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Gene expression, biochemical and physiological activities in evaluating melon seed vigor through ethanol release

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ABSTRACT

The aim of the present study was to establish parameters to evaluate melon seeds vigor by ethanol release test using a modified ethylometer and correlate with physiological seed quality, gene expression and enzyme activities alterations. To perform this trial, six seeds lots of yellow melon were submitted to determinations such as moisture content, germination assay and vigor tests. 25 and 50 seeds soaked with 0.5 mL of distilled water and tested after 1, 2, 3, 4, 5, 6 and 24 h at 40 °C. After 0, 6 and 24 h of imbibition, the high and low vigor lots were evaluated for the following enzyme activity: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and alcoholic dehydrogenase (ADH). Accumulation of ADH protein was verified using Western Blotting hybridization technique with the primary Anti-ADH antibody. The release of ethanol in 25 seeds, soaked in 0.5 mL of distilled water for 6 h, proved efficient for vigor evaluation and can be related to other traditionally used tests. Enzyme activity varied according to the vigor of the seed lot. For the protein profile, there was variation in the accumulation of proteins of specific molecular weights throughout the soaking periods. There was also variation in expression according to accumulation of ADH protein. The biochemical and molecular tests corroborate with the establishment of the ethanol test as an additional tool to evaluate seed vigor.

1. Introduction

Deterioration is the progressive process of loss of quality due to physiological, biochemical, physical and cytological changes, starting from fertilization and culminating in death of the seed or plant (Marcos-Filho, 2015). Within many alterations that occurs in seeds during this process the ones based in initial events of the hypothetical deterioration sequence proposed by Delouche and Baskin (1973), such as cell membrane degradation, respiratory activity reduction and biosynthesis reduction, are the most potential to identify quickly alterations and rank seed lots by vigor.

Ethanol release by seeds during imbibition is a potential vigor test based on the initial principles of seed deterioration, where the mitochondria incapacity to process the pyruvate from glycolysis and some enzymatic reactions results in alcoholic fermentation reactions, in which pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) act on pyruvate, releasing ethanol and CO_2, and oxidizing NADH. ADH and lactate dehydrogenase (LDH) are essential in the glycolytic cycle under anaerobic conditions, as they recycle NAD^+, reducing pyruvate to ethanol or lactate. This process of ethanol accumulation involves oxidation of NADH and produces a small amount of ATP, which is essential for survival in some species during the absence of oxygen and when mitochondrias are non-functional due to the damage (Kodde et al., 2012). Seeds are impermeable to oxygen during the first few hours of germination, thus generating an increase in the respiratory coefficient, as well as an increase in ADH activity, which activates alcoholic fermentation (Taiz et al., 2017).

High-vigor seeds maintain their cell membrane structure, making it

Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPX, guaiacol peroxidase; APX, ascorbate peroxidase; ADH, alcoholic dehydrogenase; PC, principal component

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difficult for solutes to enter and exit, due to the high selectivity of the membranes (Moncaleano-Escandon et al., 2013). In this context, less vigorous seeds tend to present membranes with lower integrity, thus facilitating the quick release of ethanol when compared to more vigorous seeds, and consequently, its quantification may provide important information on physiological problems related to seed deterioration (Buckley et al., 2016; Kodde et al., 2012).

The optimization of ethanol assay protocols may be an efficient alternative to determine the physiological potential of seeds, as the procedures are relatively simple, fast and economical, and can be reproduced, which represents an important initiative directed to the contribution to the advancement of seed technology research. This results are compatible with other vigor tests, but are quickly obtained as for cabbage (Kodde et al., 2012), malting barley (Buckley et al., 2016), and maize (Onwimol et al., 2019).

Therefore, the production of ethanol by partially imbibed seeds is a potential indicator of seed vigor, since ethanol production by deteriorated seeds is higher in relation to high-vigor seeds (Buckley and Buckley, 2009). Seeds that present a higher level of membrane degradation tend to produce more ethanol (Wendt et al., 2017). It is believed that ethanol is the least toxic final product of fermentation, as it can diffuse out of the cell, while lactate accumulates and promotes acidification of the cytosol (Taiz et al., 2017).

Through gene expression techniques, a process by which information encoded by a given gene is decoded into a protein, factors associated with physiological conditions of seeds can be understood. Regulating any of the steps in this process may induce differential gene expression, which is used to identify genes and has been used for understanding and functioning of the gene and the molecular mechanisms involved in biological events (Vasconcelos et al., 2010).

The melon, *Cucumis melo* L., belongs to the Cucurbitaceae family, which is considered one of the largest vegetable families, with morphological characteristics of herbaceous plants. In Brazil, melon production is concentrated in the Northeast region. This crop shows great social and economic importance, as it is one of the main items of Brazilian exports, relevant to the country's trade balance, besides generating income and thousands of jobs in one of the poorest regions of Brazil (Calixto et al., 2019).

Therefore, the aim of this research was to establish parameters to evaluate the vigor of yellow melon seeds through the release of ethanol, in relation to other vigor evaluation procedures, gene expression parameters of metabolic processes of interest, and enzyme activity linked to deterioration.

2. Material and methods

The assay was performed in partnership between the phytotechnology laboratory at State University of Santa Cruz (Ilhéus, Bahia State, Brazil) and the laboratory of seed analysis of Semi-arid Embrapa (Petrolina, Pernanbuco State, Brazil).

2.1. Seed lots

Six commercial seed lots of yellow melon, Gladial F1-Hybrid, were obtained from harvests in 2011/2012 from Brazil (lot 1), 2015 from Peru (lot 2), 2016 from Chile (lot 3), 2016 from Peru (lot 4 and lot 5) and 2016 from France (lot 6).

2.2. Water content determination (WC)

Water content was determined with two replicates of 50 seeds from each seed lot. The seeds were initially weighed and then placed in an oven at 105 ± 3 °C, for 24 h. After this period, the seeds were weighed again and the results were expressed as a percentage, on a wet basis (Brasil, 2009).

2.3. Germination test (GE) and first count (FC)

Four replicates of 50 seeds from each lot were distributed on two sheets of germination paper, moistened with distilled water at an amount equivalent to 2.5 times their mass. Subsequently, the seeds were covered with another sheet of germination paper, and the rolls were placed in a germination chamber at 25 °C under constant white light. The percentages of normal seedlings for each first-count (FC) and germination were calculated by counting on the 4th and 8th days after test installation, respectively (Brasil, 2009).

