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Ethanol test to evaluate the physiological quality of forest seeds

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ABSTRACT: Rapid tests that allow the assessment of physiological potential are essential parameters for quality seed and seedling production programs. The aim of this work was to establish parameters of a method for evaluating the vigor of seeds of native species through the analysis of ethanol production, measured with a modified alcohol meter. Six lots of *Myracrodruon urundeuva* were tested, and for each lot 1.0 g and 2.0 g of seeds were soaked for two, four, six, eight and 24 hours. Seven lots of *Cenostigma pyramidale* and five lots of *Amburana cearensis* were tested, where 20 seeds were soaked for two, four, six, eight and 24 h. The physiological quality of the seeds was also evaluated by germination and electrical conductivity tests. The use of 1.0 g of seeds soaked in 0.5 mL of water and evaluation of ethanol at six hours distinguished lots of *M. urundeuva* in terms of vigor. For *C. pyramidale* and *A. cearensis*, 20 seeds soaked in 1.0 mL of water and ethanol evaluation at six hours proved to be more efficient. The ethanol test proved to be fast and accurate, allowing differentiation of lots, as well as germination and electrical conductivity tests.

Index terms: alcohol meter, *Amburana cearensis*, *Cenostigma pyramidale*, *Myracrodruon urundeuva*.

RESUMO: Testes rápidos que permitam a avaliação do potencial fisiológico são parâmetros essenciais para programas de produção de sementes e mudas com qualidade. O objetivo do trabalho foi estabelecer parâmetros de um método de avaliação do vigor de sementes de espécies nativas por meio da análise da produção de etanol, medido com etilômetro modificado. Foram testados seis lotes de *Myracrodruon urundeuva*, para cada lote foram colocadas 1,0 g e 2,0 g de sementes embebidas durante duas, quatro, seis, oito e 24 horas. Testou-se sete lotes de *Cenostigma pyramidale*, e cinco lotes de *Amburana cearensis*, onde foram colocadas 20 sementes embebidas durante duas, quatro, seis, oito e 24 h. A qualidade fisiológica das sementes foi avaliada, também, pelos testes de germinação e condutividade elétrica. A utilização de 1,0 g de sementes embebidas em 0,5 mL de água e avaliação de etanol em seis horas distinguiu lotes de *M. urundeuva* quanto ao vigor. Para as sementes de *C. pyramidale*, e de *A. cearensis*, 20 sementes embebidas em 1,0 mL de água em seis horas se mostrou mais eficiente. O teste de etanol mostrou ser rápido e preciso, permitindo diferenciar os lotes, assim como os testes de germinação e condutividade elétrica.

Termos para indexação: etilômetro, *Amburana cearensis*, *Cenostigma pyramidale*, *Myracrodruon urundeuva*.

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INTRODUCTION

For forest regeneration, to combat climate change and the loss of diversity (Gilroy et al., 2014), knowledge on seed germination and propagation is of fundamental importance (Scalon et al., 2012). A significant, fast, and uniform germination of forest species is extremely necessary for sowing purposes due to the interest in their use in reforestation (Bandeira et al., 2017).

The physiological quality of forest seeds is related to several factors, being characterized by genetic, physical, sanitary, and physiological aspects (Gomes et al., 2013). Evaluating physiological quality in forest seeds is extremely important for conservation and restoration, as it determines the value of seeds from a parent tree before selecting it as a seed producer (Lima et al., 2014).

From the point of physiological maturity, the seed reaches its maximum physiological quality: vigor, germination, size, and dry matter weight (Carvalho and Nakagawa, 2012), so performing the collection before physiological maturity can lead to seeds with incomplete development of the embryonic axis, with consequent reduction in their viability and vigor (Dornelas et al., 2015).

Maturity indices are practical parameters that make it possible to infer on the development stage of seeds and can be used when the goal is to determine the appropriate harvest time of a given species (Agustini et al., 2015), as seed deterioration involves biological, physiological and biochemical changes, metabolism of nucleic acids, membrane structure and permeability, enzymatic activities, respiration intensity, lipid peroxidation, accumulation of reactive oxygen species, and rehabilitation mechanism (Wang et al., 2012). In this case, there is insufficient aerobic metabolism, reduction of oxidative phosphorylation, and consequent reduction in energy production (Benamar et al., 2003). Under such conditions of deterioration, cells can produce energy by anaerobic fermentation, which generates ethanol and lactic acid as final products (Kennedy et al., 1992).

Ethanol production by soaked seeds is a potential indicator of vigor, since ethanol production by decayed seeds is higher than by high-vigor seeds (Buckley and Buckley, 2009). Ethanol production is initiated, or increased, by the loss of mitochondrial membrane integrity, and quantification by ethanol test has good potential to examine the level of seed deterioration. Ethanol can be quantified with research methods, analytical equipment and easily measured with breath analyzers (alcohol meters) (Kodde et al., 2012), becoming a good practical candidate to evaluate seed vigor.

