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RESEARCH NOTE

Adequacy of the tetrazolium test methodology for *Stylosanthes capitata* Vogel seeds[1]

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ABSTRACT - The seed sector is increasingly dynamic and therefore demands rapid decision-making. The tetrazolium test is a rapid test that determines the viability of seeds, especially from species that show slow germination or dormancy. The methodology described in the Rules for Seed Testing for *Stylosanthes* sp. is not applied effectively to the *Stylosanthes capitata* Vogel species. Three seed lots of *S. capitata* seeds with different quality levels were used to fit the tetrazolium test methodology. In order to determine the physiological quality of seeds, the tests of germination, first count germination, emergence speed index, initial and final seedling emergence percentage, electrical conductivity, respiratory activity and seed moisture content were performed, besides the imbibition curve for the establishment of pre-imbibition periods of the seeds to perform the tetrazolium test. The pre-imbibition and staining periods of 6, 12 and 18 h, and 3, 6, 12 and 24 h at concentrations of 0.075%, 0.1% and 0.5% tetrazolium solution were tested. It was concluded that the 12-h pre-imbibition period and 6-h staining at the 0.1% tetrazolium solution is ideal for evaluating the viability of *S. capitata* seeds.

Index terms: forage seeds, viability, vigor.

Adequação da metodologia do teste de tetrazólio em sementes de *Stylosanthes capitata* Vogel

RESUMO - O setor sementeiro está cada vez mais dinâmico e por isso demanda rápidas tomadas de decisões. O teste de tetrazólio é um teste rápido que determina a viabilidade das sementes, principalmente de espécies que apresentam germinação lenta ou dormência. A metodologia contida nas Regras para Análise de Sementes para a espécie *Stylosanthes* sp. não se aplica efetivamente para a espécie *Stylosanthes capitata* Vogel. Para adequar a metodologia de condução do teste de tetrazólio, foram utilizados três lotes de sementes de *S. capitata* com níveis de qualidade distintos. Na determinação da qualidade fisiológica das sementes, foram realizados os testes de germinação, primeira contagem da germinação, índice de velocidade de emergência, estandes inicial e final, condutividade elétrica, atividade respiratória e grau de umidade, além da curva de embebição para estabelecimento dos períodos de pré-embebição das sementes para a condução do teste de tetrazólio. Foram testados os períodos de pré-embebição e coloração, de 6, 12 e 18 h e, 3, 6, 12 e 24 h, nas concentrações de 0,075%, 0,1% e 0,5% da solução de tetrazólio. Concluiu-se que o período de 12 h de pré-embebição e 6 h de coloração na solução de tetrazólio a 0,1% é o ideal para a avalição da viabilidade de sementes de *S. capitata.*

Termos para indexação: sementes forrageiras, viabilidade, vigor.

Introduction

Stylosanthes capitata Vogel is the species, which has one of the highest number of cultivars among tropical legumes used as pasture. Worldwide, there are 48 *Stylosantes* species, of which 43 are present in the American continent, and 29 can be found in Brazil, numbers that attest its great colonization capacity, possibly as consequence of their

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adaptation to low fertility soils and symbiosis with nitrogen-fixing bacteria (Karia et al., 2010). At least three species had had their potential characterized for use in the Brazilian livestock scenario: *Stylosanthes guianensis, S. capitata* and *S. macrocephala*; in fact, cultivars have been developed from genetic accessions of these species (Andrade, 2013). Both *S. capitata* and *S. macrocephala* compose the cultivar multi-line Estilosantes Campo Grande, released by the Brazilian Corporation for Agricultural Research (*Empresa Brasileira de Pesquisa Agropecuária* - EMBRAPA).

In the last few years, Estilosantes Campo Grande has been highlighted as an important forage, and in consequence, there has been a considerable increase of the area planted with the cultivar in the livestock production systems in Brazil (Barcellos et al., 2008).

Due to its forage potential and its adaptation to different environments, besides the development of new cultivars, there is an increased demand for high quality seeds in the market, which requires precise information in order to perform tests that evaluate its quality (Rodrigues et al., 2010). Thus, the use of rapid tests becomes an essential tool to evaluate the physiological quality, optimizing the decisions regarding the management of seed lots.

