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Adjustment of the methodology of the tetrazolium test for estimating viability of *Eugenia uniflora* L. seeds during storage[1]

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ABSTRACT - The study aimed to adjust the methodology of the tetrazolium test to estimate seed viability of *Eugenia uniflora* L. (Surinam Cherry). Seeds were collected in September 2012 and divided into four lots: freshly harvested (Lot I); stored in plastic bags in a refrigerator at 10 °C for: 15 days (Lot II); 30 days (Lot III); and 45 days (Lot IV). The freshly harvested seeds were preconditioned with direct immersion in water and wet paper towel, followed or not by longitudinal cutting. The seeds were immersed in a 0.1% tetrazolium solution for 4 hours at 30 °C. Appropriate soaking and preparation methods were applied to the seeds lots using three tetrazolium concentrations: 0.1; 0.5; and 1.0%; and four preconditioning periods (2, 4, 6 and 8 hours) at 30 °C. The viability results obtained by the tetrazolium test were compared with those of the germination test. Direct immersion of seeds in water for 24 hours at 25 °C, followed by a longitudinal cut was efficient for preconditioning the seeds. Seed staining with tetrazolium solution at a concentration of 0.5% for 2 hours at 30 °C can be used to estimate the viability of freshly harvested and stored Surinam Cherry seeds.

Index terms: viability test, surinam cherry, native fruit.

Adequação da metodologia do teste de tetrazólio para estimativa da viabilidade de sementes de *Eugenia uniflora* L. submetidas ao armazenamento

RESUMO - O estudo teve como objetivo adequar a metodologia do teste de tetrazólio para estimativa da viabilidade de sementes de *Eugenia uniflora* (pitanga). As sementes colhidas em setembro de 2012 foram divididas em quatro lotes: recém-colhidas (lote I), armazenadas em sacos plásticos em refrigerador a 10 °C durante 15 (lote II); 30 (lote III) e 45 dias (lote IV). As sementes recém-colhidas foram submetidas aos pré-condicionamentos: embebição direta em água e em rolo de papel umedecido, ambos por 24 horas a 25 ºC e, em seguida, submetidas ou não ao corte longitudinal. Posteriormente, as sementes foram imersas em solução de tetrazólio a 0,1% por quatro horas a 30 ºC. O método de embebição e preparo mais adequados foram aplicados aos lotes, utilizando-se três concentrações de tetrazólio: 0,1; 0,5 e 1,0% e quatro tempos de coloração (2, 4, 6 e 8 horas) a 30 ºC. Os resultados obtidos pelo teste de tetrazólio foram comparados com os de germinação. A imersão direta das sementes em água por 24 horas a 25 ºC, seguida do corte longitudinal, apresentou eficiência no pré-condicionamento de sementes de pitanga. O teste de tetrazólio a 0,5% por 2 horas a 30 ºC pode ser utilizado para estimar a viabilidade tanto de sementes recém-colhidas como armazenadas.

Termos para indexação: teste de viabilidade, pitanga, frutífera nativa.

Introduction

Brazil has a considerable area of native forest with a great diversity of fruit trees, many of them with potential use still little explored and lacking studies to allow their rational exploitation (Kohama et al., 2006). In the family Myrtaceae, numerous species are found in such condition, especially the genus *Eugenia* (Bülow et al., 1994). Among the species of this genus, *Eugenia uniflora* L., commonly known as Surinam Cherry, occupies a distinctive position. Because it is a plant that adapts to the environment very easily, it is found all over the Brazilian land and in several parts of the world (Bezerra et al., 2000; Merwe et al., 2005).

It is a species that has multiple uses; it is considered a source for the production of herbal medicines, essential oils, cosmetics and dyes. The wood has been used in the making

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of agricultural tools and luxury furniture. The fruits can be consumed either *in natura* or used in the preparation of pulps, juices, ice creams, candies, liqueurs and even in fermented beverages. In addition, the bittersweet flavor and typical aroma of the fruit attract the frugivorous fauna, which feeds on this fruit and also spreads it seeds. Given this, its use is indispensable in programs of restoration of degraded ecosystems (Lorenzi, 2002; Carvalho, 2006; Vieira et al., 2011).

Successful reforestation of plots of land with native species depends primarily on the use of seeds of high physiological quality to produce more vigorous seedlings. Often, the seeds are not used immediately after being harvested and are stored for future use because most of the native species have different cycles of seeds production. As a result, there is a need to maintain the viability of the seeds during storage by means of technologies developed and suited to each species (Kissmann et al., 2009).

