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Viability of canola seeds by the tetrazolium test

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Abstract

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Canola (Bras*sica napus L. var. oleifera* Moech) is one of the main oilseeds for edible and industrial purposes. For the evaluation of the physiological quality of a lot of seeds, the tetrazolium test has been considered as a promising alternative, due to the speed and efficiency in the characterization of viability. The present study aimed to adapt the methodology of the tetrazolium test to evaluate the quality of canola seeds. Alth B4 and Hyola 433 hybrids were used. The study involved the characterization of the initial profile of the lots by determining the moisture content, weight of a thousand seeds, first count, germination test, germination speed index, emergence, initial stand, emergency speed index. Pre-conditioning of the seeds was carried out, defining the soaking curve and the seeds placed to soak for periods of 10, 12, 14, 16, 18 and 22 hours, by the methods of hydration on paper and direct immersion in water and the adequacy of the tetrazolium test methodology as a function of different concentrations (0.075%; 0.5% and 1.0%) and immersion periods of 2, 4 and 6 hours. The results indicated that the lowest concentrations and lowest imbibition periods present a higher percentage of viability for canola seeds. It was concluded that the tetrazolium test is efficient to evaluate the viability in canola seeds with pre-conditioning of the seeds in hydration in paper for 10 hours at 25 ºC, followed by the total removal of the tegument, in the tetrazolium solution of 0.075% per 2 hours at 30°C.

Keywords: Brassica napus L.; imbibition curve; pre-conditioning; physiological quality

Introduction

Canola (*Brassica napus* L. var. *oleifera* Moech), belonging to the *Brassicaceae* family and the genus *Brassica* (Guimarães et al., 2022) it is widely used as a source of oil and protein, containing approximately 40–47% and 25–35% (Raboanatahiry et al., 2021). It has great worldwide importance because it is used in the crop rotation system, production of edible oil and because it is a source of raw material for the production of biodiesel (Secchi et al., 2023).

About 70 million tons of canola are produced each year worldwide. It is the second most cultivated oilseed, and in terms of oil production, canola occupies the third position in the world ranking, behind only soy and palm oil (FAOSTAT, 2023). However, canola production has little expression in Brazil, when it discovered the world scenario.

In order to improve the seed sector and make it less dependent on seed imports, the production of seeds with high regulatory quality is essential (Gularte et al., 2020; Milani et al., 2012), in addition to the need for well-defined quality

control, and with tests that attest to the physiological quality of canola seeds.

The tetrazolium test is a widely used method for evaluating the regulatory quality of seeds, in quality control, standing out due to its accuracy, speed and large amount of information generated (França-Neto & Krzyzanowski, 2022). With this, it has become an important tool for evaluating the viability of seeds within quality control programs. The efficiency of the tetrazolium test in the evaluation of seed viability depends on the development of suitable methods for each species, determining adequate hydration conditions, seed preparation, concentration of the tetrazolium solution, as well as conditioning time and temperature (Fantazzini et al., 2020).

According to França-Neto & Krzyzanowski (2022), the tetrazolium test determined the respiratory activity in the cells that made up the tissues of the seeds, based on the activity of dehydrogenase enzymes, particularly malate dehydrogenase, which fulfilled the tetrazolium salt (chloride of 2,3,5-triphenyl tetrazolium or TCT) in living tissues. Therefore, the colors resulting from the reaction are a positive indication of viability through the indirect detection of motors at the cellular level, since non-viable tissues do not react and therefore are not colored. Studies included with different methodologies to evaluate the efficiency of the tetrazolium test have been highlighted and proven efficiency in Brassicas seeds, such as in cabbage seeds (Sales et al., 2022), coriander (Silva et al., 2021), forage turnip (Nery et al., 2015), canola (Flores et al., 2015) and crambe (Rezende et al., 2015).

Studies carried out on canola cultivation with recent production methodologies and seed technology are scarce in the literature. Therefore, it is necessary to delve deeper and deeper into the suitability of the tetrazolium test for these seeds. Aiming to speed up the process of evaluating the physiological quality of seeds of this species, the present work aimed to adapt the methodology of the tetrazolium test to evaluate the quality of canola seeds. This involved determining the optimal concentration and soaking period to classify seeds into viable and non-viable categories.