Among the rapid tests, the tetrazolium test determines the seed viability, especially those seeds with dormancy or slow germination. The test has been widely used in several crops, mainly due to the higher number of provided information, such as the vigor level and the occurrence of different types of damage, such as mechanical, the caused by insects and by weathering at the pre-harvest stage (Krzyzanowski et al., 1999).

Despite the rapid and accurate information on the viability of a seed lot that the tetrazolium test is able to provide, the methodology is standardized for the genus *Stylosanthes* in the Rules for Seed Testing (Brasil, 2009) and not for each species belonging to the genus. Moreover, the Rules for Seed Testing was published more than eight years ago, thus before launching the cultivar multi-line Campo Grande and the great expansion of the use of *Stylosanthes*, which has demanded several laboratory analyses.

The Rules for Seed Testing indicated methodology for *S. capitata* is 18 h for pre-imbibition in water at 25 ºC and a staining period from 18 to 24 h at 30 ºC with a 1% or 0.5% tetrazolium solution (Brasil, 2009). However, when using this method, it is observed that the pre-imbibition time is too long, promoting radicle protrusion of the seeds. Moreover, with the long staining period, the seeds acquire intense staining, including the viable, similar to the deteriorated tissue, inducing to a misinterpretation on the quality level of the seed lots. Therefore, the objective of the present study was to adapt this methodology to perform the tetrazolium test in the seeds of the *S. capitata* Vogel species.

Material and Methods

The experiment was performed at the Central Laboratory of Seed Testing of the *Universidade Federal de Lavras* (UFLA), Lavras, MG, Brazil. Three seed lots of the *S. capitata* Vogel were used, all from the 2016 harvest. The seed lots had three distinct quality levels that were considered as low, medium and high quality.

The following determinations were used to evaluate the physical and physiological qualities of the seeds:

Water content: determined by the oven-drying method at 105 ºC for 24 h (Brasil, 2009) using two samples of 0.1 g of seeds.

Germination test: four replications of 50 seeds each were prepared on a blotting paper substrate, moistened with distilled water at the proportion 2.5 times the paper-weight, placed in gerbox acrylic boxes, and conditioned in BOD with alternating temperature 20-35 °C and 12 h photoperiod. The normal seedlings were evaluated in two counts, being the first count on the 4th day and the last on the 10th day after sowing, as prescribed by the Rules for Seed Testing (Brasil, 2009). Results were expressed as average percentage of normal seedlings.

Emergence test: performed with four replications of 50 seeds each, seeded in plastic trays containing sand and soil substrate in a 2:1 ratio, moistened at 60% field capacity and kept in a growth room at 25 °C with 12 h of light, during 14 days. The evaluations were performed at the 7th (initial seedling emergence) and at the 14th days (final seedling emergence). Results were expressed as a percentage of emerged seedlings. The number of emerged seedlings from the beginning of the test was computed daily for the emergence speed index (ESI), and the calculation was performed according to Maguire (1962).

Bulk electrical conductivity test: performed using four replications of 50 seeds each, which were weighed and placed to soak in 40 mL of distilled and deionized water in a disposable plastic cup and kept in BOD chamber at 25 °C for 24 h. After the imbibition period, the electrical conductivity was measured by the MS TECNOPON® electrical conductivity meter, model mCA 150, and the results were expressed in $\mu S.cm^{-1}.g^{-1}$ seeds.

Analysis of respiratory activity: a total of 50 seeds of each lot were weighed and seeded in two blotting paper sheets (11 x 11 cm), moistened with 2.5 times the paperweight in distilled water. After sowing, the rolled papers were placed in Falcon tubes (50 mL) with a slotted opening in the silicone-

sealed cap, preventing gas exchanges with the environment. After 24 h, a needle of the PBI - Dansensor CHECKPOINT O_2/CO_2 apparatus was inserted in the silicone-sealed cap, allowing the absorption of an aliquot of 15 mL of air from the samples atmosphere, which reads electrochemically the percentage of carbon dioxide (CO_2) and oxygen (O_2). The values of carbon dioxide obtained in the readings were later divided by the weight of the seeds present in the tube and by the time that remained in the tube until the reading, in this case 24 h, being the results expressed as % $CO_2.g^{-1}.h^{-1}$. As a calibration measure, a blank test was performed with four Falcons tubes containing only the moistened blotting paper sheet without the seeds (Santos et al., 2016).