But it is known that seeds from various Brazilian species of *Eugenia* have a high content of water when the fruits are dispersed, which may compromise the viability of the seeds in storage, with direct impacts on the seeds physiological quality (Neves, 1994; Andrade and Ferreira, 2000; Maluf et al., 2003; Delgado and Barbedo, 2007; Justo et al., 2007; Masetto et al., 2008; Sena et al., 2010). In this context, it is important to know the storage potential of these seeds by means of tests that determine viability in a fast, simple, effective and low-cost way to support the decision-making process.

The seeds quality is routinely evaluated by direct and indirect tests. Example of direct tests is the germination test, which is conducted under favorable conditions of temperature, light and substrate (Brasil, 2009). But, even though it helps provide optimum germination potential, the test has some disadvantages, such as having the results compromised by the presence of fungi and dormant seeds and, especially, for causing some delay in obtaining the results (França-Neto et al., 1998).

In contrast, the tetrazolium test has been proved to be one of the most efficient and complete methods to assess the physiological quality of seeds. Besides estimating the seeds viability, this test provides a quick assessment of the seeds (less than 24 hours depending on the species); it may diagnose the main causes of reduction of physiological potential; it detects deterioration at the earliest stages, allowing to determine different levels of viability of the seeds; it requires simple and low cost equipment (França-Neto et al., 1998).

The information provided by this test may help the nursery technician make safer decisions on the discard of lots with unsuitable quality for seedlings production (Azerêdo et al., 2011), or estimate the feasibility of seeds that require long time to germinate (Pinto et al., 2008), or determine the viability of seeds that have short shelf life during storage (Abbade and Takaki, 2014).

The test basically consists of staining the tissues in the presence of tetrazolium solution. Such staining, or change in the color, reflects the activity of a set of enzymes involved in respiration. These enzymes, particularly malic acid dehydrogenase, catalyze the reaction of H^+ ions released by living tissues that reduce 2,3,5-tryphenil tetrazolium chloride, forming a red-colored, stable and non-diffusible substance called triphenyl-formazan. So, the red color obtained at the completion of test indicates cell viability and, consequently, tissue viability (França-Neto et al., 1998).

For native species the tetrazolium test has been used sparingly although it has potential to be used routinely. However, some research efforts have been conducted in order to adjust the method, such as for seeds of *Peltophorum dubium* ("canafístula") (Oliveira et al., 2005), *Lafoensia pacari* ("mangaba-brava") (Mendonça et al., 2006), *Gleditschia amorphoides* ("sucará") (Fogaça et al., 2006), *Schizolobium parahyba* ("guapuruvu") (Ferreira et al., 2007), *Poecilanthe parviflora* ("coração-de-negro") (Pinto et al., 2008), *Parkia velutina* ("visgueiro") (Mendes et al., 2009), *Eugenia pleurantha* ("pitanga-do-mato") (Masetto et al., 2009) and in seeds under storage, such as *Acca sellowiana* ("Brazilian guava") (Sarmento et al., 2013) and *Tabebuia roseoalba* ("white ipe") (Abbade and Takaki, 2014).

Given this, this study aimed to suit the methodology of the tetrazolium test to estimate the viability of freshly harvested and stored seeds of *Eugenia uniflora*.

Material and Methods

Fruits of *Eugenia uniflora* were harvested manually from six adult trees in Toledo, Paraná, Brazil, in September 2012. After being harvested, they were sent to the Laboratory of Biotechnology of the Pontifícia Universidade Católica do Paraná – Toledo campus.

To extract the seeds and remove pulp wastes the method used was to frictionate the seeds in a sieve under running water. To eliminate excess water from the seeds, they were dried in shade in laboratory ambient for 24 hours. Subsequently, the seeds were homogenized and divided into four lots, according to the recommended storage time: fresh seeds (lot I); seeds stored for 15 days (lot II); 30 days (lot III) and 45 days (lot IV). For storage of lots II, III and IV, the seeds were placed in closed perforated polyethylene plastic bags (ten 0.5-mm holes) and kept in refrigerator at a temperature of 10.0 °C ± 3.0 °C (Barbedo et al., 1998).

