Article

Chia seed viability analysis protocol by tetrazolium test

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Abstract

Salvia hispanica L. is an annual herbaceous plant, belonging to the Lamiaceae family, it stands out as the natural resource of plant origin with the highest content of fatty acids known so far. To obtain success in the production of seeds it is necessary to use lots of high quality, which can be evaluated, through the vigor of the same, at present, one of the main requirements for the evaluation of vigor refers to the obtaining of reliable results in a relatively short period of time. The tetrazolium test stands out for being fast and reliable. However, the methodology for the genus Salvia is not referenced within the standards of the International Seed Testing Association (ISTA), considering these facts, it becomes important to carry out the experiment for the development of a protocol that allows this analysis. The research was carried out in the Seed Laboratory of the Federal University of Pelotas, six batches of black chia seeds were used and four concentrations of tetrazolium salt (0.075%, 0.1%, 0.5% and 1%) were tested to evaluate the seed viability. The experimental delineation was in randomized blocks, submitted to the analysis of variance through the F test and subsequently the means compared to each other by the Tukey test, at 5% probability, for comparison of the means. The tetrazolium test conducted in the concentration of 0.075% is efficient to evaluate the viability of the seeds of *S. hispanica* L., as well as to differentiate lots with different physiological quality.

Keywords: *Salvia hispanica* L., concentrations, ISTA, tetrazolium salt, vigor.

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Introduction

Salvia hispanica L., is an annual herbaceous plant, belonging to the Lamiaceae family, native to the region that extends from southern Mexico to northern Guatemala (Chicco *et al*., 2009). In the pre-Columbian period, it was one of the staple foods used by civilizations that inhabited Central America, being exceeded in importance only by corn and beans, but with greater prominence than other important crops (Ayerza and Coates, 2004).

These seeds, in addition to being used as food by the Indians for many centuries, were offered to the gods during religious ceremonies for the Aztecs. This custom disappeared 500 years ago, after the conquest of the territory by the Spanish (Ayerza and Coates. 2005). However, Jiménez (2010) states that the cultivation of chia survived in the mountainous areas of Mexico and Guatemala.

In addition to the historical importance it has, it is an excellent source of minerals, rich in proteins, lipids, energy and fibers, the content of these nutrients in the seed of this species is greater than other cereals, such as rice, barley, oats, wheat and corn (Coates and Ayerza, 1996). It stands out as the natural resource of plant origin with the highest content of fatty acids known so far, including alpha linolenic acid (omega 3) and linoleic acid (omega 6).

The fruits, mistakenly called 'seeds', are schizocarp 1-1.2 mm wide and 2-2.2 mm long, are characterized by the mixture of different shades of colors varying from black to gray, with irregular spots, flushed and brown and even some white, the reasons for the presence of different colors being unknown. When soaked in water the seeds give rise to a gelatinous liquid due to the presence of mucilage on its surface (Ayerza and Coates, 2004 and 2005).

They are currently grown in numerous countries such as Australia, Mexico, Argentina, Ecuador, Bolivia, Perú, Paraguay and other (Busilacchi *et al*., 2013). In Brazil, the western Paranaense and northwestern regions of Rio Grande do Sul began to invest in the cultivation of chia in the last crops, presenting good results, despite the lack of information about the nutritional requirements of the plant (Migliavacca *et al*., 2014).

As well as the other annual cultivated species, to obtain success in a commercial tillage it is necessary to use high quality seeds; however, there is still no methodological standardization to evaluate the viability and physiological quality for the species. At present, one of the main requirements for the evaluation of vigor in seeds refers to obtaining reliable results in a relatively short period of time, allowing the producer to make decisions more quickly during the entire production process.

Among the various procedures used for this purpose, the tetrazolium test stands out, which is characterized by a biochemical evaluation, based on the activity of the dehydrogenase enzymes that catalyze the respiratory reactions, present in the mitochondria, located inside the plant cells (França-Neto *et al*., 1998). During cellular respiration, there is release of hydrogen ions, which react with the tetrazolium salt (colorless and diffusible), forming a red and insoluble substance, called formazam, delimiting the living tissues of the seed.

