DOI: 10.5433/1679-0359.2017v38n3p1115

Carbon dioxide quantified by the infrared in respiratory activity evaluation in corn seeds

Dióxido de carbono quantificado pelo infravermelho na avaliação da atividade respiratória em sementes de milho

João Alexandre Lopes Dranski[1*]; Marlene de Matos Malavasi[2]; Ubirajara Contro Malavasi[3]; Ivan Schuster[4]; Norma Schlickmann Lazaretti[5]

Abstract

Respiratory activity is one of the first biological manifestations of vigor loss and detecting it can help the monitoring and quality control of seed production by seed industries. This research aimed to develop and validate the methodology of the carbon dioxide concentration in the evaluation of physiological quality of *Zea mays* (L.) seeds. The proposed method is grounded on the measurement of the CO_2 content by infrared through a drag system and exhaustion of the gas released by the seed maintained in a closed system up to the detection chamber of the gas meter. Samples of 15, 20 and 25 seeds of 6 lots of cultivar CD 384 Hx were incubated at temperatures of 15, 25 and 40 °C. The CO_2 content released after 1, 3, 6, 9, 12 and 24 h of incubation were quantified. In parallel, the percentage of normal seedlings emerged in the field were evaluated. The simple correlation coefficients among tests were calculated. After determining the reading conditions, the accuracy and precision of the proposed method were evaluated, using 15 seed lots. For the evaluation of respiratory activity in *Z. mays* seeds, we recommend a sample of 25 seeds, incubated at 15 °C for a maximum of 12 h, which allows to classify lots with different levels of vigor and predict the establishment of seedlings in the field, being the appropriate method for measuring CO_2 as it externalizes precision between successive measurements and agreement to the reference method.

Key words: *Zea mays* L. CO_2. Infrared spectroscopy. Validation.

Resumo

A atividade respiratória é uma das primeiras manifestações biológicas da perda de vigor e sua detecção pode auxiliar no monitoramento e no controle de qualidade da produção de sementes pelas indústrias sementeiras. Objetivou-se desenvolver e validar a metodologia da concentração de dióxido de carbono na avaliação da qualidade fisiológica de sementes *Zea mays* L. com base na espectroscopia no infravermelho. O método proposto quantifica o conteúdo de CO_2 através de um sistema de arraste e exaustão do gás liberado pelas sementes. Amostras de 15, 20 e 25 sementes, de 6 lotes da cultivar CD 384 Hx foram incubadas sob temperaturas de 15, 25 e 40 °C. Quantificou-se o conteúdo de CO_2 liberado após 1, 3 6. 9. 12, e 24 h de incubação. Paralelamente, avaliou-se a porcentagem de plântulas normais emergidas em campo. Foram calculados os coeficientes de correlação simples entre os testes.

[1] Biólogo Dr. Pesquisador, Universidade Estadual do Oeste do Paraná, Centro de Ciências Agrárias. Marechal Cândido Rondon, UNIOESTE, PR, Brasil. E-mail: joaodranski@yahoo.com.br
[2] Engª Agrª Profª Drª, UNIOESTE, Marechal Cândido Rondon, PR, Brasil. E-mail: marlenemalavasi@yahoo.com.br
[3] Engº Florestal, Prof. Dr., UNIOESTE, Marechal Cândido Rondon, PR, Brasil. E-mail: biramalavasi@yahoo.com.br
[4] Engº Agrº Dr. Pesquisador, Desenvolvimento, Produção e Comercialização Agrícola Ltda, COODETEC. Cascavel, PR, Brasil. E-mail: ivan@coodetec.com.br
[5] Engª Agrª, COODETEC, Cascavel, PR, Brasil. E-mail: nslazaretti@coodetec.com.br
[*] Author for correspondence

Received: Mar. 02, 2016 – Approved: Jan. 10, 2017

Após a determinação das condições de leitura, avaliou-se a exatidão e precisão utilizando 15 lotes de sementes. Para a avaliação da atividade respiratória em sementes de *Z. mays* recomenda-se uma amostra de 25 sementes, incubadas a 15 ºC por um período máximo de 12 h, o que possibilita classificar lotes em diferentes níveis de vigor e predizer o estabelecimento de plântulas em campo, sendo o método adequado para a mensuração de CO_2, pois externa precisão entre medidas sucessivas e concordância ao método de referência.
Palavras-chave: *Zea mays* L. CO_2. Espectroscopia no infravermelho. Validação.

Introduction

Zea mays (L.) is the main domesticated vegetable species of global economic importance among agronomic crops (BARBIERI; STUMPF, 2008). Brazil is one of the main producing countries, with expected harvesting of 81.5 million tons for the 2015/2016 growing year in the first and second crops, cultivated in 15.8 million hectares (USDA, 2016). The importance of corn drives the development in the field of seed technology, in order to guarantee greater quality control of seeds placed on the market.

Success in grain production depends in part on the quality of the seeds used (BEWLEY et al., 2013). With physiological maturity, seeds reach their maximum physiological potential based on their genetic and environmental characteristics and, at the same time, begin the process of natural and irreversible deterioration, which continues until the complete deterioration (KUMAR et al., 2015). As well as the agronomic and environmental crop conditions, the advance in the process of natural or intensified deterioration, by inadequate processing and storage, reduces seeds vigor in different intensities as a function of time (MARCOS FILHO, 2015). Therefore, evaluation of seed vigor is essential in quality control programs of seed production, in order to select lots with superior quality for commercialization.

Seed germination and seedling growth is an entirely heterotrophic phenomenon, depending on the respiratory metabolism. Oxidation of carbon from starch, lipids and proteins accumulated during maturation provides the energy required for germination, emergence and seedling development until the transition to autotrophy (MACHEREL et al., 2007).

During maturation, respiratory rate of orthodox seeds remains high and constant until deterioration process begins, followed by an abrupt decline due to dehydration, when all cellular metabolic processes enter the quiescent state (ROSENTAL et al., 2014), an adaptive strategy of anhydrobiotic organisms that makes it possible to preserve the structure and function of organelles throughout the dry state (LAW et al., 2014). Respiratory activity is resumed with the beginning of imbibition, leading to increased metabolic activity, which generates intermediate carbon compounds to support cell growth and cell division. However, the rehabilitation of the redox state is the metabolic function initially recovered, since it is necessary to promote the production of chemical energy through the transfer of electrons (WEITBRECHT et al., 2011).

The loss of seed vigor is accompanied by a change in the decarboxylation efficiency and by the number of active mitochondria with the beginning of the imbibition (ROSENTAL et al., 2014). This mitochondrial alteration occurs ahead of germination speed and seedling growth, since the production of adenosine triphosphate (ATP) is considerably lower and unable to support metabolic processes essential to germination, such as the synthesis of new proteins and nucleic acids (BEWLEY et al., 2013). Thus, seed respiration has been used as a parameter in the evaluation of seed vigor of different species (COPELAND; McDONALD, 2001).

Although studies have already presented methodological assumptions for the evaluation of CO_2 released by seeds, either via titration, micro

calorimetry, gas chromatography (MENDES et al., 2009; BUCKLEY; HUANG, 2011, SCHABES; SIGSTAD, 2011), or by infrared spectroscopy (DRANSKI et al., 2013; DANTAS et al., 2015), the proposed method suggests that modification of the collection and expression of results obtained by a gas analyzer must be preceded by methodological validation procedures (KATAOKA et al., 2011).

