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Vigor and anaerobic metabolism of soybean seeds evaluated by ethanol test

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ABSTRACT: Among the vigor tests, ethanol is an alternative that provides desirable characteristics to be applied in seed-producing companies internal quality control planning. The aim was to evaluate the vigor of soybean seeds by the ethanol test and the fermentative metabolism after its application. A completely randomized experimental design with five replications was used, with treatments consisting of 10 lots of soybean seeds, except for the variables composing the fermentative metabolism, for which five lots of soybean seeds were used. First, the initial quality of the lots was assessed by evaluating water content, germination, seedling emergence, accelerated aging, tetrazolium test (vigor), electrical conductivity, and seed respiration. For the ethanol test, the seeds were subjected to soaking times of 30, 60, 90, and 120 minutes until the moment of reading, and subsequently, the fermentative metabolism was evaluated (lactate dehydrogenase - LDH, pyruvate decarboxylase - PDC, and alcohol dehydrogenase - ADH). The ethanol test in soybean seeds efficiently ranks lots into different levels of vigor, using the soaking time of 30 minutes, showing a moderate association between the electrical conductivity test and seed respiration. Furthermore, the activity of the enzymes LDH, PDC, and ADH proves that ethanol is produced during the soaking process of soybean seeds.

Index terms: fermentation pathway, *Glycine max* L., physiological quality.

RESUMO: Dentre os testes de vigor, o do etanol constitui-se como uma alternativa que aporta característica desejáveis para ser aplicado nos planejamentos de controle interno de qualidade de empresas produtoras de sementes. Assim, objetivou-se avaliar o vigor de sementes de soja pelo teste do etanol, bem como avaliar o metabolismo fermentativo após a aplicação do teste. Foi utilizado delineamento experimental inteiramente casualizado com cinco repetições, sendo os tratamentos compostos por 10 lotes de sementes de soja, com exceção das variáveis compostas pelo metabolismo fermentativo, na qual foram utilizados cinco lotes de sementes de soja. Primeiramente, avaliou-se a qualidade inicial dos lotes por meio da avaliação de teor de água, germinação, emergência de plântulas, envelhecimento acelerado, teste de tetrazólio (vigor), condutividade elétrica e respiração das sementes. Para o teste do etanol, as sementes foram submetidas aos tempos de embebição de 30, 60, 90, e 120 minutos até o momento da leitura e, posteriormente, avaliou-se o metabolismo fermentativo (lactato desidrogenase – LDH, piruvato descarboxilase – PDC e álcool desidrogenase – ADH). O teste do etanol em sementes de soja é eficiente no ranqueamento dos lotes em diferentes níveis de vigor, utilizando o tempo de embebição de 30 minutos, sendo este tempo apresentando associação moderada entre o teste de condutividade elétrica e a respiração das sementes. A atividade das enzimas LDH, PDC e ADH comprovam que há a produção de etanol durante o processo de embebição das sementes de soja.

Termos para indexação: rota fermentativa, *Glycine max* L., qualidade de fisiológica.

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INTRODUCTION

In the soybean seed production system, the evaluation of physiological quality is a fundamental factor for decision-making in internal control and for seeds to be used as propagation material for the subsequent operations to which they are subjected (Frandoloso et al., 2017). Therefore, official research laboratories and companies need to conduct different tests to evaluate the quality of seeds, which can be physical, physiological, or biochemical, at all stages of the production system.

In this context, the new technologies application may interest the seed market as it allows for time-reducing results for producers. It also optimizes protocols in seed quality control programs, becoming critical in establishing seed lots with different vigor levels, and saving time and resources (Medeiros et al., 2020).

A methodology that includes characteristics of speed, practicality, and reliability in the results of vigor is the ethanol test (Buckley and Huang, 2013; Cavalcante et al., 2017). Ethanol accumulates in plant organs, particularly when exposed to anaerobic conditions for being a natural product (Kimmerer and MacDonald, 1987; Mustroph et al., 2006), in addition to other stresses, such as seed deterioration (Woodstock and Taylorson, 1981). The levels at which ethanol accumulates in plant organs vary according to organs and plant species (Chen et al., 2020).