2.4. Emergence in Field (EF) and Emergence Speed Index (ESI)

This evaluation is based on the principle that seeds that provide a greater percentage of emergences under field conditions, that is, uncontrolled, are more vigorous. The tests were performed with four replicates of 50 seeds per lot, sown in 2 m long grooves, 1 cm deep and spaced 0.4 m apart. Seedlings with fully expanded primary leaves were considered emerged. The ESI was performed in conjunction with the emergence test, in which daily counts of emerged seedlings were performed until emergence stabilization. For each repetition, the formula proposed by Maguire (1962) was applied.

2.5. Electrical Conductivity (EC)

The electrical conductivity test was performed using four replicates of 50 seeds from each lot. The seeds were weighed and placed to soak in 75 mL of deionized water in 200 mL plastic cups. The seeds were placed in a regulated chamber at 25 °C for 24 h. After this period, the electrical conductivity reading was measured and the mean values for each lot were expressed in $\mu S\ cm^{-1}\ g^{-1}$ (Marcos-Filho and Vieira, 2009).

2.6. Ethanol Test (ET)

Ethanol release was evaluated under a completely randomized design, in a factorial scheme 6 × 2×7 (lots × amounts of seeds × soaking periods) with four replicates. Samples of 25 and 50 seeds were placed in penicillin-type glass vials (30 mL) with 0.5 mL of distilled water, sealed with rubberized caps and metal seals with manual pliers and incubated at 40 °C for 1, 2, 3, 4, 5, 6 and 24 h (Buckley et al., 2013). The readings were performed with Dräger Alcotest® 6810 modified ethylometer and the results expressed in $\mu g\ L^{-1}$.

2.7. Enzyme activity

For the accomplishment of enzyme activity analyses, seeds from lots 1 and 6 were separated, as they were considered as having the lowest and highest vigor (based on the results of the aforementioned tests), after 0, 6 and 24 h, respectively, of imbibition. A pool was formed with five seeds per replicate, with a total of 20 seeds or ˜200 mg, per lot. The pools were macerated in the presence of liquid nitrogen and PVP (Polyvinylpyrrolidone) (0.7 g of PVP / g of tissue) to avoid oxidation of the macerate. The obtained material was stored in an ultra-freezer at −80 °C for subsequent biochemical determinations.

2.7.1. Preparation of crude protein extract

The concentration of total proteins was determined by the Bradford Method using bovine albumin (BSA) as standard (Bradford, 1976). For the enzymatic assays, the macerated pools were re-suspended in 800 µL of extraction buffer, which varied according to the type of enzyme involved in the antioxidative metabolism (50 mM sodium phosphate buffer, pH 7.0, for CAT and GPX enzyme activity, and 50 mM potassium phosphate buffer, pH 6.0, for SOD and APX enzyme activity), and briefly vortexed. Subsequently, the samples were sonicated in an ultrasonic probe sonicator (Ultrasonic processor Gex 130, 130 W) in an ice bath, until total tissue rupture under 70% amplitude for 1 min (10 s

on x 20 s off). Soon after, the material was centrifuged at 13,000 × *g* at 4 °C for 20 min. Thereafter, the supernatant was collected as crude protein extract. The same was used to determine the concentration of total soluble proteins and enzyme activity. Five experimental replicates were performed for each of the measures of enzyme activity. Absorbance readings were performed on a Spectramax Paradigm microplate spectrophotometer (Molecular Devices).

2.7.2. Superoxide dismutase (SOD) activity - EC: 1.15.1.1

The enzyme activity of SOD was determined by the inhibition of the photo-reduction of nitro blue tetrazolium chloride (NBT), according to Beauchamp and Fridovich (1971), with modifications. A total of 10 µl aliquots of crude protein extract were transferred to test tubes containing 50 mM potassium phosphate buffer pH 7.8, 0.1 mM EDTA, 13 mM L-methionine and 75 µM NBT. The enzyme activity was initiated with the addition of riboflavin (2 mM) with the reading being taken at 560 ηm. The initial reading occurred after 5 min of incubation in the dark. The second reading was performed after 10 min of 15 W fluorescent light. Reactions without the crude protein extract were considered blank. One unit of activity (UA) was considered as the amount of enzyme required to inhibit 50% of NBT photo-reduction compared to the blank. The activity of SOD was expressed in UA $\mu g^{-1} min^{-1}$.

2.7.3. Catalase activity (CAT) - EC: 1.11.1.6

CAT activity is defined by the rate of H_2O_2 consumption based on decreasing absorbance at 240 ηm for 1 min, according to Sudhakar et al. (2001), with modifications. Aliquots of 1 µL of crude extract were added to 2.95 mL of reaction medium consisting of 50 mM sodium phosphate buffer (pH 7.0) at 30 °C. The reaction was started by addition of 15 mM H_2O_2. CAT activity was expressed as mol H_2O_2 $mg^{-1} min^{-1}$.

2.7.4. Guaiacol Peroxidase (GPX) activity - EC: 1.11.1.7

Activity was determined according to the methodology described by Matsuno and Uritani (1972), with modifications. The reaction buffer containing sodium phosphate (25 mM pH 6.8), H_2O_2(15 mM) and Guaiacol (10 mM) was added to the crude extract (1 µL). Absorbance readings were performed at 470 ηm at 30 °C. GPX activity was expressed in UA $\mu g^{-1} min^{-1}$.

2.7.5. Ascorbate Peroxidase (APX) activity - EC: 1.11.1.11

For APX activity was determined though ascorbate consumption by way of decreases in absorbance at 290 ηm, according to Nakano and Asada (1981). Aliquots of crude extract (5 µL) were added to the reaction buffer containing potassium phosphate (50 mM pH 6.0) and L-ascorbate (1 mM). The reaction was started by adding H_2O_2 (2 mM) and enzyme activity was expressed in µmol ascorbato $g^{-1} min^{-1}$.