The assessment of CO_2 concentration by infrared consists of exhausting the contents of the gas released by the seeds in the detection chamber of the gas meter. The meter chamber remains with constant CO_2 flow and concentration, serving as the baseline, similar to gas chromatography [for didactic information on gas chromatography, we recommend reading Penteado et al. (2008)]. When the content is dragged, it is possible to detect and construct a peak similar to that of a chromatogram, the area of which represents its concentration, and therefore comparable.

Several factors may influence the outcome, such as temperature, seed moisture content, incubation time and sample size because temperature may alter the operation of routes and metabolic rates (TAIZ; ZEIGER, 2013), or reduce oxygen partial pressure as a function of a larger mass, influencing the availability of electron acceptors (BORISJUK; ROLLETSCHEK, 2009). The degree of humidity and incubation time are directly associated with the activation of the respiratory metabolism, the integrity and functionality of the internal membrane of the mitochondria and the capacity of remobilization of respiratory substrates (WANG et al., 2012).

Therefore, there is a need to define a methodology that makes it possible to separate seed lots more accurately, as these factors may affect respiratory rates. In addition, measurements of carbon dioxide concentration are non-destructive, allowing repeated measurements of a single seed or even a seed sample, either in the dry state or with ongoing germination, in addition to being quantified in a short period of time.

The present research had the objective of developing and validating the methodology of the concentration of carbon dioxide in the evaluation of the physiological quality of *Zea mays* L. seeds.

Materials and Methods

Sample of seeds: Were used six seed lots from S1 category of *Z. mays,* cultivar CD 384 Hx provided by the Central Cooperative of Agricultural Research – COODETEC, from the 2013/2013 crop, retained in sieves C1M and R2M, and previously treated with Derozal (60 mL Ton^{-1}), Actelic (40 mL Ton^{-1}), K-Obiol (60 mL Ton^{-1}) with a degree of humidity of 9.3 ± 0.3%, and 379.5 ± 36.7 g for the average weight of one thousand seeds. During the experiment, seeds were packed in Kraft paper bags and kept in a dry chamber adjusted to 20 ± 2 ºC with relative humidity of 40 ± 3%.

Characterization of physiological quality: germination test was conducted with four replicates of 100 seeds, using Germitest paper roll method, packed in a Mangelsdorf seed germinator and maintained at 25.0 ± 2.0 ºC with photoperiod of 12 hours. The results were expressed as percentage of normal seedlings, obtained on the seventh day after sowing, as recommended by the Rules for Seed Analysis (BRASIL, 2009).

To evaluate seed vigor, we used first germination count test, vigor by the tetrazolium test, cold test and the electrical conductivity test.

First germination count occurred simultaneously with germination test, accounting for the percentage of normal seedlings, obtained on the fourth day after sowing (BRASIL, 2009).

Seed vigor by the tetrazolium test used four replicates of 50 seeds in germination paper, soaked with water equivalent to 2.5 times its mass, for 16 h at 25 ºC. Seeds were sectioned longitudinally and placed in 100 mL plastic cups and added to the solution of 2,3,5-triphenyl tetrazolium chloride at 0.1%, and kept in a BOD-type chamber at 35 ºC

for three hours, then washed in running water and analyzed individually (DIAS; BARROS, 1999).

The cold test was conducted using the paper roll methodology, with four replicates of 50 seeds, as recommended by Dias and Barros (1995a). Seeds were placed in paper rolls soaked with deionized water, stored in sealed plastic bags and kept in a germination chamber previously regulated to 10.0 ± 2.0 ºC, for seven days. After words, the rolls were removed and transferred to a germination chamber adjusted to 25.0 ± 2.0 ºC where they remained for seven days. At the end, the percentage of normal seedlings was quantified.

The electrical conductivity test followed Dias and Barros (1995b), using four replicates of 25 seeds, packed in containers with 75 mL of deionized water and kept in a BOD chamber at 25 ºC for 24 h. The electrical conductivity values were obtained with a benchtop microprocessor conductivity meter.

At the same time, field emergency was carried out using four blocks of 100 seeds that were sown in October 2014, in grooves 5.0 m long and 3.0 cm deep, with groove spacing of 50 cm under field conditions, without irrigation. The percentage of emerged normal seedlings was computed on the fifteenth day after sowing (NAKAGAWA, 1994).

Development of the carbon dioxide concentration method

Development of the carbon dioxide concentration method: quantification of CO_2 was performed by assessing the CO_2 content released during respiration with a gas exchange meter IRGA (LI-COR 6400 XT). Coupled to the equipment, a CO_2-free air injection was fitted with an airflow compressor set at 140 mL min^{-1}, which injected air into a 50 mL vial, filled with soda lime, properly sealed and with a rubber septum. To connect the system formed by the compressor, soda lime container and sample vial were used plastic hoses with a diameter of 3.0 mm and a length of 15 cm, coupled with disposable hypodermic needles (1.6 mm x 40 mm) at the extremities. In the equipment, was attached a segment of plastic hose with the same characteristics described above, one end being fixed in the IRGA gas chamber and the other inserted in the seed sample bottle. Soon, two needles were inserted into the sample flask at the time of the reading, and then the air flow system was simultaneously run with the equipment.

We used four replicates of 15, 20 and 25 seeds per sample accommodated in 50 mL glass containers with deionized water with a volume sufficient to reach a humidity of 20% (m/m) according to Equation 1. We sealed the containers with a rubber septum for injection before incubation in BOD chambers at temperatures of 15, 25 and 40 ºC.

$$WV(g) = \left[\left(SW - \frac{SW - (SW * SM/100)}{1 - (0.01 * MCA)}\right)\right] \quad (1)$$

Where: SW – sample weight (g); SM – seed moisture content (%); MCA – moisture content to be achieved (%); WV – water volume.

Carbon dioxide concentration readings were taken after 1, 3, 6, 9, 12 and 24 h of incubation. To obtain the value of each repetition, the equipment was self-programmed to read CO_2 concentration every 0.5 s, until total exhaustion. The gas analyzer chamber was maintained at the constant concentration of 380 µmol of CO_2, with flux of 500 µmol s^{-1}. With the values of each repetition, gas peaks were adjusted to a log-normal distribution and obtained the peak area, according to Equation 2 proposed by Felinger (1998) and the results expressed in mmol of CO_2 per gram of seeds.

$$f(t) = \frac{A}{x} \quad EXP\left[-0.5\left(\frac{\ln(t - t0)}{\sigma}\right)^2\right] \quad (2)$$

Where: A = peak area (µmol CO_2);

Data analysis: evaluation of seed physiological quality followed a completely randomized design while field emergence used a randomized blocks. For the CO_2 concentration evaluation, a randomized block design was used in a factorial arrangement (6 x 6 x 3), formed by six seed lots, six time periods and three sample sizes. The incubation temperature factor was analyzed separately.

Data was tested for normality of the distribution of residues by the Shapiro-Wilk test and homogeneity of the variance by the Bartlet test. Subsequently, the data was submitted to analysis of variance. When there were statistically significant differences, batch means were grouped by the Scott-Knott test at 5% error probability. For the purpose of the time period, regression analysis was used. Subsequently, the simple correlation coefficients were obtained between the carbon dioxide concentration and field performance at 5% error probability by the t-test.

