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RESEARCH ARTICLE

PETTENKOFER METHOD FOR ASSESSING THE QUALITY OF HABANERO PEPPER SEEDS

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ABSTRACT

Quick and reproducible methods for evaluating the physiological quality of seeds are needed for decision making in quality control programs. Therefore, the objective of this study was to evaluate the efficiency and quickness of Pettenkofer method to determine the respiratory activity to differentiate the vigor of five lots of habanero pepper seeds (*Capsicum chinenses jacquin*). It also determined the water content, germination test, seedling emergence and electrical conductivity in seeds of five lots. In addition to the physiological analysis it was also examined expression of esterase enzymes, catalase, malate dehydrogenase and alcohol dehydrogenase by electrophoresis technique. The experimental design was completely randomized with four replications. There was a correlation between the results of the respiratory activity and the tests used to evaluate the physiological quality of seeds. The results allowed the classification of lots habanero pepper seeds in different levels of physiological quality. The Pettenkofer system use is efficient and quick to distinguish quality levels of lots pepper seeds, which may be considered promising method to identify differences in physiological quality of lots pepper seeds.

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INTRODUCTION

Among the domesticated species of *Capsicum*, the Habanero is the most Brazilian of all pepper species. With the increase of the market and consequently the planted area there is greater demand for high quality seeds. Quick and reproducible methods for evaluating the physiological quality of seeds are needed for decision making during the stages of seed production (Mendes *et al*., 2009). Respiration is the oxidation of organic substances in the cellular system, with release of energy. The molecular oxygen is the final electron acceptor. Among the most common respiratory substrates are carbohydrates such as starch, sucrose, fructose, glucose and other sugars; lipids, especially triglycerides, organic acids and proteins (Taiz and Zeiger, 2009).With water absorption by imbibition occur tissues rehydration. With this, the first metabolic activity of the seeds is respiration (Dode et. al., 2013). The respiration on its initiation stage is almost zero, however it is observed an increase considered in relatively short time, depending on the species, after to start the imbibitions process. The activity and mitochondria integrity of viable embryos increase from the beginning of imbibition, making more efficient the ATP production, reflecting the increase in oxygen consumption and consequently higher CO_2 release (Marcos-Filho, 2005). Methods for determining respiration activity in seeds are related to the loss of dry mass and/or with gas exchange. However, determining the dry mass variation of seeds requires large amount of material and implies in its destruction (Marenco and Lopes, 2007). As for the methods based on gas exchange require minor amount of materials and are non-destructive. According Dode *et al*., 2012, determination of the sunflower seeds respiratory activity by the Pettenkofer physico-chemical method, wherein evaluating the amount of CO_2 released from the seeds' respiration per gram of seed per hour. It is effective to separate lots with different vigor levels. The Pettenkofer method for determining the respiratory rate is an alternative method, simple, practical, since it is fast and inexpensive to differentiate lots of soybean seeds as the vigor (Mendes *et al*., 2009). The respiratory activity determination of the habanero pepper seeds by Pettenkofer physical-chemical method can be a quick test alternative for use in quality control programs seeds. It is noteworthy that this test had positive correction with the physiological analysis and

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can also be one more alternative to be used as a vigor test. In this context, the objective was to evaluate the efficiency and quickness of the Pettenkofer method in determining the respiratory activity to differentiate the vigor of five lots of habanero pepper seeds (*Capsicum chinenses* jacquin).

