Viability of barley seeds by the tetrazolium test[1]

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ABSTRACT - The tetrazolium test is used to control seed quality of various plant species since it allows a rapid evaluation of viability. Freshly harvested barley seeds show dormancy that can make the germination test ineffective for an immediate evaluation. Therefore, the development of more efficient methods, such as the tetrazolium test, is necessary. The objective of this research work was to study various procedures for performing the tetrazolium test on barley seeds. Five lots of cv. BRS 195 barley seeds were used and subjected to the following treatments: two different methods of seed preconditioning (direct immersion in H_2O and between sheets of moistened paper towels); two types of preparation for staining (longitudinal cross-section of the seed through the embryo with immersion of one half in a 2,3,5 triphenyl tetrazolium chloride solution or placing both halves on top of filter paper moistened with the tetrazolium salt solution); two methods of staining (on top of filter paper and direct immersion in the tetrazolium salt solution). Three concentrations of the tetrazolium salt solution (0.1%, 0.5%, and 1.0%) were used. It was concluded that the tetrazolium test on barley seeds may be accomplished with preconditioning by direct immersion in H_2O and staining by immersing in a 0.1% or 0.5% concentration of tetrazolium salt solution or staining on top of filter paper moistened with such solution at a 1.0% concentration.

Index terms: *Hordeum vulgare*, germination, dormancy.

Viabilidade de sementes de cevada pelo teste de tetrazólio

RESUMO - O teste de tetrazólio é empregado no controle da qualidade de sementes de várias espécies, pois permite estimar rapidamente a sua viabilidade. Sementes de cevada apresentam dormência quando recém-colhidas, o que pode tornar o teste de germinação ineficaz para avaliação imediata, sendo necessário o desenvolvimento de métodos mais eficientes como o de tetrazólio. O objetivo desse trabalho foi estudar vários procedimentos para a condução do teste de tetrazólio em sementes de cevada. Foram utilizados cinco lotes de sementes da cv. BRS 195, testando duas formas de pré-condicionamento da semente (imersão direta em água e entre folhas de papel toalha umedecidas); dois tipos de preparo para coloração (corte longitudinal da semente através do embrião com imersão de uma das metades em solução de cloreto de 2,3,5 trifenil tetrazólio ou com colocação das duas metades sobre papel de filtro umedecido com a solução do sal de tetrazólio); duas formas de coloração (sobre papel de filtro e com imersão direta na solução) e três concentrações de solução do sal de tetrazólio (0,1%, 0,5% e 1,0%). Foi concluído que o teste de tetrazólio em sementes de cevada pode ser conduzido mediante pré-umedecimento por imersão direta em água, coloração por imersão na solução de sal de tetrazólio nas concentrações de 0,1% e 0,5%, ou coloração sobre papel de filtro umedecido com essa solução, na concentração de 1,0%.

Termos para indexação: *Hordeum vulgare*, germinação, dormência.

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Introduction

Barley *(Hordeum vulgare* L.) is a winter cereal widely produced in the Southern Region of Brazil and its main producing states are Rio Grande do Sul and Paraná. Although being used for animal feeding (grazing or rations), human consumption (flour), and therapeutical uses, that cereal is specially used for malt production in the beer industries (Urchei, 1994).

Brazil produces about 30% of the malt utilized in the country (Minella, 2009). The main factors for this low production are the instability of the crop production, high productions costs and management problems, when compared to the imported product that presents better quality, resulting in nonstandard barley seed lots for malt production and leading to frequent importations of raw material.

To guarantee adequate agricultural production, the use of known quality seeds is fundamental; and their physiological potential have to be constantly monitored, starting from the pre-harvest, passing through the processing unit until the end of the storage period. For the physiological quality analysis of seed lots, the method mostly used by seed producing companies is the germination test. The period for performing this test, however, may vary from days, weeks, or even months for some species that present dormancy (Marcos-Filho, 2005). According to the Rules for Seed Testing (Brasil, 2009) the final evaluation of the germination test for barley seeds has to be performed until the seventh day after installing the test, sometimes needing to prolong that period for seven days more, since the seeds of this species present dormancy when freshly harvested, thus impairing their initial evaluation.

It is increasingly necessary to obtaining quick answers about the real situation of a given seed lot in order to give a suitable target for it. The efficiency and the rapidity for that decision-taking are directly correlated to the optimization of resources, thus reducing losses with unnecessary processing and storage.

