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RESEARCH NOTE

Optimization of tetrazolium tests to assess the quality of *Platymiscium floribundum*, *Lonchocarpus muehlbergianus* and *Acacia polyphylla* DC. seeds[1]

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ABSTRACT - The tetrazolium (TZ) test is an alternative to conventional germination tests, which usually require longer periods for seed quality evaluation. However, it is necessary to develop specific methodologies for each species, determining the concentration and time of exposure to salt. Thus, the objectives of this study were (i) to optimize the application of tetrazolium in forest seeds of *Platymiscium floribundum* Vog., *Lonchocarpus muehlbergianus* Hassl. and *Acacia polyphylla* DC., with the proposal of an evaluation protocol for tetrazolium test in order to reduce the subjectivity of interpretation and (ii) to adapt standardized methodologies to evaluate the seed quality of forest species using TZ. To perform the tests, the seeds were submitted to immersion times and concentrations (0.1, 0.5 and 1.0%) in solution of 2,3,5-triphenyl tetrazolium chloride. The ideal concentrations and immersion time for each species were: *P. floribundum* - 0.5 and 1.0% for 60 minutes; *L. muehlbergianus* - 0,1% for 6 hours and *A. polyphylla* - 0,5% for 4 hours. Each seed was evaluated by color, size and localization of colored spots in three zones of low, medium and significant impact on germinability and after classified in three vigor classes. The protocol and the proposed methodology presented high efficiency according to the established parameters.

Index terms*:* viability, seed analysis, vigor.

Otimização do teste de tetrazólio para avaliação da qualidade de sementes de *Platymiscium floribundum*, *Lonchocarpus muehlbergianus* e *Acacia polyphylla* DC.

RESUMO - O teste de tetrazólio (TZ) é uma alternativa aos testes de germinação que, geralmente, demandam de maior tempo de execução. Para sua utilização é necessário o desenvolvimento de metodologias especificas de aplicação, determinando a concentração e o tempo de exposição das sementes ao sal. Assim, com este trabalho busca-se (i) otimizar a aplicação do TZ em sementes florestais de *Platymiscium floribundum* Vog., *Lonchocarpus muehlbergianus* Hassl. e *Acacia polyphylla* DC., reduzindo a subjetividade da interpretação dos resultados e (ii) adequar metodologias para avaliar a qualidade de sementes por meio do TZ. Para a realização dos testes, as sementes foram submetidas a tempos de imersão e concentrações (0,1; 0,5 e 1,0%) em solução de 2, 3, 5 trifenil cloreto de tetrazólio. Cada semente foi avaliada por cor, tamanho e localização das manchas em três zonas de baixo, médio e alto impacto para a capacidade de germinação e, classificada em três classes de vigor. As concentrações e tempo de imersão ideais para cada espécie foram: *P. floribundum* - 0,5 e 1,0% por 60 minutos; *L. muehlbergianus* - 0,1% por 6 horas e *A. polyphylla* - 0,5% por 4 horas. O protocolo e a metodologia apresentaram alta eficiência de acordo com os parâmetros estabelecidos.

Termos para indexação**:** viabilidade, análise de sementes, vigor.

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Introduction

The time between seed harvesting to seedlings production is influenced by the extent of time among collection, extraction, sowing until the final planting. Rapid evaluations of seed lots are fundamental to evaluate the seed quality just before planting, in accordance with pre-established standards. In this context, legal requirements such as the Normative Instruction n. 56 from December 8th, 2011 (Brasil, 2011) requires that seed quality analysis is performed only by certified laboratories with standardized methods. However, for seed trading, the period between the seed purchase and the result of the seed analyzes may be excessive, causing the loss of the seed viability and of the proper time for sowing. In addition to this, it creates costs for seed storing until tests were concluded, which may also affect their quality (Figliolia et al., 1993; Piña-Rodrigues and Figliolia, 2005).

The use of rapid tests to control seed quality is an indispensable tool to evaluate their physiologic quality and, therefore, they deserved the attention of technologists, producers and researchers (Deminicis et al., 2009). The tetrazolium test is applied with this objective and it is based on the activity of the dehydrogenase enzymes involved in the respiration process, which react forming a red substance, stable and non-diffusible: triphenyl formazan (França-Neto, 1999; Costa et al., 2007). This reaction allows distinguishing the living parts, colored in red, from the dead ones, that maintain the original color (Abbade and Takaki, 2014). The intensity of the tissue color also allows determining the viability degree of seeds, since according to Roversi and Theisen (2005), the formation of the light carmine red color indicates viable tissues. On the other hand, the intense carmine red color reveals deteriorating tissue, because of the higher diffusion intensity of the tetrazolium solution over the compromised cell membranes of such tissues.