Therefore, this test is based on the ethanol released by seeds during soaking, leading to hypoxia. The hypoxia process is changed according to the structure of cell membranes. The early signs of alteration in the physiological quality of seeds occur through cell membrane degradation, reduction of respiratory activity, and reduction of biosynthesis. These factors are directly related to the mitochondria' actions. The action cannot process pyruvate from glycolysis and some enzymatic activities, resulting in alcoholic fermentation reactions. So pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) act on the pyruvate of the cellular cytosol, releasing ethanol and CO_2 and consequently oxidizing NADH. During this process, ADH and lactate dehydrogenase (LDH) are essential in the glycolytic cycle under anaerobic conditions. They recycle NAD^+, reducing pyruvate to ethanol or lactate (Taiz et al., 2017), facilitating the detection of ethanol, and the deterioration and oxidation of seed reserve substances.

High-vigor seeds maintain their structure with high membrane selectivity, hindering solute entry and exit (Moncaleano-Escandon et al., 2013). On the other hand, less vigorous seeds tend to show membranes with lower integrity, facilitating the rapid release of ethanol, so its quantification can provide information on physiological problems related to seed deterioration (Buckley et al., 2016). Some adaptations of a pre-existing instrument have already been used to measure the amount of ethanol in seeds.

The instruments used for the ethanol test, in general, are adapted from commercial alcohol meters and may methodologically compromise the analyses and results. This situation was observed in studies carried out with cowpea seeds (Cavalcante et al., 2019) and barley seeds (Buckley et al., 2016), in which the results of the ethanol test do not fully corroborate the initial quality tests. Silva et al. (2021) demonstrated that it is possible to quantify the release of ethanol in soybean using an adapted alcohol meter. However, these same authors found that the adapted instrument is inefficient in ranking lots of soybean seeds into different levels of vigor, which requires adjustments or even the design of new devices so that the test can be performed more safely and reliably.

Because of the above, the aim was to evaluate the vigor of soybean seeds by the ethanol test and analyze the fermentative metabolism.

MATERIAL AND METHODS

The study was conducted at the Didactic Laboratory of Seed Analysis of the *Universidade Federal de Pelotas*, Campus of Capão do Leão, RS, Brazil. Ten soybean seeds from five different cultivars were used for the experiment. It is worth pointing out that the nomenclatures of the cultivars were not revealed because the lots were not certified; however, the lots came from different cultivars of the same crop season and had similar physiological qualities. The

seeds remained in a cold chamber (temperature and relative humidity of 15 °C and 65%, respectively) during and/or until the experiment was conducted.

The experimental design used was completely randomized, with four replications for each treatment, except for the ethanol test, which had five replications. Treatments were established with ten lots of soybean seeds. Five lots of soybean seeds were used to evaluate the activity of fermentative enzymes.

Before applying the ethanol test, the water content and initial physiological quality of the seeds of the different lots were evaluated, through the germination (Brasil, 2009), seedling emergence, accelerated aging tests (Marcos-Filho, 2020), tetrazolium test (França-Neto and Krzyzanowski, 2019) and respiratory activity by the Pettenkofer method (Moraes et al., 2012) and electrical conductivity (Vieira and Marcos-Filho, 2020) to detect lots with low, medium and high vigor.

After the analyses of the initial quality of the seed lots, ethanol and electrical conductivity tests were performed as described above.

Ethanol test: 25 visibly intact seeds with no apparent physical damage were selected from each pre-established sample. In the next step, the seeds were weighed and placed in 150-mL glass flasks containing 40 mL of deionized water, where they remained soaked for periods of 30, 60, 90, and 120 minutes at a temperature of 41 °C in a BOD chamber.

After the soaking times, the flasks were connected to the ethanol measuring instrument. The components of the equipment were (Figure 1): glass flasks used to receive the seed and the ethanol reading sensor, digital measuring panel, syringe-type aspirator to remove ethanol from a flask with seed and take it to the one with the sensor; and tube with adjustment of ethanol inlet and outlet interconnecting the containers. Readings were performed using an MQ-3 sensor in the instrument, and the results were expressed in millivolt (mV) (Cavalcante et al., 2022). After the ethanol test, the seeds were frozen to determine the enzymatic metabolism during fermentation - Lactate dehydrogenase (LDH, EC 1.1.1.17), pyruvate decarboxylase (PDC. EC 4.1.1.17) and alcohol dehydrogenase (ADH, EC 1.1.1.1) (Bradford, 1976; Hanson and Jacobsen, 1984; Souza and Sodek, 2003).