Lots characterization

For the characterization of lot I the weight of thousand seeds was determined using 16 replications of 100 seeds, as recommended by RAS – Rules for Seed Testing (Brasil, 2009). Seed moisture content was also determined by using four replications of five grams of intact seeds. The seeds were kept in oven at 105 °C ± 3 °C for 24 hours. After the drying period, the samples were kept in a desiccator for ten minutes and then weighed in an analytical scale (±0,001 g). The results were expressed based on the wet weight of seeds (Brasil, 2009). For lots II, III and IV, the seed moisture content was also examined to determine whether there was water loss or gain in the seeds during storage.

Germination test

The germination test was performed aiming to compare results and ensure the reliability of tetrazolium test. For this test, the substrates consisted of paper germitest type, moistened to saturation with distilled water in the amount of 2.5 times its dry weight, sand and vermiculite. Both paper germitest and sand were previously sterilized in autoclave at 120 °C (1 atm), for one hour. Subsequently, the substrates were put into gerbox-type plastic boxes using three layers for the paper, and a 2-cm layer for the sand and vermiculite substrate. The seeds were disinfected with 2% sodium hypochlorite for five minutes, then rinsed under running water (Brasil, 2009) and arranged in a single layer on the substrates. Testing was conducted in BOD germination chamber at 25 °C with 12 hours of photoperiod (Wielewicki et al., 2006).

Evaluation was carried out by counting the seeds daily until stabilization of germination 35 days after sowing, considering as germinated seeds those that presented roots size equal to or greater than 2.0 mm (Hadas, 1976). When the test was completed, the percentage and average speed of germination was determined according to Labouriau (1983). The same procedure was performed in the other lots, once the test of seeds of lot I had not been concluded when the tests for storage seeds started (lots II and III), preventing the indication of the best substrate for its driving.

For the germination test, a completely randomized experimental design was employed, with four replications of 25 seeds for each substrate tested. In order to identify the best substrate for lot I (freshly harvested seeds), variances in the treatments were tested for homogeneity by the Bartlett´s test, and those which were found homogeneous were subjected to analysis of variance (F test), and the averages of the treatments were compared by the Tukey test at 5% probability level, with the aid of the Assistat 7.7 software (Silva, 1996). So, after identification of the proper substrate for the germination test of lot I, the same was used for a comparative calculation of the mean values of the stored lots (II, III and IV).

Tetrazolium test

For the tetrazolium test, preliminary studies with freshly harvested seeds of *E. uniflora* (lot 1) were conducted to identify the most appropriate method of soaking and preparation to be used for the stored seeds. Thus, for pre-conditioning two seeds soaking methods were tested: direct immersion in water at 25 °C for 24 hours and soaking in wet towel paper at 25 °C for 24 hours. The seeds were subsequently subjected to two preparation methods: intact seeds and seeds sectioned lengthwise. During preparation of the replications related to the longitudinal cut, the seeds remained under deionized water until the full sample was prepared.

After completion of this process, the seeds were immersed in a 0.1% tetrazolium solution for four hours at 30 °C in the dark (Masetto et al., 2009). After staining has been completed, the tetrazolium solution was drained, the seeds washed under running water and then were assessed in a 4 x magnifying stereoscopic microscope. During assessment, the seeds that remained intact were cut lengthwise to allow viewing the inner structure and determining whether penetration of the tetrazolium solution in the tissues had occurred.

As the proper conditions of seeds soaking and preparation were defined, they were used to assess the best concentration and exposure time to tetrazolium. For this purpose, three tetrazolium concentrations were tested: 0.1; 0.5 and 1.0 % and four periods of exposure to the salt (2, 4, 6 and 8 hours) at 30 ºC, in the dark.

After each incubation interval, the seeds were washed under running water and maintained immersed in distilled water and in air-conditioned ambient until the time of analysis. The evaluation was performed using a 4 x magnifying stereoscopic microscope, and the result was expressed in percentage of viability according to the diverse staining patterns observed in the internal structures of the seeds (Masetto et al., 2009).

For the tetrazolium test, a completely randomized experimental design, 3 x 4 factorial (three concentrations and four intervals of immersion of the seeds in the tetrazolium solution) with four replications of 25 seeds for each experiment. The results were analyzed by the Bartlett´s test and analysis of variance (F test), and the means were compared by the Tukey test at the level of 5% probability using the Assistat 7.7 statistical software (Silva, 1996). So, after selection of the appropriate methods for the tetrazolium test, they were employed in the viability analysis of the stored seeds.