Material and Methods

To carry out the experiments, five lots of canola seeds from the hybrid Alth B4 – Lot 1 (Vila Maria – RS) were used; Lots 2, 3 and 5 (Lavras – MG, differing from the harvest season) and Lot 4 (Diamantina – MG). To build the imbibition curve, two lots of hybrid Hyola 433 (Lavras – MG) were used, lots 6 and 7.

These lots were sent to the Seed Technology Laboratory of the Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM), Campus JK, located in the municipality of Diamantina, Minas Gerais, where the experiments were conducted.

Initial characterization of the batch profile

The seed lots were previously characterized by the following tests and determinations (Brasil, 2009):

1) the moisture content of the seeds, performed by the oven method at 105°C for 24 hours; 2) weight of a thousand seeds, it was determined, in which eight repetitions of 100 seeds were weighed on an analytical scale and later the standard deviation and the coefficient of variation were calculated, with the results expressed in grams;

2) Germination test was treated according to the recommendations for Brassica napus, placed under three sheets of germitest paper, moistened with distilled water, in the proportion of 2.5 the weight of substrates, placed to germinate in gerbox plastic boxes and conditioned in B.O.D germination chambers, with a temperature of 20°C. The estimates would start from the 5th day (first germination count) and end on the 7th day (final count), computing the normal seedlings;

3) The germination speed index computed daily from the germination test, the number of seeds with protrusion from 0.7 mm of radicle measured with a digital caliper, continued according to the formula of (Maguire, 1962); v) For the emergence test, the seeds were sown in plastic boxes containing sand and earth in a 2:1 ratio, moistened with distilled water and kept in a growth room at a constant temperature of 20°C and constant photoperiod.;

4) The initial stand was counted on the 5th day, the test ended when the percentage of emergence stabilized for three days in a row, evaluating the number of normal seedlings emerged;

5) For the emergence speed index, the number of seedlings emerged from the beginning of emergence were computed daily and calculated according to the Maguire formula (1962);

6) Cold test was followed as recommended by Cicero & Vieira (2020). The paper rolls containing the seeds were placed in plastic bags and kept for seven days in B.O.D at a temperature of 10°C. subsequently, removed from plastic bags and placed in a germinator at a temperature of 25°C, the percentage of normal seedlings being evaluated on the fifth day.

Soaking and Preconditioning Curve

An imbibition curve was constructed following the three-phase imbibition pattern described by Bewley et al. (2013), from two replications of 50 seeds using two canola seed lots. The seeds were placed for imbibition between germitest pa-

per, moistened in the amount of distilled water equivalent to 2.5 times the weight of dry paper in gerbox boxes, and kept in a BOD chamber at 25°C. During an evaluation, the seeds were removed from the gerbox, dried with a paper towel. The first searches were in a 10-minute interval, until the weight stabilized three times in a row. In sequence, the amount of time was doubled, until the repetition reached 50% + 1 of germinated seeds. Using an analytical balance with a precision of 0.0001 g, the weight of seed repetitions was estimated during each weighing process (Rodrigues et al., 2008). After building the imbibition curve and establishing the time in hours for each phase, the best imbibition period for the preconditioning was determined in order to precede the immersion of the seeds in the tetrazolium salt. Lots 4 and 5 were used. The canola seeds were placed to soak, with the tegument, for soaking periods of 10, 12, 14, 16, 18 and 22 hours, by methods of hydration on paper and direct immersion in water. After each period, the tegument was removed from two replicates of 25 seeds and two replicates of 25 remained with the tegument, subsequently determining the degree of moisture (Brasil, 2009).