To conduct the tetrazolium tests, procedures generating color effectiveness are necessary; they are called pre-conditioning and help the solution to penetrate inside the tissues. Numerous studies aim at standardizing the procedures for forest species; among them, as the ones developed for *Peltophorum dubium* (Oliveira et al., 2005a); *Tabebuia serratifolia* and *T. impetiginosa* (Oliveira et al., 2005b); *Gleditschia amorphoides* (Fogaça et al., 2006); *Ceiba speciosa* (Lazarotto et al., 2011); *Araucaria angustifolia* (Silva et al., 2016) among others.

However, when seeds are dormant, the tetrazolium test may present higher results than the germination test, which underestimates the results in relation to tetrazolium (Piña-Rodrigues and Valentini, 1995; Silva et al., 2012). Even if the test has a simple principle, it requires training from the analysts to use subjective criteria that are based on the color of tissues and on the knowledge about seed structures (Fogaça et al., 2011). The procedures to interpret tetrazolium take four to eight viability classes, varying from viable to unviable (Fogaça, 2011; Lazarotto et al., 2011), which makes the evaluation more subjective. However, the difficulty in differentiating the original damages of seeds from the ones caused by performing preparation cuts and seed management may result into interpretation errors by the analysts (Zucareli et al., 2001).

Considering the aforementioned, our objectives were: (a) to optimize the application of tetrazolium on forest seeds by developing a protocol to reduce the subjectivity of the tetrazolium interpretation and (b) to adequate standardized methodologies that allow evaluating the quality of forest species seeds with the use of the tetrazolium test.

Material and Methods

Species were selected due to their importance in the production of seedling to restore degraded area and their low natural longevity, being them: *Platymiscium floribundum* Vog. (sacambu) and *Lonchocarpus muehlbergianus* to require the prompt evaluation of their physiologic quality. On the other hand, *A. polyphylla* presents dormancy, but it has high demand for restoration of degraded areas.

The research was conducted in the period from September 2012 to August 2013, with newly-collected seeds of *L. muehlbergianus* Hassl. and *P. floribundum* Vog. harveted, respectively, in June and September 2012, and seeds of *Acacia polyphylla* DC., in June 2011 and after stored in a cold chamber along 2 years. In order to create the samples to be used in the germination test (GT) and in the tetrazolium test (TZ), three seed lots from each species were mixed and homogenized by the successive division manual method, following the Rules for Seed Testing (RST) (Brasil, 2009).

For tetrazolium tests, we first applied a pre-conditioning, to facilitate the diffusion of tetrazolium in seed tissues (Table 1). Times and concentrations were determined by simultaneous tests, namely: (a) a pre-test with the goal to determine the coloring time of each concentration and (b) an experimental test to evaluate immersion times in tetrazolium and concentrations. In pre-tests, seed status was observed every 15 minutes, until reaching a color that could be evaluated. In the experimental test, at the end of each period, seeds were washed, placed in water and kept under refrigeration until analysis.

In order to establish an interpretation protocol that could be replicated with other species, each seed was examined

individually under a stereoscopic magnifier (2x to 4x) defining, initially, four zones of their anatomy, namely: (a) *area 1* - near the embryonic axis, considered the most important one for the development of the embryo, because of its proximity to the hypocotyl-radicle axis structure; (b) *area 2* - median zones of the seed, considered important in the translocation of substances to the embryonic axis; (c) *area 3* - that are distant from the embryonic axis and (d) the embryonic axis itself (Figure 1).

