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Evaluation of ethanol preconditioning as a rapid seed vigour and viability test

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Abstract

It is essential that tests for evaluating seed vigour be faster and increasingly efficient to enable precise differentiation among batches. In this way, it is possible to evaluate the quality of seeds based on the anaerobic metabolism of cells when exposed to environments lacking oxygen. Thus, the objective of this study was to establish methodology for evaluating the viability and vigour of 3 lots of cowpea (*Amendoim* cultivar) seeds using the ethanol test. The treatments were carried out in a completely randomized design with four replications. For the test, 25 seeds were stored in hermetically sealed PET (Polyethylene the Ethylene) bottles containing 40 ml of distilled water and subjected to 3 soaking times in distilled water (6, 24, and 48 h) at a controlled temperature of 40°C in a germinator. The amount of ethanol produced was quantified with the aid of an adapted breath analyser. The results are expressed as mg L^{-1}; these data were then compared with data for the following: germination; the first germination count; the total length, root length and shoot length of the seedling; dry weight of the seedling; emergence in the field; emergence speed index; and electrical conductivity. The experimental design was completely randomised, and the data were subjected to analysis of variance and correlation analysis. The results were compared using the Tukey test at 5% probability. Measurement of ethanol after 6 or 48 h of soaking at 40°C was effective for determining the viability and vigour of cowpea seeds. As ethanol test results have high correlation with germination and vigour test results, this approach is a viable alternative for analysts and seed producers.

Keywords: *Vigna unguiculata* L., methodology, viability, vigour.
Abbreviations: PET_Polyethylene the Ethylene; CO_2_Carbon dioxide; NAD^+_Nicotinamide Adenine Dinucleotide; NADH_Nicotinamide Adenine Dinucleotide (reduced form).

Introduction

The development and standardisation of tests for assessing the vigour of seeds are essential for the establishment of efficient quality control in the production chain. Such tests should be increasingly efficient, including tests that quickly evaluate physiological potential and enable classification of seed vigour among different batches (Fessel et al., 2010).

In Brazil, the Ministry for Agriculture, Livestock, and Supply (Ministério da Agricultura, Pecuária e Abastecimento/ MAPA), which is the entity responsible for regulating the commercialisation of seeds, sets a minimum percentage of seeds that must be germinated in a germination test before that batch of seeds can be sold (Brazil, 2015). However, due to differing vigour levels, lots with similar germination results may have different results with respect to seedling emergence in the field. Although there is no legal minimum standard for vigour, ensuring the high quality of seeds increases credibility not only of the production company but also for the entire seed sector. Thus, vigour tests have become increasingly routine tools in the seed industry for determining physiological potential. The most commonly used vigour tests include first germination count, accelerated ageing, and field emergence (Santos et al., 2011).

These tests are based on the formation of normal seedlings, and, depending on the species, a period of 5 to 10 days is required to obtain results. However, this is an excessively long period of time, given that faster evaluation of the physiological quality of seeds streamlines decision-making in the early and final stages of production, storage, and commercialisation (Menezes et al*.,* 1994; Brazil, 2009).

To obtain faster results, tests that can quickly and efficiently evaluate seed vigour — estimating the storage capacity and emergence of seedlings in the field — have been sought and improved (Mendes *et al.,* 2010). Among these, the ethanol test is highlighted owing to its promising results in the

classification of lots of cabbage seeds with high and low levels of vigour (Buckley and Huang, 2012).

The ethanol test is based on the theory of alcohol fermentation, in which the enzymes pyruvate decarboxylase and alcohol dehydrogenase act on pyruvate to produce ethanol and CO_2, oxidising NADH in the process. Alcohol dehydrogenase and lactate dehydrogenase are essential for operating the glycolytic cycle under anaerobic conditions because they recycle NAD^+ and thereby reduce pyruvate to ethanol and lactate, respectively (Taiz et al., 2017). This process of ethanol accumulation involves oxidation of NADH and results in low levels of ATP production that are essential for the survival of certain species in the absence of oxygen. As seeds are impermeable to oxygen during the early hours of germination, they rapidly increase the respiratory coefficient, with enhanced alcohol dehydrogenase activity and alcoholic fermentation activation (Taiz et al., 2017).