Within this context, the quick tests for seeds viability evaluation have been a nowadays demand and, among the available methods, the tetrazolium test deserves an outstanding position (França-Neto, 1999). As emphasized by Tunes et al. (2009), the tetrazolium test is important on seed quality control, as it allows a fast estimate of the seed germination capacity, including the dormant ones.

The test is based on the dehydrogenase enzymes activity that catalyzes the respiratory reaction in the mitochondria, correlating seed viability with changes on the color of living tissues. In this sense, there is an oxireduction reaction with the 2,3,5-triphenyl tetrazolium chloride (França-Neto, 1999), which results in the formation of a stable and non-diffusible compound of reddish coloration, the formazan. Such formation indicates respiratory activity in the mitochondria and allows delimiting living tissues from those tissues that remain unstained or exhibit abnormal coloration (Marcos-Filho, 2005).

The methodology of the tetrazolium test has been improved over time and specific manuals are already available for its usage for the crops of corn (Dias and Barros, 1999), cotton (Vieira and Von Pinho, 1999), common bean (Bhering et al., 1999), peanut (Bittencourt and Vieira, 1999), and soybean (França-Neto et al., 1999).

The test can be affected by given conditions such as: the presence of fungi, which can impair the germination test results; focuses on the embryos physic and physiological conditions of each seed; allows for rapidly evaluating the seed viability; enables the identification of different viability levels for some species as common bean and soybean; is able to provide a diagnosis for the cause for seed viability loss; and requires simple and low cost equipments (França-Neto, 1999).

The International Seed Testing Association (ISTA, 2007) recommends the preconditioning of barley seeds by the immersion in H_2O during 4 and 18 h, at a 20 ºC temperature and then carry out the staining procedure for 3 h, at 30 ºC in a 1% tetrazolium chloride solution. In relation to splitting the seeds, a longitudinal cross-section of the embryo and ¾ of the endosperm, as well as a transversal cross-section, with the elimination of one of the halves, is recommended. The Rules for Seed Testing (Brasil, 2009) also recommend the preconditioning for 18 h, between sheets of paper towels moistened with H_2O and staining on top of filter paper moistened with a 0.5% tetrazolium salt solution.

In relation to the seeds cross-sectioning and preparation for some species of the Poaceae family, however, the methodology most used in the Seed Analysis Laboratories is the longitudinal cross-section through the middle of the embryo. The evaluation is then performed on the two halves of the seed in order to improve test interpretation, since the seeds are small, thus impairing the cross-sectioning exactly through the middle of the embryonic axis.

Because of the species economical importance and the need for developing methods allowing for the fast viability estimation of freshly harvested seeds, the objective of this research work was to study various different procedures for performing the tetrazolium test on barley seeds.

Material and Methods

The experiment was performed in the "Seed Laboratory of Fitotecnia and Fitosanitarismo Department of the Federal

University of Paraná), in Curitiba, State of Paraná, Brazil, from July to November 2010.

Five barley (cv. BRS 195) seed lots, produced in the 2009 growing season were utilized for the experiment. The seed lots were homogenized and divided into 27 subsamples of 220 seeds each (representing the 27 treatments studied), which remained stored into Kraft® paper bags under controlled temperature (15 ºC) and RH (60%) during all the experimental period.

Initially, the moisture content and the germination percentage of each seed lots were evaluated. The moisture content determination was performed using the drying oven method at 105±3 ºC, for 24 h, with two replications of 5.0 g each per seed lot, according to the Rules for Seed Testing (Brasil, 2009) recommendations. For the germination test, eight replications of 50 seeds each per treatment were used. For that, the seeds were placed into rolls made with three sheets of paper towels moistened with H_2O, in a quantity equivalent to 2.5 times the mass of the substrate and then placed in a germinator, at 20 ºC. Five days after starting the test, the counting was carried out by computing the percentage of normal seedlings according to the criteria established by the Rules for Seed Analysis (Brasil, 2009). No treatment for dormancy breaking was performed, since the seed lots used had already lost dormancy.

For studying the tetrazolium test, four replications of 50 seed each per treatment were used for evaluating the different preconditioning combinations and seed preparation and staining. Such procedures are described as follows:

a. Preconditioning – two imbibition methods were tested:

a.1 – imbibition in rolled paper towels moistened with H_2O with an amount equivalent to 2.5 times the mass of the paper, for 18 h, at 20 ºC (Brasil, 2009);

a.2 – direct immersion in 40 mL of H_2O, with seeds being placed into a 100 mL Beaker for 4 and 18 h, at 20 ºC (Brasil, 2009; ISTA, 2007).

