Capítulo II

Tetrazolium test in Pterogyne nitens Tul. seeds (Fabaceae)

10.46420/9786588319741cap2

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INTRODUCTION

Pterogyne nitens Tul. species popularly known as "carne-de-vaca", is a species of the Fabaceae family. It occurs from the Northeast to the South of Brazil, being characterized as a pioneer species, characteristic of the semi-deciduous broadleaved forest and the "caatinga". Due to its rusticity and rapidity of growth it is optimal for mixed plantations in degraded areas of permanent preservation. It presents wide but discontinuous dispersion, both in the dense primary forest and in secondary formations in several stages of succession. Its wood is used for the manufacture of fine furniture, interior of boats and wagons, casks and civil construction (Lorenzi, 2008).

P. nitens seeds present dormancy as soon as they are harvested, due to the impermeability of its coat to the water absorption (Nassif et al., 1997). As a result of this dormancy occur in most tree species, the germination becomes slow and often difficult to obtain fast and conclusive results (Fogaça, 2015).

In addition, from the occurrence of integument dormancy, other factors affect the germination test, such as temperature, which influences the biochemical reactions that regulate the metabolism necessary to initiate the germination process (Zamith et al., 2004), the moisture of the substrate, which must present the quantity of water required for germination (Brasil, 2009), oxygen, essential for the metabolic processes of respiration (Tanaka et al., 1991), light and type of substrate (Figliolia et al., 1993). Another big problem in native forest species is the infestation of microorganisms, mainly fungi that colonize the seeds, altering the result of the germination analysis (Oliveira, 2012).

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Thus, the technologists have been seeking alternative tests with faster results and less interference of the medium, wich allow the determination of the physiological quality of the evaluated lots. The search for tests with these principles resulted in several scientific studies, using the tetrazolium test to evaluate the physiological quality of forest seeds (Fogaça, 2015).

The tetrazolium test is based on the activity of dehydrogenases in living tissues. These enzymes catalyze respiratory reactions in mitochondria during glycolysis and the Krebs cycle. During respiration the release of hydrogen ions, with which the salt 2,3,5-triphenyl tetrazolium chloride, or simply tetrazolium, colorless and soluble, reacts forming a red and insoluble substance called formazam (Delouche et al., 1976; Krzyzanowski et al., 1991; França-Neto et al., 1998).

The substance formed by the reaction of the tetrazolium solution with the dehydrogenases, forming it, does not diffuse. Marking with the coloration and differentiating living and vigorous, deteriorated and dead tissues. Tissues with bright red or uniform coloration, typical of healthy tissue, white-milky or original seed coloring and flaccid appearance, dead tissues and, finally, deteriorating tissues with intense red coloration (Fogaça, 2015).

The efficiency of the tetrazolium test to evaluate seed viability depends on the use of the appropriate method for each species, with the determination of appropriate conditions for seed preparation, staining and evaluation (Pinto et al., 2008).

With respect to the comparative tests of tetrazolium with the germination, the results obtained in the tetrazolium test tend to be better than those obtained in the standard germination test, due to the absence of fungi and no identification of the occurrence of dormant seeds (Piña-Rodrigues et al., 1988). To avoid problems of discrepancy between the results of the tetrazolium and germination tests, it is recommended, if necessary, that the seeds be treated to overcome dormancy before performing the standard germination test (Delouche et al., 1976; Fogaça, 2000). In addition, it is recommended that the two tests be performed together until the methodology is proven, so that the analyst is sure to perform correct evaluation (Fogaça, 2015).

From the standardization of the tetrazolium test and comparison with the germination test, the authors found positive correlations between the two tests for forest species, which makes possible the use of the tetrazolium test as an alternative to the germination test in the evaluation of seed viability *Astronium graveolens* Jacq., *Jacaranda cuspidifolia* Mart. and *Piptadenia rigida* Benth. (Fogaça, 2003); *Sebastiania commersoniana* (Baill.) L.B. Sm. & Downs (Santos et al., 2006); *Poecilanthe parviflora* Benth. (Pinto et al., 2008); *Copaifera langsdorffii* Desf. and *Schizolobium parahyba* (Vell.) S.F. Blake (Fogaça et al., 2011); *Eugenia involucrata* DC. and *Eugenia pyriformis* Cambess. (Cripa, 2012) and *Matayba elaeagnoides* Radlk. (Freitas, 2012).

