
0.075 %	0Ba	0Ba	3ABa	7ABa	3ABa	8ABa	9Aa
CLASS 3							
	Time (hours)						
Concentration	2	3	4	5	6	7	8
0.05 %	33Aa	16Ba	14Ba	14Ba	6Ba	14Ba	19ABa
0.075 %	22Ab	15ABa	6Ba	8ABa	11ABa	3Bb	4Bb
CLASS 4							
	Time (hours)						
Concentration	2	3	4	5	6	7	8
0.05 %	13Aa	9Aa	4Aa	3Aa	4Aa	1Aa	8Aa
0.075 %	22Aa	9ABa	5Ba	3Ba	1Ba	4Ba	3Ba

[(1)] Class 1 = Viable - Embryonic axis with light pink color, Class 2 = Viable - Embryonic axis with light pink color and some dark pink spots, without compromising essential tissues such as plumule, radicle, Class 3 = Non-viable - Embryonic axis with 50 % or more with white color, Class 4 = Non-viable - Embryonic axis with 100 % white color or deformed cotyledons or empty seeds with non-existent cotyledons.
*Means followed by the same capital letter in the rows and lowercase letters in the columns do not differ from each other using the Tukey test at 5 % probability.

Different times in tetrazolium solution, as well as the different concentrations, provided variation in the color of the embryonic axis (Figure 3). It has been observed that the tetrazolium test is capable of staining viable embryonic axes, leaving these axes red (Silva-AL *et al*., 2021; Silva-RN *et al.*, 2021).

Lamarca and Barbedo (2014) identified that as the tetrazolium salt concentration and the period in solution increase, there is an increase in the intensity of red color in the axis tissues. In the present work as seen in Table 2 and Figure 3), the concentration was important for the increase in tissue color, since the seeds under 0.075 % tetrazolium for 4 hours already appeared classified in class 2 (with a slightly more intense color) unlike the treatment with lower concentration (0.05 %) at the same time where no seeds had yet appeared within this class.

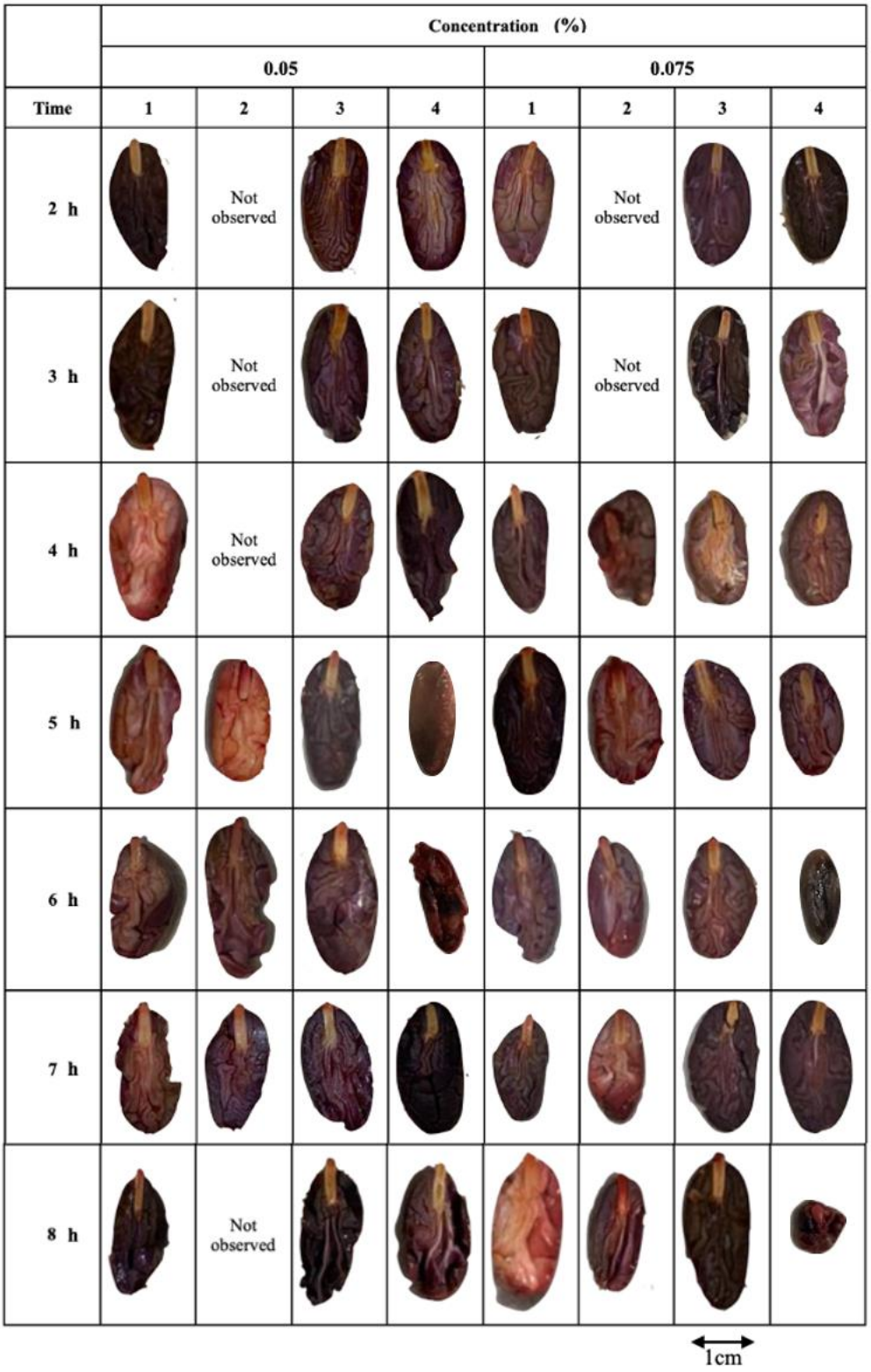

Figure 3. Staining of hypocotyl-radicle axis of *Theobroma cacao* L. seeds, by class, at different times in tetrazolium solution for concentrations 0.05 % and 0.075 %. "Not observed" indicates that there were no classified seeds in class 2.

We are highlighting that, within each class, it was possible to observe a variation in color. As an example, in Figure 4, color variations are identified within the classes of viable seeds (Class 1) over a period of 3hours for concentration of 0.05 %. Despite the variation in the intensity of the pink color on the axis, it was possible to classify all these as viable, unlike the non-viable ones (classes 3 and 4) in which the color was not observed at least in more than 50 % of the seed. This difference in seed color within the same class may be related to the penetration capacity of the tetrazolium salt into the seed tissues. This also happened in the work of Borella *et al*. (2020) in Brazil nut seeds, where it was identified that regardless of the treatment, there was a variation in the degree of penetration of the tetrazolium salt into the seed.

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Figure 4. Color variations in the hypocotyl-radicle axis in cocoa seeds (*Theobroma cacao* L.) within class 1 (viable, with light pink color) at 3 hours for 0.05 % concentration.

CONCLUSIONS

The tetrazolium test is indicated for evaluating the viability of cocoa seeds. The recommended immersion time in the tetrazolium solution is 3 hours, with a concentration of 0.05 % being the most appropriate to reduce the use of tetrazolium salt.

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