2.7.6. Alcoholic dehydrogenase (ADH) activity - EC: 1.1.1.1

ADH activity was determined according to the methodology of Chung and Ferl (1999) with modifications. Soluble proteins were extracted with the addition of extraction buffer (50 mM Tris − HCl (pH 8.0) and 15 mM DTT) to the macerate. The mixture was centrifuged at 12,000 *g* for 15 min at 4 °C. The protein concentration of the crude extract was determined as previously described. The extract obtained was used to measure ADH activity with the reaction buffer composed of 50 mM Tris − HCl (pH 8.0), NAD^+ 9.0 mM. The enzymatic reaction was initiated by the addition of ethanol to the mixture (final concentration of 20% (v/v)). The reading was monitored for 1 min with absorbance at 340 nm. Activity of the ADH enzyme was expressed as UA $mg^{-1} min^{-1}$.

2.8. Protein profile

The electrophoretic profile of total proteins of melon seeds was obtained by separating the proteins in denaturing polyacrylamide gel (SDS-PAGE). As such, 10 µg of the obtained crude extract was mixed with a sample buffer containing β-mercaptoethanol, which assists in disruption of the disulfide bonds and avoids sample oxidation (Sambrook et al., 1989), and denatured at 95 °C for 5 min. Samples were applied to pools of 12% SDS-PAGE and subjected to electric current (150 V and 30 A) for 4 h in order to determine protein mobility. The electrophoresis run system was established with buffer solution (pH 8.3) containing 50 mM Tris, 384 mM glycine, 5 mM EDTA (pH 8.8) and 0.25% SDS. All electrophoretic runs were performed with three experimental repetitions. The gels were then stained with 0.1% Coomassie Brilliant Blue for 12 h and bleached with 10% acetic acid solution, 45% methanol and 45% distilled water. The observed bands represented the total protein profiles and the bands of interest were specifically measured for accumulation through the GelQuant.NET image analysis program (version 1.8.2 - http://biochemlabsolutions.com/GelQuantNET.html), which identifies and quantifies bands on gels by means of the pixel ratio.

2.9. Western blotting (WB)

Accumulation of ADH protein was specifically verified by means of membrane immunodetection of nitrocellulose. The experiment was carried out according to the protocol of Sambrook et al (1989). SDS-PAGE was initially performed in triplicate for the separation of the proteins from the crude extract. Separated and immobilized proteins were transferred from each gel to nitrocellulose membranes using the Trans-Blot system (BioRad), according to the manufacturer's instructions. After transfer, the membranes were washed and blocked with casein in 1x TBS-T buffer. Blocked membranes were incubated with the primary (dilution 1:10,000) Anti-ADH (Agrisera®) antibody for 1 h at room temperature under gentle agitation. The membrane was further incubated with the alkaline phosphatase conjugate anti-rabbit IgG (dilution 1:5000) secondary antibody (Invitrogen®). Hybridization was shown with the colorimetric reaction of alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro blue tetrazolium (NBT) (Promega) as substrates. The theoretical molecular weight of melon ADH (ID: NP_001284398.1) was calculated using the Compute pl/Mw present on the Expasy platform (https://www.expasy.org/).

2.10. Statistical procedures

The data were tested for normality and homoscedasticity, and analyzed in a completely randomized design for the physiological quality tests. For the ethanol test, lot and seed quantity factors were compared using the Scott-Knott test ($p \leq 0.05$) and reading periods were analyzed with polynomial regression. The field experiment was set up in a randomized block design. Statistical analyses were performed using the SISVAR® 5.3 statistical program (Ferreira, 2011). The Pearson correlation (r) and principal component analysis (PCA) were performed using PAST software 3.20, considering all variables.

3. Results

3.1. Water content determination (WC)

Initial water content of the melon seeds of all lots presented uniformity, with lot 1 presenting a water content of 7.2%, which was higher than the other lots (Table 1).

3.2. Germination test (GE) and first count (FC)

The germination test for melon seeds enabled differentiation of lots at two levels. Lot 1 presented values lower than the others and was thus characterized as being of reduced quality (Table 1). For the first germination count, lot 1 presented the lowest percentage of normal seedlings, being lower than the other lots, following the results of the germination test (Table 1). It is worth noting that, for melon seeds, the

Table 1
Initial water content (WC), germination (GE), first count (FC), emergence of field seedlings (EF), emergence speed index (ESI), and electrical conductivity (EC) of six lots of melon seeds.

Lots	WC	GE	FC	EF	ESI	EC
	%					μS. cm^{-1}. g^{-1}
1	7.2	67 b*	54 b	62 b	2.8 c	75.7 a
2	7.0	92 a	71 a	91 a	5.8 b	31.6 b
3	7.0	94 a	72 a	96 a	7.3 a	30.9 b
4	6.4	92 a	71 a	96 a	6.9 a	22.3 c
5	6.2	96 a	73 a	95 a	7.1 a	20.0 c
6	6.4	87 a	69 a	92 a	6.0 b	27.2 b
CV (%)	–	4.8	32.4	5.3	4.2	8.4

* Means followed by the same letter do not differ statistically from each other. The Scott-Knott Test was applied at the 5% probability level.

germination value established for commercialization is at least 80% (Muniz et al., 2004).

3.3. Emergence in field (EF) and emergence speed index (ESI)

For seedling emergence in the field, lot1 presented lower performance (with 62% of plants emerging in the twenty-day period) in relation to the other lots. As for the emergence speed index, lot1 presented lower development over the evaluation days after emergence, and in lots 3, 4 and 5 the ESI were superior in relation to the other lots (Table 1).

3.4. Electrical conductivity (EC)

In the electrical conductivity test, lot 1 showed a significantly higher number of leached electrolytes, while in lots 2, 3 and 6 there were no significant differences between them, and 4 and 5 presented less leached electrolytes (Table 1).

3.5. Ethanol test (ET)

For ethanol test, after 1 h of imbibition, with 25 seeds, there was greater ethanol release for lot 6, while for 50 seeds there were differences between lots 1 and 6. After 2 h of imbibition, with 25 seeds, there was greater ethanol release in lot 6 in relation to lot 1, and in the 50 seeds there were variations in ethanol release, but the largest release was lot 6 from lot 1 (Table 2). The ethanol test was able to distinguish the levels of physiological quality of the lots, with results that can be evaluated from 6 h with 25 seeds partially soaked in 0.5 ml of distilled water.