Validation of the carbon dioxide concentration method

Sample preparation: We used the validation parameters of precision and accuracy proposed by ISO 5725-2 (1994). Percentage of normal seedlings were evaluate using fifteen seed lots of *Z.mays* of cultivar CD 384 Hx category S1 provided by the Central Cooperative of Agricultural Research – COODETEC from 2013/2014 crop, retained in sieves C1M and R2M, and previously treated with Derozal (60 mL Ton^{-1}), Actelic (40 mL Ton^{-1}), K-Obiol (60 mL Ton^{-1}), with degree of humidity of 10.5 ± 0.4%.

The CO_2 concentration was read from twenty replicates of 25 seeds per sample with deionized water sufficient to reach 20% humidity (according to Equation 1) and incubated in BOD chambers at temperature of 15 ºC for 12 h.

The results were subjected to Grubbs test at 95% of probability error for detection of outliers.

Precision: The precision analysis was performed by repeatability analysis by comparing critical repeatability limit (CRL), with total amplitude (A) and by analysis of variance. For that, we read ten samples on two different days for the same seed lot. For the comparison of days by seed lot, we performed an analysis of variance at 5% probability.

Accuracy: The accuracy analysis was performed by comparing methods using the gas chromatography results as the reference. For this purpose, in four replicates per seed lot were extracted 2 mL samples with the aid of a 2.5 mL analytical syringe and injected into a gas chromatograph equipped with a methanator, flame ionization detector and hydrocarbon capillary column. The concentration of CO_2 was quantified by comparing the areas of the chromatographic peaks of the samples and the analytical standard of CO_2, with results expressed in mmol CO_2 g^{-1}. Simple correlation coefficient between the results from infrared and gas chromatography (reference) was calculated, and the t-test was applied at a 5% error probability to compare methods.

Results and Discussion

Determination of the carbon dioxide concentration method

Evaluation of the physiological quality indicated that there were significant differences for the percentage of normal seedlings from germination test (Table 1). Two groups were formed: one with the lowest viability from germination of 88% and the other with a mean of 95%, with all seed lots showing a germination percentage above the minimum (85%) recommended by the Normative Instruction 45/2013 of the Ministry of Agriculture, Livestock and Supply (BRASIL, 2013).

Table 1. Percentage of normal seedlings in the germination test (NS), in the first germination count (FGC), vigor by the tetrazolium (TZv) test, cold test (CF), electrical conductivity (EC) and field emergence (FE) of six seed lots of *Zea Mays* CD 384 Hx.

Seed lot	NS	FGC	TZv	CF	EC	FE
	---------- % ----------				$\mu S\ cm\ g^{-1}$	----- % -----
1	88b	55c	57e	89c	17.89a	91c
2	94a	54c	62d	94b	18.69a	94b
3	94a	59c	71c	95b	15.25b	94b
4	95a	63b	77b	95b	16.93b	96a
5	96a	64b	81a	98a	11.93c	97a
6	97a	73a	86a	97a	11.98c	98a
CV (%)	2.2	8.9	4.2	1.9	5.5	1.3

Means followed by the same letter in the column do not differ statistically from each other at the level of 5% probability of error by Scott-Knott test.

In the evaluation of vigor (Table 1), it was detected that lots 5 and 6 had the highest physiological potential, with a tendency to loss of speed in germination, as observed in seed lot 5 through the first germination count. However, seed lot 1 had the lowest physiological potential, because of its lower membrane integrity, with a higher percentage of seeds with visual damage, and its seeds were less tolerant to low temperatures.

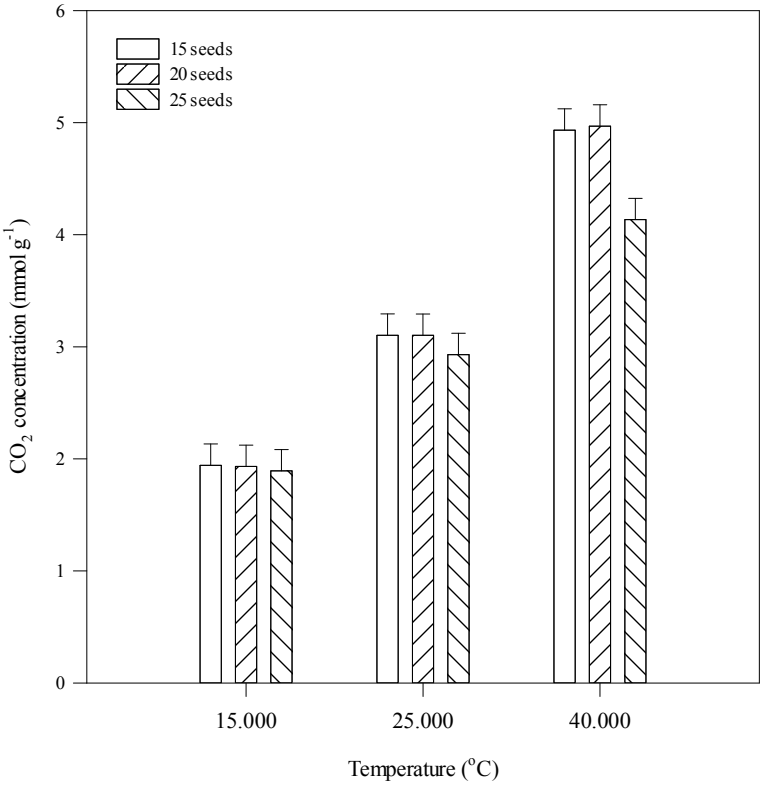
The performance in the field (Table 1), evaluated by the emergence of seedlings, allowed the formation of three groups, comprising lots 4, 5 and 6, lots 2 and 3, and batch 1, which had the lowest physiological quality in the lab evaluations. Although seed lot 4 had less vigor than seed lots 5 and 6, its field emergence was similar to those with higher vigor. Loss of viability is a result of physiological, biochemical, physical and ontogenetic changes occurring in advance, corroborating with the results of the vigor tests (BEWLEY et al., 2013).

There was an increase in the release of carbon dioxide as a function of the incubation temperature. Incubation at 40 ºC resulted in an increase of CO_2 of 144% and 53% higher in relation to samples incubated at 15 ºC and 25 ºC, respectively (Figure 1).

The positive relationship between temperature and respiratory activity reflects thermal requirement necessary for root production (PATANÈ; AVOLA, 2013), provided there is no limitation for humidity and oxygen. However, in the proposed method, the humidity is maintained at 20%, which would not be enough for germination. Therefore, under low humidity levels deterioration is encouraged and the energy requirement is now directed to the production of systems for the removal of reactive oxygen forms in seeds of lower physiological potential (DANTAS et al., 2015), because generation of redox state occurs in the first hours after imbibition (ROSENTAL et al., 2014).

Non-significant differences were detected (Figure 1) for the effect of seed number when incubated at 15 ºC or 25 ºC. The incubation at 40 ºC showed a reduction in the respiratory rate of seeds in samples with 25 seeds, indicating a higher oxygen consumption, resulting in less availability of required electron acceptors in the electron transport chain, and thus reducing the decarboxylative capacity (BORISJUK; ROLLETSCHEK, 2009).

