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AGRARIAN SCIENCES

High-throughput phenotyping of brachiaria grass seeds using free access tool for analyzing X-ray images

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Abstract: New approaches based on image analysis can assist in phenotyping of biological characteristics, serving as support for decision-making in modern agribusiness. The aim of this study was to propose a method of high-throughput phenotyping of free access for processing of 2D X-ray images of brachiaria grass (*Brachiaria ruziziensis* cv. Ruziziensis) seeds, as well as correlate the parameters linked to the physiological potential of the seeds. The study was carried out by means of automated analysis of X-ray images of seeds in which a macro, called *PhenoXray*, was developed, responsible for digital image processing, for which a series of descriptors were obtained. After the X-ray analysis, a germination test was performed on the seeds and, from this, variables related to the physiological quality of the seeds were obtained. The use of the macro *PhenoXray* allowed large-scale phenotyping of seed X-rays in a simple, rapid, robust, and totally free manner. This study confirmed that the methodology is efficient for obtaining morphometric data and tissue integrity data in *Brachiaria ruziziensis* seeds and that parameters such as relative density, integrated density, and seed filling are closely related to the physiological attributes of seed quality.

Key words: automated image analysis, *Brachiaria ruziziensis*, ImageJ, *PhenoXray,* relative density, seed X-rays.

INTRODUCTION

Food security is one of the biggest current challenges faced by the world (Godfray et al. 2010). Therefore, innovative approaches are necessary to resolve problems of food production and sustainability never experienced before (Foley et al. 2011).

The seeds used for the establishment of production fields constitute a fundamental part of this problematic and, in many agricultural crops, their stability and uniformity is strictly related to yield at the end of the cycle. Thus, phenotypic information of morphometric traits of seeds can be valuable from the perspective of plant breeding (Tanabata et al. 2012) and are often related to physiological attributes, such as vigor (Whan et al. 2014), essential for initial development of plants in the field (Finch-Savage & Bassel 2016).

Recently, non-invasive optical technologies based on electromagnetic radiation have been adapted to the large area of plant sciences and have provided a range of possibilities for applications in seed studies (Hughes et al. 2017, Rolletschek et al. 2015, Rousseau et al. 2015). Among these technologies, the X-ray test has been successfully applied for non-destructive evaluation of indicators that are not visible in seeds (Huang et al. 2015, Kotwaliwale et al. 2014, Rahman & Cho 2016). Several studies have recommended the use of the X-ray technique for analysis of inner seed morphology, seeking to obtain faster and more accurate diagnoses regarding physical characteristics of interest, often related to seed germination and vigor (Abud et al. 2018, Arruda et al. 2018, Medeiros et al. 2018, Nielsen et al. 2017, Noronha et al. 2018).

In this respect, diverse characteristics of the seeds, especially those related to inner morphology, can make important contributions to quality control programs of seed production companies, as well as to the area of plant breeding, as for example, to genome selection. The current challenge consists of developing robust tools of low cost and high throughput that are capable of generating reliable phenotypic data to measure characteristics of the seeds on a large scale (Hughes et al. 2017).

Recent progress in the field of digital image processing has allowed the development of image analysis algorithms with the use of advanced stochastic techniques to carry out high-throughput phenotyping (Kotwaliwale et al. 2014). For that purpose, open-source software, such as ImageJ®, has been used for automated image processing in plants (Costa & Yang 2009, Dhondt et al. 2014, Legland et al. 2017, Miart et al. 2018, Tello et al. 2018, Vasseur et al. 2018).

ImageJ® has stood out among the tools able to supply solutions for rapid prototyping of image processing algorithms (Schneider et al. 2012), mainly due to its ease of use, recordable macro language, and extendable plugin architecture (Rueden et al. 2017). The use of ImageJ® for development of algorithms and later creation of personalizable macros has been successfully reported for histological quantification of sections of maize stalk (Legland et al. 2017), simultaneous phenotyping of soluble mucilage and morphometric parameters of seeds (Miart et al. 2018), pollen phenotyping (Tello et al. 2018), and dynamic phenotyping of growth of *Arabidopsis thaliana* (Vasseur et al. 2018), among other studies. However, its application to process seed 2D X-ray images has been limited to manual or semi-automated analyses (Abud et al. 2018, Medeiros et al. 2018, Noronha et al. 2018, Silva et al. 2013), which can already be considered an advance compared to traditional methods of visual evaluation. However, they are limited for large-scale analysis.