Imbibition curve: four replications with 50 seeds each from each lot were placed in "gerbox" acrylic boxes, having as substrate two blotting paper sheets moistened with distilled water in a quantity equal to 2.5 times the paperweight. These boxes were conditioned in BOD chambers at 25 °C with constant light. The seeds were weighed dry before beginning of imbibition and weighed again in 3-h time intervals, the imbibition curve was finished at 80 h for the lot 1 and 3, and 140 h for the lot 2. For the evaluation, the seeds were removed from the "gerbox," the excess water was eliminated with paper towels and weighed in a 0.001 g precision scale. It was calculated the increased percentage of weight over time as a function of the initial weight of seeds (Justo et al., 2007).

Tetrazolium test: based on the imbibition curve results and from the methodology of the tetrazolium test contained in the Rules for Seed Testing (Brasil, 2009), the imbibition and staining periods, as well as the tetrazolium solution concentrations were determined. Three pre-imbibition periods of the seeds were evaluated (6, 12 and 18 h), under three staining periods (3, 6, 12, and 24 h), using three tetrazolium solution concentrations (0.075%, 0.1% and 0.5%).

After the pre-imbibition process and before being placed in the tetrazolium solution, the seeds were cut longitudinally with a blade through the seed coat to facilitate the solution penetration. After staining, the seeds were washed in running water and, then, evaluated. Subsequently, they were analyzed individually under a magnifying glass and, according to the extent and intensity of the reddish tones, presence of milky white areas, tissue appearance and location of these stains in relation to essential areas for growth, the seeds were classified in the categories of viable (with intact embryonic axis and axis-cotyledon translocation area and more than 50% stained area in the cotyledon) and non viable.

Statistical analysis: a completely randomized design was performed in a 8x3x3 factorial design, with eight treatments derived from the combination of pre-imbibition time and staining time (6 h pre-imbibition and 3 h staining; 6 h pre-imbibition and 6 h staining; 6 h pre-imbibition and 12 h staining, 6 h pre-imbibition and 24 h staining; 12 h pre-imbibition and 3 h staining; 12 h pre-imbibition and 6 h staining; 12 h pre-imbibition and 12 h staining, 12 h pre-imbibition and 24 h staining), three tetrazolium solution concentrations (0.075%, 0.1% and 0.5%) and three seed lots of *S. capitata,* with low, medium and high physiological quality. For the k-means clustering, the Scott-Knott test was performed at 5% probability using the Sisvar statistical software (Ferreira, 2011).

Results and Discussion

Initially, the seed quality was evaluated (Table 1), being important to identify the seed lots with higher and lower physiological potential in order to verify the reliability of the tetrazolium test for this purpose (Deminicis et al., 2009).

Thus, by the tests of first count germination, germination, final seedling emergence count, emergence speed index and electrical conductivity, it was possible to classify the lots into three levels of quality, being lot 1 with superior quality, followed by lot 3 with intermediate quality and lot 2 with lower quality. Despite of the respiratory activity did not separate the lots in three different levels of quality, this procedure ranked lot 1 as highest quality, as determined in the other tests (Table 1).

Table 1. Average values of the first germination count (FC %), germination (G %), initial seedling emergence (IS %), final seedling emergence (FS %), emergence speed index (ESI), electrical conductivity (EC $\mu S.cm^{-1}.g^{-1}$), respiration (RESP % $CO_2.g^{-1}.h^{-1}$) and seed moisture content (MC %) of three seed lots of *S. capitata*.