Figure 1. Concentration of CO_2 released from seeds of *Zea mays* CD 384 Hx depending on the number of seeds in the sample and incubation temperatures.



According to the results of Figure 1, volume of glass container exerts a direct effect on CO_2 release from seeds of *Z. mays*. Therefore, use of temperatures above 25 ºC requires glass containers with bigger volume, allowing the quantification of CO_2 release in a closed system. On the other hand, lower temperatures attenuate respiratory activity through modulation of enzymatic kinetics (BANDEIRA et al., 2013), as well as to exclude the effect of the unavailability of oxygen in a closed system. Therefore, temperature of 15 ºC was preferred for measurement of seed physiological quality under the experimental conditions.

Concentration of CO_2 as a function of time period was adjusted to a three-parameter sigmoidal model for the different temperatures and number of seeds per sample (Figure 2). Seeds incubated at 15 ºC (Figure 2a) allowed to obtain the point of maximum inflection of the curve with 23.3 h (Table 2). This time period indicates that from this moment on the increase in CO_2 concentration is minimal and stability begins. On the other hand, the recognition of the maximum inflection point allows determining the maximum period of time necessary for the test to be interrupted, as there will be no significant accumulation of CO_2. In seeds incubated at 25 ºC (Figure 2b), seed samples expressed the point of maximum inflection of the curve with 11.13 h. For the seeds incubated at 40 ºC (Figure 2c), samples of 15 and 20 seeds expressed the point of maximum inflection of the curve with 4.99 h, while with samples of 25 seeds the point of maximum inflexion of the curve was reached with 4.88 h.

Figure 2. Concentration of CO_2 released from seeds of *Zea mays* CD 384 Hx as a function of number of seeds per sample and incubation period. (a) 15 ºC; (b) 25 ºC; (c) 40 ºC.

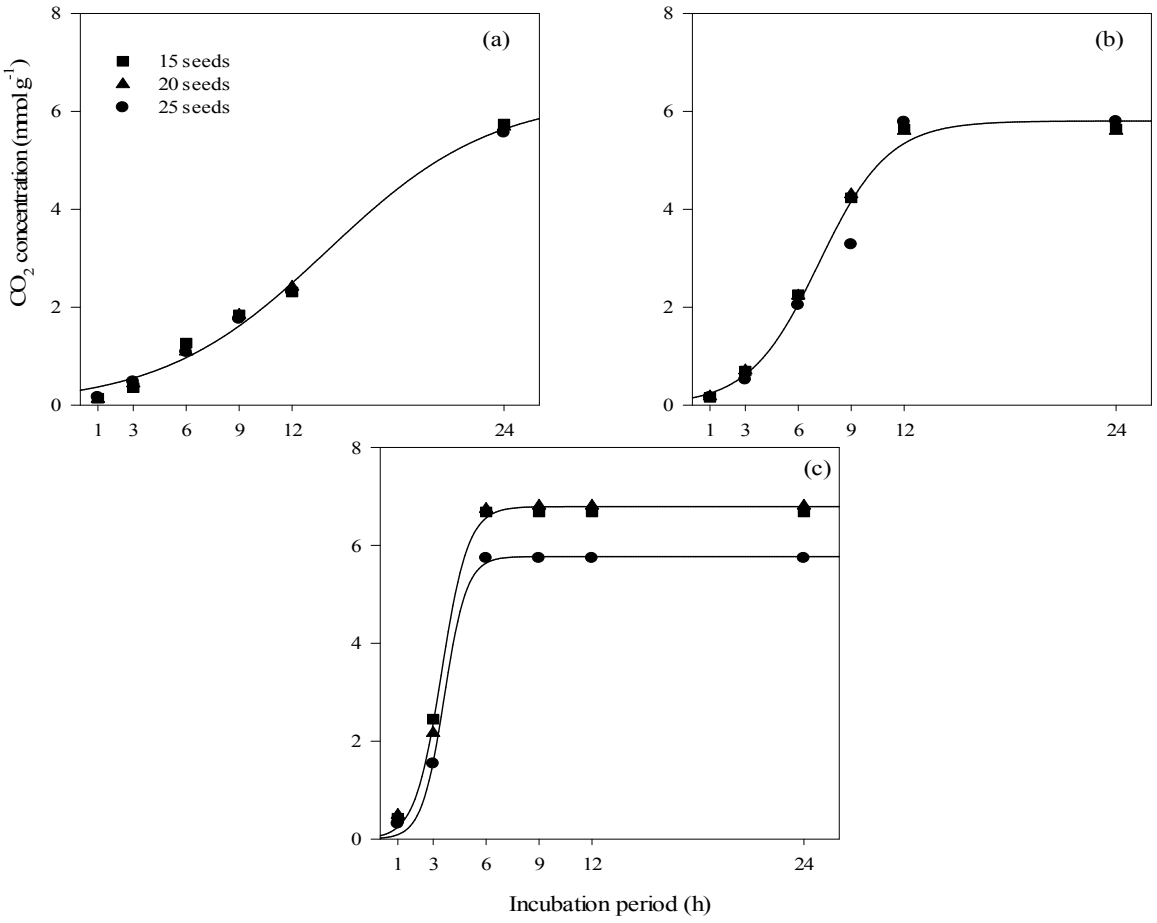


Table 2. Equations adjusted for the incubation temperature and number of seeds per sample as a function of incubation period.

Temperature (ºC)	Number of seeds	Equation	R^2	Maximum inflection (h)
15	15 20 25	f = 6.31/(1+exp(-(x-13.97)/4.68))	0.95**	23.33
25	15 20 25	f = 5.83/(1+exp(-(x-7.19)/1.97))	0.93**	11.13
40	15 20	f = 6.79/(1+exp(-(x-3.47)/0.76))	0.91**	4.99
	25	f = 5.77/(1+exp(-(x-3.62)/0.63))	0.93**	4.88

** Significant at 1% by t-test. Where (h): Period of time in decimal hours.

The maximum period of time the method allowed to quantify CO_2 was reduced with increasing incubation temperature. At temperatures of 15, 25 and 40 °C the reading time range was 24, 12 and 6 h, respectively.

The gas analyzer instrument has a working range of up to 89 mmol CO_2. Time intervals above mentioned intervals above mentioned reached the maximum limit of detection and differences in mean values occurred depending on sample weight. Seed imbibition before incubation or individual reading would be alternatives for the analysis of *Z. mays* seeds by infrared spectroscopy when the goal is to assess the respiratory activity during germination.

In order to compare the vigor of different lots, it is noted that the significant differences in seed lots incubated at 15 ºC was proportional to the number of seeds, because interpretation of the interaction between seed lots due to incubation period by number of seeds (Table 3), 15 seed sample allowed to separate the six seed lots into two groups after 12 h of incubation, while employing 25 seed sample resulted in three groups. For samples of 20 seeds and 12 h of incubation, segregation of seed lots resulted in stability indicating that lot 5 and 6 produced a lower concentration of CO_2, similar to that observed in the vigor tests (Table 1).

Table 3. Concentration of CO_2 released from seeds of *Zea mays* CD 384 Hx at 15 ºC as a function of number of seeds per sample, seed lot and incubation period.