3.6. Correlation

Correlations between physiological quality evaluations of melon seeds and the ethanol test were analyzed using the Pearson method. Highly significant positive and negative correlations were obtained for germination, electrical conductivity and seedling emergence results in the field and emergence speed index. There were high positive and negative germination correlations with the ethanol test. The ethanol test also obtained correlations of high and medium significance, both positive and negative, with electrical conductivity, emergence of field seedlings, emergence speed index, seedling length and fresh and dry matter weight (Fig. 1).

3.7. Enzyme activity

Enzyme activity analysis confirmed the differences between the level of vigor between the two lots evaluated, which also presented differences in physiological quality tests. SOD and CAT activities were similar, with higher values in the most deteriorated seeds after 6 h of imbibition (Figs. 2A and 2B). The increase in SOD activity in the lower vigor lot occurred at 6 and 24 h of imbibition (Fig. 2A). The highest activity of CAT also occurred in the lower vigor lot, after 6 and 24 h of imbibition (Fig. 2B).

The GPX and APX activity showed higher values in high vigor seeds with 6 h of imbibition (Figs. 2C and 2D). GPX showed higher activity in lot 6 (higher vigor) in all imbibition periods (Fig. 2C). APX showed higher activity at 6 h of imbibition for lot 1 (lower vigor), whereas lot 6 (higher vigor) presented lower activity at 24 h of imbibition, however, there was a decrease in the activity of lot 1 (lower vigor) and an increase in the activity of lot 6 (higher vigor) (Fig. 2D).

The ADH activity in the lower vigor lot increased according to the imbibition time of the seeds. This data is in accordance with that observed for the measurement of ethanol release in the lots (Table 2). ADH activity was higher for the most vigorous lot, at 0 -h imbibition time, with decreasing activity at 6 h of imbibition. After this period there was an increase in ADH activity (24 h of imbibition) (Fig. 3).

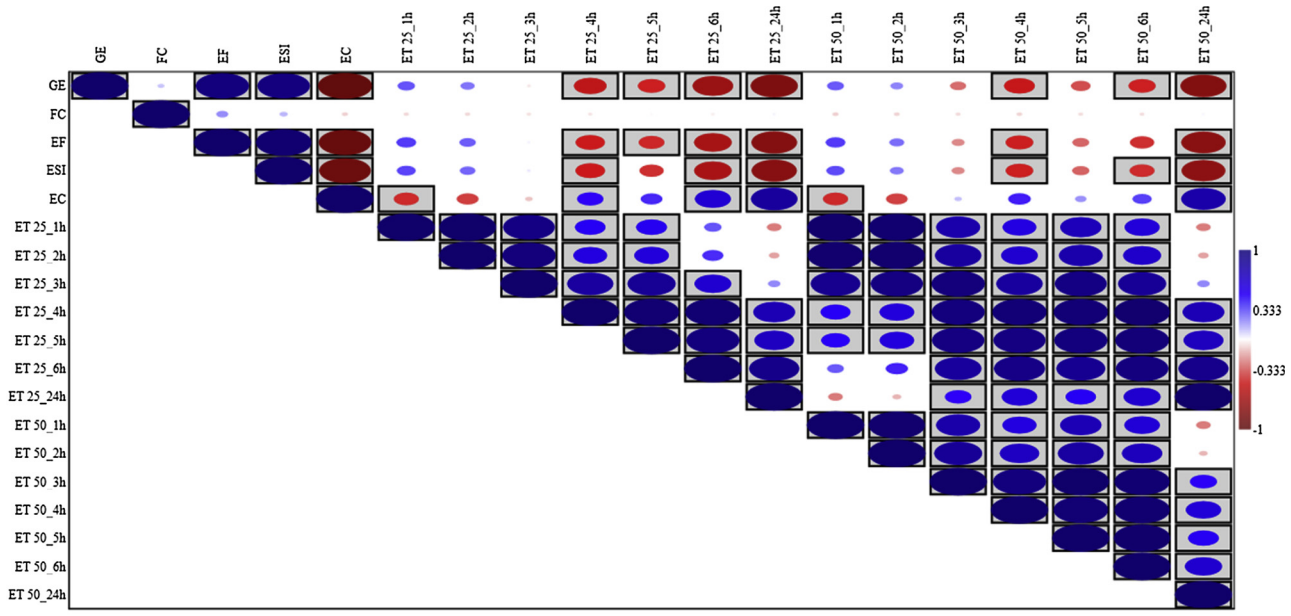
3.8. Principal components analysis (PCA)

It was performed to correlate the characteristics of enzyme activity (SOD, CAT, GPX, APX and ADH) and the ethanol test for seeds from lots 1 (lower vigor) and 6 (higher vigor) at periods of 0, 6 and 24 h of imbibition in melon seeds. The first two principal components (PC)

Table 2
Ethanol content released by 25 and 50 melon seeds soaked in 0.5 ml of distilled water as a function of time.

Lots	1h		2h		3h		4h		5h		6h		24h	
	25	50	25	50	25	50	25	50	25	50	25	50	25	50
							Seeds							
1	32.5 bB*	72.5 cA	57.5 bB	180.0 bA	195.0 bB	445.0 aA	255.0 aB	400 aA	385 aB	477.5 aA	477.5 aA	530 aA	1007.5 aA	780 aB
2	27.5 bB	62.5 cA	40.0 bA	100.0 cA	72.5 cA	95.0 cA	17.5 bB	140 bA	70.0 cA	22.5 cA	37.5 cA	7.5 dA	17.5 bA	2.5 bA
3	47.5 bB	75.0 cA	65.0 bA	107.5 cA	130.0 cA	150.0 bA	42.5 bA	42.5 bA	130 cA	40.0 cB	7.5 cA	12.5 dA	25 bA	12.5 bA
4	45.0 bB	112.5 bA	65.0 bB	185.0 bA	115.0 cA	185.0 bA	40.0 bA	87.5 bA	112.5 cA	97.5 bA	50.0 cA	52.5 dA	10 bA	10 bA
5	45.0 bB	112.5 bA	57.5 bB	227.5 bA	107.5 cB	202.5 bA	40.0 bA	140 bA	112.5 cA	137.5 bA	45.0 cB	107.5 cA	2.5 bA	17.5 bA
6	127.5 aB	280.0 aA	185.0 aB	595.0 aA	292.5 aB	457.5 aA	177.5 aB	385 aA	302.5 bB	442.5 aA	225 bB	397.5 bA	90 bA	60 bA
CV (%)	20.1	20.1	30.7	30.7	25.8	25.8	53.2	53.2	28.9	28.9	25.2	25.2	46.9	46.9

* Means followed by the same letter, lowercase in lines and upper case in columns, do not differ using Scott-Knott's test at the 5% probability level.