To standardize interpretations referring to the color, the hexadecimal codes (COLORHEXA, 2015) and RGB were used, in accordance with Van de Sande et al. (2010) to compare the seed color with the color standards, aiming at reducing the subjectivity of color interpretation. After that, each seed was classified based on the step-by-step analysis of the following parameters: (a) *tissue color* - observation of the color intensity of tissues; (b) *stain position* - analysis of the position of dark red stains - #660000- RGB (R=102; G=0; B=0) to #990000- RGB (R=153; G=0; B=0) (indicating deterioration) and/or white ones – #FFFFFF- RGB (R=255; G=255; B=255) to #FFFF99- RGB (R=255; G=255; B=153) (dead tissues); (c) *stain or spots extension and size* - analysis of the proportional size of color of stains or spots (dark red or white) in relation to the seed size itself and (d) *tissue aspect* - observation of the turgescence of tissues in each seed development area. Next, to analyze the parameters, the characteristics of each seed were pointed out to be framed in the three viability classes defined in the protocol (Table 2).

The seeds evaluation was based on the following criteria: *Class 1* - viable seeds, presenting tissues with a bright red even color, considered as typical of healthy seeds; *Class 2* - they present characteristics that may occasionally interfere in the germination process, but they keep their germination potential. They present distinct colors in parts with little influence and/or stains of small extension or that may often affect their vigor, but without causing interferences in the development of the seedling; *Class 3* - seeds considered as the ones with the lowest germination potential and dead seeds, seeds with extended stains or totally discolored tissues and with an appearance indicating degradation with exudates. Seeds from class 1 and 2 were considered as viable, using the sum of the seed number in the class and its total percentage to calculate the percentage of "viable seeds" per treatment.

Germination tests (GT) were conducted at the same time of TZ ones, in chamber germinators (BOD), with a photoperiod of 12 hours, under white light at 25 °C, according to the methodologies established by Brasil (2013) (Table 3). Seeds were sowed on vermiculite or paper previously sterilized at 105 °C for 24 hours. Before sowing, seeds were treated with detergent during five minutes and rinsed under running water until all residues was removed. When they germinated, the seeds emitting a primary root at least 2.0 mm long were counted; counts were made every two days starting from the beginning of germination, and ending on day 30.

Table 1. Pre-conditioning of forest seeds for the application of the tetrazolium test (OP = on paper; W = immersion in water; L.C = Longitudinal Cut).

Species	Pre-dampening		Preparation for coloring	Color			Preparation method for the evaluation
	Type	Time (h)		Solution (%)	Time (h)	Temp. (°C)	
Platymiscium floribundum	OP	24	Tegument removal	0.1; 0.5 and 1	0.5; 1 and 2	35	L.C
Lonchocarpus muehlbergianus	W	2	Tegument removal	0.1 and 0.5	3, 5 and 6	35	L.C
Acacia polyphylla	W	24	Tegument removal	0.1;0.5 and 1	4, 6 and 8	30	L.C

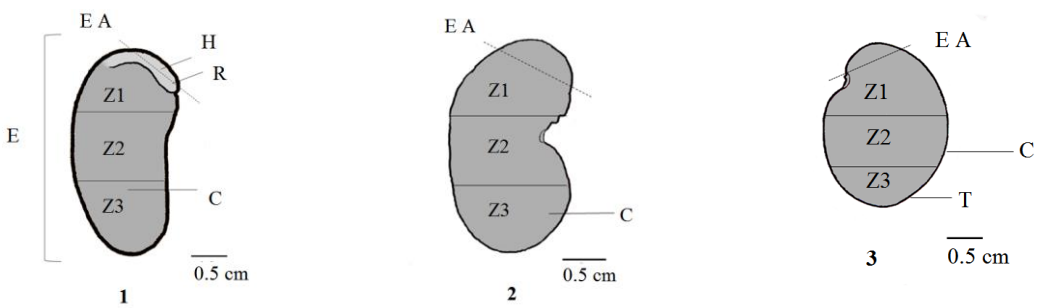

Figure 1. Schematic representation of the definition of analysis areas to interpret the tetrazolium test on exalbuminous seeds applied for seeds of (1) *Platymiscium floribundum* Vog, (2) *Lonchocarpus muehlbergianus* Hassl. and (3) *Acacia polyphylla* DC (E = Embryo, EA = Embryonic axis, H = Hypocotyl, R = Radicle; C = Cotyledon, T = Tegument, Z1, Z2, Z3 = Zones).

Table 2. Description of the seed characteristics and classification system for the tetrazolium test evaluation.

