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Ethanol vigor test to assess physiological quality of annual ryegrass seeds

Teste de vigor pelo etanol para avaliar a qualidade fisiológica de sementes de azevém

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Content

ABSTRACT:
This preliminary study aimed to establish a procedure to evaluate ryegrass seed vigor by ethanol test. Five annual ryegrass seed lots, cv. BRS Ponteio, were used. Ethanol content was quantified using an adapted ethyl meter (mg.L-1). Results were compared to germination test, first germination count, seedling emergence, electrical conductivity, and respiratory activity of the seeds. On the basis of the preliminary results of this study, it can be concluded that the ethanol technique can be used to determine seed vigor. This technique allows for precise data collection in less time than standard germination test and field emergence. The data collected using the ethanol test were in agreement with data obtained from tests of seed viability and vigor.
Keywords: *Lolium multiflorum* Lam., seed analysis, seed physiological potential.

RESUMEN:
Objetivou-se com este estudo preliminar estabelecer um procedimento para avaliar o vigor de sementes de azevém pelo teste de etanol. Foram utilizados 5 lotes de sementes de azevém, cv. BRS Ponteio. O etanol foi quantificado utilizando um etilômetro adaptado (mg.L-1). Os resultados foram comparados com o teste de germinação, primeira contagem de germinação, emergência de plântulas, condutividade elétrica e atividade respiratória das sementes. Com base nos resultados preliminares, pôde-se concluir que a técnica do etanol pode ser usada para determinar o vigor da semente. Esta técnica permite a coleta de dados precisos em menos tempo do que o teste de germinação e emergência de campo. Os do teste de etanol foram concordantes com dados obtidos de testes de viabilidade e vigor de sementes.
Palavras-chave: *Lolium multiflorum* Lam.; análise de sementes, potencial fisiológico de sementes.

1. Introduction

Seed germination, seedling emergence, and the establishment of cultivars in the field are important for the successful crop production. These factors are associated with the initial plant growth. In this sense, a major concern is the deterioration of seed lots before sowing, which leads to a drastic decrease in vigor; however, these are issues that can be detected before planting. Current vigor tests that could quickly detect seed vigor vary regarding their credibility and applicability to individual crops (Buckley and Huang, 2011).

The development of tests to evaluate seed vigor as well as their standardization is essential to build up an efficient quality control system. Such tests must be increasingly efficient with time and include protocols that can quickly evaluate the physiological potential as well as allow the detection of precise differences between seed lots (Fessel et al., 2010).

Current standard germination test is not sufficient to precisely identify lots that have different quality levels. For this reason, vigor tests are important tools in the routine of the seed industry to determine physiological potential. The first germination count test, accelerated aging test, and the field emergence test are among the most commonly used tests (Santos et al., 2011). However, these tests are based on normal seedling development and require a minimum of ten days to obtain some results, which many consider too long because a quick physiological seed quality evaluation test can speed up decision-making processes during the initial and final steps of production, storage, and commercialization of products (Menezes et al., 1994).

The ethanol test is a promising method that can be used to differentiate seed lots with different vigor levels. Buckley and Huang (2011) applied this method to cabbage seeds and observed that the seeds with higher vigor had less ethanol production than seeds with lower vigor. The ethanol test is based on the alcoholic fermentation theory, where the enzymes pyruvate decarboxylase and alcohol dehydrogenase act on pyruvate, producing ethanol and CO2 as well as oxidizing NADH during this process. Alcohol dehydrogenase and lactate dehydrogenase are essential to operate the glycolytic cycle under anaerobic conditions, especially because they recycle NAD+, reducing pyruvate to ethanol or lactate. This process of accumulation of ethanol involves the oxidation of NADH and produces a small amount of ATP that is essential for survival in some species during the absence of oxygen. Seeds are impermeable to oxygen during the first hours of germination, so they quickly generate an increase in the respiratory coefficient. This event also increases alcohol dehydrogenase activity, which activates alcoholic fermentation (Taiz and Zeiger, 2017).

Seed deterioration is associated with the seed exudate concentration in the solution. Such exudates are the result of membrane decomposition (Copeland and McDonald, 1995). According to Powell and Matthews (1978), and Delouche (2002), membrane damage is the initial degenerating event that causes seed alteration. In this way, seeds that present a higher level of membrane degradation tend to produce a higher amount of ethanol.

The aim of this study was to explore the possibility of using the ethanol test to evaluate vigor in different seed lots of ryegrass as a way to increase efficiency in detecting seed vigor in comparison to other conventional tests.