Brachiaria ruziziensis is a forage grass of high nutritional value that is promising for plant breeding programs (Moreira et al. 2018). Nevertheless, studies on advanced techniques for monitoring seed quality for this species are few. Thus, new methods that are less subjective and that make automatization of analyses possible would contribute to selection of higher quality seeds, which leads to significant advances for the forage sector and ensures faster response to investments.

Considering that combined use of multiple technologies of image analysis for description of traits of interest in seeds is a key point for modernization of agriculture, the aim of this study was to propose a method of high-throughput phenotyping, using free access tools for processing of 2D X-ray images of brachiaria seeds, as well as to relate the parameters obtained with seed physiological quality.

MATERIALS AND METHODS

The study was performed in the Seed Analysis and X-ray Analysis Laboratories belonging to the Universidade Federal de Viçosa, Viçosa, state of Minas Gerais, Brazil. We used seven lots of *Brachiaria ruziziensis* cv. Ruziziensis seeds produced in the 2017 and 2018 crop season in comercial seed production fields. Each analysis was composed of 200 seeds per lot, which were divided into four replications of 50 seeds.

Initially, the moisture content of the seeds was determined by the laboratory oven method at 105 ± 3°C for 24 hours, based on the Rules for Seed Analysis (Brasil 2009). Two 5-g subsamples of seeds for each lot were used for this purpose. The results were expressed in percentage (wet basis, w.b.).

Samples preparation and X-ray images acquisition

The seeds were placed and fixed in an ordered way on contact adhesive paper to allow later individual identification in subsequent analyses. After that, the seeds were placed within the digital X-ray equipment Faxitron MX-20 (Faxitron X-ray Corp., Wheeling, IL, U.S.A) to generate 2D X-ray images. The following adjustments were made: exposure time of 10 seconds to radiation, a voltage of 23 kV, focal distance of 27.8 cm, and image contrast calibrated at 13915 (width) x 5374 (center). The digital images generated were stored on the computer and then analyzed automatically by the protocol described below.

Automated X-ray image analysis

The image processing software ImageJ® (Schneider et al. 2012) was used to develop a macro (automated sequential analysis) called *PhenoXray* and subsequent image analysis. ImageJ® was chosen to host the macro because it is software in the public domain and is commonly used by the scientific community. ImageJ® is presented as the fastest image-processing program in the world, written in pure Java (https://imagej.nih.gov/ij/features.html). It supports and works with a wide range of file formats and functions in many operating systems. In addition, the Fiji open code platform (Fiji is just ImageJ) (Schindelin et al. 2012) provides many powerful solutions of image analysis and a selection with diverse plugins. *PhenoXray* was thus written in the Java macro language of ImageJ® to be easily implemented on the Fiji platform as a module. The workflow projected for X-ray analysis of the seeds is shown in Figure 1. This sequential analysis was executed in batch mode on the entire set of images stored in a predefined directory.

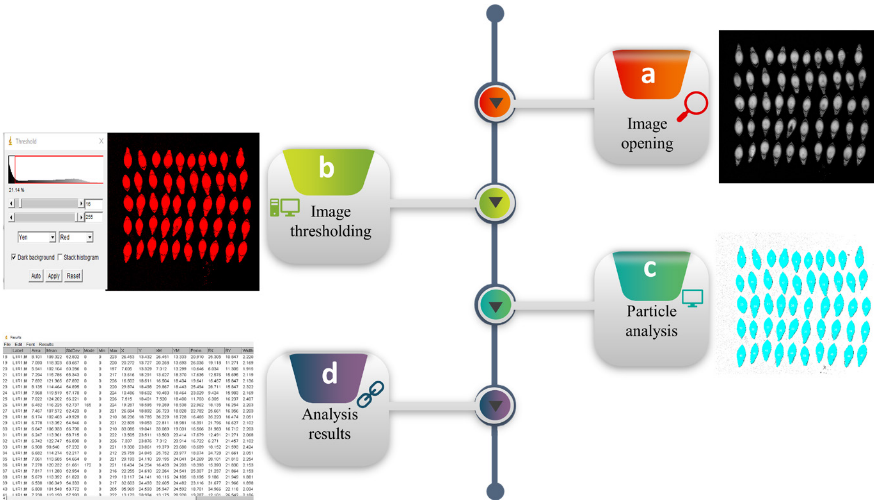


Figure 1. Schematic representation of the processing made by the macro *PhenoXray* in automated analysis of X-ray images of brachiaria seeds. The main commands implemented by the macro are indicated in the boxes. The input image is open (a) and then segmented (b). After segmentation, particle analysis (c) is performed, in which the results of analysis (d) are generated at the end, with various measurements for each individual seed. For simplification, only one area representative of one of the processed images is presented.