MATERIALS AND METHODS

The research was conducted at the Seed Center Laboratory of the Agriculture Department - Federal University of Lavras (UFLA), in Lavras, MG - Brazil. Five lots of Habanero pepper seeds (*Capsicum chinenses* Jacquin) produced by Agristar seed company in the Goiás state were used. The respiratory activity of different seed lots were evaluated by Pettenkofer physical-chemical method. The device used consists of four bottles, the two first containing sodium hydroxide (NaOH) at 25%, which is intended to retain CO_2 of the ambient air; the third containing 100 grams of seeds being analyzed (storage bottle) free from CO_2 of the ambient air, and the fourth and final bottle containing barium hydroxide Ba $(OH)_2$ at 0.1 N concentration, which reacts with CO_2 from respiratory activity of the seeds, resulting in a white precipitate, the barium carbonate ($BaCO_3$). The bottles were sealed with a silicone stopper, connected by a hose with controlled air flow, allowing the regulation of its speed by observing bubbles formed in the bottles. The seeds of different lots, 100 grams, initially were imbibed between papers for two hours in BOD at 25 °C. This period previously was determined where test different imbibition periods every two hours, in a range from 0 to 10 hours, with the imbibition periods of two hours sufficient to measure the beginning of respiratory activity in the seeds. Then, they were placed in the storage bottle of the Pettenkofer device, where they remained for more two hours. After the stay period in the device were placed two aliquots of 10mL of the supernatant for titration. In each aliquot were added two drops of phenolphthalein reagent and then subjected to titration with hydrochloric acid (HCl) 0.1 N in burette 50 mL. The HCl volume spent until the turning point was noted. This volume is directly related to the CO_2 intensity determined by the Ba $(OH)_2$ solution, and CO_2 set from the respiration process. The results were expressed as amount of carbon dioxide released per gram of seed per hour ($\mu g.CO_2.g\ seed^{-1}.h^{-1}$). The device was installed inside a camera with controlled temperature (25 ºC ± 1), with no interference of this factor in the results. The simplified formula used:

$$(Lb - La) * \frac{1{,}1.10^5}{h * g}$$

where:

Lb: reagent blank reading (mL)
La: Sample reading (mL)
h: time spent on the device (hours)
g: mass seeds used (grams)

The five lots of seeds used in the respiration test were also subjected to the following tests and determinations: Water content: determined by oven method at 105 °C per 24 hours (Brazil, 2009), using two replicates one gram of seeds of each treatment. After this period, the seeds were maintained in desiccators until the samples cooling, and then determined dry weight. The results were expressed as a percentage. Germination test: sowing was carried out on two sheets of blotting paper, moistened with water at a ratio 2.5 times the weight of dry substrate, in plastic boxes type gerbox. The boxes were maintained in BOD under alternate scheme of light and temperature (20 °C/16 h in the dark and 30 °C/8 h in the light presence). Counts were performed at seven and fourteen days according to the Rules for Analysis of Seeds - RAS (Brazil, 2009). Each treatment consisted of four replicates with 50 seeds. The results were expressed as percentage of normal seedlings. To calculate the germination speed index were carried out daily readings of the number of normal seedlings (Maguire, 1962). Seedling emergence: sowing was carried out in plastic trays containing mixture of soil and sand. The trays were maintained in a growth chamber at 25 °C and watered daily.

The evaluations were daily from beginning seedling emergence, counting up the number of seedlings with cotyledonary leaves above the soil surface. The percentage of normal seedlings at 14 and 21 days after sowing was considered. It was also estimated the emergency speed index seedlings (Maguire, 1962). Each treatment consisted of four replicates with 50 seeds. Electrical conductivity: was conducted in the mass system with four replications of 50 seeds per treatment. The seeds were weighed and then placed in disposable plastic glasses with 25 mL of deionized water. After 24 hours of imbibition at a temperature 25 °C, the electrical conductivity was determined with aid of a conductivimeter, being the results expressed as µS/cm/g (Vidigal *et al*., 2008). Enzyme activity assessment: two samples were collected of 50 seeds from each treatment and were macerated in the PVP presence and liquid nitrogen in melting pot onto ice, and subsequently stored at -86ºC temperature. For the enzymes extraction was added the extraction buffer (Tris HCl 0.2 M pH 8 + 0.1% of β-mercaptoethanol) in a ratio 250µL per 100 mg powder of the seeds. The homogenized material in vortex and kept in the refrigerator overnight followed by centrifugation at 14000 rpm per 30 minutes at 4 °C. It was applied 60 µL of supernatant onto the gel. The electrophoretic run was on polyacrylamide gels system 7.5% (separating gel) and 4.5% (concentrating gel). The run was performed at 150 V per 4 hours. At the end of the run, the gels were revealed for Esterase (EST), Malate Dehydrogenase (MDH), Alcohol Dehydrogenase (ADH), and Catalase (CAT) according Alfenas *et al*. (2006). The gel/electrode system used was Tris-glycine pH 8.9.

The gels' evaluation was performed on transilluminator and is considered the variation of bands intensity. Statistical analysis: the experimental design was completely randomized with four replications in all tests. Pearson's simple linear correlation analysis for each vigor test and germination used to evaluate the physiological quality with Pettenkofer test, by Assistat software (Silva and Azevedo, 2009). Data were subjected to variance analysis with the aid of Sisvar® software and compared by Scott-Knott test at 5% probability.