Number of seeds	Seed lot	Incubation period at 15 ºC (h)					
		1	3	6	9	12	24
		---------- mmol g^{-1} ----------					
15	1	0.15a	0.43a	1.40a	2.20a	2.53a	7.03a
	2	0.13a	0.38a	1.38a	1.91a	2.52a	6.23b
	3	0.11a	0.36a	1.35a	2.01a	2.50a	5.96b
	4	0.19a	0.39a	1.27a	1.70b	2.26b	5.39b
	5	0.11a	0.26a	1.10a	1.51b	2.06b	4.79c
	6	0.15a	0.31a	1.08a	1.72b	1.99b	5.03c
20	1	0.14a	0.56a	1.39a	2.22a	2.78a	6.68a
	2	0.11a	0.42a	1.20a	2.02a	2.46b	5.99b
	3	0.10a	0.45a	1.11a	1.92a	2.50b	5.96b
	4	0.17a	0.53a	1.08a	1.61b	2.47b	5.47c
	5	0.11a	0.31a	0.85b	1.58b	2.30c	5.09d
	6	0.12a	0.36a	0.91b	1.70b	1.96c	4.91d
25	1	0.20a	0.59a	1.46a	2.06a	2.58a	6.82a
	2	0.14a	0.44a	1.15b	1.96a	2.49a	6.00b
	3	0.15a	0.54a	1.06b	1.89a	2.34b	5.55c
	4	0.22a	0.53a	1.03b	1.47b	2.31b	5.07d
	5	0.12a	0.30a	0.92b	1.67b	2.08c	4.92d
	6	0.14a	0.47a	0.89b	1.51b	1.75c	5.02d
CV (%)		12.6					

Means followed by the same letter in the column do not differ statistically from each other at the level of 5% probability of error by Scott-Knott test.

For samples incubated at 25 °C (Table 4), it was observed that independent of the number of seeds in the sample, only two groups were formed after 9 h of incubation. We inferred that at 25 °C seeds with intermediate vigor were not classifiable as observed in the other vigor tests (Table 1). The temperature of 25 ºC, considered optimal for seed germination, may have made it difficult to segregate seed lots, since the ability of a seed to germinate under a broad thermal limit is a manifestation of its vigor (PATANÈ; AVOLA, 2013). Thus, seeds that tolerate a greater thermal variation are the most vigorous and susceptible to segregation by respiratory activity.

Table 4. Concentration of CO_2 released from seeds of *Zea mays* CD 384 Hx at 25 ºC as a function of number of seeds per sample, seed lot and incubation period.

Number of seeds	Seed lot	Incubation period at 25 ºC (h)					
		1	3	6	9	12	24
		---------- mmol g^{-1}----------					
15	1	0.23a	0.73a	2.19a	5.15a	6.61a	6.61a
	2	0.12a	0.79a	2.49a	4.71a	5.85b	5.85b
	3	0.13a	0.72a	2.16a	4.39a	5.71b	5.71b
	4	0.18a	0.71a	2.37a	4.19b	5.99b	5.99b
	5	0.14a	0.58a	2.22a	3.57b	5.49b	5.49b
	6	0.15a	0.64a	2.09a	3.39b	4.18c	4.18c
20	1	0.22a	0.81a	2.34a	5.16a	6.70a	6.70a
	2	0.13a	0.76a	2.48a	4.81a	5.81b	5.81b
	3	0.19a	0.71a	2.13a	4.43a	5.89b	5.89b
	4	0.24a	0.74a	2.26a	4.11b	5.82b	5.82b
	5	0.13a	0.60a	2.28a	3.61b	4.83c	4.83c
	6	0.14a	0.62a	1.91a	3.40b	4.54c	4.54c
25	1	0.18a	0.64a	2.18a	3.66a	6.38a	6.38a
	2	0.13a	0.51a	2.13a	3.59a	6.22a	6.22a
	3	0.18a	0.57a	2.17a	3.44a	6.08a	6.08a
	4	0.20a	0.49a	2.08a	3.29b	6.07a	6.07a
	5	0.14a	0.44a	1.93a	3.03b	4.99b	4.99b
	6	0.14a	0.45a	1.76a	2.69b	4.96b	4.96b
CV (%)		10.9					

Means followed by the same letter in the column do not differ statistically from each other at the level of 5% probability of error by Scott-Knott test.

Although there was a reduction in CO_2 concentration because of the increase in the number of seeds incubated at 40 ºC (Figure 1) and limitations in the quantification of CO_2, by exceeding equipment working range after 6 h of incubation (Figure 3c), using a sample with 20 and 25 seeds incubated for 3 h allowed to separate the most vigorous seed lots (Table 5). Samples with 15 seeds could not classify seed lots at 40 ºC.

The simple correlation analysis between concentration of CO_2 under 15 ºC with the percentage of normal seedlings emerged in the field revealed a significant negative correlation (p<0.05) independent of the number of seeds from 6 h of incubation (Table 6). For samples incubated at 25 °C for 9 h and at 40 °C for 3 h, were calculated correlation coefficients higher than –0.82 a very high value as suggested by Davis (1971). These results indicate that field emergence was inversely proportional to the concentration of CO_2 released, so seed lots that release less CO_2 per mass unit are the ones with the best performance in the field.

Table 5. Concentration of CO_2 released from seeds of *Zea mays* CD 384 Hx at 40 ºC as a function of number of seeds per sample, seed lot and incubation period.

Number of seeds	Seed lot	Incubation period at 40 ºC (h)					
		1	3	6	9	12	24
		---------- mmol g^{-1}----------					
15	1	0.50a	2.89a	7.54a	7.54a	7.54a	7,54a
	2	0.33a	2.68a	6.80a	6.80a	6.80a	6,80a
	3	0.52a	2.48a	6.73a	6.73a	6.73a	6,73a
	4	0.46a	2.29a	6.46a	6.46a	6.46a	6,46a
	5	0.29a	2.22a	6.49a	6.49a	6.49a	6,49a
	6	0.45a	2.12a	6.06a	6.06a	6.06a	6,06a
20	1	0.63a	2.54a	7.51a	7.51a	7.51a	7.51a
	2	0.44a	2.43a	7.22a	7.22a	7.22a	7.22a
	3	0.63a	2.43a	7.02a	7.02a	7.02a	7.02a
	4	0.52a	2.16a	6.80a	6.80a	6.80a	6.80a
	5	0.33a	1.69b	6.05b	6.05b	6.05b	6.05b
	6	0.41a	1.72b	5.85b	5.85b	5.85b	5.85b
25	1	0.37a	1.75a	6.62a	6.62a	6.62a	6.62a
	2	0.22a	1.62a	6.00b	6.00b	6.00b	6.00b
	3	0.31a	1.81a	6.30b	6.30b	6.30b	6.30b
	4	0.42a	1.46a	5.88b	5.88b	5.88b	5.88b
	5	0.28a	1.13b	5.26c	5.26c	5.26c	5.26c
	6	0.27a	1.17b	4.37c	4.37c	4.37c	4.37c
CV (%)		9.8					

Means followed by the same letter in the column do not differ statistically from each other at the level of 5% probability of error by Scott-Knott test.

A lower rate of CO_2 release may favor a higher carbon economy, resulting in greater seedling growth and development, such as observed in the field emergence (Table 1). Additionally, it would be responsible for generating precursors for several biosynthetic routes directed to growth, not resulting however in the complete oxidation of organic compounds to CO_2 (HOPKINS; HÜNER, 2009).