In brief, each monochromatic input image (Figure 1a) was duplicated and only the generated image was segmented using the *Threshold* mode of ImageJ**®** (Figure 1b). The image threshold was defined automatically using the method *automatic multilevel thresholding* of Yen (Yen et al. 1995) to segment the images in individual regions of interest (in this case, the braquiaria seeds). Following, the selections in the binarized image was directed to the original image. Then, the individual seed images were analysed using the *Analyze Particles* command (Figure 1c). Only particles with a surface area from 3 to 12 mm^2 were considered in the images. After processing, the results of the automatic measurements were stored on a dynamic table and automatically saved in a file in the TXT format (Figure 1d). In addition, processed images were created in the same directory in JPEG format files. In these images, the seeds detected and analyzed are shown outlined in cyan. *PhenoXray* proposes an intuitive manner of validating segmentation processes, attributing specific numerical identification to each seed analyzed, which allows the user to easily search for the parameters in the tables of results.

The variables obtained from image analysis and used in this study were the following: area (delimited surface of the selection, expressed in mm^2); perimeter (length in millimeters of the outer limit of the selection, expressed in mm); circularity (obtained through the equation: $C = 4\pi * \frac{area}{(perimeter)^2}$; in this equation, values equal to 1.0 indicate a perfect circle, and the extent to which values tend to 0 suggests an elongated shape); width (the width of the smallest rectangle that encompasses the selection, expressed in mm); height (the height of the smallest rectangle that encompasses the selection, expressed in mm); solidity (obtained through the equation: $\text{Solidity} = \frac{\text{Area of the object}}{\text{Area of the convex hull}}$; in this equation, values equal to 1.0 indicate that the object is solid, and less than 1.0, objects with irregular edges and with holes); relative density or mean gray level (defined as the mean of the gray values of all the pixels in the area selected, expressed in gray pixel^1); integrated density (the sum of values of the pixels in the image or selection, expressed in gray mm^2 pixel^{-1}); and seed filling (percentage of seed area effectively filled by high density material – i.e., the percentage of the seed area that comprises grey levels above the initially defined threshold).

Manual X-ray image analysis

Manual or semi-automated analyses of the X-rays images were performed using the Fiji platform (Schindelin et al. 2012) for the purpose of validating the segmentation of the automated process. The process began with individual opening of the images, following adjustment of the scale. It was done by means of a radiographic image of a reference object with known dimensions, that was radiographed under the same conditions (equipment model, focal length and calibration of contrast) as the seeds. Then, the cursor of the computer was used to make individual selection of each seed using the wand tool of ImageJ**®**. It was necessary to click on each seed to define the limit of selection, and then the “Ctrl + M” command was applied for particle analysis. At the end of the process, the dynamic tables generated containing the variables analyzed were saved individually.

Evaluation of physiological quality

The germination test was conducted on the same seeds that had undergone the X-ray test, maintaining the same seed arrangement originally used. The germination test was conducted on rolls of paper toweling for germination (*Germitest* paper) moistened with water in the amount of 2.5 times the weight of the paper towel and kept in a seed germinator under temperature alternating from 15°C (sixteen hours) to 35°C (eight hours) (Brasil 2009). Germination (normal seedlings) and radicle emergence counts were made daily up to the twenty-first day after setting up the test. From these data, the following were calculated: percentages of radicle emergence (radicle longer than 2 mm) and germination, speed of radicle emergence, germination speed index, mean time for radicle emergence, mean germination time, and the synchronization of radicle emergence and germination, according to formulas described in Silva et al. (2019). All the germination variables were calculated through use of the *Germcalc* function of the SeedCalc package of the R software.