RESULTS AND DISCUSSION

Lots of assessed pepper seeds were separated into four levels of quality when evaluated through germination tests and first

germination count (Table 1). For these tests, it was also possible to classify the lots 1 and 2 as of higher quality. Based on data from the first count, percentage and germination speed index (Table 1), lot 5 was classified as lower quality seeds.

Table 1. Average data of first germination count, germination percentage and germination speed index of lots of habanero pepper seeds

Lots	1st Count (%)	Germination (%)	Germination Speed Index (GSI)
1	86 a	98 a	11.60 a
2	84 a	91 a	10.21 a
3	68 b	86 b	9.76 a
4	53 c	73 c	6.90 b
5	36 d	66 d	3.76 c
CV (%)	9.96	3.73	11.20

Averages followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

Table 2 shows that the lots were separated into four statistically different levels by the seedling emergence test, lot 5 being considered the less vigorous, result also verified by the emergence speed index.

Table 2. Average data emergence first count, emergence and emergence speed index of lots of habanero pepper seeds

Lots	1st Count (%)	Emergence (%)	Emergence Speed Index (ESI)
1	19 a	88 a	9.87 a
2	12 b	76 a	9.66 a
3	11 b	67 b	8.38 a
4	6 b	58 c	4.74 b
5	3 b	27 d	1.69 c
CV (%)	55.38	13.49	19.84

Averages followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

By the seedling emergence test lots 3 and 4 were intermediate as quality. With the electrical conductivity test results (Table 3) it was possible to distinguish the lots into five vigor levels, and that with higher vigor lot 1, and the less vigor lot 5.The vigor is inversely proportional to the electrical conductivity reading. This test has been used in different species to the distinction of seed lots in relation to vigor, as example physic nut (Araujo *et al*., 2011) and ryegrass (Lopes and Franke, 2010).

Table 3. Average data electrical conductivity and respiratory activity by Pettenkofer method of lots of habanero pepper seeds

Lots	Electrical Conductivity ($\mu S\ cm^{-1}\ g^{-1}$)	Pettenkofer ($\mu g\ CO_2\ g\ seed^{-1}\ h^{-1}$)
1	5.10 a	39.5 a
2	6.60 b	37.0 b
3	8.40 c	34.0 c
4	10.31 d	28.3 d
5	13.22 e	15.5 e
CV (%)	7.23	4.79

Averages followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

In castor beans seeds were not possible to separate, by the individual method, lots of castor beans (Souza *et al*., 2009) at different deterioration levels. In the Pettenkofer's method respiration test results (Table 3) was observed the same trend for the other tests used to assess physiological quality of seeds, ie, could distinguish the lots of different vigor levels being possible to infer that the lots 1 and 2 had higher vigor than the others. The most vigorous seeds, beginning the imbibition process, probably took less time for the synthesis of new RNAs and DNA repair, thus, presenting greater respiratory activity. The activity and integrity of mitochondria of viable embryos increase from the beginning of imbibition, which makes more efficient the ATP production, reflecting the increase in oxygen consumption and consequent increase in carbon dioxide production (Bewley and Black, 1994).Thus, the most vigorous lot tends to breathe more than one lot with less vigor in the same period of time. By the Pettenkofer test was observed higher respiration with 39.5µg CO_2 g $seed^{-1}$ h^{-1}, at lot 1.As for lot 5 the lowest respiration value was found, 15.5µg CO_2 g $seed^{-1}$ h^{-1}, compared to other lots evaluated. Through other tests used to evaluate the physiological quality of five lots of seeds it was also observed worse physiological quality lot 5, and overall better quality of lots 1 and 2.There was significant correlation of test results used to evaluate the physiological quality of Habanero pepper seeds (Table 4). Table 4 - Simple linear correlation coefficients (r) among the analyzed tests.

Emergency (EM), emergence first count (1st EM), emergence speed index (ESI), germination (GER), germination first count (1st GER), electrical conductivity (EC) and Pettenkofer (PETT). * and ** significant at 5 and 1% probability, respectively.