According to Copeland and McDonald (1995), the degree of seed deterioration is associated with the concentration of seed exudate in solution, which is a reflection of membrane degradation. Notably, membrane damage is an initial event among degenerative changes to seeds (Powell & Matthews, 1977; Delouche, 2002). Theoretically, when subjected to an anaerobic environment, seeds with a higher degree of membrane degradation tend to produce more ethanol.

Therefore, the aim of the present study was to establish methodology for preconditioning cowpea seeds for the rapid evaluation of vigour using the ethanol test.

Results

Initial quality of seed lots

Significant differences among the germination results for cowpea batches were observed (Fig. 1A). Batch 1 had a better result than did lot 3; however, neither significantly differed from lot 2. Conversely, no significant difference in the vigour of the seed batches was observed when evaluated by tests for first germination count (Fig. 1B) or emergence speed index (Fig. 1D). Nonetheless, at 5 days, a higher percentage of emerged seedlings was found for lot 1 compared to lot 3, though neither differed significantly from lot 2 (Fig. 1C).

The seeds from lot 1 generated seedlings with longer roots than those generated from lots 2 and 3, which did not differ from each other (Fig. 2A). However, shoot length was unable to differentiate the lots of seeds with respect to vigour (Fig. 2B). As with tests for first germination count (Fig. 1B), the emergence speed index (Fig. 1D), shoot length (Fig. 2A), and dry weights of roots and shoots (Fig. 2B) did not effectively distinguish the different lots of cowpea seeds. In contrast, the EC test did provide stratification of the 3 lots of cowpea seeds: lot 1 showed the least solute leaching, followed by lot 2 and then lot 3.

Ethanol test

When subjected to an anaerobic environment at high temperature, irrespective of soaking time, cowpea seeds produced ethanol as an alternative means for ATP production (Fig. 3).

An ethanol test conducted after 6 h of soaking in distilled water in a controlled environment at 40°C enabled lots 1 and 3 to be distinguished with respect to physiological quality, yet neither differed significantly from lot 2 (Fig. 3). These results are in agreement with the results of germination tests (Fig. 1A) and data for seedling emergence (Fig. 1C) and root length (Fig. 2A), given that less ethanol released in the 6-h period represents higher physiological quality.

The results of ethanol measurement after 24 h of soaking in distilled water at 40°C differed from those of ethanol measurement after 6 or 42 h of soaking under the same temperature conditions. Although the 24-h test was not able to distinguish batches with regard to vigour, the results corroborated the data for the first germination count, emergence speed index, seedling shoot length and seedling dry weight (Fig. 3).

After 42 h of soaking, determination of the amount of ethanol released by the seeds enabled the lots to be differentiated into different levels of vigour (Fig. 3). Lot 1 released the greatest amount of ethanol, followed by lot 2 and then lot 3. Similarly, lot 1 exhibited better physiological quality, followed by lot 2 and then lot 3. Seeds with greater vigour tend to produce more ethanol if anaerobic conditions are sustained, and because all of the ethanol produced by seeds has been released by 42 h irrespective of membrane integrity, a higher concentration of ethanol in the medium after 42 h is a sign of greater seed vigour. Quantification of ethanol in solution after seeds were subjected to 42 h of soaking (Fig. 3) was more rigorous than was typical standard tests for viability and vigour, and the results were similar to those of the EC test (Fig. 2C).

Correlation analysis

The variable ethanol at 6 h of soaking (Fig. 4) showed a negative correlation ($p \leq 0.01$) with the results of the germination test (Fig. 4A) and with data for the first germination count (Fig. 4B), seedling emergence (Fig. 4C), root length (Fig. 4D), and root dry weight (Fig. 4E). This result indicates an inverse relationship between ethanol release and germination and vigour. In addition, the 6-h ethanol test displayed a positive correlation of 95% with the EC test ($p \leq 0.01$), indicating a similar trend for the results of both tests (Fig. 4F). In contrast, the other vigour tests did not exhibit a significant correlation with the results of the ethanol test after 6 h of soaking. The 24-h ethanol test showed positive correlations of 46% and 78% for emergence in the field and EC, respectively (Fig. 5A and B), but significant correlations with the other variables were not found.

Correlations corresponding to the ethanol test when seeds were soaked in water for 42 h (with comparative variables) were significant at 1% probability for germination (70%), first germination count (56%), shoot length (69%), root length (76%), and root dry weight (69%). There were no significant correlations regarding the variable dry weight of the shoot (Fig. 6).