Among the forest species, *Myracrodruon urundeuva* Allemão, *Cenostigma pyramidale* Tul., and *Amburana cearensis* Allemão A.C. Smith can be highlighted for their biological importance and multiple use due to their multiple use, resilience and tolerance to harsh environmental conditions (Nascimento et al., 2021). These tree species, with potential for both logging and fruits, are tools in studies that aim to improve techniques for their development, so the quality of seeds to achieve success in seedling production stands out (Pinheiro et al., 2016). These species are also widely used for medicinal purposes and may constitute one of the only therapeutic resources in poorer regions (Coradin et al., 2018).

Thus, the aim of this study was to establish the parameters of a method to evaluate the vigor of *M. urundeuva*, *C. pyramidale* and *A. cearensis* seeds through the analysis of ethanol production.

MATERIAL AND METHODS

Plant material

The study was conducted at the Seed Analysis Laboratory (LASESA) of Embrapa Semi-Arid Region, municipality of Petrolina, PE, Brazil.

Seed lots were collected in different years and placed in cloth bags in a cold chamber (10 ± 1 °C, RH of 40%) until the beginning of the experiment (Table 1). The differences between the lots regarding the year of harvest and origin are desired to improve the representation and variability to explain the results.

Table 1. Seed lots used in the experiment. Species, origin, geographic coordinates of parent trees, seasons and identifications attributed to the seasons.

Species	Origin	Coordinates	Season	ID*
Myracrodruon urundeuva	Jutaí District - Petrolina - PE	8°34'13.1" S and 40°11'02.2" W	2010	Jutaí 2010
			2012	Jutaí 2012
			2013	Jutaí 2013
			2014	Jutaí 2014
			2015	Jutaí 2015
			2016	Jutaí 2016
Cenostigma pyramidale	Massaroca District - Juazeiro - BA	9°52'09" S and 40°16'00" W	2012	Massaroca 2012
			2013	Massaroca 2013
Cenostigma pyramidale	Juremal District - Juazeiro - BA	9°43'51.12" S and 40°21'02.52" W	2010	Juremal 2010
			2011	Juremal 2011
			2014	Juremal 2014
Cenostigma pyramidale	Salitre District - Juazeiro - BA	9°42'30.89" S and 40°34'48.89" W	2015	Salitre 2015
			2016	Salitre 2016
Amburana cearensis	Lagoa Grande - PE	8°34'04.00" S and 40°10'18.00" W	2009	Lagoa 2009
			2012	Lagoa 2012
			2014	Lagoa 2014
			2016	Lagoa 2016
			2017	Lagoa 2017

*ID - Denomination assigned to the seasons.

Moisture content (MC)

Moisture content was determined with two replications of 50 seeds of each lot of *M. urundeuva*, *C. pyramidale*, and *A. cearensis.* The seeds were initially weighed and then placed in a oven at 105 ± 3 °C where they were kept for 24 hours. After this period, the seeds were weighed again and the results were expressed as a percentage on wet basis (Brasil, 2009).

Germination test (GE) and first count (FC)

The tests of germination, first count (*M. urundeuva* – 14 days, *C. pyramidale* – 7 days, *A. cearensis* – 10 days, after setting up the germination test) and final germination count (*M. urundeuva* – 25 days, *C. pyramidale* - 10 days, *A. cearensis* – 30 days, after setting up the germination test) were evaluated in a completely randomized experimental design, with four replications of 50 seeds per lot. For *M. urundeuva* seeds, different lots were sown in acrylic boxes (11 cm × 11 cm × 3.5 cm), using two sheets of blotter paper as substrate, and the seeds were germinated in a B.O.D. (Biochemical Oxygen Demand) chamber at 25 °C and photoperiod of 12 h (Brasil, 2013). For *C. pyramidale* and *A. cearensis*, germination paper moistened with distilled water in a proportion of 2.5 times the dry paper weight was used. Seeds of *C. pyramidale* were germinated in B.O.D. chamber at 25 °C under photoperiod of 12 h (Brasil, 2013), while *A. cearensis* seeds were germinated at 30 °C under photoperiod of 12 h (Brasil, 2013)

Electrical conductivity (EC)

The electrical conductivity of *M. urundeuva* seeds was evaluated with a DIGIMED CD-21 conductivity meter, expressed in $\mu S.cm^{-1}.g^{-1}$, using four replications of 50 seeds soaked in 25 mL of deionized water for 24 h at 25 °C (Vieira

and Carvalho, 1994). For *C. pyramidale* and *A. cearensis* seeds, four replications of 25 seeds were soaked in 75 mL of deionized water for 24 h at 25 °C (Vieira and Krzyzanowski, 1999).