Parameter	Description of the characteristics	Class 1	Class 2	Class 3
Color	Tissues with bright red and/or pinkish color	•	•	
	Tissues with intense red color		•	•
	Tissues with yellowish to red color			•
Stain position	Stain absence	•		
	Stains located in areas 2 and 3 of cotyledons		•	•
	Stains located on the radicle		•	•
	Stains located on area 1			•
	Stains located on the insertion of the hypocotyl-radicle axis			•
Stains extension and size	Single stain, less than 20% of the seed size		•	
	Stains whose summed area is lower than 20% of the seed size		•	
	Stain/s whose area is 20-40% of the seed size			•
	Stain/s occupying higher than 40% seed area/s			•
Aspect of seed tissues	Firm tissues, without exudates, intact cells	•	•	
	Tissues with the presence of exudates, milky aspect and with signs of deterioration			•

Table 3. Conditions used in the germination test of the forest species.

Species	Substrate	Temperature (°C)	Replications	Seeds/replication	Time (days)
Platymiscium floribundum Vog.	Vermiculite	25	4	25	45
Lonchocarpus muehlbergianus Hassl.	Vermiculite	25	4	15	30
Acacia polyphylla DC.	Paper roll	25	4	15	15

The used experimental design was the factorial completely randomized one, with four replications per treatment. Data on viable seeds (TZ) and number of germinated seeds in the germination test (GT) were transformed into percentages and applied in the analysis of variance (ANOVA) and average comparison by Tukey's test (p = 5%). When data did not showed normality in the error distribution, verified through the Shapiro-Wilk test, the Kruskal-Wallis non-parametric test or the Wilcoxon test were used followed by the Bonferroni test to compare averages, both calculated with the PAST 3.14 program (Hammer et al., 2016).

For the analysis of the protocol and its effectiveness in interpreting the tests, the evaluators (n=3) attributed notes from zero (difficult) to three (easy) for each protocol parameter (Table 2), considering the following indicators: (a) *precision* – effectiveness of interpretation; (b) *robustness* – interpretation and easiness; (c) *sensitivity* - ability to predict the results; (d) *replications* - degree of response variability in the analysis of the same material. After that, the sum of the assigned notes was calculated and the effectiveness was defined, such as: low (from 0 to 16 points), medium (from 16.1 to 32 points) or high (from 32.1 to 48 points).

Results and Discussion

There was a significant difference in the times of immersion of *P. floribundum* (F= 6.42; p= 0.006), and the lowest rate of viable seeds was obtained for the seed immersion in TZ for 30 minutes (Table 4). This may be attributed to the difficulty in distinguishing the classes due to the short-time for penetration of the salt into seed tissues (Figure 2). Only at 60 minutes, in the concentrations 0.5 and 1.0%, the tissue color allowed the differentiation of viability classes. Although there was no significant effect of concentrations for *P. floribundum*, for another Fabaceae *Schizolobium parahyba*, the 0.5% tetrazolium solution had easier visual analysis than the 0.1% (Ferreira et al., 2007), highlighting the need of developing

Table 4. Percentage of living (TZ) and germinated (GT) seeds of *Platymiscium floribundum* Vog.

TZ (%)				
Concentration (%)	Time in contact with salt (minute)			
	30	60	120	Average
0.1	53 Ba	67 Aa	62ABa	61
0.5	57 Ba	68 Aa	62ABa	62
1	62 Ba	68 Aa	63ABa	64
Average	57	68	62	62
GT (%) = 72				

Averages followed by the same capitol letter do not present significant difference among themselves at 5% probability, within each tetrazolium concentration and germination condition. Lowercase letters indicate the differences for the averages between germination and tetrazolium tests.

standard tetrazolium procedure for each species.

P. floribundum seeds presented high germination rate (72%), and there was no significant difference between the result of GT and TZ (Table 4). The use of TZ for an hour at the concentration of 0.5% provided suitable conditions to interpret the protocol, with the distinction of colored tissues and the differentiation between them (Figure 2), associated to reduction of immersion time and salt concentration.