After the last count, the seedlings and non-germinated seeds from each replication were scanned, and the images generated were evaluated on the ImageJ® software. The images were used to check seedling length, expressed in mm seedling^{-1}. The data regarding length were inserted in the SeedCalc package of R, to which the *Plantcalc* function was applied to generate the total length of the seedlings, the growth indices (adapted from Sako et al. (2001)), uniformity (Castan et al. 2018), vigor (Sako et al. 2001), and corrected vigor (Medeiros & Pereira 2018), according to the formulas described below:

$$\mathrm{GI}=\left(\frac{\mathrm{TLmean}}{\mathrm{Xmax}}\right)\times 1000$$

where GI is the growth index, TLmean is the mean total length of seedlings of the replication, and Xmax is the mean total length of the 5% largest seedlings of the experiment.

$$\mathrm{UI}=\left[1-\frac{\sum_{i=1}^{n}\left|\mathrm{Xi}-\bar{\mathrm{X}}\right|}{\mathrm{n}\times\bar{\mathrm{X}}}\right]\times 1000-\left[\mathrm{n}_{\mathrm{dead}}\times\left(\frac{50}{\mathrm{n}_{\mathrm{total}}}\right)\right]$$

where UI is the uniformity index, Xi is the length of the seedling analyzed, X is the mean length of seedlings of the seed lot analyzed, n is the variable of total number of seedlings evaluated, n_{dead} is the number of non-germinated or dead seeds in the lot evaluated, and n_{total} is the total number of seedlings.

$$\mathrm{VI}=(\mathrm{GI}\times 50)+(\mathrm{UI}\times 50)$$

where VI is the vigor index, GI is the growth index, and UI is the uniformity index.

$$\mathrm{CVI}=\frac{\left[(\mathrm{GI}\times 50)+(\mathrm{UI}\times 50)\right]\times \mathrm{Ger}}{100}$$

where CVI is the corrected vigor index, GI is the growth index, UI is the uniformity index, and Ger is the germination percentage of the seed lot.

Adaptation of the growth index described above consisted of change in the formula originally proposed by Sako et al. (2001) in which first the mean values of the shoots and roots were taken into consideration, and specific weights were attributed to these variables as described in the formula below.

$$\mathrm{GI}=\min\left(w_h * l_h + w_r * l_r, 1000\right)$$

where GI is the growth index, l_h is the mean hypocotyl length, l_r is the mean length of the radicle, w_h is the specific weight attributed to the hypocotyl, and w_r is the specific weight attributed to the radicle.

This adaptation was necessary since the specific weights used in the original formula are not standardized for all the species and, depending on the size of the seedlings, these weights generalize the index in an inadequate way, making it less sensitive in differentiation of lots, especially when the seedlings are larger. Thus, with the adaptation, the total length of the seedlings would be taken into consideration, and the index demarcated by selection of the 5% largest seedlings of the experiment. That way, the index tends to be more sensitive and adequate for the specific data of the experiment, allowing a more refined comparison among the seed lots.

Experimental design and statistical analysis

The experiment was conducted in a completely randomized experimental design, with four replications. To validate automated segmentation of the X-ray images of the brachiaria seeds with the macro *PhenoXray*, manual measurements made on the Fiji platform were used, and then linear regressions and scatter plots were applied to obtain correlation between the methods. After that, the data obtained from automated analysis of the X-rays and from evaluation of seed physiological quality were subjected to analysis of variance (ANOVA), and the mean values were clustered by the Scott-Knott test ($p \leq 0.05$). Pearson linear correlation analysis (r) was performed on the data, in which the significance of the r values was determined by the t test ($p \leq 0.05$). Multivariate principal component analysis was also carried out, using all the variables evaluated. Normality and homoscedasticity of the data sets were checked by the Shapiro-Wilk and Bartlett tests, respectively. The software used in the statistical analyses was R 3.5.1.

RESULTS AND DISCUSSION

Visual analysis of the X-ray images

The small variation in moisture content among the seed lots evaluated (8.2% to 8.9%), combined with the configurations of voltage, exposure time, focal distance, and contrast of the X-ray equipment adopted in this study, allowed to obtain high-quality X-ray images (Figure 2). The X-rays allowed visualization of the main internal structures of the seeds (Figure 2a), as well as identification of embryonic malformation (Figure 2b[1,2,3]) and physical damages such as cracks (Figure 2b[4]), which may have arisen from the harvest or drying process. In addition, tissue deterioration was visualized at different stages, possibly brought about by pathogenic agents (Figure 2b[5,6,7]).