Ethanol Test (ET)

Ethanol formation was evaluated with a modified Dräger Alcotest® 6810 alcohol meter. For *M. urundeuva*, 1.0 and 2.0 g of seeds were placed in penicillin-type glass vials (30 mL), which received 0.5 mL of distilled water and were sealed and incubated at 40 °C for two, four, six, eight and 24 h. For *C. pyramidale*, 20 seeds were placed in penicillin-type glass vials (30 mL), which received 0.5 mL and 1.0 mL of distilled water and were sealed and incubated at 40 °C for two, four, six, eight and 24 h. For *A. cearensis*, 20 seeds were placed in penicillin-type glass vials (30 mL), which received 0.5 and 1.0 mL of distilled water and were sealed and incubated at 40 °C for two, four, six, eight and 24 h (Ornellas et al., 2019). The results were expressed in $\mu g.L^{-1}$ after the reading stabilized.

Statistical analysis

The normality and homoscedasticity of the from the physiological quality tests were tested and analyzed. For the ethanol test, the factors lots and amount of seeds were compared by the Scott-Knott Test ($p \leq 0.05$). The experimental design was completely randomized, according to the species. *M. urundeuva* seeds were evaluated in a 6×2×5 factorial scheme (lots × weights of seeds × soaking periods) with four replications. *C. pyramidale* seeds were evaluated in a 7×2×5 factorial scheme (lots × volumes of soaking water × soaking periods), with four replications. *A. cearensis* seeds were evaluated in a 5×2×5 factorial scheme (lots × volumes of soaking water × soaking periods) with three replications. Statistical analyses were carried out with the statistical program SISVAR® 5.6 (Ferreira, 2011). Pearson's correlation (r) was performed using PAleontological STatistics 3.20 software (PAST 3.20), considering all variables.

RESULTS AND DISCUSSION

The initial moisture content of *M. urundeuva*, *C. pyramidale* and *A. cearensis* seeds showed uniformity between the lots. In *M. urundeuva* seeds, the 2012 and 2016 lots had moisture content of 11.3%, being superior to the others (Table 2). In *C. pyramidale* seeds, the 2012 lot showed moisture content of 10.2%, being inferior to the others (Table 2). In *A. cearensis* seeds, the 2014 lot had moisture content of 10.3%, being superior to the others (Table 2). The moisture content of the seed influences its metabolic activity, interfering in germination and deterioration processes (Peske et al., 2003). The higher the moisture content in seeds, the faster the deterioration process will be, because there is the formation of products that cause immediate damage to cells, such as free radicals, so the lots need to be tested for their moisture content in order to show the least possible variation (Marcos-Filho, 2015).

The lot of *M. urundeuva* seeds collected in 2010 showed 62.7% germination, while the 2016 lot obtained 85% germination. Between the 2012 and 2013 lots, no significant difference was observed in the germination values (Table 2). For the first germination count, the 2010 lot had a lower percentage of normal seedlings, following the results of the germination test (Table 2). Between the 2014, 2015 and 2016 lots, no significant difference was observed in germination values (Table 2). Regarding the electrical conductivity of *M. urundeuva* seeds, there was greater loss of electrolytes in the 2010 lot, with no significant difference between the 2012, 2013, 2014 and 2015 lots (Table 2).

The germination percentages of *C. pyramidale* lots from 2011, 2012, 2014, and 2016 were higher than those of the lots from 2010 and 2013. The 97% germination of the 2014 lot was significantly higher than the values of the 2010 and 2013 lots. The 2011 and 2012 lots, despite the long storage period, showed germination of 80% and 88%, respectively (Table 2). The highest level of vigor was obtained by the increase in the rate of seedling formation, and the values of first germination count of seeds collected in 2014 differed statistically from those of the other lots (Table 2). In *C. pyramidale* seeds, there was a greater amount of electrolytes released in the 2010 and 2015 lots, while the 2014 lot showed higher vigor due to the lower amount of electrolytes released, thus being able to differentiate the lots (Table 2).

Table 2. Moisture content (MC), germination (GE), first count (FC), and electrical conductivity (EC) of different lots of *M. urundeuva, C. pyramidale and A. cearensis* seeds.