For *L. muehlbergianus*, TZ [1.0%] became unviable, with the seeds fast acquiring an intense red color [#4d0000 (R= 77, G= 0; B= 0) to #990000 (R= 153, G= 0; B= 0)]. Even though the 0.1% and 0.5% concentration (Figure 3) provided identifiable tissue color typical of the respiratory activity (deterioration process), the 0.5% concentration was the one allowing a clear analysis of the physiological conditions of seeds, facilitating the use of the protocol (Figure 3). According to Krzyzanowski et al. (1999), the choice of the proper methodology for TZ must be based on the easiness to differentiate viable and unviable tissues, and the capacity to separate lots with distinct physiological quality. Other researches with Fabaceae seeds, such as *Pterodon pubescens* (Ferreira et al., 2001), *Senna multijuga* and *Senna macranthera* (Ferreira et al., 2004), indicated that lower tetrazolium salt concentrations provided suitable results with higher correspondence with the germination tests.

There were significant differences in the percentage of viable seeds of *L. muehlbergianus*, between the tested concentrations (F=10.083; p=0.005) (Table 5). It was observed in TZ that 60% of the evaluated seeds were considered viable; this is higher than seed germination observed in GT (47%). Dormancy by tegument impermeability in Fabaceae is reported by various authors (Roversi et al., 2002; Oliveira et al., 2005a; Oliveira et al., 2008; Pereira and Ferreira,

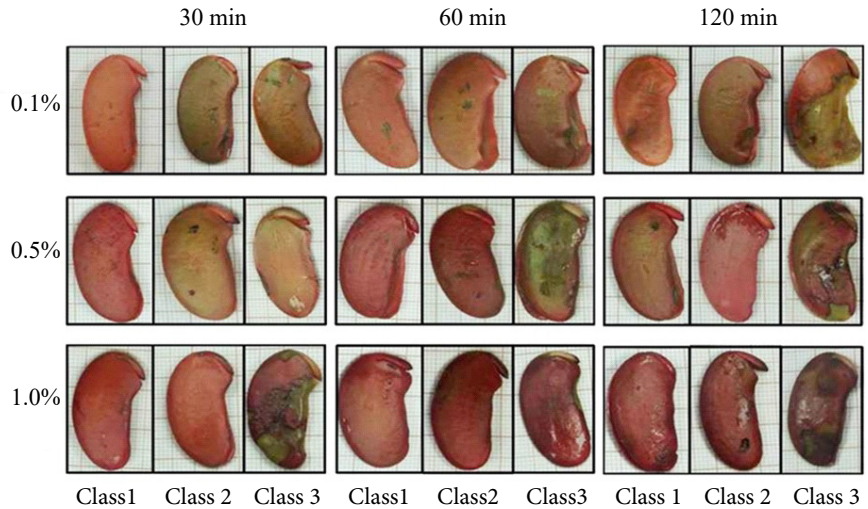

Figure 2. Color of *Platymiscium floribundum* Vog. seeds submitted to tetrazolium test at concentration (0.1, 0.5 and 1%) and times (30, 60 and 120 minutes) of immersion at the temperature of 35 °C.

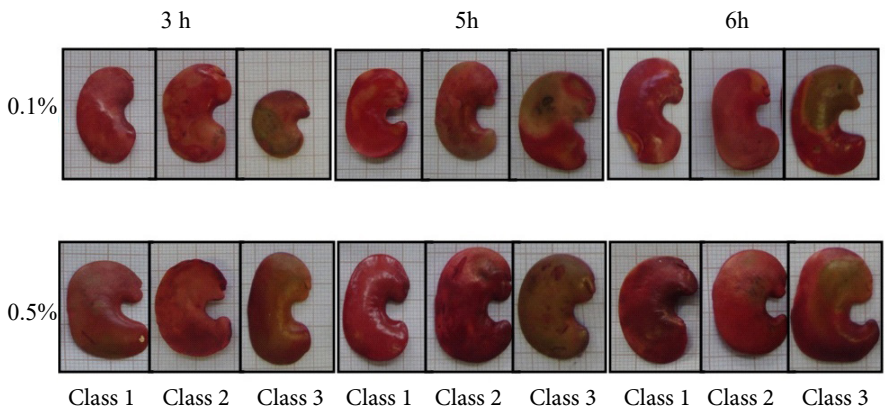

Figure 3. Color of *Lonchocarpus muehlbergianus* Hassl. seeds submitted to tetrazolium test at concentration (0.1% and 0.5%) and times (3, 5 and 6 hours) of immersion at the temperature of 35 °C.