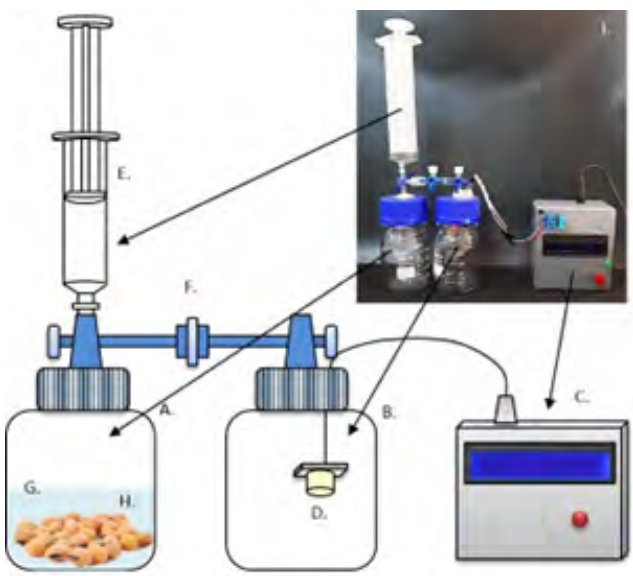

Figure 1. Sketch of the alcohol meter for analysis of soybean seeds. Glass flask A (A), Glass flask B (B), electronic system (C), sensor (D), syringe-type aspirator (E), central valve (F), water (G), soybean seeds (H) and alcohol meter for seed analysis (I).

The data of initial quality, ethanol test, and fermentative enzymes were subjected to analysis of variance ($p < 0.05$) and, when significant, compared by the Scott-Knott test at a 5% probability level. In addition, principal component analysis (PCA) and analysis of the relationship between variables by Pearson's correlation test (r) at 1% and 5% probability levels were performed. The data were evaluated by the statistical program R® (R Core Team, 2022).

RESULTS AND DISCUSSION

The results regarding the initial water content of the seeds ranged from 11.9 to 13.9%, with a maximum difference of 2.0 percentage points between the lots (Table 1). This proximity between the values of the initial water content is essential so that the tests are not affected by differences in metabolic activity (Steiner et al., 2011) since the water content of the seeds directly influences membrane integrity and can also interfere in other analyses of physiological quality (Barbosa et al., 2012).

In the initial characterization of the lots, the seeds showed germination above the standard used for commercialization according to Brazilian legislation, which recommends a minimum of 80% (Brasil, 2013). Almost all lots showed similar germination, except lot 8, with lower germination than the others (Table 1). These lots demonstrate the need to use a vigor test to better detect the quality of seeds since the germination test does not allow detection of the progress of their deterioration, indicating only the final stages of the process (Marcos-Filho, 2015).

The seedling emergence, accelerated aging, and tetrazolium tests allowed ranking the lots into two levels of vigor, high and low. Lots 1, 2, 5, 6, and 10 showed more vigorous seeds than others; however, the seedling emergence and tetrazolium tests indicated low vigor in lot 7, but accelerated aging detected high vigor, which may characterize a mixture of seeds rapidly deteriorating in specific samples within the same lot. Thus, it can be deduced that the seeds do not start deteriorating evenly.

Table 1. Water content (WC), germination (GER), seedling emergence (EME), accelerated aging (AA), tetrazolium (TZ), electrical conductivity (EC) and respiration (RES) of ten lots of soybean seeds.

LOT	WC	GER	EME	AA	TZ	EC	RES**
		---------- % ----------				$\mu S.cm^{-1}.g^{-1}$	
1	11.9	95 a*	93 a	94 a	93 a	91.35 a	0.90 a
2	12.2	96 a	92 a	93 a	92 a	105.83 b	1.83 b
3	12.7	96 a	86 b	88 b	89 b	106.30 b	2.52 b
4	12.6	97 a	86 b	86 b	88 b	109.14 b	2.33 b
5	12.1	93 a	90 a	91 a	92 a	98.41 b	1.64 b
6	12.1	93 a	92 a	92 a	91 a	112. 76 b	1.59 b
7	12.8	93 a	89 b	90 a	88 b	118.29 c	2.97 b
8	13.9	88 b	85 b	87 b	85 b	137.02 c	4.96 c
9	13.2	93 a	84 b	86 b	85 b	133.63 c	3.87 c
10	12.0	96 a	93 a	92 a	94 a	81.18 a	0.86 a
Mean	12.6	94	89	90	88	109.01	2.34
CV (%)		3.37	5.26	4.43	3.19	8.83	14.2

*Means followed by the same lowercase letter in the column do not differ from each other by the Scott-Knott test at 5% probability level. ** $\mu g\ CO_2$ released g^{-1} seed h^{-1}; CV: coefficient of variation.