If the respiratory rate exceeds cellular demand by ATP (low ATP / ADP ratio) the level of reduction in the mitochondria will be high and the alternative oxidase path will be activated, whose energy loss will occur by heat and not for energy conservation in the form of ATP. Therefore, the alternative oxidase enables mitochondria to adjust their relative rates of ATP production and synthesis of carbon skeletons according to metabolic requirements in order to conserve organic compounds, resulting in lower CO_2 release (TAIZ; ZEIGER, 2013). On the other hand, a high demand of chemical energy to support maintenance processes is necessary to keep mature cells in a viable state, since the use of energy for maintenance respiration includes the *de novo* synthesis of proteins and mRNAs for repair mechanisms, whose greater energy is released by organisms with higher level of deterioration (SIEDOW, 2010).

Table 6. Simple correlation coefficient between percentage of field emergence with the number of seeds, temperatures and periods of incubation.

Temperature (ºC)	Number of seeds	Incubation period (h)					
		1	3	6	9	12	24
15	15	0.11	-0.77	-0.91**	-0.88**	-0.92**	-0.85**
	20	-0.03	-0.69	-0.93**	-0.91**	-0.92**	-0.99**
	25	-0.37	-0.57	-0.94**	-0.91**	-0.90**	-0.94**
25	15	-0.49	-0.67	-0.22	-0.97**	-0.84**	-0.84**
	20	-0.39	-0.85**	-0.63	-0.97**	-0.94**	-0.94**
	25	-0.33	-0.94**	-0.88**	-0.93**	-0.85**	-0.85**
40	15	-0.34	-0.98**	-0.71	-0.71	-0.71	-0.71
	20	-0.73	-0.91**	-0.94**	-0.94**	-0.94**	-0.94**
	25	-0.17	-0.82**	-0.91**	-0.91**	-0.91**	-0.91**

** Significant at 5% probability of error by the t-test.

Loss of viability is the result of advanced degree of deterioration. However, before that state is reached, seeds lose their vigor at different rates. Such trend is observed when comparing field emergence results with CO_2 concentration in samples of 25 seeds at 15 ºC for 12 h, considering that three seed lots (lots 4, 5 and 6) with the highest emergence rate (97%) differ significantly in the respiratory rates, with a tendency to increase CO_2 release with the decline in viability. Therefore, quantification of CO_2 from respiration allows determination of seed deterioration before visualization of physiological manifestations that result in reduction of germinative power in the field.

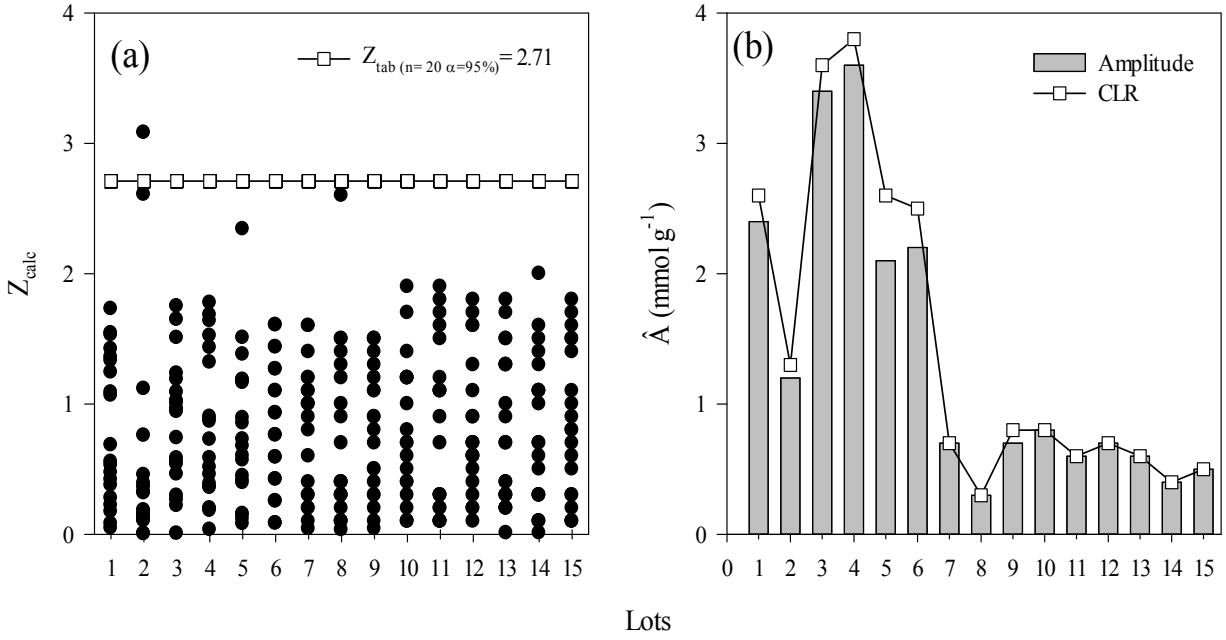
In the present research, the respiratory activity of seeds from different seed lots was higher the lower the physiological quality, in accordance with Dode et al. (2013) with seeds of *Glicine max* L., Martins et al. (2014) with seeds of *Z. mays* and Dode et al. (2016) with seeds of *Triticum aestivum* L., which observed an inverse relationship between CO_2 concentration released by seeds and their physiological quality.

The high correlation coefficients between evolved CO_2 with the percentage of emerged seedlings suggested that the methodology was efficient to predict the establishment of seedlings in the field independent of temperature. However, lower incubation temperature provided the greatest separation of seed lots resulting in greater sensitivity to detect differences in physiological potential of *Z. mays* seeds undetected by viability test.

Validation of the Analytical Method

The Grubbs test detected the presence of outliner in the seed lot 2 (Figure 3a) which was removed from the other analyses. This discrepant value is associated with the deterioration level of the seed lot which had an average viability of 37% (Table 7), with infestation by *Sitotroga cerealella* Oliver. Pest insects during storage reduce physiological quality of seeds, either by the consumption of their reserves or by the increase in respiratory activity (SMIDERLE; CICERO, 1999), leading to an increase in the variability of respiratory activity between seeds of the same lot. The level of mechanical damage and lesions of embryonic tissues resulting from predation by *S. cerealella* is inherent to each seed, which favors the diffusion of water by the tissues in damaged seeds.

Figure 3. (a) Extreme value test (Grubbs test) applied to the results of CO_2 concentration in fifteen seed lots of *Z. mays* CD 384 Hx. (b) Precision analysis by comparing the critical limit of repeatability (CLR) with the total amplitude (Â).



Therefore, the passage from dry state to the fluid of the cell membranes favors the rapid diffusion and capillarity of the water in seeds with higher level of deterioration (SHABAN, 2013), and the advance in the faster absorption of water causes the mitochondria present in dry seeds to resume their metabolic activity more quickly, resulting in greater release of CO_2 in corn seeds.

The evaluation of accuracy by comparison of critical limit of repeatability (CLR) indicated an acceptable repeatability for the 15 seed lots tested, since individual total amplitude results remained below the critical limit (Figure 3b). These results expressed the degree of agreement between the results of successive measurements of the same measurement (represented by seed lots) carried out under the same predetermined conditions.