According to Berry et al. (2018), a big challenge associated with automated image analysis is variation in image quality, which may inadvertently affect the results. In this respect, the configurations of the X-ray equipment used in this study proved to be adequate and standardizable for future experiments with this equipment. Nevertheless, it is known that different X-ray systems might not produce similar results due to the types of X-ray detectors used

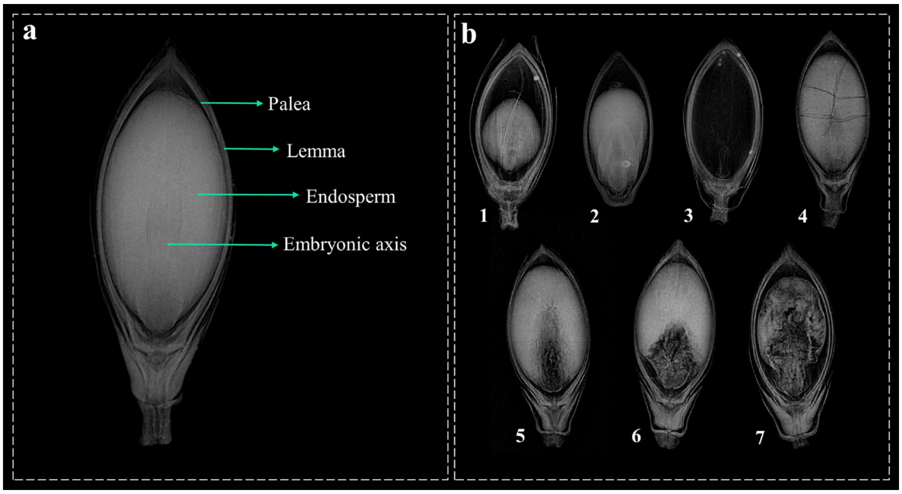


Figure 2. **X-rays of *Brachiaria ruziziensis* cv. Ruziziensis seeds with indications of the basic structures that compose the seed (a) and of embryonic malformation or physical damage (b). 1, 2, and 3 – embryonic malformation; 4 – cracks; 5, 6, and 7 – tissue deterioration.**

(Kotwaliwale et al. 2014), and adaptations would be necessary to generate good quality images.

Other studies have shown that the information generated by simple visual evaluation of the X-rays are highly valuable for analysis of the physical quality of the seeds, and it is a good tool for monitoring germination (Nielsen et al. 2017), selection of more vigorous seed lots (Abud et al. 2018, Medeiros et al. 2018), and identification of polyembryony (Arruda et al. 2018). In this study, the ability to use the X-ray images to visualy identify tissue deterioration brought about by pathogenic agents at different levels is another promising result, which may contribute to fortification of sanitary barriers, avoiding contamination of crops by infested seeds (Figure 2).

Nevertheless, although visual evaluation of X-rays performed by an analyst is the simplest and most sensitive approach, it is a slow process, subject to errors, and not practical on a large scale. Therefore, automation of analyses is necessary to optimize the entire process of extraction of information from the X-rays and simultaneously offer more immediate and precise diagnoses regarding characteristics of interest. For that reason, the macro *PhenoXray* on ImageJ® was developed.

Automated analysis of the X-ray images

To validate the efficiency of *PhenoXray* for precise automated segmentation of the X-ray images of the seeds, all the parameters were measured in all the seeds of the seven lots using the macro and then correlated with manual measurements performed using the Fiji platform (Figure 3). Significant correlations were observed for all the parameters ($p < 0.001$), with strong correlations between the methods for relative density, seed filling, integrated density (r = 0.99), area (r = 0.87), perimeter (r = 0.87), circularity (r = 0.84), width (r = 0.86), and height (0.90). There was, however, a moderate correlation for solidity (r = 0.62), a variable that indicates how the edges of