At the same time of the imbibition procedure, the seed moisture contents were determined after preconditioning. Determination was performed using the oven-drying method at 105 ± 3 °C, for 24 h (Brasil, 2009).

b. Seed preparation – the following seed preparations were studied:

b.1 – seeds were longitudinally cross-sectioned through the embryo with disposal of one of the halves and staining the other half (Brasil, 2009; ISTA, 2007);

b.2 – seeds were longitudinally cross-sectioned through the embryo, staining the two halves.

c. Staining procedures– seeds were placed in the dark for 2 and 3 h with a tetrazolium salt solution at the concentrations of 0.1%, 0.5%, and 1.0%. The staining method varied according to the type of preparation:

c.1 – immersion of one seed half into 5 mL of a 2,3,5 triphenyl tetrazolium chloride (TTC), in a 100 mL capacity Beaker, for staining at 30 ºC temperature (Brasil, 2009; ISTA, 2007);

c.2 – the two seed halves were placed on top of a sheet of filter paper moistened with the tetrazolium salt solution equivalent to 2.5 times the mass of the paper and put for staining under 40 ºC temperature.

For the first method (item c.1), once reached the ideal staining, the seeds were removed from the chamber, washed under tap water and then submerged in H_2O under refrigeration (5 ºC to 10 ºC) until evaluation. For the second method (item c.2), the seed were maintained on top of filter paper, under refrigeration (5 ºC to 10 ºC), until evaluation.

For seed viability evaluation, their structures were observed under stereomicroscope, following the Rules for Seed Testing (Brasil, 2009) recommendations. Seeds were classified as viable and non-viable according to the coloration of the embryonic axis, computing only the percentage of viable seeds.

A completely randomized experimental design, with four replications, was used for the experiment. The means were compared by the Tukey test at 5% probability. The Spearman model was also used for performing the correlations between means of the tetrazolium test and the germination test. Data for moisture content were not statistically analyzed.

Results and Discussion

After the initial evaluation of the physiological quality of seeds, performed by the germination test (Table 1), the seed lots were sorted into three viability levels: high (seed lot 1); intermediate (seed lots 2 and 3); and low (seed lots 4 and 5).

Table 1. Mean data on germination and moisture content of five lots of barley seeds.

Seed lots	Germination (%)	Moisture content (%)
1	91 a	12.5
2	86 ab	12.6
3	84 ab	12.5
4	83 b	12.8
5	82 b	12.7
CV (%)	2,48	-

Means followed by the same letter in the column are not statistically different between each other (Tukey test, 5% probability level).

As far as initial moisture content of seeds is concerned, the five lots studied presented similarity, varying from 12.5% to 12.8% (Table 1). That is very important for performing the tests, since the uniformity of seed moisture content is essential for the evaluation standardization and achievement of consistent results (Marcos-Filho, 1999).

On Table 2 are presented the results found for viability determined by the tetrazolium test performed with preconditioning by immersion in H_2O during 4 h and staining on top of filter paper moistened with tetrazolium chloride solution and by immersion in that solution. It can be observed that the procedure of staining on top filter paper moistened with tetrazolium salt solution, at the concentration of 1%, was able to sort the seed lots similarly to the results obtained in the germination test, whose data are presented on Table 1, classifying the lot 1, as of high quality; the lots 2 and 3 as of intermediate quality; and the lots 4 and 5 as the ones with the lowest seed quality. That methodology allows performing and obtaining the final result for seed viability in approximately seven hours. That is very important; once the internal quality control of the seed producing companies requires a fast responses on the true status of a given seed lot, in order to give a suitable destination to it.

Table 2. Viability (%) determined by the tetrazolium test performed on five lots of barley seeds carried out with preconditioning by immersion in H_2O for 4 h and staining on top filter paper moistened with a 2,3,5 triphenyl tetrazolium chloride solution and by immersion in that solution, using different concentrations of the salt.