*Blue - positive correlation; red - negative correlation; size of the ellipses - correlation intensity; rectangles - significant correlation at the 5% probability level.

Fig. 1. Correlation between ethanol test (ET), first count (FC), germination (GE), emergence of field seedlings (EF), emergence speed index (ESI), and electrical conductivity (EC) of melon seeds.
*Blue - positive correlation; red - negative correlation; size of the ellipses - correlation intensity; rectangles - significant correlation at the 5% probability level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

enabled explanation of 97.1% of the variance contained in the original elements (Fig. 4). PC 1 and PC 2 contributed 69.4% and 27.7%, respectively, of the remaining variance. In PC 1, the SOD and CAT enzymes and the ethanol test had a higher association with seeds imbibed for 6 and 24 h for lot 1 (lower vigor). In PC 2, the GPX, APX and ADH enzymes were associated with seeds from the 24 -h imbibition of lot 6 (higher vigor). For the other periods of soaking, it was not possible to verify associations in the represented components, 1 and 2. There was a higher ratio of CAT and SOD enzyme activity with the ethanol test, due to the oxidative stress in the most deteriorated seeds, in which there is a greater action of ROS, and consequently the activity of these enzymes to fight free radicals. Furthermore, higher ethanol release was observed

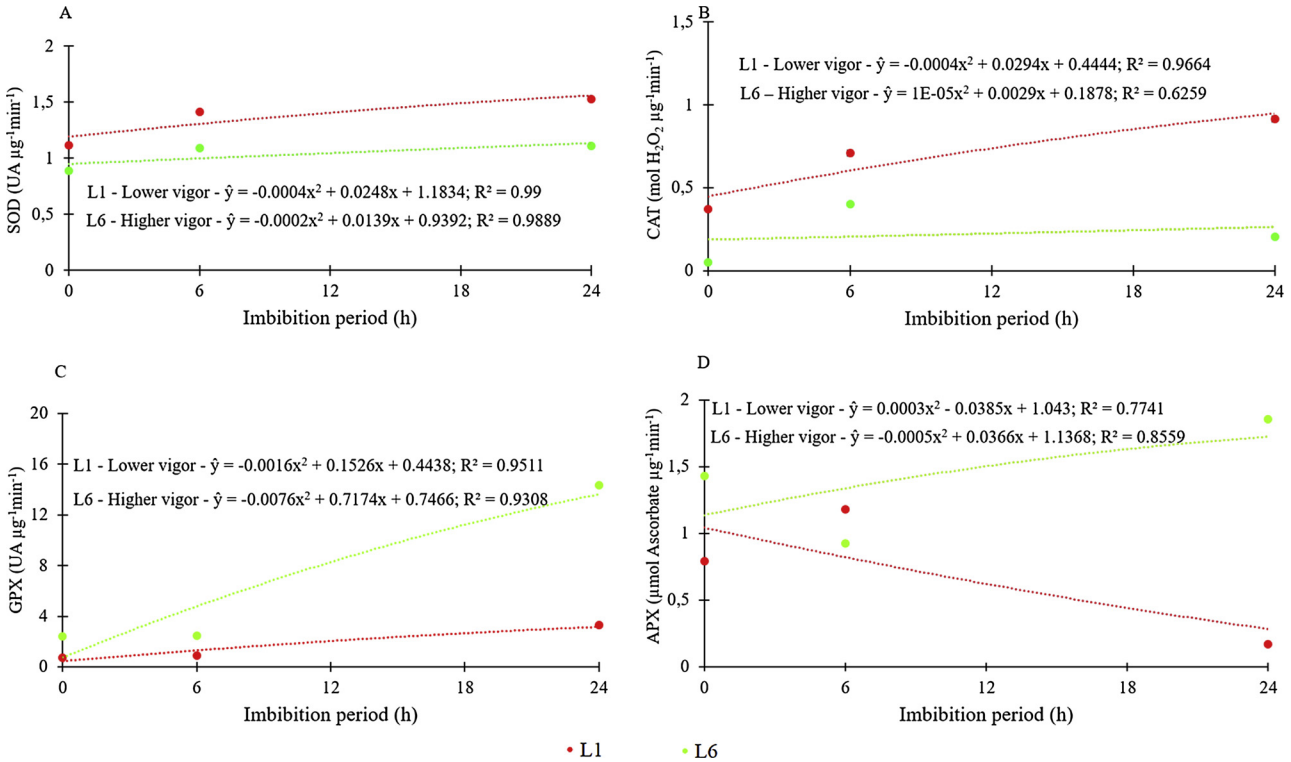


Fig. 2. Activity of antioxidant enzymes. A - Superoxide Disodium (SOD), B - Catalase (CAT), C - Guaiacol Peroxidase (GPX), and D - Ascorbate Peroxidase (APX) from lots L1 (lower vigor) and L6 (higher vigor) of the imbibition period.

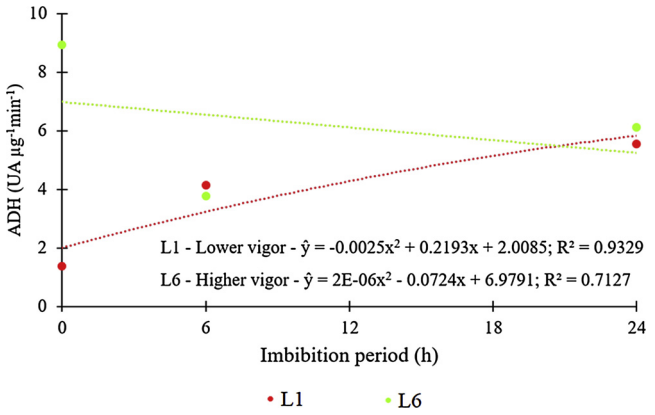


Fig. 3. Activity of fermentative enzyme. Alcoholic Dehydrogenase (ADH) from lots L1 (lower vigor) and L6 (higher vigor) as a function of imbibition period.

through the ethanol test.