2010); however, other species from the *Lonchocarpus* genus did not present dormancy by impermeability, but by slow and irregular germination (Sautu et al., 2007), as well as the presence of fibers in the endocarp, which may restrict water exchanges (Souza, 1988). On the other hand, in this test it was possible to observe low germination in the GT of *L. muehlbergianus*, which was not submitted to any treatment to break dormancy. Meanwhile, in the tetrazolium test, the tegument was removed (Table 1); this allowed the diffusion of salt in seed tissues. According to this, there may be some mechanism or barrier in the seed, and in this case, tetrazolium expresses the viability of seeds or their germination potential, if this barrier is removed.

Only the 0.1% concentration along 6 hours of immersion in tetrazolium salt did not differ from the GT. This may characterize the difficulty to analyze and differentiate seeds to categorize them in the classes established by the protocol. On the other hand, the use of TZ at 0.1% along 3 hours was effective for evaluate seed viability of *L. muehlbergianus*.

Table 5. Percentage of considered viable (TZ) and germinated (GT) seeds of *Lonchocarpus muehlbergianus* Hassl.

TZ (%)				
Concentration	Time in contact with salt (h)			
(%)	3	5	6	Average (%)
0.1	55 AaA	53 AaA	52 AbB	53
0.5	68 AaA	65 AaA	63 AaA	66
Average	62	59	58	60
GT (%) =B 47				

Averages followed by the same capital letters do not present significant difference (p=0.05) within the tetrazolium concentrations and lowercase letters indicate the differences for the averages of exposure time.

For *A. polyphylla* seeds, the 0.1% and 0.5% concentrations provided a well-defined color (pinkish) [#CC6666 (R=204; G=102; B=102) to #CC3333 (R=204; G=51; B=51)] and visual distinction between immersion times (Figure 4). After four hours in the 1% concentration, seeds showed an intense red color [#4d0000 (R= 77, G= 0; B= 0) to #990000 (R= 153, G= 0; B= 0)] making it difficult to evaluate seed physiological status. However, the 0.1% concentration provided easiness in distinguishing the color tones in the three evaluated immersion times, with typical spots of intense respiratory activity (deterioration process).

In TZ, there were significant differences for viable seeds between the tested concentrations ($F = 4.87$; $p=0.015$) but not for immersion times ($F = 2.16$; $p=0.13$). The concentrations of 0.5% and 1%, did not differ between themselves and showed the highest viability rates (Table 6). However, the color intensity at 1% affected the analysis of seeds turning difficult the identification and distinction of the possible necrotic areas (Figure 4).

A. polyphylla seeds had low viability, with an average of 28% (Table 6). This GT result was lower than the ones found by Silva et al. (2007) with higher than 80% values. Considering that seeds were stored along two years; their low viability and germination rates may have occurred due to storage conditions. According to studies conducted by Neto et al. (2005), *A. polyphylla* seeds, when stored in a normal laboratory environment may have their germination reduced mainly from the fourth month; and the germination percentage and speed may be reduced practically by half.

The seed quality obtained by GT and TZ were similar

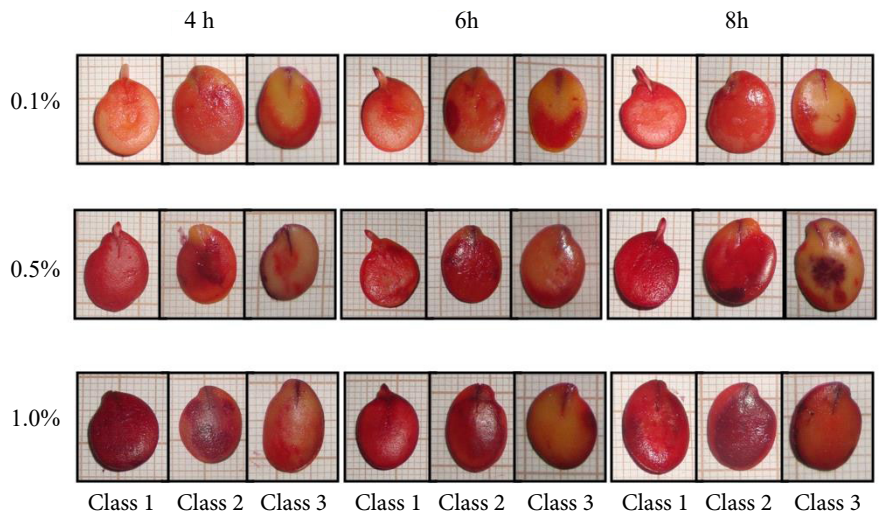

Figure 4. Color of *Acacia polyphylla* DC. seeds submitted to tetrazolium test at concentration (0.1, 0.5 and 1%) and times (4, 6 and 8 hours) of immersion at the temperature of 35 °C.