The coloration resulting from the reaction of the tetrazolium solution with the hydrogen ions is an indication of the viability of the tissues, which occurs through the detection of the respiration of the plant cells (AOSA, 1983; França-Neto *et al*., 1998). The physiological quality assessment through the tetrazolium test has been disseminated for some species and used in seed quality control programs (Costa *et al*., 2007). According to Deswal and Chand (1997), the tetrazolium test stands out in this context, as it is a technique that, in addition to evaluating the viability of the seeds, can also estimate its vigor.

For some species, such as soybeans, the technique is well studied and well calibrated, being a routine procedure in many laboratories, even serving as a tool for decision-making related to the crop (França-Neto, 1998). However, the methodology for the genus Salvia is not referenced within the norms of the International Seed Testing Association (ISTA, 2003), nor in the guidelines for analysis of seeds (RAS) (Brasil, 2009). Considering these facts, it becomes important to carry out the experiment for the development of a protocol that allows this analysis. Therefore, the objective of this work was to evaluate methodology to evaluate the viability in seeds of *Salvia hispanica* L. using the tetrazolium test.

Materials and methods

The work was carried out in the period from July to December, in the Seed Analysis Laboratory of the Department of Phytotechnics-Postgraduate Program in Seed Science and Technology of the Federal University of Pelotas. Six batches of black chia seeds (*S. hispanica*) were used for the work.

The seeds were subjected to the germination test, for which 200 seeds were used per treatment, divided into four sub-samples of 50 seeds, placed in gerboxes, using as a substrate two sheets of mataborron paper moistened with distilled water (2.5 times dry paper weight). Subsequently, they were placed in the germination chamber (germinator or BOD) at alternate temperatures of 20-30 ºC, according to the recommendations of the (Brasil, 2009) seed analysis rules for other species of the same genus.

Germinated seed evaluations were performed at four and seven days. The first germination count test was carried out jointly with the germination test and evaluated on the fourth day after planting, the results were expressed as a percentage of normal seedlings. To evaluate the viability of the seeds, four concentrations of 2,3,5-triphenyltetrazolium chloride (0.075%, 0.1%, 0.5% and 1%) were used, and were subjected to the following methodological procedure:

Solution preparation

Initially a 1% standard solution was prepared, mixing 10 g of the tetrazolium salt in 1 liter of distilled water. And for working solutions, dilutions were made until the desired concentration was obtained. These solutions were stored in amber glass jars, in a dark and cool place.

Pre-conditioning

220 seeds were immersed in water per treatment and kept under these conditions for a period of 8 hours, at a temperature of 20 °C, recommended temperature for species of the genus *Ocimum* spp., belonging to the same botanical family of the Sage, Lamiaceae (Brasil, 2009). After that time, they were placed to dry on a sheet of paper at room temperature for a period of one hour or until the mucilage formed during the imbibition disappeared, which makes cutting difficult.

Coloration

After pre-conditioning, the seeds were sectioned longitudinally through the center of the embryonic axis with the help of a scalpel and placed in vessels, completely submerged in different concentrations of tetrazolium salt (0.075%, 0.1%, 0.5% and 1%) maintained in these conditions for 2 h at 30 °C, recommended temperature for the species *Ocimum* spp. (Lamiaceae) (Brasil, 2009).

Sample washing

After the red color was reached, the seeds were removed from the environment and washed with water and kept submerged until the moment of the evaluation.

Interpretation

Stereoscope (10x) was used for interpretation. Four sub-samples of 50 seeds were evaluated for each concentration and the following classifications were considered: carmine red: live and vigorous tissue and milky white: dead tissue.

Statistical procedures

The experimental delineation was completely randomized, for the classification of the lots in terms of germination the data were submitted to the ArcSen transformation (root x/100) and subsequently to the means compared to each other by the Tukey test at 5% chance For the evaluation of salt concentrations, a 6 x 4 factorial experiment (six batches x four salt concentrations) was considered. The statistical procedure was identical to the one described above, with the differential of observing the significance of the interaction between the factors and the realization of the respective splittings.