3.9. Protein profile

Protein profile analysis showed that there was variation in the accumulation of proteins of specific molecular weights throughout the imbibition time. Seeds with lower vigor showed higher protein accumulation (˜ 20 to 45 kDa) for 6 and 24 h of imbibition (Fig. 5). Quantification of specific bands observed from the obtained protein profile (Fig. 5, red arrows 1–5) was carried out. The bands of interest presented between ˜ 20–45 kDa and represent five groups of proteins for which it was possible to verify variation in the intensity of the bands between the lots and the evaluated soaking periods. The quantification carried out with the aid of the GelQuant program showed an increase in the intensity of the bands of all five groups for the 24 h period of imbibition in the lower vigor lot. Group 1 of this lot did not present any relevant variation, except for the 6 h imbibition period. Analysis of the variation in the groups of proteins between the lots enabled different patterns of band intensity to be observed, generating different profiles between the lots for the five groups.

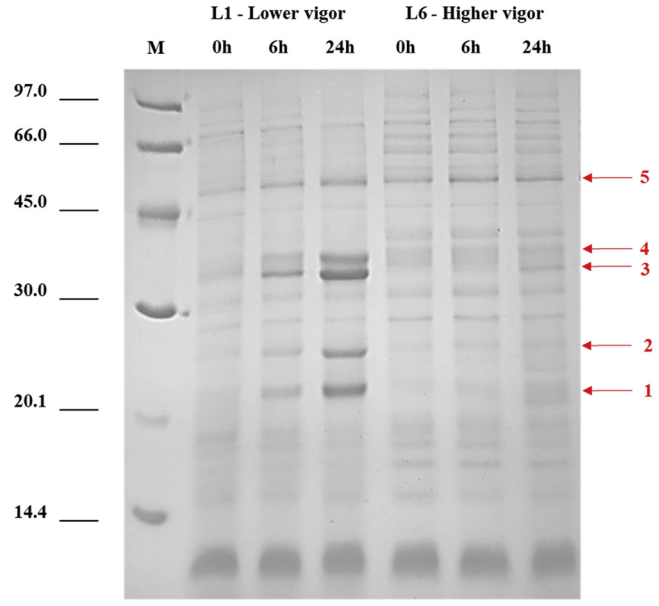


Fig. 5. Protein profile. SDS-PAGE of 10 µg of total soluble melon seed proteins, by lot, at different imbibition times. M- Molecular Marker (kDa): Low Molecular Weight Marker in kDa- GE Healthcare. Red arrows represent the groups of proteins of interest by the intensity variation of the bands. Quantification performed by the Bradford method with standard curve $R^2 = 0.99$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.10. Western blotting (WB)

Accumulation of ADH protein was verified by the Western Blotting hybridization technique with the primary Anti-ADH antibody (Agrisera®) (Fig. 6). In the membranes, single marks with molecular weight of approximately 35 kDa were observed for the 24 h imbibition period in the less vigorous lot. As the antibody used was commercial and defined based on the ADH protein of *Arabdopsis thaliana*, it was necessary to confirm the specificity of the antibody used in the experiment for the validation of the bands on the membranes. This

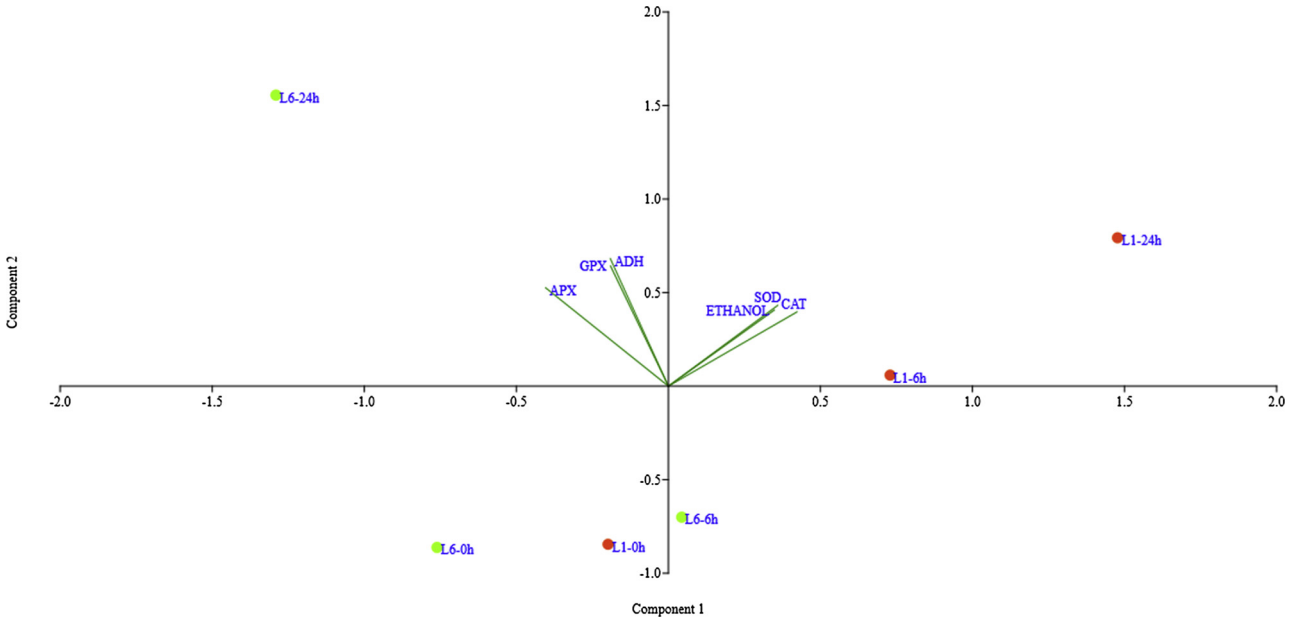


Fig. 4. Principal component analysis. Biplot showing the projection of the variables of the first two principal components with distinction in enzymatic activities (SOD, CAT, GPX, APX and ADH) and ethanol test of lots B1 (lower vigor) and B6 (higher vigor) in the periods of 0, 6 and 24 h of imbibition in melon seeds.