To ensure the reliability of the results of the tetrazolium test, expressed as percentage of viable seeds, they were

compared with the results of the germination test, expressed as percentage of germinated seeds, through a completely randomized design, in a 2 x 4 factorial arrangement (two viability tests, tetrazolium and germination tests, for the four lots in different storage times. The results were subjected to the Bartlett´s test and analysis of variance (F test), and when statistical differences were observed in the treatments, the means were compared by the Tukey test at 5% probability level using the Assistat 7.7 statistical software (Silva, 1996).

Results and Discussion

Lots characterization

The mean weight of 1000 seeds was 347.70 g, corresponding to 2,876 seeds/kg or 0.35 g/seed, which is in agreement with the values proposed on Instructions for analyses of forest species seeds (Brasil, 2013), i.e., 2,300 to 5,000 seeds/kg, but above the values reported by Lorenzi (2002) (2,350 seeds/kg). According to Piña-Rodrigues and Aguiar (1993), the seeds size and weight are extremely plastic features, differing from place to place, year to year and between and within individuals, which explains the result found, once the seeds were provided from matrices of different locations.

The water content of *E. uniflora* seeds at the harvesting time was 43.4% (lot I), 42.8% (lot II); 41.8% (lot III) and 40.6% (lot IV). The high water content in these seeds is a common characteristic in recalcitrant species, especially those of the genus *Eugenia,* which present moisture content varying from 40-70% when dispersed, as observed for *E. involucrata* ("cherry of the rio grande") (Barbedo et al., 1998), *E. dysenterica* ("cagaita") (Andrade et al., 2003), *E. rostrifolia* ("batinga") (Santos et al., 2004) and *E. cerasiflora* ("guamirim") (Delgado and Barbedo, 2007).

Germination test

The germination rate (%) and mean speed obtained for the freshly harvested seeds of *E. uniflora* (lot I) are shown in Table 1. The germination rate (%) and speed were not affected by the substrates tested, with mean values that varied from 98% for germination in paper and vermiculite and 96% in sand. Regarding the germination speed, the mean values found for sand and vermiculite substrates were of 0.09 germinated seeds/day and 0.08 for paper.

It has been observed high germination rate for some native species in more than one type of substrate. *E. uniflora* is a species that adapts easily, with occurrence in the most diverse soils and climates, which can explain the behavior shown in this study. This was also the case of the seeds of *Maquira sclerophylla* ("bacabinha"), which showed good performance in sand and vermiculite (Miranda and Ferraz, 1999) and *Dalbergia nigra* ("jacarandá-da-bahia"), in vermiculite and paper towel (Andrade et al., 2006). Thus, the three substrates tested can be used for standard germination test of *E. uniflora* seeds in germination chamber at 25 °C and photoperiod of 12 hours.

Table 1. Mean values of germination (%) and germination speed of freshly harvested seeds (lot I) of *Eugenia uniflora* L. (Surinam Cherry).

Substrate	Germination (%)	Mean speed (seeds/day)
Sand	96 a*	0.09 a
Paper	98 a	0.08 a
Vermiculite	98 a	0.09 a
DMS	3.72	0.015
CV (%)	1.94	8.16

*Means followed by the same letter in the column do not differ by the Tukey test (p >0.05).

Tetrazolium test

In the method defined as appropriate to conduct the tetrazolium test in seeds of *E. uniflora*, it was found that the seeds that remained intact, irrespective of the preconditioning used, did not present staining or change in color. It is suggested that the presence of tegument attached to the seeds might have worked as a physical barrier, preventing the direct contact of the internal tissues with the tetrazolium solution, and so an additional preparation of these seeds aiming to allow penetration of the tetrazolium solution was necessary.

Thus, it was found that when the seeds were preconditioned directly in water for 24 hours at 25 ºC, followed by a longitudinal cut, a greater diffusion of the salt through the tissues occurred, causing the development of homogeneous staining. As the main goal of this step is to facilitate the penetration and diffusion of the tetrazolium solution through the tissues to provide homogeneous staining (França-Neto et al., 1998), preconditioning of *E. uniflora* seeds consisted of immersing the directly in water for 24 hours at 25 ºC, and as preparation prior to staining the seeds were bisected lengthwise.

In addition to the seeds preconditioning and preparation steps, the use of a solution with different concentrations of tetrazolium, the staining time and temperature, and the proper interpretation of the seeds color are also crucial steps to ensure successful testing (Abbade and Takaki, 2014).