Lots	FC (%)	G (%)	IS (%)	FS (%)	ESI	EC	RESP	MC (%)
1	52 a	63 a	37 a	50 a	6.22 a	349.38 a	0.250 a	7.70
2	16 c	17 c	8 b	10 c	1.23 c	715.44 c	0.064 b	8.61
3	36 b	40 b	28 a	34 b	4.62 b	534.72 b	0.100 b	7.66
CV (%)	18.78	18.09	25.57	20.38	21.98	13.41	44.77	-

Means followed by the same letter within each column do not differ among themselves by the Scott-Knott test at 5% probability.

Regarding the data on the seed moisture content, it was verified that the difference in moisture among the evaluated lots varied less than 1%. This standardization of seed water content is essential for the evaluations and to obtain consistent results (Marcos-Filho, 2015).

For the characterization of the hydration pattern of the three evaluated lots and establishment of the pre-imbibition periods of seeds for the adequacy of the tetrazolium test methodology, the imbibition curves (Figures 1, 2 and 3) were calculated. By the observed imbibition curves, it was possible to clearly notice the three phases of the water absorption pattern by the seeds (Bewley et al., 2012) from lots 1 and 3 and the first two phases for lot 2 (Figures 1, 2 and 3). Phase I, considered as the fastest, lasted 5 h and phase II extended to approximately 40 h, when radicle protrusion was noticed. Vigor and viability are among the factors that affect the rate and the period of water absorption by the seeds (Cavariani et al., 2009), which may explain this differentiated behavior from lot 2 in relation to its imbibition pattern, since this lot has worse physiological quality, according to the results of the physiological tests presented in Table 1.

After the establishment of the hydration curves, the experiments involving the adequacy of the tetrazolium test methodology were started. To reach satisfactory results in this test, the tetrazolium solution should be absorbed adequately by the seeds. Thus, some species require preparatory steps to be soaked in this solution (Costa and Santos, 2010), such as *S. capitata*. Imbibing the seeds in water before being subjected to the staining process is known as preconditioning or pre-moistening, as described in the Rules for Seed Testing (Brasil, 2009).

Pre-moistening becomes necessary to rehydrate the tissues, allowing the diffusion of the tetrazolium solution and to activate the dehydrogenase enzymes responsible for the release of hydrogen ions that react with the tetrazolium solution, forming the formazan that allows developing of red-pinkish staining of the test (Nery et al., 2007).

The pre-moistening should comprise phase II of the imbibition curve, thus preventing radicle protrusion (Marcos-Filho, 2015). For the determination of the pre-moistening times, the 6 h periods were defined, since the seeds would already have entered the second stage of the imbibition, a 12 h intermediate period and a 18 h period, being the latter recommended by the Rules for Seed Testing (Brasil, 2009). However, the 18-h period of pre-moistening was not efficient for the *S. capitata* seeds, since it favored radicle protrusion (Figure 4).

Regarding the tetrazolium solution, three concentrations were tested, being 0.075%, 0.1% and 0.5%, and the pre-moistening and staining temperatures were maintained at 25 and 30 °C, as recommended by the Rules for Seed Testing (Brasil, 2009).

The seed viability results determined by the tetrazolium test as a function of the combination of the pre-moistening and staining times and the concentration of tetrazolium salt in the different lots are expressed in Table 2.

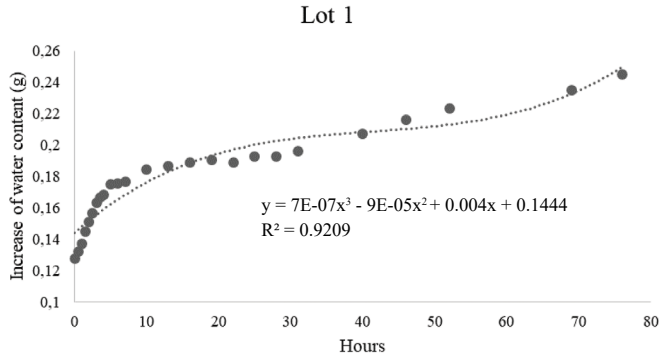

Figure 1. Imbibition curve of lot 1 of *Stylosanthes capitata* seeds.