Vigor tests can identify less advanced stages of seed deterioration so decision-making does not generate doubts and facilitates the correct destination of seed lots (Wendt et al., 2017). Therefore, companies generally perform vigor tests associated with germination tests for internal quality control, seeking to estimate the potential for performance in the field for sowing under both favorable and unfavorable conditions (Grzybowski et al., 2015; Tillmann et al., 2019).

Regarding the tests of electrical conductivity and seed respiration by the Pettenkofer method, it was observed that both ranked the lots into three levels of vigor (low, medium, and high). Lots 1 and 10 showed high vigor, and lots 7, 8, and 9 showed low vigor in both tests (electrical conductivity and respiratory activity of the seeds). Except for lot 7, which was classified as low vigor only in the electrical conductivity test (Table 1), the other lots (2, 3, 4, 5, and 6) were characterized by these two tests as medium vigor for both variables. The results of electrical conductivity corroborate those found for soybean (Mendes et al., 2009; Fessel et al., 2010), cowpea (Aumonde et al., 2012), and okra (Leite et al., 2018) when used for ranking the seed lots into levels of vigor.

Regarding the ethanol test, it was found that the soaking times of 30 and 60 minutes allowed ranking the lots into three levels of vigor (Figure 2), showing performance with a criterion superior to those of traditional vigor tests, as it allowed ranking the lots into three levels of vigor. It was also observed that in the ranking between lots of high, medium, and low vigor, only the time of 30 min showed results similar to those of electrical conductivity and seed respiration (Table 1).

In the initial periods of the ethanol test (30 and 60 minutes), it was possible to observe that soybean seeds with superior vigor release ethanol more slowly. Hence, its quantification is less in lower-vigor seeds, whose release is faster and increases (Figure 2). However, as the soaking time increased, ethanol production was reduced in low-vigor seeds. The fermentation process began to accelerate in high-vigor seeds, inverting the understanding of the test (Figure 1), which may have occurred due to two factors: low-vigor seeds at a given time may cease ethanol release, reaching maximum degradation, and high-vigor seeds, when imbibed, accelerate the process of degradation of membranes, consequently increasing the release of ethanol.

It should emphasize that high-vigor seeds have greater resistance to membrane degradation, making the gas exchange more difficult (Moncaleano-Escandon et al., 2013). On the other hand, seeds with lower vigor may have membranes with fragile integrity, allowing a rapid release of ethanol compared to seeds with higher vigor (Kodde et al., 2012; Buckley et al., 2016). Onwimol et al. (2019) investigated the rapid ethanol test potential in maize seeds stored under two conditions (controlled and uncontrolled) for six months of storage. They observed that the ethanol measured was higher at six months, concluding that the ethanol test can be used in seeds with different levels of vigor and membrane structuring.

These physiological events can be observed in the initial quality of lots 8 and 9, characterized as low vigor (Table 1). Moreover, high membrane degradation levels may favor the glycolytic inactivation and fermentative activation pathway to reduce seed germination. Also, membrane degradation is associated with natural aging, loss of organic solutes, and increased respiratory activity (Moncaleano-Escandon et al., 2013).

In ryegrass seeds (Cavalcante et al., 2017) and beans (Cavalcante et al., 2019), the authors observed that the ethanol test could differentiate the vigor of seed lots in two ways. The first is with a short time of imbibition, in which the seeds of higher vigor produce few ethanols, and with a long time of imbibition, in which these same seeds produce more ethanol, the opposite occurring for low-vigor seeds.

Besides containing a greater amount of reserves in their tissues, the seeds of higher vigor have greater membrane integrity (Chaengsakul et al., 2019). Buckley and Huang (2011) observed that ethanol production by seed is initiated or enhanced by the loss of mitochondrial membrane integrity in the absence of O_2. Rocha et al. (2010) report that ethanol diffuses rapidly out of cells, leading to considerable carbon loss during hypoxia.

This process of ethanol accumulation involves the oxidation of NADH. It produces a small amount of ATP, which is essential for survival in some species during the absence of oxygen and when mitochondria do not work due to damage (Kodde et al., 2012). The seeds are impervious to oxygen during the first hours of germination, generating an increase in the respiratory coefficient and increased activity of ADH, which activates alcoholic fermentation (Taiz et al., 2017).

Regarding the soaking times of 90 and 120 minutes, it was observed that seeds subjected to these soaking times had their lots ranked in only two levels of vigor (Figure 2). Seeds of lower vigor, subjected to the times of 90 and 120 minutes, had a lower ethanol production, while those with higher vigor had a higher ethanol production than the others.