2. Methodology

Five ryegrass seed lots, cultivar BRS Ponteio were used to conduct this experiment. The seeds were subjected to viability, vigor, and respiratory activity tests, and then they were subjected to the ethanol test.

2.1. Variables

Germination: Test performed according to Association of Official Seed Analysts (AOSA, 1992, 1993), conducted in four repetitions, each composed of four replicates with 50 seeds each. Seeds were placed in germination paper (Germitest®) previously moisturized with distilled water, where the water mass was 2.5 times the dry paper mass. The seeds were kept in the

growth chamber at a temperature of 25 °C. Seeds were evaluated according to the published protocols for seed analyses. The results were expressed as the percentage of normal seedlings.

First germination count: This test was conducted along with the germination test. It detects the percentage of normal seedlings recorded five days after sowing (AOSA, 1993).

Germination speed index: Performed along with the germination test, where, the number of seeds with primary root protrusion at least 3,0 mm long were recorded daily until the stabilization, and the result calculated according to the formula described by Maguire (1962).

Seedling emergence: Seeds were planted in six-meter-long and one-meter-wide (6x1 m) plots that were filled with soil collected from the A1 horizon of a Solodic Eutrophic Haplic Planosol at the Pelotas mapping unity. Evaluations were performed 21 days after sowing; evaluated plants were ≥ 1.0 cm long. The results were expressed as percentage of emerged seedlings.

Electrical conductivity: The seeds were weighed and placed in a beaker with 70 mL of deionized water and kept in the growth chamber at a temperature of 25 °C. The electrical conductivity of deionized water was determined. After 24 hours, the conductivity readings were taken using a Schott LF613T bench conductivity meter. To obtain the electric conductivity values of the solution containing seeds, the conductivity value read on the conductivity meter was subtracted from the deionized water conductivity and then divided by the mass of 50 seeds. The results were expressed as μS.cm-1g-1 of seeds (Krzyzanowski 1991).

Respiratory activity: This was determined by following the method developed by Pettenkofer method as adapted by Moraes et al. (2012). A total of 4 g of seeds previously used in the ethanol test was selected for this test. These seeds were placed on a system composed of the following: two gas washing flasks containing sodium hydroxide, aiming to retain atmospheric CO_2; a flask free of atmospheric CO_2 for seed storage; and a flask containing barium hydroxide that reacted with the CO_2 that originated from the seed respiratory activity, which resulted in the formation of barium carbonate that was then quantified by titration. These flasks were connected by a silicone hose coupled to an air-sucking tube. The airflow was regulated by a faucet in a way that allowed air speed control by counting the bubbles formed within the flasks.

Three aliquots of $BaCO_3$ were collected after each repetition of the experiment. These aliquots received two drops of phenolphthalein, and then they were subjected to titration with 0.1 N hydrochloric acid (HCl). The volume of HCl used in each repetition was registered at the turning point, and it was directly related to the amount of CO_2 fixed by the BaOH solution, which was used to determine respiratory activity in the seeds.

The final calculation of respiratory activity was made based on the average of four repetitions. The result was expressed as the released CO_2 per seed gram per hour (μg released CO_2 g-1 seed h-1). The following equation was used: N * D * 22, where N = the normality of the used acid (0.1 N HCl); D = the difference between the blank proof and the sample; and 22 = the normality of CO_2 for respiratory activity (RA) determined by the Pettenkofer machine.

Ethanol test: A total of 4 g of seeds was used to conduct this test. The seeds were initially stored in tightly closed polyethylene bottles containing 60 mL of distilled water under a controlled temperature of 40 °C. After 24 hours, the amount of ethanol produced by the seeds was measured with the aid of an ethyl meter (INSTRUTHERM BFD-60) adapted with a needle, which was introduced into the container. The results were expressed in mg.L-1.

2.2. Statistical Analysis

A completely randomized design with four replications was used for each of the five lots, constituting 20 experimental units. The collected data were submitted to analysis of variance ($p < 0.05$) and then compared using Tukey's test with 5% probability. In addition, a correlation analysis was conducted between the principal components using Pearson's test with 1% probability. Data were evaluated using R (R Development Core Team, 2006).