Adequacy of the tetrazolium test methodology

From the results obtained in the preconditioning, the tegument was removed from each seed with the aid of a scalpel and tweezers. There were staining and imbibition periods of 2, 4 and 6 hours in tetrazolium solution (Triphenyl tetrazolium chloride, CAS number: 298.96-4) at concentrations of 0.075%, 0.5% and 1.0% kept in the dark at room temperature of 30°C in BOD, so that there was a process of coloring the seeds and thus evaluations as viable or non-viable. Canola seeds were classified into Category A (viable) and Category B (non-viable). Those belonging to Category A were subdivided into: A1 – well-projected embryonic structures, intact and with light crimson red colors and A2 – less than 50% of the embryo discolored, without reaching the embryonic axis and tissue with normal appearance. Category B were subdivided into: B1 – embryo with intense red colors; B2 – more than 50% of cotyledons discolored; B3 – only the region of the discolored/deteriorated embryonic axis; B4 – completely discolored embryo; B5 – totally discolored cotyledons and B6 – when the seed has more than one type of damage (classes described above) (Figure 1).

Statistical analysis

The experiments were followed in a completely randomized design (DIC), with four attempts. In the preconditioning phase, a 2×2×6 factorial was used (2 – with and without tegument x 2 methods – hydration on paper and direct immersion in water × 6 periods of imbibition – 10, 12, 14, 16, 18 and 22 hours). The data were previously controlled by the test for normality of errors using the Shapiro-Wilk method and homogeneity of variances using the Bartlett method, both at 5% significance level. All variables met the assumptions and then an analysis of variance and Tukey test of means were performed, at 5% significance. The evolution data of the tetrazolium test methodology were analyzed in a 4×3×3 factorial scheme (4 lots × 3 concentrations of tetrazolium solution – 0.075%, 0.5% and 1.0% x 3 imbibition periods – 2, 4 and 6 hours), and tolerated by the Shapiro-Wilk normality test and the analysis of variance and Tukey's test, at 5% probability. Linear and multiple regression methods and Pearson's correlation test between quality, regulation and viability parameters diagnosed by the tetrazolium test were also used. All statistical analyzes were performed using the "R. 4.1.2".

Result and Discussion

The profile characterization of the canola seed lots is shown in Table 1. The moisture content of the seed lots ranged from 8.12% to 9.95%, this variation is within the maximum tolerable limit, which should be 2 ,0 percentage

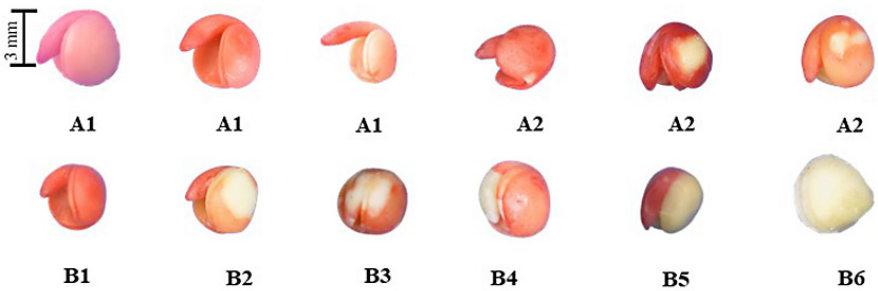

Fig. 1. Tetrazolium test in canola seeds:
Category A subdivided into A1 and A2 (viable) and Category B subdivided into B1, B2, B3, B4, B5, B6 (non-viable)

points, being important for the standardization of the analyzes and reliable evaluation of the physiological potential of the batches (Marcos-Filho, 2015).

There was a variation for the weight of a thousand seeds, between seed lots from 0.28 to 0.40 g. This variation can be attributed to batch production under different environmental and biological conditions.

The tests of first germination count, germination, germination speed index and emergence speed index, did not allow to separate the lots in levels of vigor and viability (Table 1).

All lots met the minimum acceptable germination standard for canola seed production, fortunately established by Normative Instruction No. 42, which is 80% (Brasil, 2013).

Based on the results of the vigor, emergence, initial stand and cold test tests (Table 1), it was possible to evaluate the regulatory quality of canola seed lots by classifying the lots into three levels of vigor. Lot 4 smaller stood out with greater vigor, due to better performance in the testicles, while lot 2, with less vigor, for presenting indexes in the testicles, when compared to the other lots. Finally, batches 1 and 3 with intermediate physiological potential. With this, it was possible to identify which lots have better field potential, initial development and resistance to adverse conditions, crucial characteristics to achieve high yields.

Milani et al. (2012) emergence was more sensitive when assessing canola seed quality, quality changes between batches that the germination test had not identified.