According to the results obtained for normal seedlings (Table 7), the group that had an average germination rate of 97% resulted in the formation of two groups regarding concentration of CO_2 with averages of 2.50 and 2.23 mmol g^{-1} of CO_2 respectively. The above indicated sensitivity of the proposed method to detect differences in physiological potential of seeds with similar germination.

For seed lots from category S1 that had a germination rate below the minimum recommended by the Normative Instruction 45/2013 of MAPA, the values obtained for the CO_2 concentration ranged from 5.76 mmol g^{-1} with 80% germination to 8.50 mmol g^{-1} with 35% germination. Therefore, the increase in CO_2 release in seeds of lower vigor can reach twice that obtained from higher physiological potential.

According to the analysis of variance there were no significant differences from readings of different days (Table 8) for the fifteen seed lots indicating a high degree of precision. The results for the accuracy analysis showed agreement between the quantification by the infrared gas analyzer and the one obtained by gas chromatography (reference

method) considering that no significant differences were detected (p>0.05) between the methods by the t-test (Table 7). In addition, the correlation between the tests resulted in a simple correlation coefficient of 0.99 (p<0.0001) suggesting a linear and directly relationship to that obtained by gas chromatography.

Table 7. Accuracy analysis between mean concentration of CO_2 from the reference method and the proposed method by t-test, the simple correlation (R) and comparison of means of the proposed method and the percentage of normal seedlings (NS) of fifteen seed lots of *Z. mays* CD 384 Hx.

Seed lot	Reference method (n=3)	Proposed method (n=20)	NS	$p_{t\text{-test}}$
	---------- mmol g^{-1} ----------		--- % ---	
1	8.23 ± 1.56	8.50 ± 0.73 a	35 c	0.81
2	8.60 ± 1.25	8.25 ± 0.37 a	37 c	0.67
3	6.37 ± 1.00	6.70 ± 0.99 b	75 b	0.62
4	5.93 ± 1.17	6.52 ± 1.05 b	76 b	0.47
5	6.44 ± 1.99	6.43 ± 0.73 b	77 b	0.99
6	5.93 ± 1.24	5.76 ± 0.68 c	80 b	0.91
7	2.20 ± 0.17	2.55 ± 0.22 d	96 a	0.06
8	2.17 ± 0.26	2.50 ± 0.24 d	98 a	0.08
9	2.10 ± 0.26	2.43 ± 0.23 d	97 a	0.18
10	2.00 ± 0.20	2.30 ± 0.24 e	97 a	0.10
11	2.00 ± 0.26	2.28 ± 0.18 e	97 a	0.22
12	2.00 ± 0.10	2.24 ± 0.21 e	97 a	0.10
13	1.93 ± 0.15	2.22 ± 0.17 e	99 a	0.06
14	1.96 ± 0.38	2.21 ± 0.12 e	98 a	0.38
15	1.90 ± 0.20	2.17 ± 0.15 e	96 a	0.15
R	0.99 p<0.0001			

Where: $p_{t\text{-test}}$ e p = Probability of significance by the two-tailed t-test. Means followed by the same lowercase letter in the column do not differ statistically from each other at 5% error probability by Scott-Knott test.

Table 8. Evaluation of repeatability from different days of *Z. mays* CD 384 Hx seeds.

Seed lot	First day (n=10)	Second day (n=10)	ANOVA		
	---------- mmol g^{-1} ----------		Mean square	F_{calc}	p
1	8.49 ± 0.67	8.51 ± 0.82	0.0026	0.0046	0.946
2	8.35 ± 0.41	8.17 ± 0.32	0.4500	3.2400	0.089
3	6.71 ± 0.99	6.69 ± 1.05	0.0008	0.0007	0.978
4	6.74 ± 1.06	6.32 ± 1.06	0.8450	0.7510	0.397
5	6.35 ± 0.80	6.49 ± 0.70	0.1000	0.1770	0.679
6	5.51 ± 0.78	6.02 ± 0.51	1.2750	2.9460	0.103
7	2.47 ± 0.21	2.61 ± 0.19	0.1080	2.7100	0.117
8	2.46 ± 0.11	2.56 ± 0.32	0.2000	0.7830	0.389
9	2.36 ± 0.14	2.50 ± 0.29	0.8000	0.2222	0.074
10	2.33 ± 0.28	2.28 ± 0.18	0.0106	0.1870	0.679
11	2.27 ± 0.12	2.29 ± 0.22	0.0034	0.1090	0.745
12	2.27 ± 0.24	2.22 ± 0.17	0.0135	0.3130	0.583
13	2.19 ± 0.11	2.26 ± 0.22	0.0268	0.8900	0.358
14	2.16 ± 0.10	2.25 ± 0.12	0.0404	3.2830	0.087
15	2.20 ± 0.13	2.11 ± 0.15	0.0407	2.0930	0.165

Conclusions

For the evaluation of respiratory activity in seeds of *Z. mays* by means of CO_2 concentration, it is recommended a sample of 25 seeds, incubated at 15 ºC for a maximum period of 12 h, which makes it possible to classify seed lots with different levels of vigor and to predict the establishment of seedlings in the field.

Based on intra-laboratory validation, this methodology is adequate for measurement of CO_2 in seeds due to external precision between successive measurements and agreement to a reference method.

Acknowledgement

We thank the Central Cooperative of Agricultural Research – COODETEC for the donation of seed lots, the Araucária Foundation for Support to the Scientific and Technological Development of Paraná – Fundação Araucária (grant number 35098 – 285/2013), the Coordination for the Improvement of Higher Education Personnel – CAPES and the National Council for Scientific and Technological Development – CNPq for granting scholarships and financial support to the project.

References

BANDEIRA, J. M.; MARINI, P.; MARTINS, A. B. N.; BORBA, I. C. G.; AMARANTE, L.; MORAES, D. M.; Relationships between the physiological and biochemical modifications in soybean seeds under different temperatures. *African Journal of Agricultural Research*, Lagos, v. 8, n. 43, p. 5369-5375, 2013.

BARBIERI, R. L.; STUMPF, E. R. T. *Origem e evolução de plantas cultivadas*. Brasília: Embrapa Informação Tecnológica, 2008. 909 p.

BEWLEY, J. D.; BRADFORD, K. J.; HILHORST, H. W. M.; NONOGAKI, H. *Seeds*: physiology of development, germination and dormancy. New York: Springer, 2013. 392 p.

BORISJUK, L.; ROLLETSCHEK, H. The oxygen status of the developing seed. *New Phytologist*, Lancaster, v. 182, n. 1, p. 17-30, 2009.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n. 45, de 17 de setembro de 2013. *Diário Oficial [da] República Federativa do Brasil,* Brasília, 18 set. 20013. Seção 1. p. 16-37.

_____. Ministério da Agricultura, Pecuária e Abastecimento. *Regras para análise de sementes.* Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília, DF: MAPA/ACS, 2009. 395 p.

BUCKLEY, W. T.; HUANG, J. An ethanol-based seed vigour assay for canola. *Seed Science and Technology,* Cambridge, v. 39, n. 2, p. 510-526, 2011.

COPELAND, L. O.; MCDONALD, M. B. *Principles of seed science and technology*. 4th ed. Massachusetts: Kluwer Academic Publishers, 2001. 488 p.

DANTAS, B. F.; SILVA, R. C. B.; RIBEIRO, R. C.; ARAGÃO, C. A. Respiration and antioxidant enzymes activity in watermelon seeds and seedlings subjected to salt and temperature stresses. *American Journal of Experimental Agriculture,* Wilmington, v. 7, n. 2, p. 70-77, 2015.