When considering significant correlations at 5%, only the variable seedling emergence (54%) showed positive significant correlations with the results obtained from the ethanol test when seeds were soaked for 42 h at a temperature of 40°C (Fig. 6C).

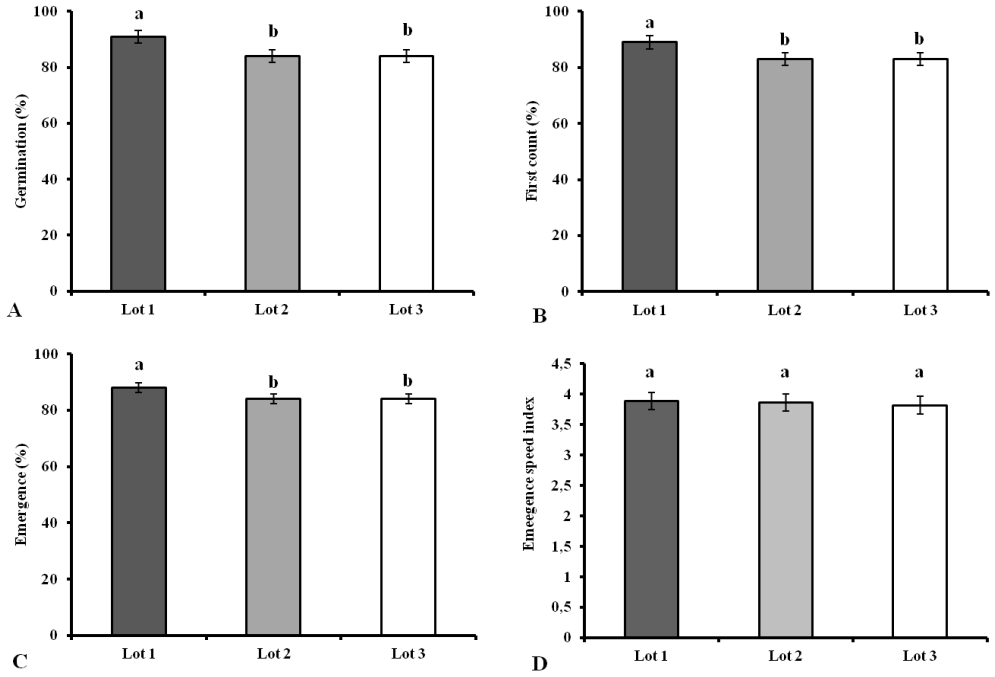

Fig 1. Germination (A), first germination count (B), seedling emergence (C) and emergence speed index (D) of 3 lots of cowpea (*Amendoim* cultivar) seeds. Means followed by the same letter upper case in each bar do not differ by Tukey's test at 5% probability (p≤0.05).