To establish the preconditioning periods for carrying out the tetrazolium test, the imbibition curve was established for two lots of canola seeds (Figure 2).

In the water absorption process of canola seeds, it was observed that the curves followed the triphasic imbibition pattern proposed by Bewley et al. (2013), as was also observed for seeds of *Raphanus sativus* L. (Nery et al., 2014) and cabbage (Armondes et al., 2015). In both seed lots, phase I happened around 22 hours, characterized by an initial phase of rapid water absorption. Then, phase II occurred, characterized by a stationary phase, starting around 22 hours to 32 hours. However, in *B. napus* L. (rapeseed) seeds (Lin et al., 2019), they report that phase I was perceived during the first 8 hours of imbibition, and phase II, from 8 to 24 hours. This same period for phase I occurred in seeds of *B. oleracea* L., being soaked

Table 1. Results of moisture content – MC (%); weight of one thousand seeds – TSW (g); first germination count – FG (%); germination – G (%); germination speed index – GSI; emergency – E (%); initial stand – IS (%); emergence speed index (ESI) and CT cold test (%) of four canola lots, for the characterization of the seed profile

Lots		Tests							
	MC	TSW	FG	G	GSI	E	IS	ESI	CT
1	8,72a	0,28c	85a	95a	17,88a	91ab	58b	16,01a	82b
2	8,33a	0,33b	89a	94a	17,31a	84b	37b	12,76a	47c
3	9,95a	0,33b	85a	90a	15,57a	88ab	84a	12,70a	87ab
4	8,12a	0,40a	87a	89a	14,78a	97a	94a	13,50a	96a
CV (%)	8,82	2,14	7,90	4,66	17,89	6,66	16,47	12,77	6,84

Means followed by the same lowercase letter in the column do not differ statistically by Tukey's test ($p < 0.05$). CV (%) – Coefficient of variation

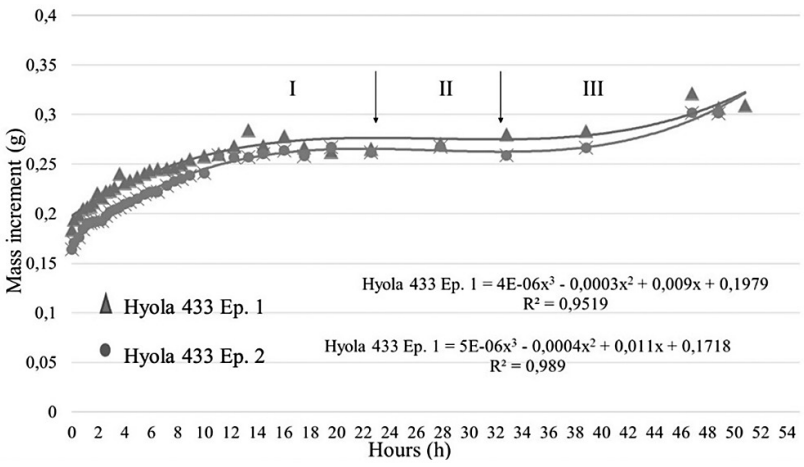

Fig. 2. Mass increment (g) of seeds of Hyola 433 hybrid canola lots over a period of 54 hours

for another 43.5h to complete phase II, and with 45 hours for root protrusion to occur (Dell'Aquila et al., 2000).

Finally, phase III, occurred with the emission of the radicle with 1 mm in length, being observed 41 hours after the beginning of the test. In cabbage seeds, phase III of the water imbibition curve was reached after 32 hours of imbibition (Sales et al., 2022), while in crambe seeds, radicle protrusion occurred after 42 hours (Silva et al., 2019). Cunha & Gomes (2015) pointed out that the imbibition time is important not only for the initiation of physiological activities in the seed, but also to favor the removal of the integument and the exposure of the embryo to contact with the tetrazolium solution.