According to Buckley and Buckley (2009) and Buckley et al. (2013), ethanol production is likely to be initiated or enhanced by the loss of mitochondrial membrane integrity, and ethanol quantification trials have the potential as a test to analyze the level of seed deterioration. Although ethanol can be quantified with research-oriented analytical equipment, the test should be simple for practical purposes at the field level.

The initial quality evaluation tests (germination, seedling emergence, accelerated aging, tetrazolium, electrical conductivity, and respiration) were correlated with the ethanol test at the soaking times (30, 60, 90, and 120 min) by a Principal Component Analysis (PCA) (Figure 3). The first two Principal Components (PC) allowed explaining 68.1% of the variance contained in the original element (Figure 3), with the contribution of PC 1 and PC 2 of 50.0 and 17.0% of the remaining variance, respectively. In PC 1, the variables electrical conductivity and respiration had the greatest association with the ethanol test, regardless of the soaking time (Figure 3).

In PC 2, it was observed that the variables germination, seedling emergence, accelerated aging, and tetrazolium was not associated with the ethanol test, even when compared with all soaking times (Figure 3). This low association can be attributed to the ranking of the lots since biochemical tests showed a greater capacity to separate them into different levels of vigor.

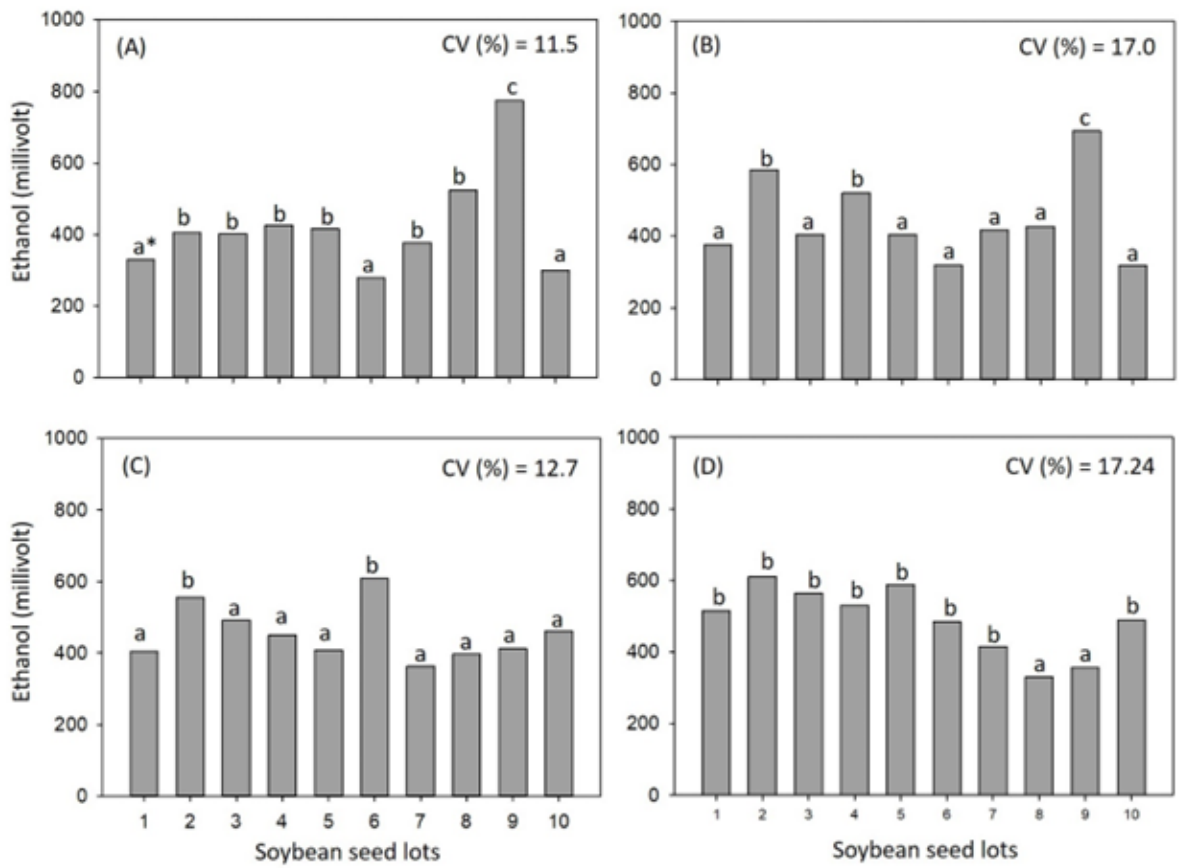

Figure 2. Ethanol tests with different soaking times (A - 30 min; B - 60 min; C - 90 min; D - 120 min) of soybean seeds from different lots using an instrument subject to the obtaining of patent. *Means followed by the same lowercase letter in the column do not differ from each other by the Scott-Knott test at 5% probability level. CV: coefficient of variation.