Thus, the objectives of this study were to standardize the tetrazolium test and to evaluate its applicability to estimate the viability of *Pterogyne nitens* seeds.

MATERIAL AND METHODS

The present work was carried out in two stages in the Forest Ecology Laboratory of the Center for the Restoration of Degraded Areas (CRAD / Dry Forest) of the Department of Agrarian Sciences of the State University of Montes Claros (UNIMONTES), Janaúba, MG, Brazil. For both, it was used a lot of seeds collected in the School Nursery of CRAD / Dry Forest in July 2017, which was handled, packed in plastic bags and kept in a refrigerator until the evaluation. For the seed lot characterization, the water content and the weight of one thousand seeds were determined according to the requirements of the Rules for Seed Analysis (Brasil, 2009).

In the first stage, it was tested the combination of the treatment of seed preparation, concentrations and periods of exposure of the seeds in the tetrazolium solution.

Seed preparation consisted of mechanical scarification (sandpaper nº 80, in the region opposite the embryo) followed by soaking in distilled water for 24 hours, conditioned at 30 °C, with subsequent removal of the integument, in order to avoid damage to the embryo.

After preparation, the seeds were conditioned in 200 mL plastic containers with a solution of 2,3,5-triphenyl tetrazolium chloride (pH of 6.5 to 7.0) at concentrations of 0.075; 0.10 and 0.20% in sufficient quantity to cover them for 1, 3 and 5 hours and kept in a controlled chamber at 35 °C in the dark. Four replicates of 25 seeds were used for each combination of tetrazolium solution concentration and staining period.

After the staining periods, the solutions were drained and the seeds cleaned in running water, with subsequent immersion in water and kept in a refrigerated environment until the moment of the evaluation. The seeds were analyzed one by one by sectioning them longitudinally through the center of the embryonic axis with the aid of a scalpel. The visualization of all the details of the seeds counted on the aid of a table magnifying glass with a six-fold fluorescent lamp (6x).

For the characterization of the staining classes, a representation of six seed coloration diagrams was elaborated, observing the presence and location of the damages, besides the physical conditions of the embryonic structures.

The definition of the best staining conditions was based on the aspects of the tissues and on the intensity and uniformity of coloration that allowed the differentiation of the tissues according to established criteria for the tetrazolium test: bright red or pink (living and vigorous tissue); strong red-carmine (deteriorating tissue) and milky or yellowish white (dead tissue) (França-Neto et al., 1998).

In the second stage of this study, the efficiency of the tetrazolium test in estimating the viability of *P. nitens* seeds was evaluated by comparing the results of the tetrazolium and germination tests.

The germination test was performed with four replicates of 25 seeds, in paper roll, kept in a germination chamber at constant temperature of 25 °C and photoperiod of 12 hours. The evaluation was performed on the eleventh day after implantation, when the seedlings presented all their developed parts and the results expressed as percentage of normal seedlings.

Parallel to the germination test, the tetrazolium test was performed using the protocols established in the first stage of this work. Four replicates of 25 scarified seeds were used for each protocol, submitted to 24 hours of soaking in distilled water, at 30 °C, with subsequent removal of the tegument. The seeds were then immersed in tetrazolium solutions at concentrations of 0.20% for 3 hours and 0.075, 0.10 and 0.20% for 5 hours at 35 °C in the dark. After these periods, the seeds were cleaned in running water and kept immersed in water in a refrigerated environment until the moment of the evaluation. The seeds were analyzed individually, sectioned longitudinally through the center of the embryonic axis, with the aid of a scalpel, and observed with the aid of a table magnifying glass with a six-fold fluorescent lamp (6x). The number of viable seeds was calculated and the results were expressed as percentage of viability.

The results obtained in the germination and tetrazolium tests were submitted to analysis of variance and the averages were compared using the Dunnett test, with a probability of 5%, using the germination test as a control. The experimental design was completely randomized. Statistical analyzes were performed using ASSISTAT software version 7.7 (Silva et al., 2016).

RESULTS AND DISCUSSION

The lot of *P. nitens* seeds had a water content of 10% and weight of one thousand seeds of 102 g.

The different staining patterns obtained by the seeds according to the preparation and staining conditions are shown in Figure 1.