The realization of the imbibition curve in canola seeds allowed establishing the minimum period of preconditioning for the tetrazolium test, suggesting that the time to be used for the activation of the enzymatic system, with intensification of respiration and other metabolic activities, should be less than 22 hours. The preconditioning aims to facilitate the penetration of the tetrazolium solution and the development of a clearer and more evident color, soften the tissues, facilitating the removal of the integuments and activate the respiratory enzymatic systems, characterized by phase II (Lamarca et al., 2009). In phase II, the metabolic process is activated, and in this phase despite the slow absorption of water, the embryonic axis still cannot start its growth (Bewley et al., 2013).

Inadequate preconditioning can lead to obtaining seeds with stains, cracks, color problems and, consequently, to unreliable results for the tetrazolium test (Oliveira et al., 2011).

After pre-conditioning the canola seeds in water, it was observed that when the seeds were soaked in a tetrazolium solution with the tegument, they did not absorb. Therefore, a second proposal of pre-conditioning was carried out with the seeds with and without the seed coat, based on the results of the moisture content (Table 2).

Table 2. Moisture content (%) of canola seeds remaining in pre-conditioning for periods (hours) and tegument (with and without) (Lot 5)

Hours (h)	Tegument	
	With	Without
10	34,56 aA	37,89 aA
12	40,77 aA	27,13 aB
14	46,67 aA	24,93 aB
16	41,52 aA	29,58 aB
18	43,22 aA	34,09 aA
22	41,04 aA	36,87 aA
CV (%)	17,50	

Means followed by the same lowercase letter in the column do not differ statistically by Tukey's test ($p < 0.05$). CV (%) – Coefficient of variation.

There was no variation in the moisture content of the seeds between the imbibition periods of seeds with or without seed coat. However, when comparing seed conditions, within the periods of 12h, 14h and 16h of imbibition, seeds with seed coat showed a rapid increase in seed moisture content compared to seeds without seed coat. The function of the integument in the seed soaking process is to regulate the entry of water and ensure that the soaking is slow and uniform, favoring adequate hydration of the tissues, avoiding the appearance of uneven coloration and stains by the tetrazolium test (Oliveira et al., 2011; Woodstock, 1988).

Therefore, the removal of the integument after the soaking period is reported in the literature in castor bean seeds (Oliveira et al., 2011), sunflower (Silva et al., 2013) and *Tamarindus indica* L. (Cordeiro et al., 2022), for subsequent imbibition in the tetrazolium salt.

It was found that for batch 4 (Table 3), the average moisture content for seeds with seed coat were higher when compared to seeds without seed coat for both hydration methods.

These water contents emphasize the importance of reaching phase II of germination, corroborating Carvalho & Nakagawa (2012), who report that when endospermic seeds reach water contents from 25% to 30% and cotyledons from 35% to 40%, water absorption stabilizes or increases very little, initiating a stationary phase (phase II), in which digestion and active transport of reserve substances will occur. In this phase, the water potentials of the medium and of the seeds are very close, and with that, the absorption of water by the seed stabilizes. Thus, according to the results of the average moisture content, it was decided to use hydration in paper and seeds with tegument in a period of 10 hours, as a pre-conditioning condition for canola seeds, corroborating with Sales et al. (2022) in cabbage seeds, who indicates hydration between paper for 10 hours. After the imbibition period, the integument was removed, since the removal of the integument facilitates the absorption of the tetrazolium salt and the color of the tissues (Pereira et al., 2020). According to Guedes et al., (2010) the removal of the integument, after pre-wetting, may allow greater uniformity and speed in color development.

Table 3. Moisture content (%) of canola seeds remaining after pre-conditioning by methods (hydration on paper and in water) and tegument (with and without) (Lot 4)

Methods	Tegument	
	With	Without
Hydration in paper	39,86 aA	31,64 aB
Hydration in water	43,32 aA	25,00 aB
CV (%)	22,60	

Means followed by the same lowercase letter in the column do not differ statistically by Tukey's test ($p < 0.05$). CV (%) – Coefficient of variation.

Table 4 shows the viability of canola seeds by the tetrazolium test. The viability of canola seeds in batch 3 is lower in all tetrazolium concentrations, as well as in the 1.0% concentration for batch 1 (Table 4).