Preconditioning by immersion in H_2O (4 h) Staining procedure									
	On filter paper (2 h)			By immersion (2 h)			By immersion (3 h)		
	Concentration of the tetrazolium salt								
	0.1%	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %
Seed Lots	Viability (%)								
1	75 a	85 a	94 a	98 a	95 a	95 a	93 a	90 a	94 a
2	83 a	82 ab	85 ab	86 b	82 b	85 b	82 ab	83 ab	78 b
3	72 a	73 b	86 ab	80 b	87 ab	79 b	85 ab	83 ab	79 b
4	71 a	76 ab	82 b	86 b	80 b	82 b	80 b	78 b	84 b
5	76 a	79 ab	81 b	82 b	85 b	79 b	79 b	75 b	80 b
C.V. (%)	10.50	6.73	5.14	5.10	5.07	5.28	6.04	8.76	4.43

Means followed by the same letter in the column are not statistically different between each other (Tukey test, 5% probability level).

The preparation procedure using the two halves of seeds for staining on top of filter paper also allowed improving seed viability evaluation, since the two halves are analyzed. When only one of the halves is evaluated, doubts may be raised about seed viability, once cross-sectioning the embryo exactly in the middle it is difficult due to the small seed size. Such fact has also been verified by Dias and Alves (2009) for seeds of *Brachiaria* spp. (2008a) and *Panicum maximum* (Jacq.) R.D. Webster; and verified by Souza et al., for seeds of black oats (2009), triticale (2010a) and white oats (2010b).

Using the methodology of preconditioning the seeds by immersion in H_2O for 4 h and performing the staining procedure by immersion in the tetrazolium chloride solution for 3 h (Table 2), the seed lots were sorted in: high viability (lot 1); intermediate viability (lots 2 and 3); and low viability (lots 4 and 5), similar to results achieved in the germination test (Table 1), both for the concentrations of 0.1% and 0.5%. The ISTA (2007) also recommends this methodology for barley seeds, although at a concentration of 1%. Results achieved within this research work emphasize the possibility of using the tetrazolium chloride solution at lower concentrations (0.1% or 0.5%) that besides being more economical, allows for an adequate staining of the seed tissues, without impairing the viability visualization. The reduction on the concentration of the tetrazolium salt solution used in the staining procedure for seed viability evaluation was also suggested for other crop species such as: 0.1% for cotton seeds (Vieira and Von Pinho, 1999); 0.2% for castor bean

seeds (Gaspar-Oliveira et al., 2009); and 0.075% for seeds of peanut (Bittencourt and Vieira, 1999), common bean (Bhering et al., 1999), corn (Dias and Barros, 1999), and soybean (França-Neto et al., 1999).

On Table 3, the results of the tetrazolium test on the viability of barley seeds, obtained with preconditioning by immersion in H_2O, for 18 h, and staining on top of filter paper moistened with tetrazolium salt solution and by immersion in that same solution are shown. It can be verified that in all the combinations tested, the identification of differences on viability of the seed lots studied was not possible and they were not sorted as in the germination test (Table 1).

With the preconditioning between sheets of H_2O moistened paper towel and staining on top of filter paper moistened with tetrazolium salt solution and by immersion in this same solution for two different periods (Table 4) it is again possible to observe that none of the procedures tested was efficient in sorting the lots by seed viability, as occurred in the germination test (Table 1).

Table 3. Viability (%) determined by the tetrazolium test in five lots of barley seeds, carried out with preconditioning by immersion in H_2O for 18 h and staining on top of filter paper moistened with a 2,3,5 triphenyl tetrazolium chloride solution and by immersion in that solution, using different concentrations of the salt.

Preconditioning by immersion in H_2O (18 h)									
Staining method									
	On filter paper (2 h)			By immersion (2 h)			By immersion (3 h)		
	Concentration of the tetrazolium salt solution								
	0.1 %	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %
Seed lots	Viability (%)								
1	88 a	82 a	92 a	95 a	94 a	94 a	99 a	94 a	95 a
2	78 a	82 a	85 a	86 a	87 a	85 a	88 b	88 bc	83 b
3	84 a	86 a	80 a	86 a	86 a	88 a	89 b	85 c	86 b
4	70 b	74 a	84 a	87 a	88 a	87 a	91 ab	92 ab	88 ab
5	80 ab	72 a	79 a	88 a	93 a	87 a	86 b	87 bc	83 b
CV (%)	6.05	9.49	7.25	4.92	5.15	6.25	5.03	2.85	4.70

Means followed by the same letter in the column are not statistically different between each other (Tukey test, 5% probability level).

Table 4. Viability (%) determined by the tetrazolium test performed on five lots of barley seeds, carried out with preconditioning between sheets of H_2O moistened paper towels (18 h) and staining on top of filter paper moistened with a 2,3,5 triphenyl tetrazolium chloride and by immersion in that solution, using different concentrations of the salt.