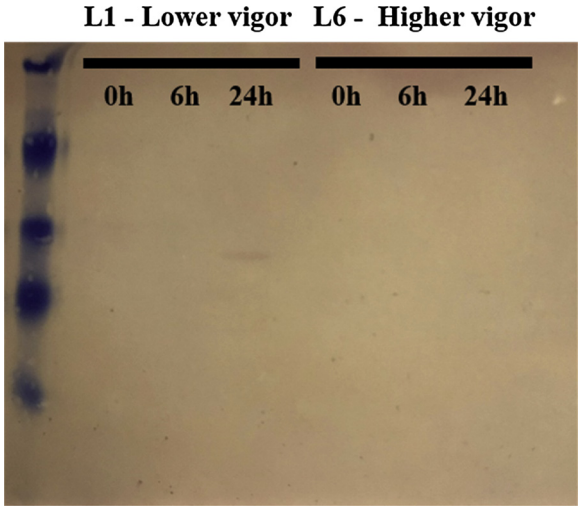


Fig. 6. Western Blotting: ADH accumulation in melon seed with lower and higher vigor at different imbibition times. M - Molecular Marker (kDa): Low Molecular Weight Marker - GE Healthcare.

analysis indicated that the melon ADH sequence obtained on the genomic information database (NCBI - https://www.ncbi.nlm.nih.gov/pubmed) has 41 kDa. The observed proximity between the calculated theoretical molecular weight values and the bands obtained with the hybridization enabled consideration of specific marking for melon ADH. Marking sensitivity was also observed for the antibodies in the membranes, as for the concentration of 10 µg of total proteins used, band intensity in the 24 h imbibition period for L1 was considerably clear. In the evaluation of gene expression, the protein profile showed that, among the lots of lower and higher vigor, there is synthesis and accumulation of differentiated proteins, according to imbibition time. For the ADH protein, linearity was shown only for the lot of lower vigor (24 h), as this can be attributed to the low amount of extract of this protein. In this case, more extract and longer exposure time would extend the linear dynamic range, but these data show that to use ADH as a reliable and quantifiable load control, a total protein load of not less than 20 µg is required. For the images this can already be attributed to the bands of interest, since the ADH bands of interest already reveal a protein load above 10 µg.

4. Discussion

The higher the moisture content in stored seeds, the greater the speed of the deterioration process, since the formation of products that cause immediate damage to the cells, such as free radicals, is accelerated. Thus, the water content of the lots needs to be tested, aiming at the lowest possible variation (Marcos-Filho, 2015). The degree of moisture influences the metabolic activity of the seed, interfering in the processes of germination and deterioration. However, the seed water content is in line with the recommendations, that is, it does not exceed 9%, for this vegetable (Peske et al., 2003). This is important for the execution of the ethanol and other vigor tests, when water content of the seeds is relatively low and homogeneous, there is greater reliability of the results obtained from physiological quality tests, because hydrolytic enzymes become more efficient as the seeds reach water content close to that necessary to begin germination (Tunes et al., 2011). However, if the minimum degree of moisture is not reached, the seed may deteriorate, since energy consumption to perform the mechanisms of repair or accumulation of digested products without the subsequent mobilization makes it impossible to continue the germination process (Copeland and Mcdonald, 1995).

The germination test does not detect seed deterioration progression, and only indicates the final stages of the germination process (Marcos-Filho, 2015). Using one or more vigor tests complements the results of the germination test, enabling better characterization of the physiological quality of a seed lot (Nakagawa, 1999). However, when vigor tests were used, such as the first count of germination that can distinguish marketable lots with germination at commercial standards (Kikuti and Marcos-Filho, 2012). There was a distinction between two levels of melon seeds. Lot 1 demonstrated as being lower than the other lots, which, in turn, did not differ from one other. In the case of the less vigorous lot, these conditions may have initiated the process of inactivation of the glycolytic pathway and activation of the fermentation pathway, since a reduction in seed germination is associated with natural aging and consequent loss of organic solutes, as well as increased respiratory activities (Moncaleano-Escandon et al., 2013).

The field seedling emergence test is an indicator of the efficiency of the tests to evaluate the physiological quality of seed lots (Marcos-Filho, 1999). According to Nakagawa (1999), deteriorated seeds tend to be more sensitive to biotic and abiotic factors in the field, there being excessive variation of temperature and relative air humidity, besides being subject to the attack of pests and diseases. In this regard, the field emergence test shows the real influence of vigor on germination. The higher the seedling emergence results in the field, the better the vigor of a given seed lot. Oliveira et al. (2009) state that ESI evaluates seedling emergence velocity under field conditions, that is, uncontrolled conditions, as the faster the emergence of seedlings, the greater the vigor of the seeds, the observations being carried out on a daily basis until emergence stabilization.

Seed vigor is directly related to the integrity of the cell membrane system (Silva et al., 2014). The greater the deterioration, the higher the amount of leachate, that is, seeds with low vigor release more electrolytes into the solution (Rosa et al., 2000).

In cabbage seeds, the results were similar, since the seeds with lower vigor showed higher ethanol production (Buckley et al., 2013). Quantification of the ethanol produced and released can provide important information related to the physiological quality of seeds, and it is an efficient, fast and relatively economical alternative potential indicator of vigor. Studies on the production of ethanol from seeds use gas chromatography or other laboratory techniques that require significant resources. The technology using the ethylometer, used for most analyses of detection and quantification of ethanol in the present study, is a quick and relatively inexpensive technique. The analytical potential of the ethylometer is adequate for the analysis of ethanol produced by seeds at the beginning of the germination process (Buckley et al., 2013).

SOD and CAT activities were similar as they were involved in the early events of germination and seedling growth, removing H_2O_2 during β-oxidation of fatty acids (Bewley et al., 2013). Additionally, these enzymes are involved in preservation and protection against ROS in the cells and tissues of the seeds, and the balance between these antioxidant enzymes is fundamental in the regulation of ROS levels within the cells (Abreu et al., 2014). SOD acts on the first line of defense against ROS, disrupting the superoxide radical ($O_2^{\bullet -}$) in hydrogen peroxide (H_2O_2) (Harter et al., 2014), followed by CAT, which neutralizes the toxic effect of H_2O_2, converting it into water and oxygen (Mhamdi et al., 2010). CAT is involved in the removal of hydrogen peroxides (H_2O_2) from cells, with increased activity associated with decreased mechanisms of oxidative damage prevention (Bailly et al., 1996).