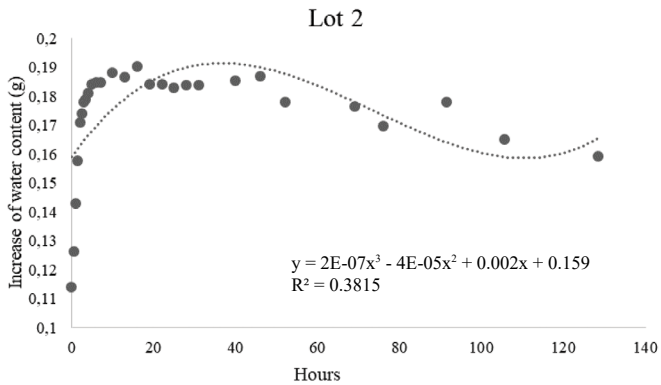

Figure 2. Imbibition curve of lot 2 of *Stylosanthes capitata* seeds.

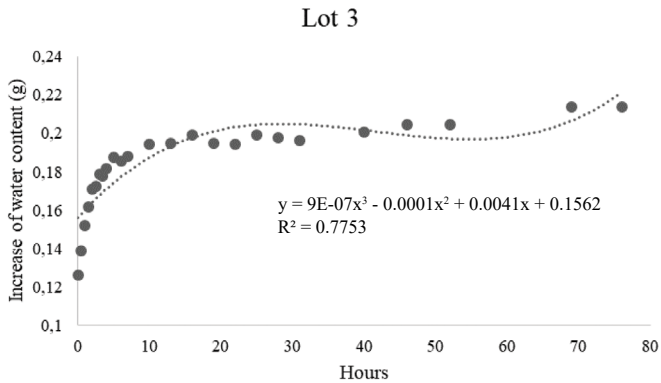

Figure 3. Imbibition curve of lot 3 of *Stylosanthes capitata* seeds.

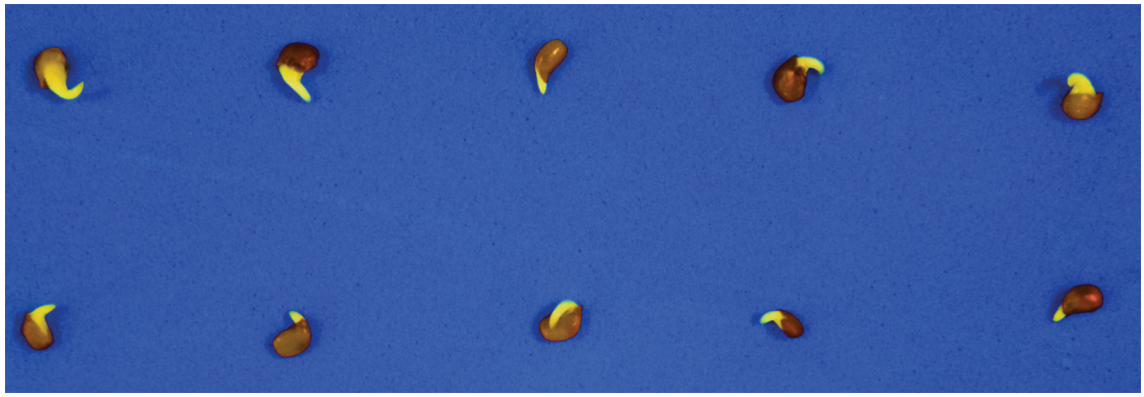

Figure 4. Seeds of *S. capitata* after 18 h soaking.

Table 2. Percentage of viable seeds from three seed lots of *Stylosanthes capitata*, estimated by the tetrazolium test in different combinations of pre-imbibition times and staining times at different concentrations of the tetrazolium salt.