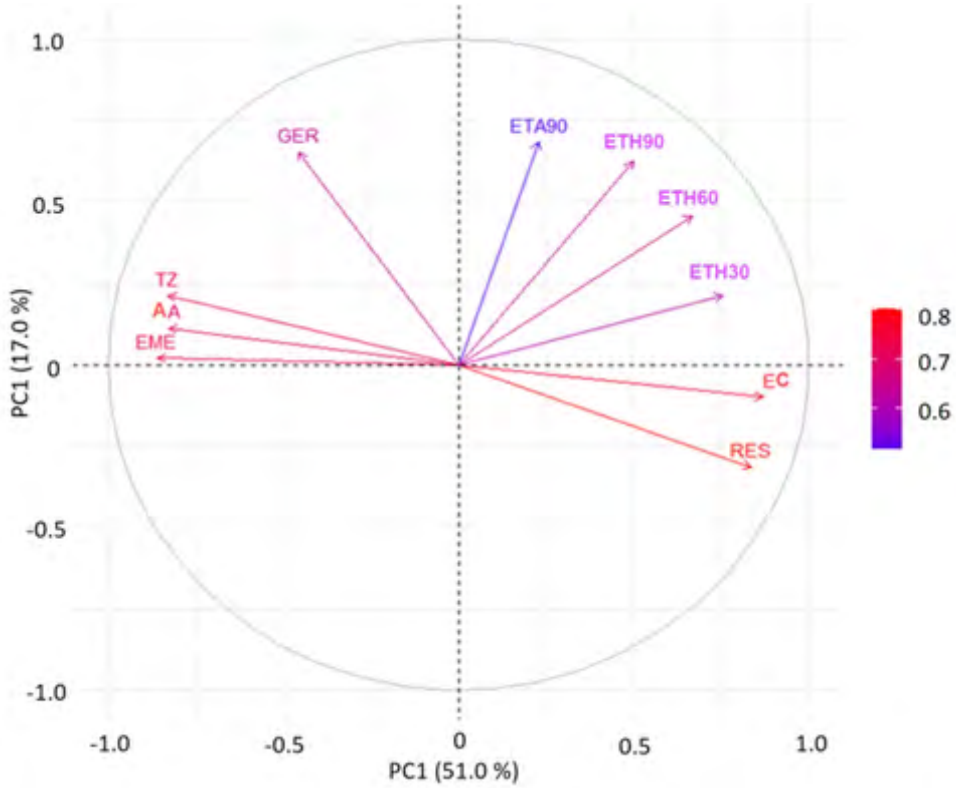

Figure 3. Principal Component Analysis (PCA) via biplot to demonstrate the projection of the variables of the two principal components, initial quality (germination - GER; seedling emergence - EME, accelerated aging - AA, tetrazolium test - TZ, electrical conductivity - EC and respiration - RES) and ethanol test (ETH) with different soaking times (30 - ETH30, 60 - ETH60, 90 - ETH90 and 120 minutes - ETH120).

Five lots (1, 4, 6, 8, and 10) were selected using a criterion that did not differ in the ethanol test, regardless of the soaking time. The exception is lot 8, which differed from the others at all soaking times evaluated to have amplitude in the results based on the metabolism of fermentative enzymes (Figure 4).

Initially, it was found that there was an activity of the fermentative enzymes LDH, PDC and ADH, confirming that there was ethanol production during the immersion of soybean seeds in water and an environment at 41 °C, regardless of soaking time (Figure 4). Regarding the LDH enzyme in soybean seeds soaked for 30 minutes in water, its activity was more intense in lots 1 and 6. At 60 minutes, LDH activity showed higher intensity in lots 8 and 10. For the soaking times of 90 and 60 minutes, the highest LDH activity was observed in lot 8 (Figure 4A).

Under hypoxia conditions to which soybean seeds were subjected, the synthesis of many cell proteins is suppressed, while the synthesis of a specific group of proteins increases (Zabalza et al., 2009; Christianson et al., 2010). This group uses pyruvate as a substrate to produce lactate and includes lactate dehydrogenase (Ren et al., 2017).

This change in metabolism is confirmed by the activity of the PDC enzyme (Figure 3B), and seeds of lot 8 resulting from lactate production, characterized as the ones with the lowest initial quality (Table 1), had the highest PDC activity at the soaking time of 30, 90 and 120 minutes compared to the other lots (Figure 4B).