In relation to the peroxidases, two plant compounds, guaiacol and ascorbate, were tested in this study. These two compounds act as electron-donating substrates for the degradation of H_2O_2 by the ascorbate peroxidase (APX) enzyme and guaiacol peroxidase (GPX), giving rise to H_2O (Asada, 1999). The peroxidases of ascorbate (APX) and guaiacol (GPX), as well as CAT, also act in the removal of H_2O_2 (Perl-Treves e Perl, 2002) with the aid of reducing agents.

GPX and APX require molecular reducing agents for intracellular dismutation of the accumulated peroxide. The presence of glutathione reductase (GR) in chloroplasts produces high levels of reduced

glutathione (GSH), which, in addition to regenerating ascorbate in APX activity, also participates in the regeneration of glutathione in an oxidized state (GSSG), formed in the reduction of H_2O_2 to H_2O by GPX (Apel and Hirt, 2004). The GPX enzyme preferably uses aromatic phenols, such as guaiacol or pyrogallol, although GPX can also oxidize ascorbate at a rate of 1% and are much lower than the consumption of guaiacol (Asada, 1999; Gill and Tuteja, 2010; Sharma et al., 2012). GPX is associated with many biosynthetic processes, including cellular wall lignification, indoleacetic acid degradation (AIA), ethylene biosynthesis, and defense against biotic and abiotic stresses in plants (Gill and Tuteja, 2010; Sharma et al., 2012).

Unlike CAT, ascorbate peroxidase (APX) requires cellular reducing agents with high specificity for ascorbate (or ascorbic acid) as a reducing substrate, catalyzing the neutralization reaction of H_2O_2 in H_2O (Carvezan, 2008), with formation of the monodehydroascorbate (MDA) molecule. This radical can be directly reduced to monodehydroascorbate reductase (MDAR), using NADPH as a reducing agent, or undergo dismutation and form dehydroascorbate (DHA). DHA is reduced to ascorbate by the action of DHAR, which uses GSH as an electron donor. This reaction results in GSSG, which is reduced again to GSH by NADPH, in a reaction catalyzed by the GR enzyme. This cycle is known as glutathione-ascorbate (Gratão et al., 2005).

According to Mittler (2002), the APX enzyme has a high affinity with H_2O_2, suggesting that it is responsible for the control of ROS levels for the purpose of molecular signaling, since it acts at low concentrations of H_2O_2. The research results reinforce that stated by Corte et al. (2010) and Nakada et al. (2010), who verified an increase in lipid peroxidation and increased seed deterioration. Therefore, a reduction in CAT activity can make the seeds more sensitive to the effects of free radicals, increasing the formation of peroxide in the cells, thus making the seeds subject to loss of viability, which is confirmed by the reduction in germination. In summary, the differences observed in the activities of the antioxidative enzymes in relation to the different times of imbibition of the seeds, shows a series of factors that interfere in or favor the activity of these enzymes, (Sivritepe et al., 2008; Kavas et al., 2013) and the balance between ROS levels and antioxidative systems in the prevention of oxidative stress in cells (Scandalios, 2005).

The action of the enzymes related to the energetic and antioxidant metabolism of the cell guarantees high seed vigor, since they present maximum physiological quality at maturity. Degenerative processes then begin to occur, whereby the loss of germination capacity is one of the final consequences (Spinola et al., 2000). In addition, these results also suggest that oxidative stress responses are more complex than a simple increase or decrease in the expression level of antioxidant enzymes (Wu et al., 2013).

Alcoholic dehydrogenase (ADH) enzyme is related to anaerobic respiration, catalyzing the conversion of acetaldehyde to ethanol, and vice versa, during fermentative metabolism, significantly reducing the accumulation of these toxic compounds. Said compounds accelerate seed deterioration, thus increasing ADH enzyme activity, which helps to protect these seeds against the damaging action of these compounds (Marcos-Filho, 2015). They also act in the respiratory process that removes toxic substances from the seeds, such as acetaldehyde and ethanol, which are produced when cells begin anaerobic respiration (Lima, 2008). The energy yield produced under low concentrations of O_2 is only two molecules of ATP per molecule of glucose in the glycolytic pathway, whereas 36 molecules of ATP are produced by oxidative phosphorylation under normal O_2 conditions (Zabalza et al., 2009). According to Bailey-Serres and Voesenek (2008), the low oxygen level causes changes in gene transcription, synthesis, and protein degradation in the cellular metabolism in *Arabidopsis thaliana* seeds, thus increasing ADH activity, which is related to expression of the ADH gene (gene involved in the fermentation) (Christianson et al., 2010).

The technique of principal components has been used to discard characters of low contribution and thus reduce labor, time and costs (Tobar-Tosse et al., 2015).

In Western Blotting (WB) the plateau observed for the same blot is not due to saturation of the detector, but is the result of protein saturation within the gel itself and consequently of the blot with the multiple layers of the target protein bound to the membrane (Taylor et al., 2013). To understand these proteins, it would be necessary to isolate these bands with the possible targets and perform mass spectrophotometry sequencing for said identification. Western Blotting (WB) served to quantify ADH-specific proteins. For most analytical techniques, the key to obtaining accurate and reproducible results is to understand the limits of the tools employed. WB involves complex steps and to obtain quantifiable results requires that all steps be performed rigorously, as most are interdependent.

In conclusion, this study showed that ethanol test, by modified ethylometer, when conducted with 25 seeds, partially soaked in 0.5 mL of distilled water, for 6 h, is a fast and efficient method, and can classify the seed vigor. There is a correlation between the germination tests, emergence of field seedlings, emergence speed index, and electric conductivity, with the ethanol test. There is a strong relationship between CAT and SOD activity with the ethanol test, due to the oxidative stress in the deteriorated seeds, with higher action of ROS, and consequently greater activity of these enzymes to combat free radicals and greater ethanol release. The variation in ADH protein accumulation demonstrates the adjustment of gene expression related to seed vigor and corroborates the biochemical activity observed for the antioxidant and fermentative metabolism proteins, as well as the total protein profile. The ethanol test can be used as a biochemical test to evaluate seed vigor in different melon cultivars as well as the cultivars of different species.

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