Preconditioning between sheets of paper towels (18 h)									
Staining method									
	On filter paper (2 h)			By immersion (2 h)			By immersion (3 h)		
	Concentration of the tetrazolium salt								
	0.1 %	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %	0.1 %	0.5 %	1.0 %
Seed lots	Viability (%)								
1	89 a	92 a	93 a	96 a	94 a	97 a	93 a	96 a	94 a
2	79 ab	83 ab	83 b	83 a	86 ab	85 b	92 a	89 ab	87 a
3	62 b	81 b	86 ab	87 ab	87 ab	87 b	90 a	88 b	89 a
4	76 ab	84 ab	86 ab	88 ab	84 b	83 b	89 a	86 b	89 a
5	79 ab	83 ab	83 b	86 ab	88 ab	88 b	86 a	87 b	91 a
CV (%)	12.41	5.28	5.12	5.72	3.93	3.92	5.88	3.91	3.65

Means followed by the same letter in the column are not statistically different between each other (Tukey test, 5% probability level).

Thus, through comparison of means it is verifiable that the most efficient methodologies for fast evaluation of barley seeds viability were the preconditioning by immersion in H_2O for 4 h (Table 2) and staining on top of filter paper moistened with the tetrazolium salt solution at a 1% concentration, for 2 h.

On Table 5, are shown the Spearman correlation coefficients between data obtained in the seed germination and in the seed viability tests by using the tetrazolium method, aiming at refining data analysis. It is evident that besides the methodologies already recommended, starting from comparison of means, the procedure of preconditioning between sheets of paper towel and staining by immersion in tetrazolium salt solution for 3 h, the 1% concentration has presented higher correlation (statistically significant at 1% probability). This methodology provided the best seed lots sorting as compared to the germination test (Table 1). This is indeed an important result, since the possibility of using tetrazolium salt solution at lower concentration (0.1%) is more economical, allowing for an adequate staining of seed tissues, without impairing the visualization of the tissues structure.

Table 5. Spearman correlation coefficient (ρ) among mean data obtained in the tests of germination and viability by the tetrazolium method, in five lots of barley seeds, with three different preconditioning methods.

Staining Method	Preconditioning		
	By immersion in H_2O (4 h)	By immersion in H_2O (18 h)	Between moistened paper towels (18 h)
	Correlation coefficient (ρ)		
SOP (2 h) [0.1 %]	0.20	0.50	0.48
SOP (2 h) [0.5 %]	0.60	0.68	0.38
SOP (2 h) [1.0 %]	0.90*	0.90*	0.55
SBI (2 h) [0.1 %]	0.63	0.08	0.30
SBI (2 h) [0.5 %]	0.50	0.10	0.30
SBI (2 h) [1.0 %]	0.83	0.38	0.30
SBI (3 h) [0.1 %]	0.90*	0.50	1.00**
SBI (3 h) [0.5 %]	0.98**	0.60	0.90*
SBI (3 h) [1.0 %]	0.10	0.48	0.08

SOP = Staining on top of filter paper moistened with tetrazolium salt solution; SBI = staining by immersion in a 2,3,5 triphenyl tetrazolium chloride solution; [0,1 %], [0,5 %] e [1,0 %] = concentration of the tetrazolium salt solution.

** Significant at 1% probability by the T test

* Significant at 5% probability by the T test

According to Spearman correlation coefficient (Table 5), the preconditioning by immersion in H_2O for 18 h and staining on top of filter paper moistened with tetrazolium salt solution at the concentration of 1.0% was also efficient, although only at 5% probability.

On Table 6, the moisture content of the barley seed lots after preconditioning by immersion and between sheets of H_2O moistened paper towels are shown. As all seed preconditioning methods studied were efficient for performing the viability test by using the staining procedure with tetrazolium salt, it is possible to notice that with moisture content varying from 26.8% to 28.0%, obtained with preconditioning between sheets of paper towel, the barley seeds activated their enzymatic metabolism, thus displaying and adequate staining for the evaluation procedure. Therefore, when the moisture content accomplished with the preconditioning between paper sheets (26.8% to 28.4%) and immersion in H_2O for 4 h (30.6% to 32.2%) are compared, it is possible to conclude that for the water immersion procedure the preconditioning period can be reduced without impairing the tetrazolium test performance for viability determination on barley seeds.