The following figures show the steps necessary to perform the tetrazolium test, whose main stages are pre-moistening, mucilage drying, cutting, coloring and evaluation of the viability of the seeds (Figure 1).

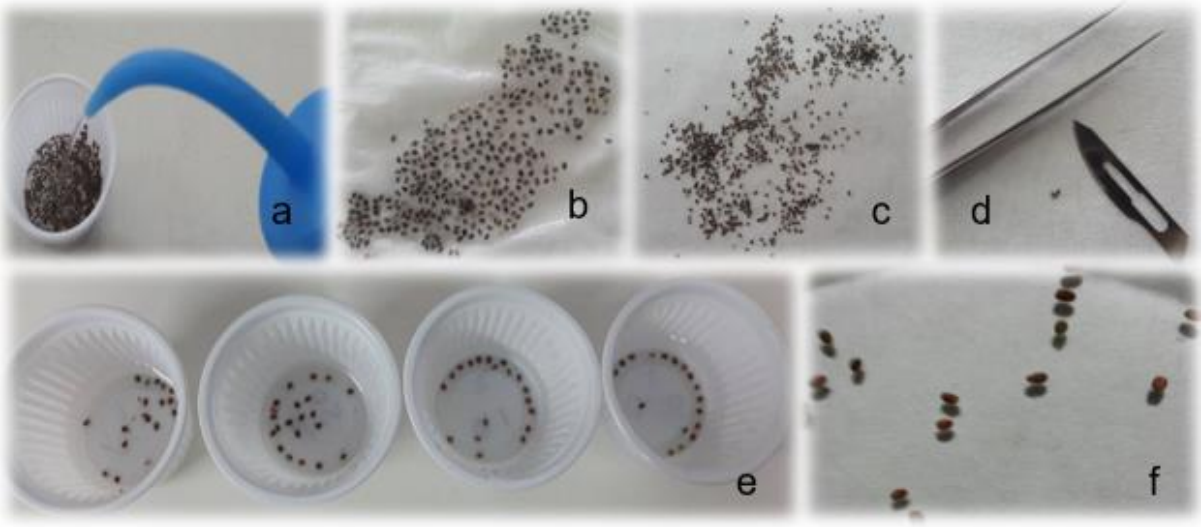

Figure 1. a) pre-humidification; b) seed drying at room temperature; c) mucilage free seeds; d) longitudinal cutting of seeds; and f) seeds after coloring.

Results and discussion

The coloration of the seeds with tetrazolium salt 0.075% and 0.1% allowed to evaluate the viability without difficulty, it was observed that the embryo has a stronger and more prominent coloration in relation to the rest of the seed; however, in the concentration of 0.5% the coloration was more pronounced, but still the reading was made without difficulty. When the 1% solution was used, it was observed that the high concentration hindered the interpretation of the viability and caused excessive coloration. This was probably because this concentration influenced the process of coloring the seeds, since the embryo tissues instead of bright pink colors had an intense red hue (similar to the color of the injured tissues).

The coloration patterns found are presented in Figure 2, where it is possible to observe variations in the hue from the white∗unviable seeds (Figure 2g), through the different shades ranging from bright pink, superficial, uniform and without lesions of the embryo until deep and deep red coloration (Figure 2 h-l).

Therefore, it was verified that all dilutions showed yield in terms of the tetrazolium solution used; however, the biggest highlight is for the use of 0.075% tetrazolium salt. In dead tissues, where there is no respiratory activity, dehydrogenase enzymes are inactive; therefore, the reaction with the tetrazolium solution does not occur and consequently, the seeds remain discolored. For seeds in deterioration process the development of the coloration is faster, generating a more intense and deep red tone, while the vigorous seeds have a bright appearance with pink to red coloration (Filho *et al*., 1987; França-Neto *et al*., 1998).

Figure 2. g) non-colored, non-viable seeds; h) viable seeds chlorination 0.075%; i) viable seeds concentration 0.1%; j) viable seeds, concentration 0.5%; k) and l) seeds with intense red coloration, 1% concentration of tetrazolium salt.