M. urundeuva				
Lot	MC	GE	FC	EC
		----------%----------		$\mu S.cm^{-1}.g^{-1}$
Jutaí 2010	11.1	62.7 b	62.7 b	120.2 b
Jutaí 2012	11.3	77.3 ab	72.0 ab	102.7 ab
Jutaí 2013	11.2	77.3 ab	68.7 ab	97.0 ab
Jutaí 2014	11.1	83.3 a	78.7 a	104.7 ab
Jutaí 2015	11.0	85.3 a	82.0 a	100.2 ab
Jutaí 2016	11.3	85.0 a	79.0 a	99.0 a
CV (%)	-	8.4	7.7	10.3
C. pyramidale				
Lot	MC	GE	FC	EC
		----------%----------		$\mu S.cm^{-1}.g^{-1}$
Juremal 2010	10.4	40.0 e	40.0 e	80.2 a
Juremal 2011	10.7	80.0 bc	79.0 bc	67.2 b
Massaroca 2012	10.2	88.0 ab	88.0 ab	63.8 b
Massaroca 2013	10.6	66.0 d	65.0 d	65.0 b
Juremal 2014	10.7	97.0 a	96.0 a	48.7 c
Salitre 2015	10.7	73.0 cd	70.0 cd	85.7 a
Salitre 2016	10.4	89.0 ab	88.0 ab	69.7 b
CV (%)	-	6.3	6.3	11.5
A. cearensis				
Lot	MC	GE	FC	EC
		----------%----------		$\mu S.cm^{-1}.g^{-1}$
Lagoa 2009	10.2	0.0 c	0.0 c	21.2 a
Lagoa 2012	9.9	7.5 b	1.5 c	9.5 b
Lagoa 2014	10.3	7.5 b	0.2 c	9.7 b
Lagoa 2016	10.2	93.0 a	31.5 a	5.0 c
Lagoa 2017	9.0	96.0 a	23.0 b	5.1 c
CV (%)	-	3.8	19.4	35.3

*Means followed by the same letter in the column do not differ from each other by Scott-Knott test at 5% probability level.
CV: Coefficient of variation.

The seeds of *A. cearensis* showed no statistical difference in the high germination values between the 2016 and 2017 lots. The 2012 and 2014 lots showed no differences in germination, and the 2009 lot had no germination (Table 2). For the first germination count, the 2016 lot showed a higher percentage of normal seedlings when compared to the others (Table 2). The electrical conductivity test confirmed differences between the seeds of *A. cearensis*. It was possible to differentiate the more vigorous lots, which showed lower electrical conductivity, namely 2016 and 2017, while seeds from the 2009 lot were classified as of low vigor, due to the high electrical conductivity and greater release of leachates (Table 2). Germination test is the most used mechanism to evaluate the physiological quality of seeds of several forest species. The first count of the germination test better expresses the differences in germination speed between lots than germination speed indices (Medeiros et al., 2014). Additionally, vigor tests are important tools to

evaluate the physiological quality of forest seeds, being constantly improved. One of the fundamental objectives of the vigor tests is to detect significant differences in the physiological quality of seed lots with similar germination, in order to complement the information provided by the germination test (Marcos-Filho, 2015). The electrical conductivity test, for being faster, easy to execute and for the possibility of being standardized as a routine test due to its high reproducibility, can be used for efficient separation of seed lots in terms of vigor, being considered one of the most important tests (Lemes et al., 2015), which explains the need for studies for standardization in forest species, but the use of the electrical conductivity test allowed the identification of lots with different levels of vigor, regarding the concentration of exudates, revealing its importance for a dynamic and effective quality control program.

In the evaluation of the vigor of *M. urundeuva* seeds, through the ethanol test, the seed lots harvested in 2010, 2012 and 2013 showed higher ethanol release than the others. Ethanol test is a promising method capable of distinguishing seed lots with different vigor levels, and for this species, using 1.0 g of seeds soaked in 0.5 mL of distilled water, was able to distinguish physiological quality levels in the lots with results that can be evaluated from six hours of soaking (Figures 1A and 1B).