(Table 6). Thus, based on the obtained results and the color evaluation, the 0.5% concentration is recommended with four hours of immersion in TZ for *A. polyphylla* seeds. This condition, as well as enabling the application of the protocol and the differentiation of tissues, provided compatible results to the GT.

The correlation between data obtained with GT and TZ was high (r = 0.942; $R^2_{adjusted}$ = 0.876) (Figure 5). This high correlation between data shows that TZ results corresponded to the GT ones in the evaluation of the quality of *P. floribundum*, *L. muehlbergianus* and *A. polyphylla* seeds. This result reinforces the potential of using the proposed procedure for the application of TZ on these species. Also, Custódio et al. (2012) analyzing the effectiveness of the TZ evaluation by digitalizing images of colored seeds verified a high correlation (0.9938) between the data obtained by digitalization and the ones analyzed conventionally. In this research, the authors concluded that the evaluations performed by digitalized images are equivalent to the ones performed with a stereo microscope.

Regarding protocol effectiveness, the parameters related with spots were difficult to interpret, such as their position (∑= 4 points) and their extension and size (∑= 6 points). In contrast, color (∑=11 points) and tissue aspect (∑=12 points) were easy to interpret. The score obtained for the protocol (∑=33 points) indicated high effectiveness. The reduction of the number of classes contributed to help the standardization and use of the protocol on a large scale. Nevertheless, more details about the parameters related to the presence of spots need to be revised.

Table 6. Percentage of viable (TZ) and germinated (GT) seeds of *Acacia polyphylla* DC.

TZ (%)				
Concentration (%)	Time in contact with salt (h)			
	4	6	8	Average
0.1	15 Ba	25 Ba	22 Ba	21
0.5	28 ABa	32 ABa	33 ABa	31
1	27 Aa	40 Aa	33 Aa	33
Average	23	32	29	28
GT (%) = 28				

Averages followed by the same capital letters do not present significant difference (p=0.05) within the tetrazolium concentrations and lowercase letters indicate the differences for the averages of exposure time.

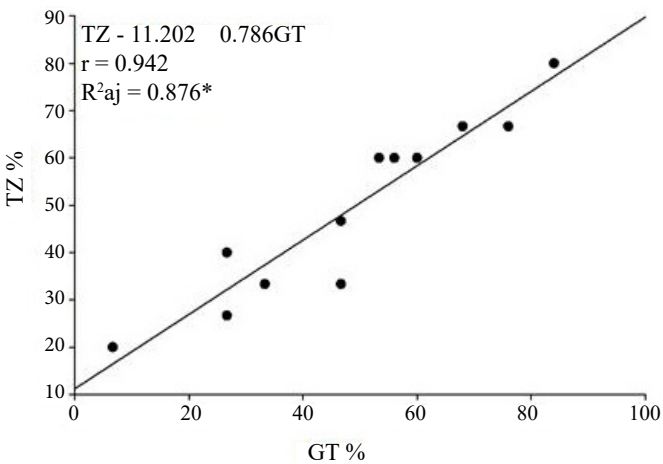

Figure 5. Linear correlation between germination test (GT) and tetrazolium test (TZ) results of the three species (*Platymiscium floribundum* Vog*, Lonchocarpus muehlbergianus* Hassl. and *Acacia polyphylla* DC.). * Significant linear equation by F test (p<0.01).

Conclusions

For the evaluation of *P. floribundum* seeds by TZ, we recommend the use of concentrations of 0.5 or 1% for 60 minutes; for *L. muehlbergianus* seeds, 0.1% for 6 hours of immersion in salt and for *A. polyphylla* seeds, the concentration 0.5% for 4 hours.

The proposed protocol for TZ was effective to optimize and facilitate the interpretation of the seed viability parameters, reducing the dependency of individual perceptions and contributing for seeds comparison due the pre-defined criteria of analysis.

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