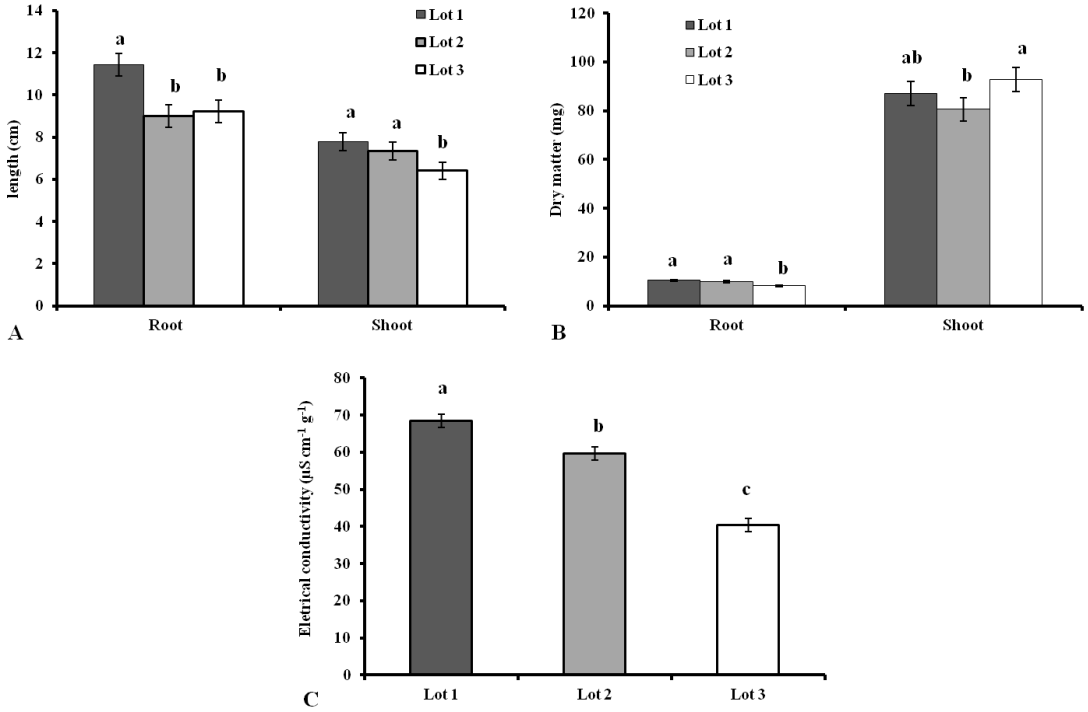

Fig 2. Length of root and length of shoot (A), dry weight of the root and dry weight of the shoot (B) and electrical conductivity (C) of 3 lots of cowpea (*Amendoim* cultivar) seeds. Means followed by the same letter upper case in each bar do not differ by Tukey's test at 5% probability (p≤0.05).

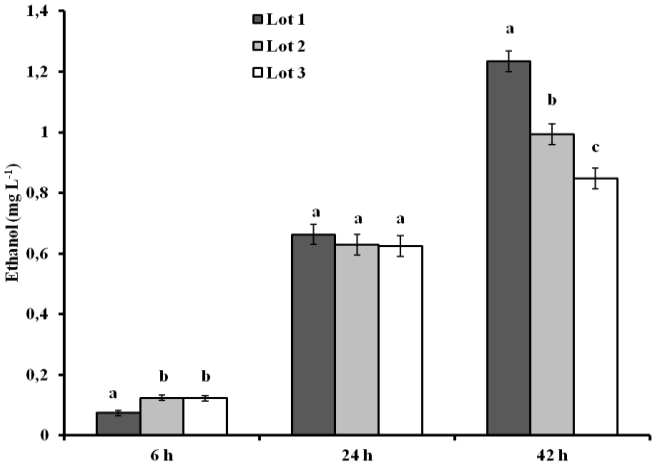

Fig 3. Ethanol test of 3 lots of cowpea (*Amendoim* cultivar) seeds under different soaking times (6, 24 and 42 hours) in a hermetically sealed environment at 40°C. Means followed by the same letter upper case in each bar do not differ by Tukey's test at 5% probability (p≤0.05).