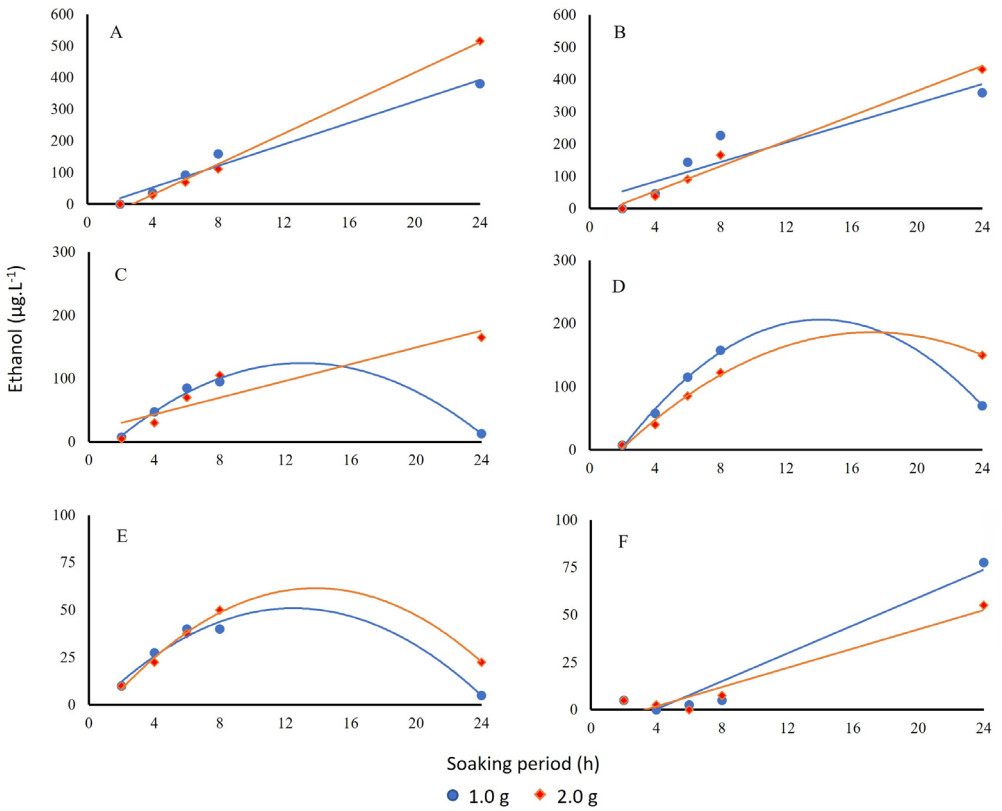
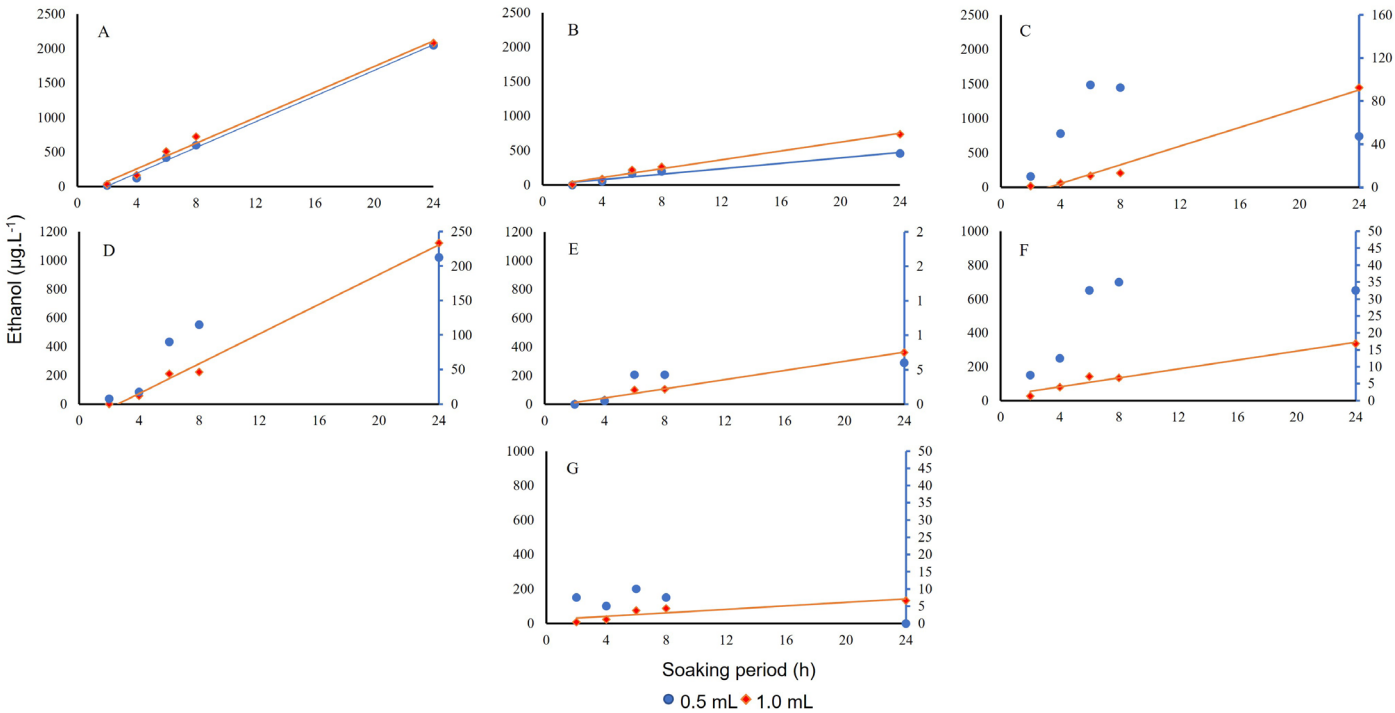


Figure 1. Ethanol content released by 1.0 g and 2.0 g of *M. urundeuva* seeds in 0.5 mL of soaking water as a function of the period in the different lots. (A) Jutaí 2010, (B) Jutaí 2012, (C) Jutaí 2013, (D) Jutaí 2014, (E) Jutaí 2015, (F) Jutaí 2016.