Table 4. Percentage of viable canola seeds treated by the tetrazolium test as a function of solution concentrations (%) and lots

Lots	Concentration (%)		
	0,075	0,5	1,0
1	87a	88 a	74c
2	88a	87a	87 a
3	81 b	61 c	72c
4	90 a	82 b	79 b
CV (%)	6,58		

Means followed by the same lowercase letter in the column do not differ statistically by Tukey's test ($p < 0.05$). CV (%) – Coefficient of variation.

Comparing the germination test (Table 1) with the viability of the lots, a greater similarity was found for the 0.075% concentration (Table 4). Corroborating these results in crambe seeds (Rezende et al., 2015), sesame (Jesus et al., 2015) and okra (Souza et al., 2018), which found that at a concentration of 0.075%, the results of the viability presented greater correspondence with the germination tests when detected with higher concentrations of the tetrazolium solution.

A significant reduction in viability is established as the concentration of the tetrazolium solution increases, except for lot 2, which recorded a percentage above 80% (Figure 3).

At the 1% concentration of tetrazolium salt, lower averages related to the viability of canola seeds were observed. Thus, the viability results show that the higher the viability for the batches, the lower the concentration of the tetrazolium salt solution.

Regarding the evaluated periods of exposure, the tolerated viability for batch 3 was lower than the others. For the period of 2 hours, greater viability was observed for lot 2 (Table 5). The two-hour immersion period in solution was efficient to assess the viability of leucaena (Costa & Santos, 2010) and carrot (Lima et al., 2018) seeds. Apparently, the one lived for 2 hours did not promote enough colors to identify the viability of gherkin seeds, possibly due to the short time of exposure to the tetrazolium solution (Paiva et al., 2017).

Table 5. Percentage of viable canola seeds cultivated by the tetrazolium test as a function of lots and periods (hours)

Lots	Periods (hours)		
	2	4	6
1	82 b	82 a	85 a
2	89a	87 a	85 a
3	75 c	71 b	68 b
4	82 b	86 a	83 a
CV (%)	6,58		

Means followed by the same lowercase letter in the column do not differ statistically by Tukey's test ($p < 0.05$). CV (%) – Coefficient of variation.

The batch 2 was negatively high, as observed in the regression symptomatology. Suggesting that the greater the viability, the shorter the periods of imbibition of the seeds by the tetrazolium test. The same behavior was observed for batch 3 (Figure 4). Thus, as the concentration increases there is a loss of viability and likewise, when the immersion period increases, it is possible to observe a drop in viability. Therefore, the concentration of 0.5% and 1.0% tetrazolium solution would not be recommended for canola seeds.

Figure 5 represents the graphs of the response surface for each lot, in order to obtain the definition of the combination

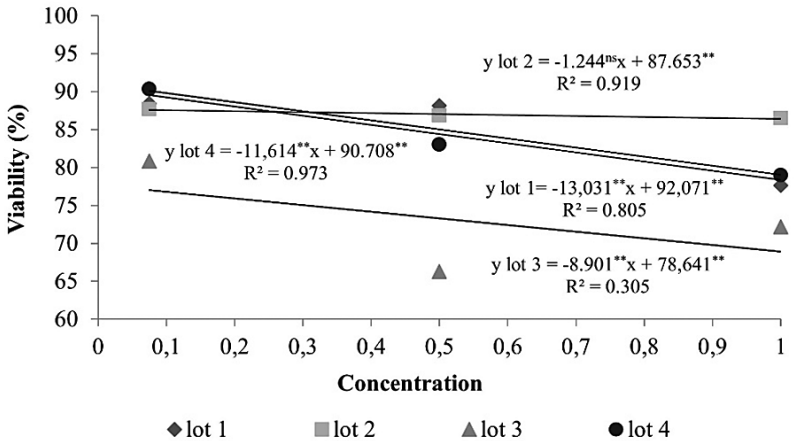

Fig. 3. Viability of canola seeds as a function of tetrazolium salt concentrations