3. Results and discussion

The ryegrass seed germination potential varied significantly ($p < 0.05$) between the seed lots (Table 1). Seed lot number 3 presented a significantly higher germination percentage than all the other lots. It was 62.5% higher than lot number 2, the one with the lowest germination rate. The first germination counting (FGC) and the germination speed index (GSI) presented the same tendency, with significant reductions ($p < 0.05$) of 82% and 91%, respectively, from the lot with highest to the one with lowest physiological quality. These results were confirmed by the data obtained in the seedling emergence test, where only lot number 3 presented an emergence percentage higher than 50%, significantly different from the other lots ($p < 0.05$). The germination test, first germination count, and germination speed index are indicators of seed viability and vigor. In this study, these test allowed us to rank seed lots accoding to their physiological potential, as illustrated by lot 2, which presented lower viability and vigor than the other lots. For several years, the germination test was the only method to evaluate seed physiological quality (Moncaleano-Escandon et al., 2013). Currently, biochemical tests are being studied in a wide range of cultivars, revealing important characteristics regarding seed viability and vigor.

Cell membrane deterioration was significantly higher in lot number 2 (Table 1) than in the other lots; it was 60% higher than lot number 3, which presented the higher physiological quality. Electrical conductivity indirectly estimates the level of cell membrane damage by determining the amount of leached ions. This technique has been studied in different crops, such as sunflower (Maeda et al., 1986), onion (Rao et al., 2006), corn (Fessel et al., 2006), lettuce (Peñaloza et al., 2005), and cotton (Mendonça et al., 2008). The increase in the amount of leached ions in lot 2 indicated extensive damage to cell membranes and consequently a less vigorous lot, which is in agreement with the information obtained in the viability tests. Considering the least vigorous lot, such conditions could have initiated the process of inactivation of the glycolytic pathway and activation of the fermentation pathway, as the reduction in seed germination is associated with natural aging and the consequent loss of organic solutes and increased respiratory activity of these seeds (Moncaleano-Escandon et al., 2013).

Respiratory activity was also increased by 63% in the lot with the lowest quality when compared to the best-performing lot (Table 1). Moreover, ethanol production was significantly different between the lots ($p < 0.05$), with an increase of 46% in lot number 2 compared to the highest-quality lot. The significant increase of respiratory activity in lot 2 and the level of exudates found in the electrical conductivity analysis suggest that the increase in the ethanol level in this lot resulted from the mitochondrial stress generated by the initial process of seed deterioration (Sershen et al., 2014). Some authors have observed the same tendency, attributing the increase in seed respiration to an early use of seed storage that jeopardizes mitochondrial activity, which depends on the oxygen absorbed by seed tissues, causing deterioration of seeds in crops such as soybean (Leopold 1980) and cotton (Woodstock et al., 1985). In this sense, the changes in ethanol metabolism are related to decreased mitochondrial function as a result of membrane oxidation. These changes mainly impact internal membranes that contain phospholipids with polyunsaturated acyl chains, which are reportedly more sensitive to oxidation (Moreau 1974).

Electric conductivity and respiratory activity analyses were conducted on the seeds, as respiration is one of the crucial factors for successful germination (Wang et al., 2012). Furthermore, such analyses are related to important characteristics regarding processes of loss of vigor such as cell membrane integrity and ethanol pathway activation during cellular respiration. The search for reliable and fast methods to estimate seed performance in the field must consider a range of factors that, in the majority of the cases, cannot be measured by only one test. Therefore, several tests are necessary to obtain a broad and reliable evaluation of seed physiological quality, which will determine the success of seed yield. In this way, seed

industry needs a quick test to determine seed quality in order to optimize postharvest processes. This is especially important because seeds can accumulate damage during storage, where the level of seed deterioration varies according to the stress levels imposed by such processes (Kodde et al., 2012). Therefore, we aimed to obtain consistent data regarding seed vigor as well as to assess the efficiency of the ethanol test compared to standard tests.

Table 1
Germination (GER), first germination count (FGC), germination speed index (GSI), seedling emergence (SE), electrical conductivity (EC), respiratory activity (RA) and ethanol test (ET) of different batches of annual ryegrass seeds (*Lolium multiflorum* Lam.)

Lots	GER %	FGV %	GSI	SE %	EC µS.cm-1g-1	RA Mg.CO2 g-1 h-1	ET mg.L-1
1	33 c	17 cd	4,36 b	44 b	122,57 c	3,027 b	0,494 c
2	21 d	11 d	1,21 d	20 c	171,70 d	4,010 a	0,596 d
3	88 a	62 a	13,0 a	75 a	67,44 a	2,460 c	0,321 a
4	51 b	36 b	2,35 c	37 b	103,39 b	2,968 b	0,402 b
5	44 bc	29 bc	2,80 c	36 c	99,81 b	3,222 b	0,475 bc
CV	7,03	18,3	9,18	14,6	5,16	9,96	9,05

Means followed by same letter in the column not differ at 5% probability by Tukey.