In *C. pyramidale* seeds, the 2010 lot, with 1.0 mL of distilled water, from soaking period of six hours, showed higher ethanol release, differing significantly from the other lots (Figure 2A). For the 2011, 2012, 2013, and 2014 lots, the seeds soaked with 1.0 mL of distilled water, from the soaking period of six hours, showed high levels of ethanol release (Figures 2B-E). On the other hand, seeds from the 2012, 2013, 2014, 2015 and 2016 lots, when soaked in 0.5 mL of distilled water, showed similar responses (Figures 2C-2G).

For *A. cearensis* seeds, in the 2009, 2012 and 2014 lots, with seeds soaked, from six hours, in 1.0 mL of distilled water, there was a significantly greater release of ethanol, when compared to the other lots, hence showing the initial stages of deterioration, which occur progressively, and determining the decline in the physiological quality of the seeds (Figures 3A, 3B, 3C).

In melon seeds, the results were similar, because the seeds with lower vigor showed higher ethanol production, with a relationship of the alcoholic dehydrogenase enzyme (ADH) with anaerobic respiration, which catalyzes the conversion of acetaldehyde into ethanol, and vice versa, in the fermentative metabolism, caused by the lack of integrity of the membranes and, mainly, of the mitochondria. The quantification of the ethanol produced and released can provide important information related to the physiological quality of seeds (Ornellas et al., 2019). Moncaleano-Escandon et al. (2013) state that seeds of high vigor keep their cell membranes structured, hindering the entry and exit of solutes, from the high selectivity of the membranes. In this context, the less vigorous seeds



A – Juremal 2010
0.5 mL - ŷ = 93.102x - 179.3; R^2 = 99.75**
1.0 mL - ŷ = 92.591x - 112.8; R^2 = 99.11**

B – Juremal 2011
0.5 mL - ŷ = 19.566x + 2.3187; R^2 = 99.24**
1.0 mL - ŷ = 31.94x - 18.07; R^2 = 99.69**

C – Massaroca 2012
0.5 mL - ŷ = ns
1.0 mL - ŷ = 18.364x + 45.729; R^2 = 99.91**

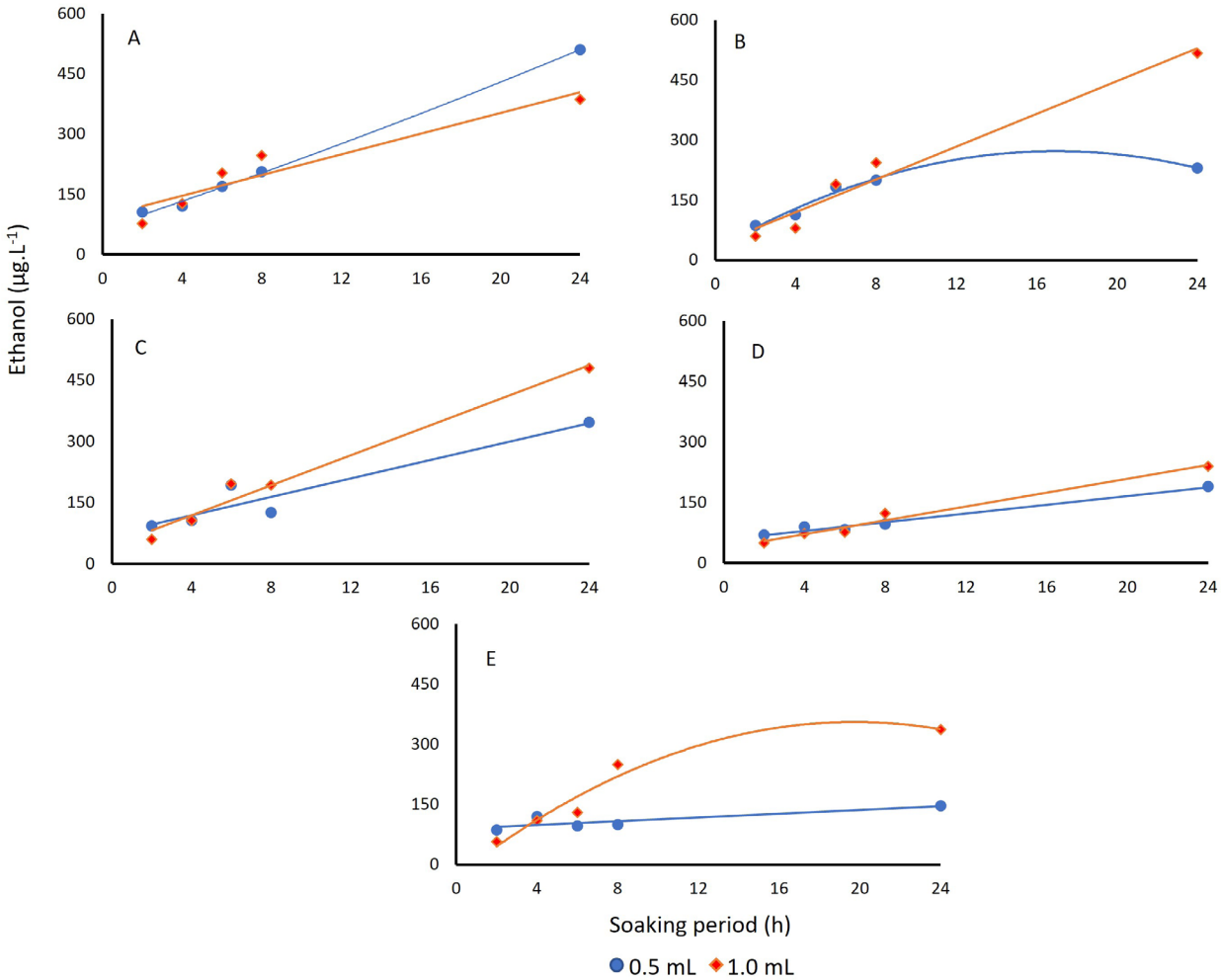
D – Massaroca 2013
0.5 mL - ŷ = ns
1.0 mL - ŷ = 8.558x + 37.354; R^2 = 99.58**