It was observed that seed tissues in lot 8 (Figure 4B) accelerated the oxidation process and pyruvate decarboxylation to acetaldehyde to modify its metabolic pathway. To inhibit the effect of lactate on the cytosol for having low carbohydrate

content and cell membranes with a higher level of disorganization. Lactate induces the PDC enzyme's activity to alter the conversion of pyruvate into acetaldehyde, a product with a lower potential for cell damage (Bui et al., 2019).

Regarding the activity of the ADH enzyme in soybean seeds, it was found, at the soaking time of 30 minutes, that lots 1 and 6 did not differ from each other and that the seeds of lot 8 showed the lowest activity at this time (Figure 4C). Therefore, for 60, 90, and 120 minutes, the seeds of lot 8 showed the lowest activity of ADH, except for lot 10 at 90 minutes of soaking, which did not differ from lot 9 (Figure 4C).

In terms of efficiency, the ethanol formation pathway is more important than the lactate formation pathway, as this pathway promotes a higher generation of NAD^+ and the consumption of protons (Kato-Noguchi, 2000). Therefore, seeds under hypoxia start to produce ethanol to have greater structuring of their organelles, well-structured membranes, and higher content of reserves. So the reduction in cytosolic pH signals ethanol production since the PDC enzyme is activated when the pH decreases and ADH is activated under hypoxia conditions (Kursteiner et al., 2003).