According to the analysis of variance, there was a significant interaction for the factors, tetrazolium concentration and soaking time, in determining the viability of freshly harvested seeds of *E. uniflora* (lot I), as it can be seen in Table 2. The highest

percentage of viable seeds was 96%, which was obtained at a concentration of 0.5% of the tetrazolium salt for 2 hours, differing significantly from 4, 6 and 8 hours (71%, 60% and 49% of viable seeds, respectively).

Table 2. Viability (%) of freshly harvested seeds (lot I) of *Eugenia uniflora* L. (Surinam Cherry) as estimated by the tetrazolium test in different solution concentrations and soaking times.

	Time (hours)				
Concentration	2	4	6	8	Means
0.1%	22 cC*	54 bB	61 aAB	69 aA	51
0.5%	96 aA	71 aB	60 aC	49 bD	69
1.0 %	55 bA	42 cB	15 bC	10 cC	30
Means	57	55	45	42	
DMS	9.02				8.18
CV (%)	9.41				

*Means followed by the same uppercase letter in the row and lowercase in the same column do not differ by the Tukey test (p >0.05).

Similar results were reported by Mendes et al. (2009) for seeds of *Parkia velutina* ("visgueiro") and Kalil Filho et al. (2008) for *Ocotea porosa* ("imbuia"). Both authors recommend the use of 0.5% tetrazolium for two hours to estimate the seeds viability.

The concentration of 0.1% of tetrazolium solution in 6 and 8 soaking hours provided similar viability results (61 and 69%, respectively) (Table 2), but a mild and uneven staining was developed making it difficult to view abnormalities and to distinguish viable and non-viable tissues in the seeds. In addition, the longer exposure time to the solution caused some delay in conducting the test, preventing the analysis of an optimal number of samples per day.

Lower rates of viable seeds were observed for the 1% of tetrazolium solution for 6 and 8 hours (15% and 10%, respectively), which did not differ. These results are likely related to the development of an intense and dark staining of the tissues, caused by high concentration of the salt used, hindering a proper interpretation of the test.

The staining time should be completed when the mean intensity of the color is considered optimal to allow interpretation (Delouche et al., 1976). Based on that, the optimal combination of tetrazolium solution and exposure time was considered as that of soaking in 0.5% solution for two hours. Such condition allowed the development of a homogeneous staining, facilitating the interpretation of the results. Added to that, the results found in this condition were similar to those found in the germination test, regardless the substrate used (Tables 1 and 2). So, this was also the condition used to test *E. uniflora* stored seeds (lots II, III and IV).

According to Marcos-Filho (2005), the staining pattern found at completion of the tetrazolium test indicates seeds viability, deterioration or non-viability, or even an excessive or insufficient time of exposure to the salt solution. As a consequence, a detailed examination of each seed, observing stained and unstained regions carefully regarding their site and extension, allows to identify viable and non-viable seeds easily.

However, the analyst should also know the seed structures, be familiar with vital regions, have experience and imagination, besides critical judgment, because the interpretation may become subjective (França-Neto et al., 1998).

The seed of genus *Eugenia* have conferruminate embryo, eugenoide type, with completely fused cotyledons without trace of hypocotyl-radicle axis (Barroso et al., 1999). As a result, both the seeds staining estimation and definition of the viability classes used as reference parameter the stain intensity throughout the inner seed region.

Thus, observing each one of the seeds that were stained, it was possible to determine the viability levels (Figure 1), as follows: light red (indicating living tissue); intense dark or purplish red (tissue in deterioration or mechanically damaged) and white the milky and/or no staining (dead tissue).