E – Juremal 2014
0.5 mL - ŷ = ns
1.0 mL - ŷ = 15.965x - 20.492; R^2 = 99.14**

F –Salitre 2015
0.5 mL - ŷ = ns
1.0 mL - ŷ = 40.479x - 148.72; R^2 = 98.09**

G – Salitre 2016
0.5 mL - ŷ = ns
1.0 mL - ŷ = 40.479x - 148.72; R^2 = 96.98**

Figure 2. Ethanol content released by 20 seeds of *C. pyramidale* in 0.5 mL and 1.0 mL of soaking water as a function of the period in the different lots. (A) Juremal 2010, (B) Juremal 2011, (C) Massaroca 2012, (D) Massaroca 2013, (E) Juremal 2014, (F) Salitre 2015, (G) Salitre 2016.



A – Lagoa 2009
0.5 mL - $\hat{y} = 0.0831x^2 + 16.54x + 65.41$; $R^2 = 99.66^{**}$
1.0 mL - $\hat{y} = 12.893x + 94.537$; $R^2 = 99.73^{**}$

B – Lagoa 2012
0.5 mL - $\hat{y} = -0.8555x^2 + 29.016x + 26.402$; $R^2 = 96.77^{*}$
1.0 mL - $\hat{y} = 20.513x + 37.479$; $R^2 = 96.52^{**}$

C – Lagoa 2014
0.5 mL - $\hat{y} = 11.269x + 74.158$; $R^2 = 90.22^{**}$
1.0 mL - $\hat{y} = 18.364x + 45.729$; $R^2 = 97.78^{**}$

D – Lagoa 2016
0.5 mL - $\hat{y} = 5.4059x + 58.426$; $R^2 = 97.99^{**}$
1.0 mL - $\hat{y} = 8.558x + 37.354$; $R^2 = 97.91^{**}$