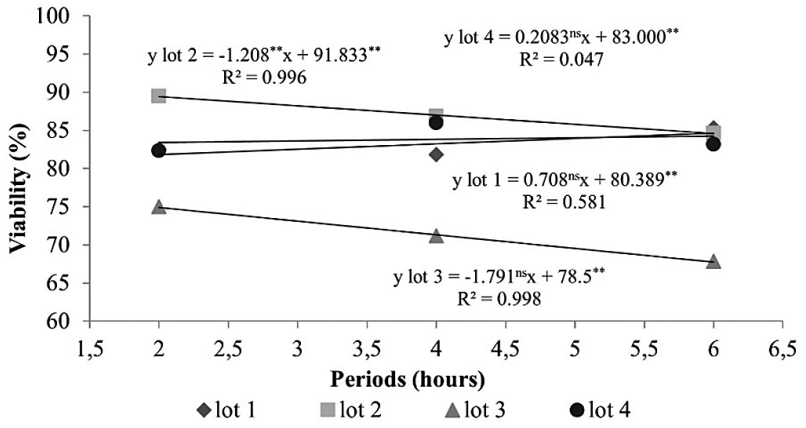

Fig. 4. Viability of canola seeds as a function of periods (hours) of imbibition by tetrazolium salt

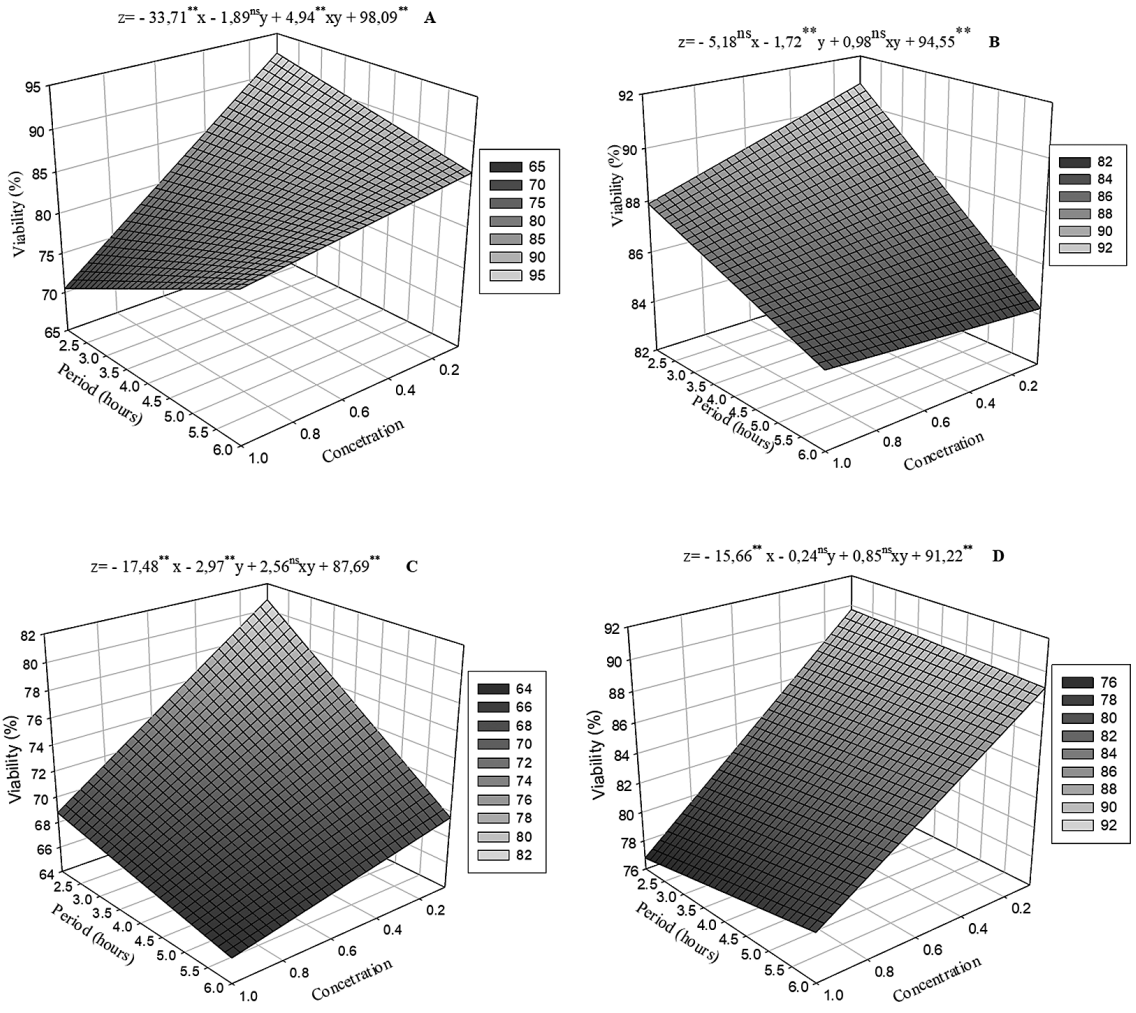

Fig. 5. Response surface for the significant interaction between concentrations (0.075%, 0.5% and 1.0%) and periods (2, 4 and 6 hours), for Lot 1 (A), Lot 2 (B), Lot 3 (C) and Lot 4 (D)

of period and concentration that is providing a greater relationship, and consequently viability, according to the results of the tetrazolium test.

The smaller concentration of the tetrazolium solution combined with the imbibition periods, provide higher percentages of viability of canola seed lots (Figure 5). Thus, the tetrazolium salt concentration of 0.075% at 2 hours showed the best results to estimate the viability of canola seeds. For batch 1 (Figure 5A) and batch 2 (Figure 5B) this concentration presented a percentage of about 92% for viability, batch 3 (Figure 5C) had a percentage above 80% and batch 4 (Figure 5D) had a percentage above 88%. In this study, a combination of concentration and period of the tetrazolium test was not found that presented a significant association with all characteristics simultaneously (Figure 6). It was also verified that the methodology that considers the concentration of 0.075% for 2 hours is more adequate, because, in this situation, there are correlations experienced with important physiological parameters such as germination, first germination count and germination speed index. According to Silva et al. (2013) efficient methodologies, using tetrazolium solution