Pre-imbibition time/Staining time (h)	Concentration (%)	Lots		
		1	2	3
6/3	0.075	2 Ac	2 Ab	5 Ab
	0.1	70 Aa	22 Ca	39 Ba
	0.5	22 Ab	17 Aa	12 Ab
6/6	0.075	40 Ab	15 Bb	34 Ab
	0.1	69 Aa	25 Ca	53 Ba
	0.5	42 Ab	34 Aa	32 Ab
6/12	0.075	63 Aa	38 Ba	45 Ba
	0.1	61 Aa	28 Bb	53 Aa
	0.5	64 Aa	24 Cb	46 Ba
6/24	0.075	67 Aa	24 Cb	42 Ba
	0.1	66 Aa	37 Ba	44 Ba
	0.5	72 Aa	37 Ba	46 Ba
12/3	0.075	30 Ab	7 Bb	17 Bb
	0.1	56 Aa	21 Ca	41 Ba
	0.5	30 Ab	11 Bb	22 Ab
12/6	0.075	55 Ab	18 Ca	37 Ba
	0.1	74 Aa	27 Ca	45 Ba
	0.5	44 Ab	22 Ba	37 Aa
12/12	0.075	71 Aa	26 Ca	45 Ba
	0.1	78 Aa	30 Ca	49 Ba
	0.5	66 Aa	29 Ca	46 Ba
12/24	0.075	72 Aa	30 Ca	45 Bb
	0.1	73 Aa	27 Ba	38 Bb
	0.5	73 Aa	36 Ca	61 Ba
CV (%)			19.79	

Means followed by the same capital letter within each row and lowercase within each column do not differ among themselves by the Scott-Knott test at 5% probability.

Based on the obtained results, the following 12 treatments were efficient in separating the three lots at different levels, corroborating with the tests of first germination count, germination, final count of seedling emergence, emergence speed index and electrical conductivity: 6 h pre-moistening and 3 h staining at 0.1% concentration; 6 h and 6 h at 0.1% concentration; 6 h and 12 h at 0.5% concentration; 6 h and 24 h at 0.075% concentration; 12 h and 3 h at 0.1% concentration; 12 h and 6 h at 0.0075% concentration; 12 h and 6 h at 0.1% concentration; 12 h and 12 h at all concentrations of the tested tetrazolium solution; and 12 h and 24 h at 0.075% and 0.5% concentrations.

However, treatments with the combinations of 6 h and 3 h, and 6 h and 6 h of pre-imbibition and staining at 0.1% concentration, despite confirming the results of physiological quality tests, also presented a very weak coloration, making it difficult to interpret the test (Figure 5).

Seeds with uniform and adequate staining are essential for safe and efficient interpretation in the tetrazolium test, and preconditioning is critical in obtaining these characteristics (Bhering et al., 2005), being observed that this result was not achieved by the tested methodology.

Nevertheless, the treatments combining 6 h and 12 h; 12 h and 12 h; and 12 h and 24 h of pre-imbibition and staining, at 0.5% concentration presented very dark seeds and could hinder the interpretation of the test (Figure 6).

For Pinho et al. (2011), the choice of methodology suitable for using tetrazolium test should be based on the easiness for the differentiation of viable and non-viable tissues and on the ability to differentiate the lots based on its physiological quality. This aspect was not observed when the 0.5% concentration was used, in which the stained seeds showed a very intense staining, supposedly to be all non-viable seeds.

The treatments in the combinations of 12 h and 3 h at the 0.1% concentration and 12 h and 6 h at 0.075% concentration, despite having separated the lots in three levels of viability,