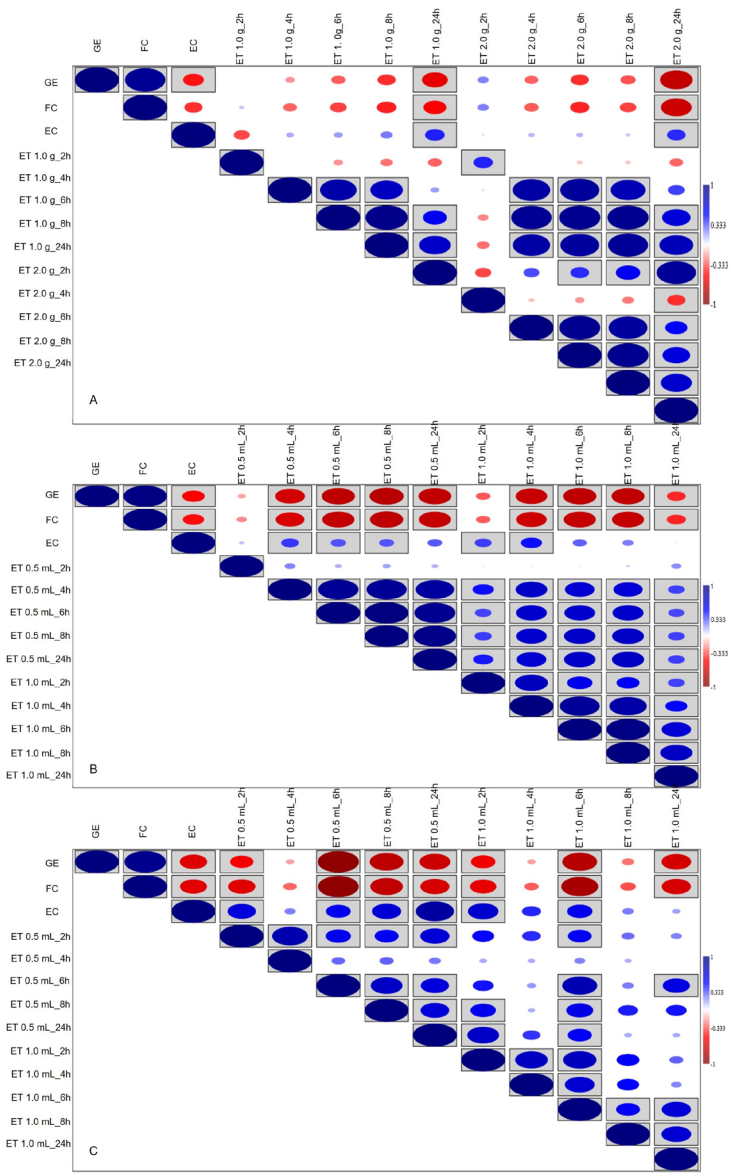
E – Lagoa 2017
0.5 mL - $\hat{y} = 2.3099x + 89.669$; $R^2 = 72.70^{*}$
1.0 mL - $\hat{y} = -0.9891x^2 + 39.012x - 28.952$; $R^2 = 94.99^{**}$

Figure 3. Ethanol content released by 20 seeds of *A. cearensis* in 0.5 mL and 1.0 mL of distilled water as a function of the period in the different lots. (A) Lagoa 2009, (B) Lagoa 2012, (C) Lagoa 2014, (D) Lagoa 2016 and (E) Lagoa 2017.

tend to show membranes with lower integrity, thus facilitating the exit of ethanol more quickly when compared to more vigorous seeds. It is believed that ethanol is the least toxic end product of fermentation, as it can diffuse out of the cell, while lactate accumulates and promotes cytosol acidification (Taiz et al., 2017). The ethanol test allowed observing the efficiency, speed and reliability of the method.

Based on the results, seeds with lower vigor level, that is, with higher level of membrane deterioration and higher respiratory activity, tend to show higher ethanol release. On the other hand, seeds with lower level of deterioration, that is, with greater vigor, tend to show lower respiratory activity, leading to lower ethanol production under anaerobiosis conditions.

Pearson's correlations between evaluations of physiological quality of *M. urundeuva*, *C. pyramidale* and *A. cearensis* seeds and ethanol test were analyzed. In *M. urundeuva* seeds, negative correlations were observed between the ethanol test and the germination and first count tests, and positive correlations were observed between the ethanol test and the electrical conductivity test, when 1.0 g of seeds were soaked (0.5 mL) in distilled water for 24 h (Figure 4A). In *C. pyramidale* seeds, there were high negative correlations between the ethanol test and the germination and first count tests, containing 20 seeds soaked in 0.5 mL of distilled water, in the periods of four, six, eight and 24 h, and low positive correlations between the ethanol test and the electrical conductivity test, containing 20 seeds soaked in 0.5 mL of distilled water, in the periods of four, six and eight h (Figure 4B). In *A. cearensis* seeds, there was a high negative correlation between the ethanol test and the germination and first count tests, containing 20 seeds soaked in 0.5 mL of distilled water, in the period of six hours, and a high positive correlation between the ethanol test and the electrical conductivity test, containing 20 seeds soaked in 0.5 mL of distilled water, in the period of 24 h (Figure 4C).



*Blue - positive correlation; Red - negative correlation; Ellipse size - correlation intensity; Rectangles - significant correlation at 5% probability level.

Figure 4. Correlation between ethanol test (ET), first count (FC), germination (GE), electrical conductivity (EC) of seeds of (A) *M. urundeuva*, (B) *C. pyramidale*, (C) *A. cearensis*.

CONCLUSIONS

The ethanol test, by modified alcohol meter, for *M. urundeuva* when performed with 1.0 g of seeds soaked in 0.5 mL of distilled water for six hours, and for *C. pyramidale* and *A. cearensis* when performed with 20 seeds soaked in 1.0 mL of distilled water for six hours, is a fast and efficient method, showing high sensitivity to evaluate the physiological potential of seeds, and is able to distinguish the vigor of the lots.

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