There was a positive correlation between respiratory activity and ethanol production ($p < 0.01$). The electrical conductivity showed positive correlations with both respiratory activity and ethanol production ($p < 0.01$). Seed germination presented negative correlations ($p < 0.05$) with respiratory activity, electrical conductivity, and ethanol production. The same pattern was observed for the first germination count test; however, this measurement was positively correlated ($p < 0.01$) with seed germination. The germination speed index and seedling emergence were negatively correlated ($p < 0.05$) with respiratory activity, electrical conductivity, and ethanol production and positively correlated with the other tests ($p < 0.01$) (Table 2). It is important to note that one of the main functions of mitochondria is the oxidation of NADH to NAD+. Thus, a decrease in mitochondrial activity will result in the production of reduced NAD+; this will not only affect glycolysis, but it will also limit the ethanol breakdown capability. Furthermore, fermentation processes are highly dependent on the availability of free sugars, such as hexoses. For example, there is higher usage of oxygen in lipid respiration than in the catabolism of carbohydrates, in which the seeds are subjected to an excess or shortage of oxygen, with a consequent loss of viability (Crawford 2003). In this way, the level of ethanol degradation and the respiratory activity of seeds can then be used as indicators of a decline in seed quality.

High levels of ethanol and the ethanol precursor acetaldehyde have been detected in soybean seeds 30 minutes after the soaking process had begun (Woodstock and Taylorson, 1981). These seeds were incapable of generating the adequate energy level (ATP) through aerobic respiration. Thus, anaerobic respiration or fermentation was activated as an alternative mechanism to generate energy at the beginning of soaking. When mitochondria become non-functional due to damage, the beginning of anaerobic respiration is expected to provide metabolic energy for basic processes, at least for the repair of cellular structures. A relationship between ethanol production and seed deterioration has been suggested, as noted by a decrease

in germination or in the growth of seedlings (Taylor et al., 1999; Kataki and Taylor, 2001; Bicanic et al., 2003; Akimoto et al., 2004; Rutzke et al., 2008).

Table 2
Pearson correlation analysis between the main components ethanol test (ET), respiratory activity (RA), electrical conductivity (EC), Germination (GER), first germination count (FGC), germination speed index (GSI), seedling emergence (SE), and of different batches of annual ryegrass seeds (*Lolium multiflorum* Lam.)

Variáveis	RA	EC	GER	FGC	GSI	SE (%)
ET (mg.L-1)	0,84**	0,86**	- 0,87**	- 0,88**	- 0,71**	- 0,78**
RA (mg.CO2 g-1 h-1)		0,82**	- 0,81**	- 0,78**	- 0,75**	- 0,77**
EC (µS.cm-1g-1)			- 0,88**	- 0,83**	- 0,72**	- 0,81**
GER (%)				0,95**	0,88**	0,90**
FGC (%)					0,84**	0,83**
GSI						0,92**

** significant at 1% probability by Pearson test.

Several authors have reported the sequence of events that take place during seed deterioration, culminating with germination and field performance losses. The first event in this model is cell membrane deterioration, as suggested in the present study. According to Kood et al. (2012), ethanol production is strengthened by the loss of mitochondrial membrane integrity, and an ethanol assay would have the potential to detect the level of seed deterioration. The practical effect found in the present study corroborates the findings of Kood et al. (2012), who proposed quantification using simple analysis tools with practical effects, as observed in their work with *Brassica oleracea* L. seeds. They analyzed the variation in seed quality by measuring ethanol production with a portable breathalyzer (Buckley and Huang, 2011).

The method described in this study was created to determine the ethanol content released by the seeds, aiming to validate the information obtained by conventional tests in a precise way and attempting to significantly reduce the time to obtain results. By applying this technique, we confirmed the results obtained by other analyses. This is a promising method that provides information related to ethanol metabolism, which is an early indicator of the seed deterioration process (Buckley and Buckley, 2009; Kodde 2012).

4. Conclusions

On the basis of the preliminary results of this study, it can be concluded that the ethanol technique can be used to determine seed vigor. This technique allows for precise data collection in less time than standard germination test and field emergence. The data collected using the ethanol test were in agreement with data obtained from tests of seed viability and vigor.

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