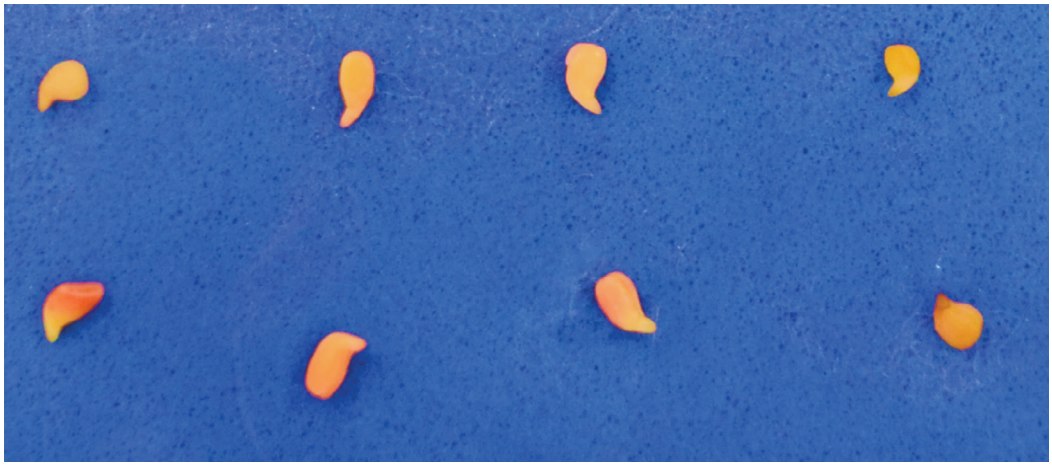

Figure 5. Seeds of *S. capitata* after staining at 0.1% tetrazolium solution.

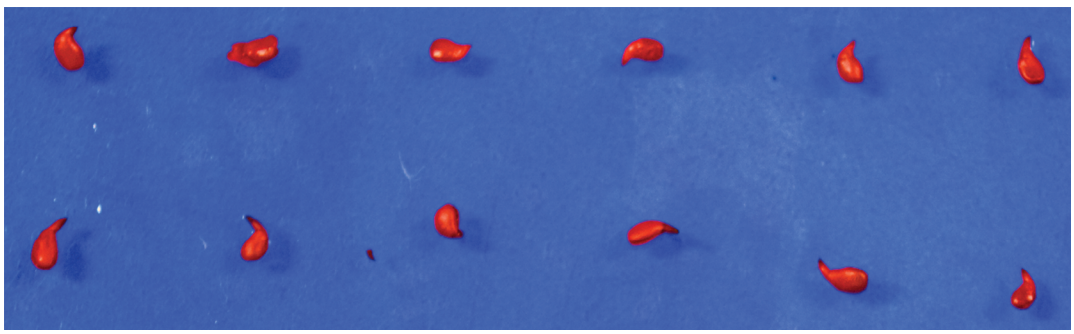

Figure 6. Seeds of *S. capitata* after treatment at 0.5% tetrazolium solution.

such as in the already mentioned tests, underestimated the seed viability of lot 1, showing results lower than the germination test.

These observed results differ from the literature, since it is reported that when comparing the results of the tests that evaluate the seed physiological quality, the tetrazolium test generally overestimates the seed viability by considering only the embryo and not the influence of external seed structures (Oliveira et al., 2005).

Thus, the best treatments were 6 h and 24 h, 12 h and 12 h / 12 h and 24 h at 0.075% concentration of tetrazolium solution and 12 h and 6 h / 12 h and 12 h at 0.1% concentration of tetrazolium solution, since they show a uniform staining and easy identification of viable and non-viable seeds.

Efficient methodologies using low concentrations of tetrazolium solution are important to optimize the application of resources within the laboratories and allow analyzing more samples with lower cost (Silva et al., 2013). Recent researches, with several species, have proven the efficiency of low concentrations of the tetrazolium solution. This is the case, e.g., of the recommended use of 0.2% solution for *Ricinus communis* (Gaspar-Oliveira et al., 2009); at 0.15% for *Leucaena leucocephala* x *L. diversifolia* (Costa and Santos, 2010); at 0.01% for embryos of *Olea europaea* (Souza et al., 2011); and at 0.05% for *Arachis hypogaea* (Santos et al., 2012).

Furthermore, according to Ferreira et al. (2004), the results of germination and tetrazolium viability should be similar, with a margin of 5% difference between them. This statement is confirmed for the studied species, whose methodology for the test was established and the results allow recommending them.

One of the tetrazolium test advantages is the speed in obtaining results. Thus, based on the present results, it is suggested a 12-h pre-imbibition period with 6 h staining using a 0.1% tetrazolium solution to obtain a faster result for the viability of *S. captata* seeds. Additionally to obtain the best staining for evaluation, this treatment also presented increased correlation with germination results.

Conclusions

The 12 h period of pre-imbibition and 6 h staining at 0.1% concentration of the tetrazolium solution is ideal to evaluate the viability of *Stylosanthes capitata* seeds.

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