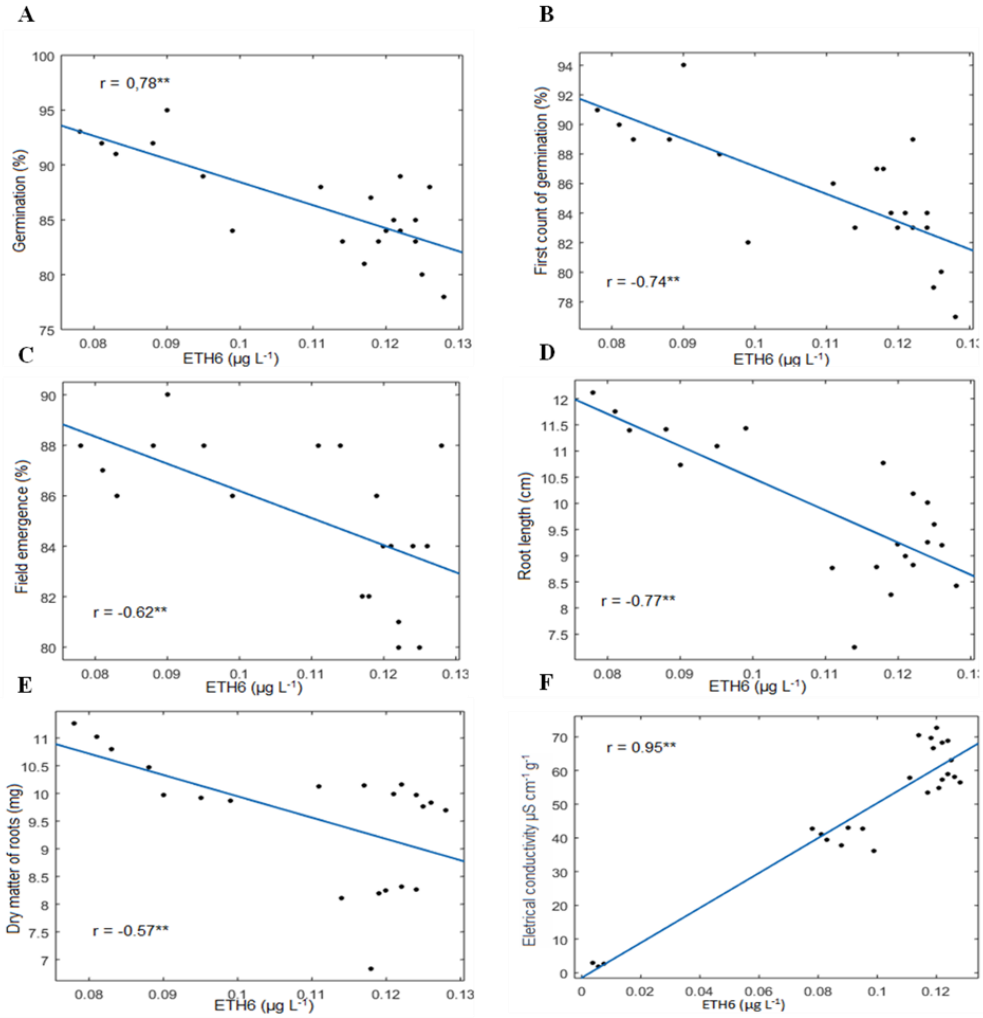

Fig 4. Pearson correlation between the ethanol test for 6 hours (ETH6) of soaking at 40°C with the variables germination (A), first germination count (B), seedling emergence (C) length of the root (D), dry weight of the root (E) and electrical conductivity (F) for 3 lots of cowpea (*Amendoim* cultivar) seeds.

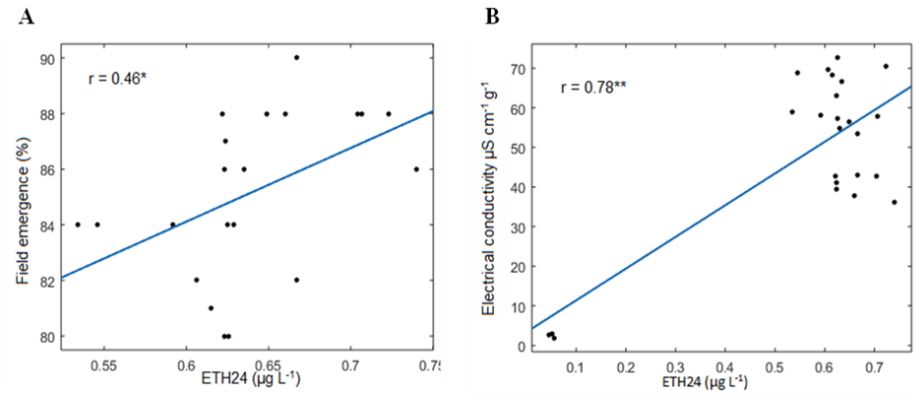

Fig 5. Pearson correlation between the ethanol test for 24 hours (ETH24) of soaking at 40°C with the variables field emergence (A) and electrical conductivity (B) for 3 lots of cowpea (*Amendoim* cultivar) seeds.