at low concentrations, are important to optimize the application of resources within laboratories and enable the analysis of more samples at a lower cost. Studies carried out to determine the viability of canola seeds through the tetrazolium test are limited in the literature. Flores et al. (2015), determined criteria for preparing the tetrazolium test, by soaking the seeds for 16 hours at 20°C followed by hours of immersion in a tetrazolium solution for colors. In the present study, when comparing the research by Flores et al. (2015), the test execution time with the increase of the temperature of the imbibition period to 25°C accounts for 62% (6h) and the amount of tetrazolium salt was 67% making the removal of the seed coat. The results helped with the development of methodologies for analysis of canola seeds, reducing analysis time and costs, without compromising their accuracy and effectiveness in assessing the regulatory quality of seeds.

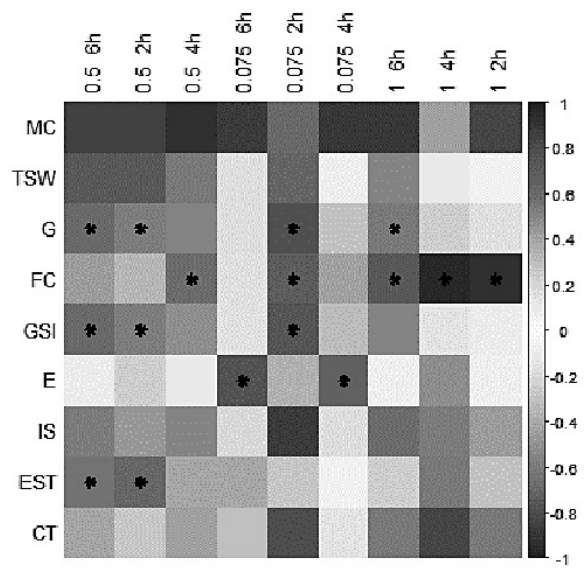

Fig. 6. Graphic dispersion of the correlations between the parameters of quality, regulation and viability of viability tested by the tetrazolium test with different concentrations and immersion times in canola seeds. * Indicates correlations by t-test at 5% probability

Conclusion

The tetrazolium test is efficient to evaluate the viability in canola seeds with preconditioning of the seeds in hydration in paper for 10 hours at 25°C, followed by the total removal of the tegument, in the 0.075% tetrazolium solution for 2 hours at 30°C.

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