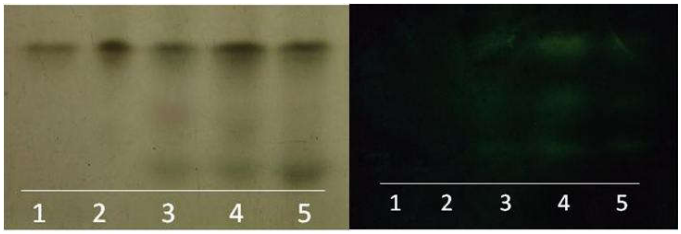

Figure 1. Enzymatic profile of esterase (A) and catalase (B) in habanero pepper seeds

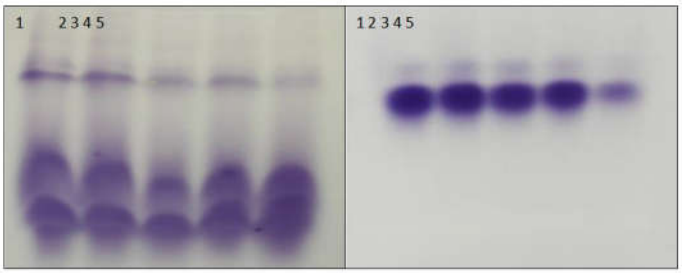

Figure 2. Enzymatic profile of malate dehydrogenase (A) and alcohol dehydrogenase (B) in habanero pepper seeds

The correlation values were higher than 0.8 being well accepted as a good correlation. Greater correlation between the emergence and Pettenkofer test (r = 0.9916) was observed. Lot 1 presents higher CO_2 value, then could be explained by the greater respiration of seeds with greater efficiency in the ATP production, this lot showed also high germination percentage, ie, their high performance in favorable conditions and the Pettenkofer method also offers these conditions (temperature and ideal humidity), the result was consistent. Regarding the

enzymes expression in habanero pepper seeds, it was observed variations in patterns in the different lots evaluated. The expression of esterase enzyme (EST) was higher in seeds of lots 4 and 5, characterized as lower quality, when evaluated by germination and vigor tests used in this study (Figure 1A).Such as EST is an enzyme that participates of the hydrolysis membrane esters and is directly linked to lipid metabolism and many of these lipids are constituents of membranes, the highest expression of this enzyme can be associated to the highest seeds deterioration observed in the seeds of lots 4 and 5.For the enzyme catalase (CAT), there was a higher expression in seeds of lots 3, 4 and 5, also characterized as having the worst quality by germination and vigor tests (Figure 1B).CAT for being an enzyme involved at process of removing hydrogen peroxide performs control of these endogenous peroxides via oxide-reduction cycle. Thus, the highest enzyme expression in seeds deteriorated can be due to the H_2O_2 formation, this function, also associated to the catalase.

It was possible to observe in lots of habanero pepper that there was higher activity of malate dehydrogenase (MDH) for the seeds of lots 1 and 2 (Figure 2A), classified as higher quality through germination and vigor tests. The MDH is an enzyme of the Krebs cycle which transforms malate into oxaloacetate producing a NADH, which is used to generate energy. Thus, this enzyme is linked to the power generation important metabolic processes such as seeds germination. Furthermore, participates in the malate movement through the mitochondrial membrane and CO_2 fixation of the plants (Taiz and Zeiger, 2009). As a result of the seeds deterioration process, there is a compromise of the respiratory activity of these, so observing a lower expression of this enzyme in the seeds of lot 5. Cruz *et al*. (2013) observed no changes in MDH activity in crambe seeds with vigor differences. Marini *et al*. (2012) found that there were changes in the action of this enzyme in rice seeds submitted to heat stress. For alcohol dehydrogenase (ADH) (Figure 2B), it was not possible to observe changes in the enzyme activity in seeds of lots 1, 2, 3 and 4 of seeds. In lot 5, due to its lower physiological quality, there was a decrease in the anaerobic respiration being less expressed the ADH enzyme activity. It is noteworthy that this enzyme reduces acetaldehyde to ethanol in anaerobic metabolism (Veiga *et al*., 2010). With the increased of ADH activity, the seeds are more protected against the acetaldehyde deleterious action (Albuquerque *et al*., 2009), thus justifying the lower enzyme activity in the seeds of the lot with the worst quality. Given these results infer that measurement of seeds' respiration by Pettenkofer method is a promising alternative to evaluate vigor in lots of habanero pepper seeds.

Conclusion

The Pettenkofer method use for determining the respiratory activity is efficient, fast and provides vigor differentiation in lots of habanero pepper seeds.

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