DAVIS, J. A. *Elementary survey analysis.* Englewood: Prentice-Hall, 1971. 206 p.

DIAS, M. C. L. L.; BARROS, A. S. R. *Avaliação da qualidade de sementes de milho.* Londrina: IAPAR, 1995a. 43 p. (IAPAR. Circular, 88).

DIAS, D. C. F. S.; MARCOS FILHO, J. Testes de vigor baseados na permeabilidade das membranas celulares: I – Condutividade elétrica. *Informativo ABRATES*, Londrina, v. 5, n. 1, p. 26-36, 1995b.

DIAS, M. C. L. L.; BARROS, A. S. R. Metodologia do teste de tetrazólio em sementes de milho. In: KRZYZANOWSKI, F. C.; VIEIRA, R. D.; FRANÇA NETO, J. B. (Ed.). *Vigor de sementes*: conceitos e testes. Londrina: ABRATES, 1999. p. 8.4.1-8.4.10.

DODE, J.; ALMEIDA, A. S.; DEUNER, C.; BORGES, C. T.; MENEGHELLO, G. E.; VILLELA, F. A.; MORAES, D. M. Respiratory activity in wheat seeds as related to physiological quality. *Bioscience Journal*, Uberlândia, v. 32, n. 5, p. 1246-1253, 2016.

DODE, J.; MENEGHELLO, G. E.; TIMM, F. C.; MORAES, D. M.; PESKE, S. T. Teste de respiração em sementes de soja para avaliação da qualidade fisiológica. *Ciência Rural*, Santa Maria, v. 43, n. 2, p. 193-198, 2013.

DRANSKI, J. A. L.; PINTO JÚNIOR, A. S.; HERZOG, N. F. M.; MALAVASI, U. C.; MALAVASI, M. M.; GUIMARÃES, V. F. Vigor of canola seeds through quantification of CO_2 emission. *Ciência e Agrotecnologia*, Lavras, v. 37, n. 3, p. 229-236, 2013.

FELINGER, A. *Data analysis and signal processing in chromatography.* Amsterdam; Oxford: Elsevier, 1998. 414 p.

HOPKINS, W. G.; HÜNER, N. P. A. *Introduction to plant physiology*. 4. ed. Hoboken: John Wiley & Sons, 2009. 528 p.

INTERNATIONAL STANDARD ORGANIZATION – ISO. Accuracy (trueness and precision) of measurement methods and results. Part 2. *Basic method for the determination of repeatability and reproducibility of a standard measurement method* (*ISO 5725–2:1994*). Geneva: International Organization for Standardization, 1994. 42 p.

LAW, S. R.; NARSAI, R.; WHELAN, J. Mitochondrial biogenesis in plants during seed germination. *Mitochondrion*, Birmingham, v. 19, Part B, p. 214-221, 2014.

KATAOKA, V. Y.; CARVALHO, M. L. M.; OLIVEIRA, M. S.; CALDEIRA, C. M. Validação de metodologia para o teste de germinação em sementes de nabo forrageiro (*Raphanus sativus* L. var. *oleiferus*). *Revista Brasileira de Sementes*, Londrina, v. 33, n. 1, p. 69-79, 2011.

KUMAR, S. P.; PRASAD, S. R.; BANERJEE, R.; THAMMINENI, C. Seed birth to death: dual functions of reactive oxygen species in seed physiology. *Annals of Botany,* Exeter, v. 116, n. 4, p. 663-668, 2015.

MACHEREL, D.; BENAMAR, A.; AVELANGE-MACHEREL, M.-H.; TOLLETER, D. Function and stress tolerance of seed mitochondria. *Physiologia Plantarum*, Lund, v. 129, n. 1, p. 233-241, 2007.

MARCOS FILHO, J. *Fisiologia de sementes de plantas cultivadas*. 2. ed. Londrina: Abrates, 2015. 560 p.

MARTINS, A. B. N.; MARINI, P.; BANDEIRA, J. M.; BORBA, I.C. G.; QUINEPER, R. R.; MORAES, D. M.; VILLELA, F. A. Vigor: separação de lotes de sementes pela atividade respiratória. *Interciência*, Caracas, v. 39, n. 4 p. 260-265, 2014.

MENDES, C. R.; MORAES, D. M. de; LIMA, M. G. S.; LOPES, N. F. Respiratory activity for the differentiation of vigor on soybean seeds lots. *Revista Brasileira de Sementes,* Londrina, v. 31, n. 2, p.171-176, 2009.

NAKAGAWA, J. Testes de vigor baseados na avaliação das plântulas. In: VIEIRA, R. D.; CARVALHO, N. M. (Ed.). *Testes de vigor em sementes.* Jaboticabal: FUNEP, 1994. p. 49-85.

PATANÈ, C.; AVOLA, G. A seed respiration-based index of cold-sensitivity during imbibition in four macrothermal species. *Acta Physiologiae Plantarum,* Nungambakkam, v. 35, n. 3, p. 911-918, 2013.

PENTEADO, J. C. P.; MAGALHÃES, D.; MASINI, J. C. Experimento didático sobre cromatografia gasosa: uma abordagem analítica e ambiental. *Química Nova*, São Paulo, v. 31, n. 8, p. 2190-2193, 2008.

ROSENTAL, L.; NONOGAKI, K.; FAIT, A. Activation and regulation of primary metabolism during seed germination. *Seed Science Research,* Cambridge, v. 24, n. 1, p. 1-15, 2014.

SCHABES, F. I.; SIGSTAD, E. E. Monitoring soybean seed germination by calorimetry. *Journal of Thermal Analysis and Calorimetry,* Berlin, v. 104, n. 1, p. 5-11, 2011.

SHABAN, M. Review on physiological aspects of seed deterioration. *International Journal of Agricultural and Crop Sciences,* Ballards Lane, v. 6, n. 11, p. 627-631, 2013.

SIEDOW, J. N.; MOLLER, I. M.; RASMUSSON, A. Does respiration reduce crop yields? In: TAIZ, L.; ZEIGER, E. (Ed.). *A Companion to plant physiology.* 5. ed. Sunderland: Sinauer Associates, 2010. p. 7-11.

SMIDERLE, O. J.; CICERO, S. M. Tratamento inseticida e qualidade de sementes de milho durante o armazenamento. *Scientia Agricola*, Piracicaba, v. 56, n. 4, p. 1245-1254, 1999.

TAIZ, L.; ZEIGER, E. *Fisiologia vegetal*. 5. ed. Porto Alegre: Artmed, 2013. 918 p.

UNITED STATES DEPARTMENT OF AGRICULTURE – USDA. World Agricultural Production. Washington: Circular Series, 2016. 16 p.

WANG, W. Q.; CHENG, H. Y.; MOLLER, M.; SONG, S. Q. The role of recovery of mitochondrial structure and function in desiccation tolerance of pea seeds. *Physiologia Plantarum*, Lund, v. 144, n. 1, p. 20-34, 2012.

WEITBRECHT, K.; MÜLLER, K.; LEUBNER-METZGER, G. First off the mark: early seed germination. *Journal of Experimental Botany*, Oxford, v. 62, n. 10, p. 3289-3309, 2011.