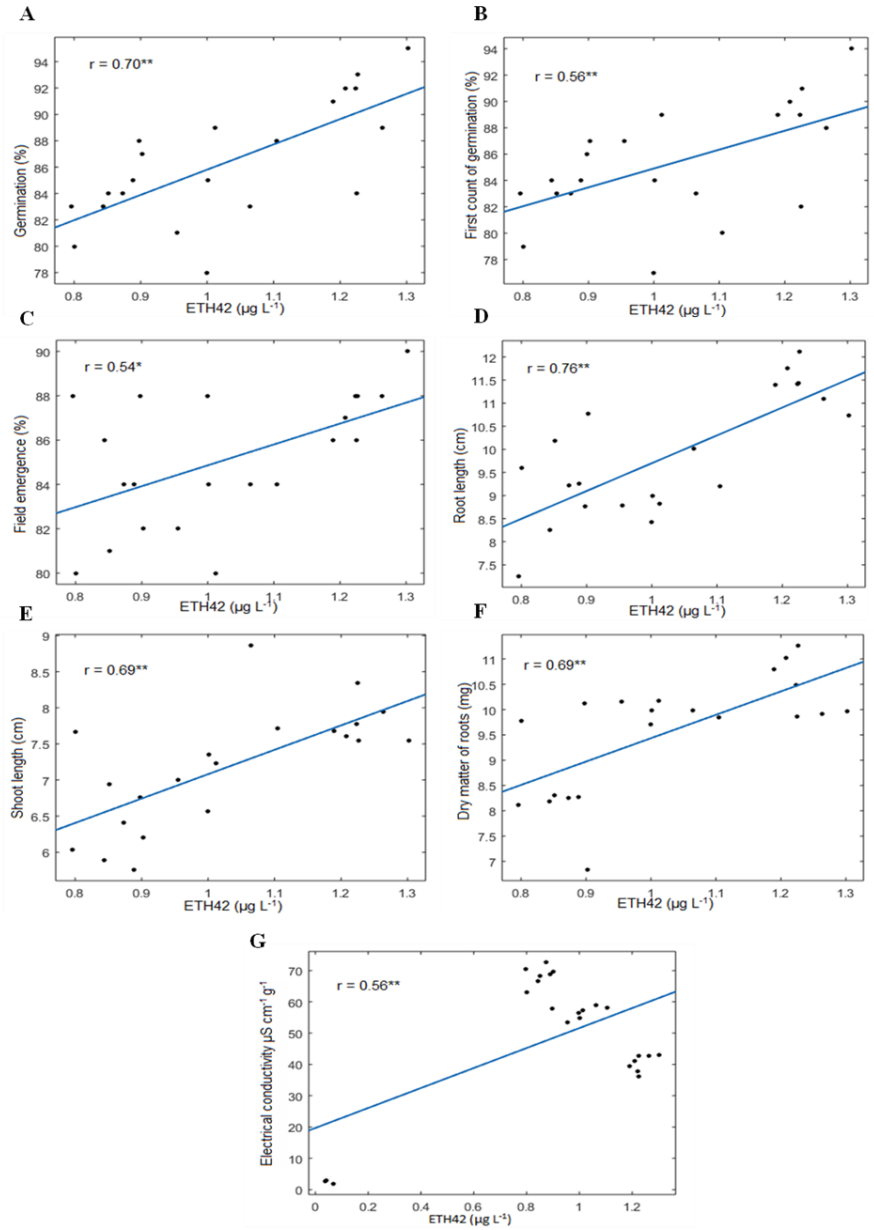

Fig 6. Pearson correlation between the ethanol test for 42 hours (ETH42) of soaking at 40°C with the variables germination (A), first germination count (B), seedling emergence (C), length of the root (D) and length of the shoot (E), dry weight of the root (F), and electrical conductivity (G) for 3 lots of cowpea (*Amendoim* cultivar) seeds.