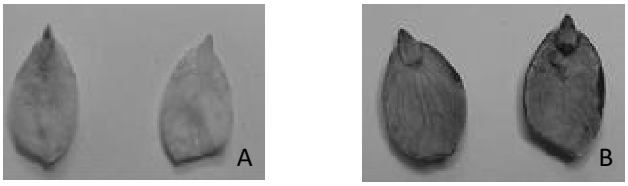

Figure 1. Patterns obtained from staining in *Pterogyne nitens* seeds. A - Seeds with weak and uneven coloring; B - Seeds with adequate and uniform coloring. Source: The Authors.

The seeds when submitted to mechanical scarification and soaking for 24 hours at 30 °C, after removal of the tegument, immersed in solution of the tetrazolium, independent of the concentration, in the period of 1 hour at 35 °C presented weak and uneven coloration, not allowing the differentiation of living, damaged and dead tissues (Table 1).

Table 1. Colors obtained by subjecting *Pterogyne nitens* seeds to the tetrazolium test in different combinations of concentrations and staining times. Source: The Authors.

Coloration process	Coloration obtained

All concentrations for 1 hour	Weak and uneven color
0.075% and 0.10% for 3 hours	Weak and uneven color
0.20% for 3 hours	Proper and uniform coloring
All concentrations for 5 hours	Proper and uniform coloring

Similar results were observed in *Enterolobium contortisiliquum* (Vell.) Morong seeds, where the same preparation (mechanical scarification, soaking for 24 hours and subsequent tegument removal) was used in seeds exposed for one hour at concentrations of 0.050, 0.075 and 0.10%, observed inadequate staining, assuming a very clear pattern, which hindered interpretation of the results (Nogueira et al., 2014).

For *Gleditschia amorphoides* Taub. seeds, it was verified that the seeds scarified and soaked in water for 48 hours, with removal of the tegument, when immersed in 0.10% solution of tetrazolium for one hour presented ideal coloration, allowing the differentiation and evaluation of the conditions of the seeds tissues (Fogaça et al., 2006).

Evaluating the same preparation with the increase of the staining time for 3 hours, verified that in the concentrations of 0.075 and 0.10%, the seeds presented weak and uneven coloration, that is, the use of these concentrations in the period of staining mentioned was not sufficient to promote proper coloring.

The standardization of the test should be performed for each species, as standardizing the methodology of this test for *Caesalpinia echinata* Lam. seeds, the authors concluded that the combination of 2 hours of staining at the concentration of 0.075% proved to be efficient for the evaluation of seed viability (Lamarca et al., 2009). For the *Enterolobium contortisiliquum* species, the immersion of the seeds in 0.075% tetrazolium solution for 3 hours was more effective for assessing viability (Nogueira et al., 2014).

The best results obtained using mechanical scarification followed by soaking for 24 hours, with subsequent removal of the integument, were the treatments that used solutions with concentrations of 0.20% for 3 hours and 0.075, 01.0 and 0.20% for 5 hours of coloring, which allowed the obtaining of adequate and uniform coloration. For the safe and efficient interpretation of the tetrazolium test is directly related to obtaining a uniform and adequate coloration that allows differentiation of living, damaged and dead tissues (Bhéring et al., 2005).

In a study on the standardization of the tetrazolium test to evaluate the viability of *Copaifera langsdorffii* and *Schizolobium parahyba* seeds, the authors recommended mechanical seed scarification followed by soaking for 24 and 48 hours at 35 °C, with subsequent tegument removal, respectively. After preparation, they recommended the immersion of seeds in tetrazolium solutions at concentrations of 0.20 and 0.10%, respectively, kept in the chamber at 35 °C for 4 hours (Fogaça et al., 2011).

For *Peltophorum dubium* (Spreng.) Taub. seeds, mechanical scarification followed by soaking for 14 hours at 25 °C with subsequent removal of the tegument and immersion of 0.10% tetrazolium solution for 5 hours at 25 °C (Oliveira et al., 2005) is recommended.

These results confirm that seed preparation, tetrazolium solution concentration and staining time are species specific (Fogaça et al., 2006).

Figure 2 shows the classification of the viability levels established in the tetrazolium test for *P. nitens* seeds considering the following characteristics as a criterion for the classification of seeds: 1. Tissues with bright red or pink coloration are typical of healthy tissues, 2. Tissues with intense red coloration are deteriorating tissues; 3. White or yellowish and flaccid tissues are dead tissues.