Table 6. Moisture content (%) of samples of barley seeds removed from five different lots, after preconditioning by immersion in H_2O for 4 e 18 h and between sheets of moistened paper towel for 18 h, at 20 °C temperature.

Seed Lots	Preconditioning		
	By immersion		Between paper towels
	4 h	18 h	18 h
	Moisture content (%)		
1	31.0	41.4	26.8
2	32.1	40.9	28.4
3	30.6	40.8	27.7
4	32.2	40.8	27.5
5	31.7	40.3	27.2

The procedure of preconditioning the seeds has the objective of activating the enzymatic metabolism; improve the feasibility of seed preparation; and benefit the development of an adequate seed staining during the contact with the tetrazolium salt solution (Marcos-Filho, 2005). Normally, the results of the tests are associated with the moisture content achieved by the seed during the preconditioning procedure, providing or not an adequate enzymatic activation.

The structural appearance of the barley seeds after staining on top of filter paper can be visualized on Figure 1, where the viable and non-viable seeds are shown. On Poaceae seeds as barley the vital areas for viability evaluation are: plumule; coleoptile; central region of the scutellum; radicle; and seminal roots region (Dias and Barros, 1999). In that way, the viable seed (Figure 1A) displayed a coloration ranging from light carmine to red at the vital areas and the non-viable seed (Figure 1B) displayed white color tissues, because the reduction of the tetrazolium salt did not occur.

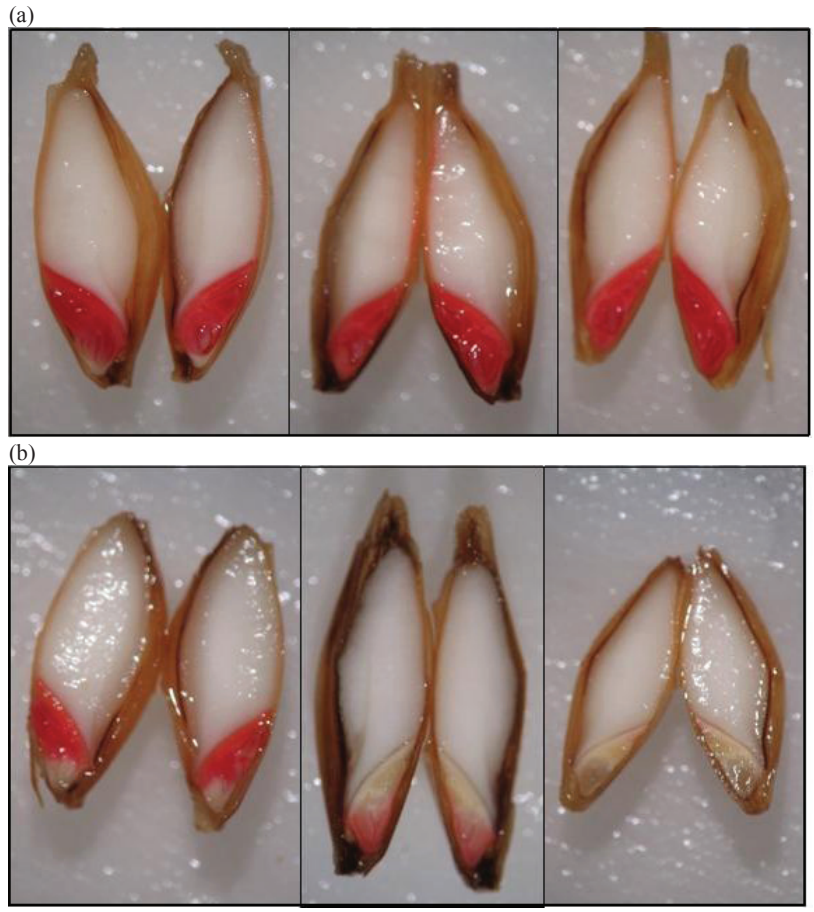

Figure 1. Barley seeds after staining on top of filter paper moistened with a 2,3,5 triphenyl tetrazolium chloride. Viable seeds (a) and non-viable seeds (b).

Conclusions

The tetrazolium test is promising for the fast viability evaluation of barley seeds using the methodology of preconditioning by direct immersion in H_2O for 4 h, at 20 ºC temperature; staining by immersion in tetrazolium salt solution for 3 h, at 30 ºC temperature, in the concentrations of 0.1% or 0.5%; and staining on top of filter paper moistened with that solution for 2 h, at 40 ºC temperature, in the concentration of 1.0%.

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