Discussion

Many plant species can produce ethanol (Cossins end Beevers, 1963). It has been observed that cowpea seeds produce ethanol in an anaerobic environment, and the amount of ethanol produced depends on the level of seed vigour. Similar results have also been observed for the seeds of soybean, which is also a member of the Fabaceae family with a high protein content. Soybean seeds release high levels of ethanol and acetaldehyde, its precursor, after 30 min of soaking (Woodstock and Taylorson, 1981). When performed after cowpea seeds were soaked for 6 h in a controlled environment at 40°C, the ethanol test showed great potential for assessing vigour. For example, the ethanol test was able to classify the tested lots into 2 different vigour levels, with lot 1 being superior to the others (Fig. 3). Additionally, the results of the ethanol test were in accord with the classification of the lots evaluated using tests for the first germination count (Fig. 1B), seedling emergence (Fig. 1C) and root length (Fig. 2A). The Pearson correlation test indicated highly negative correlations between the 6-h ethanol test results and the first germination count (Fig. 4B), emergence in the field (Fig. 4C), root length (Fig. 4F) and root dry weight (Fig. 4E). These negative associations likely occurred because lower ethanol concentrations are associated with greater vigour and are highly and positively correlated with EC test results (Fig. 4F). For the EC test, the correlation was at 95%, and it was significant at 1%. The high positive correlation with EC test results is likely because in the ethanol test, cowpea seeds that release less ethanol after 6 h of soaking due to greater membrane integrity are more vigorous, which is similar in principle to the EC test. Dias et al. (2006) stated that seeds with high levels of vigour maintain the structure of their cell membranes, thereby impeding the entry and exit of solutes (due to the high membrane selectivity). Notably, membrane damage is an initial event among degenerative changes in seeds (Powell & Matthews, 1977; Delouche, 2002). The inability of the 24-h ethanol test (Fig. 3) to stratify the different lots of cowpea seeds in comparison to standard vigour tests may be associated with the level of membrane degradation and, in particular, with the vigour level of the seeds during the 24 h of soaking. For example, the tested lots with less vigour (lots 2 and 3) would produce less ethanol due to the lower reserves; however, the lot with the most vigour (lot 1) also retained the greatest membrane integrity. With a greater energy reserve compared to the other lots, the seeds of lot 1 intensified ATP production via alcoholic fermentation, which consequently increased ethanol release to an amount equivalent to that of lots 2 and 3, thereby preventing stratification of the batches with respect to vigour. According to Marcos Filho (2015), at this moisture level (24 h) and above, which is determined by the soaking time, there is a change in the physiological activity of seeds that favours an increase in oxidative damage.

The poor correlation between the results of the 24-h ethanol test and the seedling emergence test (Fig. 5A) underscores the inefficiency of the former for cowpea seeds under our test conditions, as each test displayed different performance in terms of vigour evaluation. Nonetheless, the positive correlation between the 24-h ethanol and EC tests confirms the influence of membrane integrity on the amount of ethanol released by seeds. Theoretically, seeds that have a high degree of membrane degradation, coupled with the presence of a greater reserve, tend to produce more ethanol when subjected to an anaerobic environment. According to Copeland and McDonald (1995), the degree of seed deterioration is associated with the concentration of seed exudates in solution, which reflects membrane degradation. However, we observed that after 42 h of soaking, the cowpea seeds with greater vigour tended to produce more ethanol. One reason may be because the seeds released ethanol into the medium irrespective of their membrane integrity after this time period; regardless, the greater energy reserves of the most vigorous seeds resulted in the production of more ethanol via anaerobic respiration. In the absence of O_2, the less-vigorous seeds may have exhausted their reserves and lost the ability to transform pyruvate into ethanol, causing death. Buckley and Huang (2012) re-established the idea that ethanol production in the seed begins with, or is enhanced by, the loss of mitochondrial membrane integrity in the absence of O_2.

When mitochondria become non-functional, an alternative mechanism for NAD^+ regeneration is necessary, and such a mechanism is provided by the conversion of pyruvate into ethanol or lactic acid during fermentation. The positive correlation between the ethanol test after 42 h of soaking and the first germination count (Fig. 6B), emergence in the field (Fig. 6C), root length (Fig. 6D), shoot length (Fig. 6E), root dry weight (Fig. 6F) and EC (Figure 6G) tests supports the hypothesis that membrane integrity becomes less important after a long period of anaerobiosis, and higher ethanol production levels are associated with greater reserves that can be converted into ethanol. Based on this principle, the ethanol test after 42 h of soaking was shown to be effective for classifying the relative vigour of lots of cowpea seeds (Fig. 3).

However, the ethanol test results differed from those for emergence in the field, as the former classified the lots into 3 levels of vigour and the latter classified them into only 2 groups (Fig. 1C). Regardless, conditions for emergence in the field are not always adverse and thus may not require seeds to completely exhaust their reserves; thus, results for the ethanol test after 42 h of soaking may be considered to be highly rigorous and to correlate well with controlled deterioration test results. When assessing the quality of cabbage seeds using the ethanol test, Buckley and Huang (2012) found that seeds subjected to controlled deterioration for long periods produced a large amount of ethanol when subjected to 24 h of anaerobiosis at a controlled temperature of 40°C. These same authors also stated that the physiological quality of cabbage seeds can be evaluated with the ethanol test. When conducting a preliminary study aimed at establishing a procedure for evaluating ryegrass seed vigour by the ethanol test, Cavalcante et al. (2017) observed that the technique allows for precise data collection in less time than the standard germination test and field emergence. The data collected using the ethanol test were in agreement with the data obtained from tests for seed viability and vigour.