The description of the classes follows:

Class 1 - Viable: seed with uniform pink color, presenting normal and firm appearance;

Class 2 - Viable: seed with uniform pink coloration and in the cotyledons presenting intense red color, without reaching the embryonic axis;

Class 3 - Viable: seed presenting more than 50% of the cotyledonar region with uniform pink coloration, intense red coloration in the radicle without reaching the cylinder;

Class 4 - Inviable: embryonic axis with intense red color, characterizing deteriorating tissues;

Class 5 - Inviable: seed with intense red color, indicating deterioration process;

Class 6 - Inviable: totally white seed with flaccid tissues.

Comparing the results of the tetrazolium test with the germination test, no significant difference was observed, demonstrating that the methodologies of evaluated tetrazolium test efficiently estimated the viability of the analysed seeds (Table 2).

The acceptable difference between the tetrazolium test results and the standard germination test may be 3-5%. If the difference exceeds 10-15%, it is recommended to revise the standards used when classifying them as viable or unviable (Piña-Rodrigues et al., 1995). In the present study, the use of the concentration of 0.20% for 5 hours showed a percentage difference of more than 5%, so it was not considered effective for evaluating the viability of P. nitens seeds.

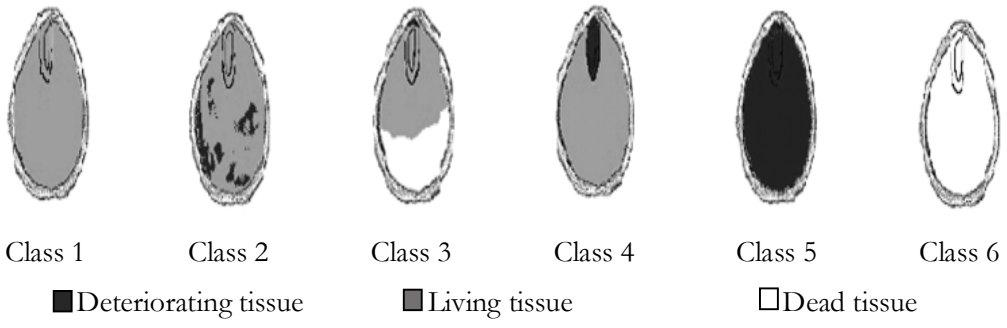

Figure 2. Diagrammatic representation of classes viability for *Pterogyne nitens*: Viable seeds (Class 1 – 3); Inviable seeds (Class 4 – 6). Source: The Authors

Table 2. Germination test and tetrazolium test to evaluate the viability of *P. nitens* seeds

Coloration process	**Viability (%)**[1]
Solution 0.20% for 3h, at 35 °C	84 a
Solution 0.075% for 5h, at 35 °C	84 a
Solution 0.10% for 5h, at 35 °C	83 a
Solution 0.20% for 5h, at 35 °C	82 a
Germination test	88 a

[1] Means followed by the same control letter (germination) did not differ significantly by Dunnet's test, at 5%. Source: The Authors

From the standardization of the tetrazolium test and comparison with the germination test, authors found positive correlations between the two tests for forest species, which makes possible the use of the tetrazolium test as an alternative to the germination test in the evaluation of seed viability, such as: *Albizia hasslerii* (Chodat) Bur.) (Zucareli et al., 2001); *Astronium graveolens*, *Jacaranda cuspidifolia* and *Piptadenia rigida* (Fogaça, 2015); *Peltophorum dubium* (Oliveira et al., 2005); *Gleditschia amorphoides* (Fogaça et al., 2006); *Sebastiania commersoniana* (Santos et al., 2006); *Poecilanthe parviflora* (Pinto et al., 2018); *Copaifera langsdorffii* and *Schizolobium parahyba* (Fogaça et al., 2011); *Eugenia involucrata* and *Eugenia pyriformis* (Cripa, 2012) and *Matayba elaeagnoides* (Freitas, 2012).

CONCLUSIONS

The procedures with mechanically scarified seeds, soaked in water for 24 hours with subsequent removal of the tegument and exposed to the 0.20% tetrazolium solution for 3 hours and 0.075 and 0.10% for 5 hours at 35 °C in the dark, are suitable to evaluate the viability of *Pterogyne nitens* seeds.

Due to the rapid obtained results, the tetrazolium test is a good option for seed quality control of the studied species, and can be used as a complement to the germination test.

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ISBN 978-658831974-1

9 786588 319741

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