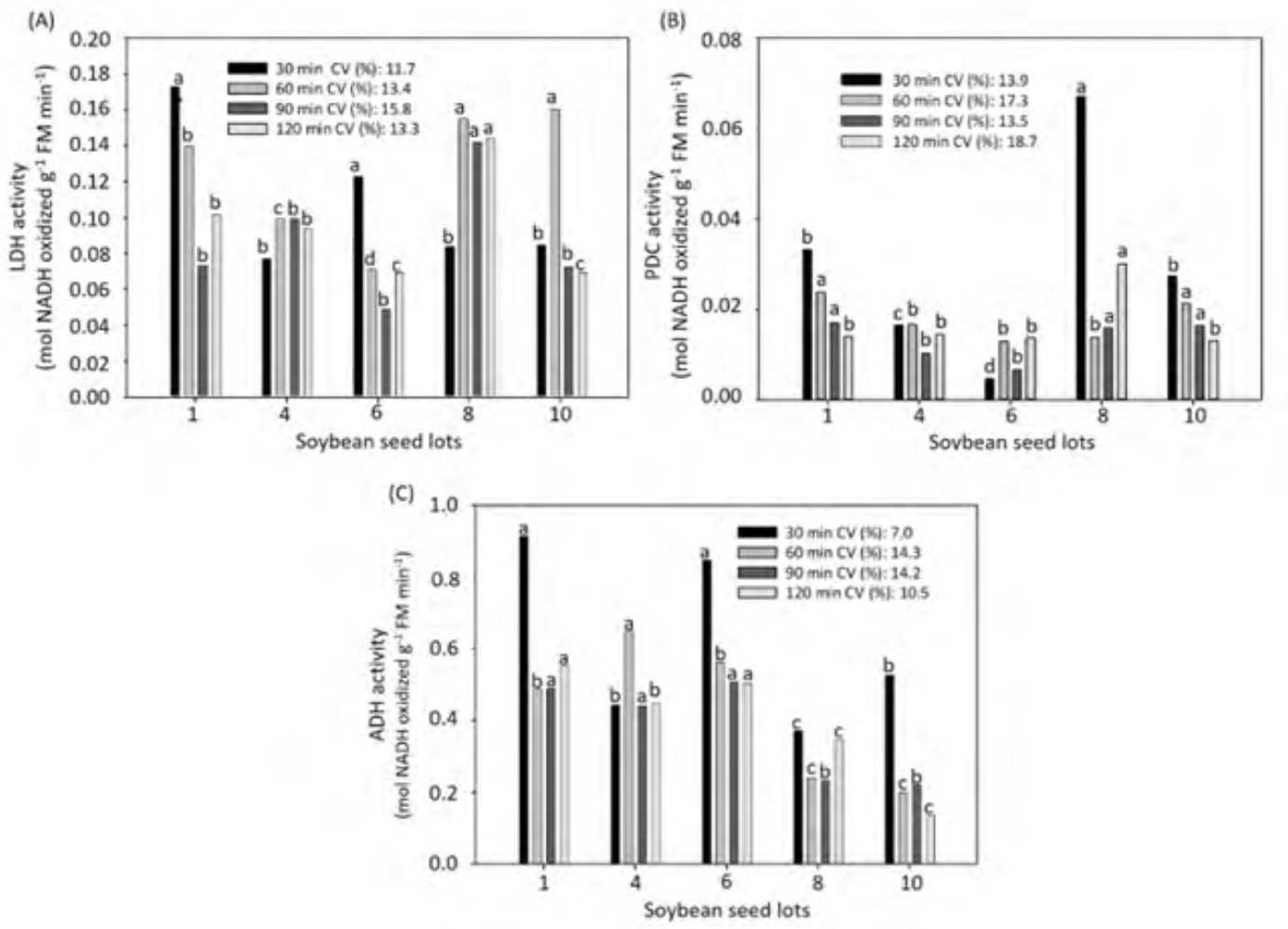

Figure 4. Activity of lactate dehydrogenase (LDH - A), pyruvate decarboxylase (PDC - B) and alcohol dehydrogenase (ADH - C) of soybean seeds from different lots, previously subjected to different soaking times in the ethanol test (30, 60, 90 and 120 minutes). *Bars of the same color between lots and followed by the same lowercase letter do not differ from each other by the Scott-Knott test at 5% probability level. CV: coefficient of variation.

Regarding the correlation between the ethanol test and the fermentative enzymes, there was a significant correlation between the ethanol test at the times of 60, 90, and 120 minutes and the LDH enzyme; as the activity of this enzyme remains active, ethanol production is reduced. These reductions occur because LDH is the first protein to manifest during hypoxia; however, its product, lactate, for being a weak acid and dissociates rapidly, causing cytoplasm acidification. Acidification of the cytoplasm of cells, caused by lactate dissociation, inhibits the activity of LDH and induces the activation of the PDC enzyme (Christianson et al., 2010).

The correlations corresponding to the ethanol test with seeds imbibed in water for 30 and 60 minutes and the comparative variables PDC and ADH were significant at a 5% probability level, with a positive effect on the ethanol test (Table 2). At the soaking times of 90 and 120 minutes, there was no significant correlation for the PDC enzyme. However, for the ADH enzyme, these soaking times showed a high positive correlation; as the activity of the ADH enzyme intensified, ethanol production increased (Table 2).

This ethanol accumulation process involves the oxidation of NADH and results in small but essential ATP production for the survival of some species in the absence of oxygen. During the first hours of germination, the seeds are impervious to oxygen, so they quickly increase their respiratory coefficient, increase ADH activity, and activate alcoholic fermentation (Taiz et al., 2017). This high oxidation level is a sequential process to the activity of PDC.

Although, the induction of ADH, PDC, and LDH activity contributes to the survival and overcoming of energy scarcity through the fermentation of carbohydrates to maintain the production of ATP in the absence of oxygen (Wang et al., 2009). The benefit under such conditions will depend on the type of tissue, stage of development, species, genotype, and severity and duration of stress (Sousa and Sodek, 2003; Fukao and Bailey-Serres, 2004; Wang et al., 2009).

According to Silva et al. (2021), it is possible to quantify the release of ethanol in soybean using an adapted alcohol meter. However, when evaluating the vigor of soybean seeds with an alcohol meter, the authors concluded that the adapted instrument is inefficient in ranking lots into different levels of vigor, which requires adjustments or even the development of devices to perform the test more safely and reliably.

Table 2. Pearson's correlation between ethanol test and lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes of seeds subjected to different soaking times (30, 60, 90 and 120 minutes).

	ETH 30	ETH 60	ETH 90	ETH 120	LDH	PDC	ADH
ETH 30	1	0.973**	0.308^{ns}	0.940**	-0.374^{ns}	0.845**	0.657*
ETH 60		1	0.415^{ns}	0.991**	-0.482*	0.765*	0.544*
ETH 90			1	0.494^{ns}	-0.606*	0.421^{ns}	0.345*
ETH 120				1	-0.554*	0.309^{ns}	0.436*
LDH					1	0.634**	0.897**
PDC						1	0.932**
ADH							1

*Significant at 1%; ** significant at 5%; ^{ns} not significant.

CONCLUSIONS

Applying the ethanol test in soybean seeds ranks the lots into different levels of vigor, using the soaking time of 30 minutes.

Soaking times showed a moderate association between the electrical conductivity test and seed respiration in the PCA, indicating that plasma membrane integrity influences ethanol release by soybean seeds.

The activity of enzymes LDH, PDC and ADH proves the existence of ethanol production during the soaking of soybean seeds.

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