Materials and methods

Location of the experiment and choice of cultivar

This study was developed in the Didactic Laboratory for Seed Analysis at the Eliseu Maciel Agronomy School, Federal University of Pelotas (Universidade Federal de Pelotas), Rio Grande do Sul State (RS), Brazil. Three lots of cowpea seeds (*Amendoim* cultivar), which had been harvested in 2015 and were obtained from crops established by farmers of the Nortense Family Farmers Cooperative (Cooperativa de Agricultores familiares Nortense - COAFAN), were used in different regions of the municipality of São José do Norte in RS.

Ethanol test

For the ethanol test, seeds were subjected to 3 soaking times (6, 24, and 42 h) in hermetically sealed PET bottles containing 40 ml of distilled water. The amount of ethanol produced by the seeds was then determined with the aid of an adapted INSTRUTHERM BFD-60 breath analyser (Company INTRUTHERM Ltda, 2016, São Paulo, Brazil), with the results expressed in mg L^{-1}.

Physiological quality test

The following evaluations were conducted to compare the results obtained in the ethanol test.

Germination (GER) was assessed in 4 subsamples of 50 seeds for each repetition. The seeds were placed in the substratum of "germitest" germination paper, which had been moistened beforehand with an amount of distilled water 2.5 times the weight of the dry paper, and incubated at a temperature of 25°C. The evaluations were performed according to the Rules for Seed Analysis (Brazil, 2009), and the results are expressed as “percentage of normal seedlings”.

First germination count (FGC) consisted of determining the percentage of normal seedlings 5 days after sowing in the germination test (Brazil, 2009).

Seedling emergence (SE) was evaluated using 4 subsamples of 50 seeds per replication sown in plastic trays containing commercial substrate and incubated at a temperature of 25°C. The evaluations were performed 5 days after sowing and involved identification of seedlings with lengths equal to or greater than 1.0 cm. The results are expressed as "percentage of seedlings that have emerged".

Emergence speed index (ESI) was evaluated concomitant with the emergence test, and counting was performed daily from the first day after sowing until the last day. The emergence speed index was calculated from the daily values of emerged seedlings according to Maguire (1962).

Length of the root (LR) and shoot (LS) of the seedling were evaluated in the first germination count. Ten seedlings per subsample were used for measurement with a ruler, with the results expressed in millimetres.

Dry weight of the root (DWR) and dry weight of the shoot (DWS) were assessed using 10 seedlings from each subsample in the first germination count. The seedlings were placed in paper bags and heated for 48 h at a temperature of 60°C in a forced ventilation oven. The dry weights of the seedlings were subsequently determined using an analytical balance, and the result is expressed in grams (g).

Electrical conductivity (EC) testing involved 4 subsamples of 25 seeds per repetition. The seeds were weighed, placed in a beaker with 70 ml of deionised water and kept in a germinator at a constant temperature of 25°C. After 24 h, readings were obtained with a Schott LF613T bench conductivity meter. To obtain the EC value of the solution containing the seeds, the conductivity value measured by the conductivity meter was subtracted from the value of the water reading, and the resulting value was then divided by the weight of the 50 seeds, with the results expressed as µS $cm^{-1}g^{-1}$ of seeds (Krzyzanowski, 1991).

Experimental design and statistical analysis

A completely randomised experimental design was used, with 7 replications for each treatment. The average germination and vigour values of the cowpea seed batches were compared via the Tukey test at 5% probability. The results of the ethanol tests after 6, 24, and 48 h of soaking were correlated with the other evaluations using Pearson's test at 5 and 1%. For statistical evaluations, the R program (version 3.1.1.) and the “ExpDes.pt” data package were used (Banzato & Kronka, 2006; R Core Team, 2014).

Conclusion

There is a need for a rapid test to evaluate the physiological potential of seeds. The ethanol test at 6 and 42 h of soaking at 40°C provides a rapid and practical alternative for evaluating the vigour of cowpea seeds. The ethanol test can be used in laboratories and seed producers to differentiate batches with different levels of vigour and can be applied to crops.

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