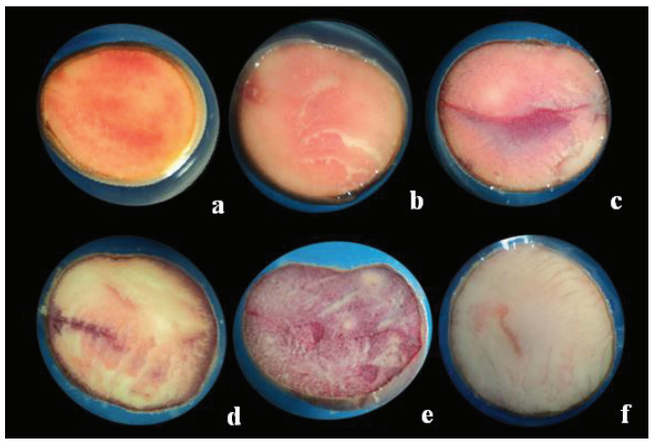

Figure 1. Viability classes of *Eugenia uniflora* L. (Surinam Cherry) found in tetrazolium test: (a) viable seed with homogeneous light pink color; (b and c) viable seed with light pink color in more than 50% of the embryo tissue, mottled white and purplish red stains; (d) non-viable seed with more than 50% of the embryo tissue in white color and intense purplish, brownish red edges; (e) non-viable seed with intense carmine, dark brownish red color; (f) non-viable, unstained seed.

Lots comparison

The mean values found in the germination and tetrazolium tests of *E. uniflora* seeds for the four lots of different storage times are shown in Table 3. According to the analysis of variance, there was no significant interaction for the viability tests factors (germination and tetrazolium) and different storage times (lots), with mean values of 93% for the germination test and 91% for the tetrazolium test.

Table 3. Mean values found in germination test (germinated seeds) and tetrazolium test (viable seeds) of seeds of *Eugenia uniflora* L. (Surinam Cherry) seeds in different storage times.

Storage time	Germinated seed (%)	Viable seeds (%)	Means
Lot I (freshly collected)	98	96	97 a
Lot II (15 days)	97	92	94 a
Lot III (30 days)	94	91	92 a
Lot IV (45 days)	86	85	85 b
Means	93 A*	91 A	
DMS	3.11		5.88
CV (%)	4.61		

*Means followed by the same uppercase letters in the row and lowercase in the column do not differ by the Tukey test ($p > 0.05$).

The results found in the germination and tetrazolium tests did no differ for all tested storage times (Table 3). This is important because to ensure the applicability of the tetrazolium test, the germination and viability results should be close, allowing differences of up to 5% between both the tests (Ferreira et al., 2001).

For *E. uniflora* seeds it is possible to obtain the results of the tetrazolium test in 30 hours, being 24 hours for seeds soaking, one hour for cutting, two hours for soaking in the tetrazolium solution, and two hours on average to assess a sample of 100 seeds. On the other hand, in the germination test the results were obtained only after 35 days. Based on this, the tetrazolium test can be recommended as a reliable method to replace the standard germination test for a quick estimation of the viability of freshly collected and stored *E. uniflora* seeds.

Regarding different storage times (lots), it can be seen that the mean percentage values of germination and viable seeds were higher for freshly harvested seeds (lot I: 97%) and stored seeds for 15 and 30 days (lot II: 94% and lot III: 92%). However, as the storage time extends, these values decrease, causing a significant reduction of the viability of seeds stored for 45 days (lot IV: 85%) (Table 3). This suggests that this behavior is associated with high water content and high respiratory activity of the seeds in this period. These conditions favor proliferation of microorganisms and accelerate the respiratory metabolism and culminate in the seeds deterioration process.

The high respiratory rates of recalcitrant seeds are due to the fact that the seeds do not undergo dehydration at the late stage of maturation and the metabolism remain active after detachment from of the mother plant (Pammenter and Berjak, 2000). However, it is important to identify whether seeds deterioration promotes proliferation of microorganisms or the microorganisms themselves increase the seeds deterioration rate or even whether both processes may occur simultaneously, so that control measures to minimize the loss of quality during storage may be implemented.

Deciding on the seeds storage method depends on the purpose and shelf life required. According to Vieira et al. (2001), all stored seeds suffer deterioration, which can be faster or slower depending on the storage ambient conditions and the seeds characteristics, which can prolong their longevity or not.

Thus, to prevent damages to the physiological quality of *E. uniflora* seeds, it is suggested that storage in semipermeable packages and refrigeration at 10 ºC do not exceed 30 days. Storage of seeds for 30 days is a good alternative for propagation and preservation of the germoplasm, once it favors the production of seedlings, not requiring sowing right after harvest, which is an indispensable practice for a great number of recalcitrant species.

Conclusions

Direct immersion of seeds in water for 24 hours at 25 ºC followed by lengthwise cutting proved to be effective in the preconditioning of *Eugenia uniflora* seeds for conducting the tetrazolium test.

Tetrazolium test with a 0.5% solution for two hours at 30 ºC is a fast and reliable method to estimate the viability of freshly harvested and stored seeds of de *E. uniflora,* as a complement to the germination test.

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