It is emphasized that in studies of this nature the use of lots with differentiated quality is fundamental, since a certain methodology must be sufficiently sensitive and at the same time robust to detect subtle differences between lots of both high and low quality.

According to the evaluation of the physiological quality of the seeds, carried out by the germination test taking into account the results of the tetrazolium test in the different salt concentrations (Table 1), the lots used can be separated into different quality levels , called high (lots 2 and 3), medium (lot 5) and low viability (lots 1, 4 and 6).

Table 1. Germination percentage of viable seeds subjected to germination and viability tests, using different concentrations of tetrazolium salt. FAEM/UFPel.

Lots	Germination (%)	PC (%)	Viability in different concentrations (%)			
			0. 075%	0.1%	0.5%	1%
L1	67 C	52 C	76 C a	78 C a	66 E b	76 C a
L2	94 A	85 A	98 A a	96 A a	96 A a	94 A a
L3	95 A	87 A	96 A a	94 A a	88 B b	92 A ab
L4	67 C	59 B	70 D a	66 D ab	68 E ab	64 E b
L5	79 B	43 D	90 B a	84 B b	82 C b	84 B b
L6	68 C	36 D	74 CD ab	76 C a	74 D ab	70 D b
CV (%)	3.75	4.48	2.61			

Means followed by the same uppercase letter in the column and lower case in the line, differ from each other by the test of Tukey ($p< 0.05$).

It was observed that, with the use of the 0.075% solution, the results were similar with those obtained by the germination test (Table 1). The use of 0.075% tetrazolium solution has been used in the analysis of the viability in seeds of several species, such as soybean (França-Neto *et al*., 1998) or corn (Días and Barros, 1995), zucchini (Barros *et al*., 2005) and watermelon (Bhering *et al*., 2005), allowing the analysis of the viability of the seeds of these species with high yield of tetrazolium solution during the evaluations.

For the evaluations made with 0.1% and 0.5% tetrazolium solution (Table 1), the results were also similar to those obtained in the germination test. However, it should be noted that the proven efficiency of the use of more dilute tetrazolium solutions becomes more suitable options because it allows the higher yield of the tetrazolium salt during the analyzes. Opposite results were observed in studies with marigold seeds, where the concentration of 0.5% tetrazolium salt solution was very low, and it was not possible to correlate with the other colorations.

When the treatment with 1% salt concentration was used, the coloration was adequate for the embryo, indicative of viability. Possibly the chemical constitution of the species is very different from each other, which refers to the need to use salt concentrations also differentiated. Craviotto *et al*. (2011) confirmed that the method with 0.5% tetrazolium at 30 °C was considered efficient for soy coloring. The data presented in Table 2 demonstrate that there is significance for the positive linear correlation between germination and first germination count in all salt concentrations studied.

Table 2. Pearson's simple correlation coefficients between PCG and G with the different salt concentrations studied.

Concentrations (%)	First count (%)	Germination (%)
0.075	0.67 **	0.95 **
0.1	0.66 **	0.92 **
0.5	0.67 **	0.94 **
1	0.66 **	0.91 **

**= significant at 1% probability by the T test.

However, despite being significant, the correlation coefficient is substantially higher in the germination variable compared to the first count, demonstrating efficiency in determining viability, for vigor tests and calibration of the methodology is necessary. According to Filho (2005), the choice of suitable methodology for the use of the tetrazolium test in seeds is based on the ease of differentiation of viable and non-viable tissues in the economic study and on the ability to differentiate lots of different physiological quality.

In this way, the concentration of 0.075% meets the economy requirements and lends itself to differentiation of lots in relation to viability and vigor. According to Santos *et al*. (2006), the importance of the tetrazolium test, as an instrument for evaluating the viability of seeds, is due to the rapidity in obtaining its results that may be useful in areas of commercialization, benefit, storage and production, without this means the commitment of the germination test that functions as a reference test.

Conclusions

The tetrazolium test using the concentration of 0.075% at a temperature of 30 °C for 2 h in an oven is efficient to evaluate the viability of seeds of *Salvia hispanica* L., as well as to differentiate lots with different physiological quality.

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