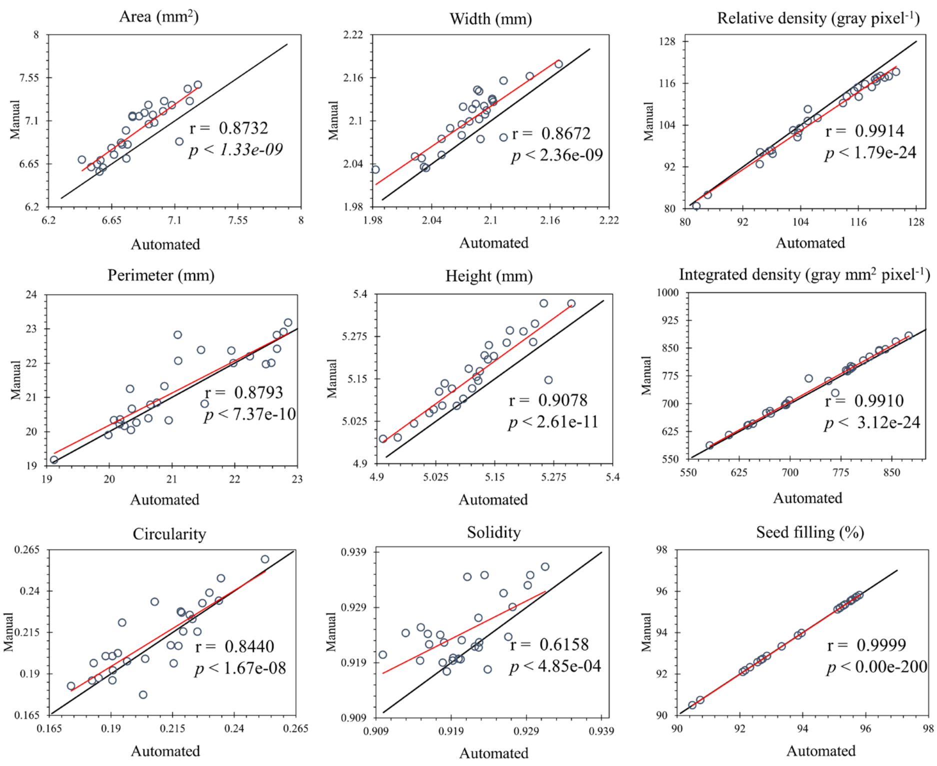


Figure 3. Statistical validation of the macro *PhenoXray* for analysis of X-ray images of *Brachiaria ruziziensis* seeds. Correlation analysis of the automated measurements *(PhenoXray)* versus manual measurements of area, perimeter, circularity, width, height, solidity, relative density, integrated density, and seed filling using the ImageJ® software. The Pearson correlation coefficients were determined by analyses of linear regression (n = 28, corresponding to the replications of all the lots).

the seeds are curved. The moderate correlation between the manual and automated methods for evaluation of solidity can be explained from adjustment of the threshold variable by the manual method, which may lead to greater variations in the curvature of edges for this method.

The high correlations between the manual and automated method is an important result since the use of the manual or semi-automated technique in ImageJ® has already been reported as successful in recent studies with analysis of X-ray images (Abud et al. 2018, Medeiros et al. 2018, Noronha et al. 2018, Silva et al. 2013).

Table I shows the variables obtained from use of the macro *PhenoXray* in evaluation of the X-ray images of the seeds of the seven seed lots. Different groups were formed for all the variables evaluated.

For the variables related to seed size and shape (area, perimeter, width, height, circularity, and solidity), different characteristics were observed among the lots, with clustering on two or three levels (Table I). These variations may be a response to environmental conditions since the lots came from different production fields. Descriptors of seed size and shape are important components of basic studies on plants since seed formation and development is a fundamental aspect of plant reproduction (Whan et al. 2014). The possibility of phenotyping these characteristics in a reliable way and on a large scale represents a significant advance for forage species breeding programs, with potential to simultaneously act with molecular profile technologies.

The integration of image tools with genome approaches and modeling has made it possible to create models that more adequately encompass patterns of gene expression, contributing to understanding of plant development processes (Sozzani et al. 2014). Upon using phenotypes of seed size and shape derived from image analysis, Tanabata et al. (2012) and Moore et al. (2013) mapped quantitative trait loci (QTL) in rice and Arabidopsis, respectively. Thus, the phenotyping of seed characteristics by means of image analysis opens a range of possibilities for deeper analyses, such as anatomical,

Table I. Mean values of morphometric data obtained by automated analysis of X-ray images of seven lot of *Brachiaria ruziziensis* seeds with use of the macro *PhenoXray*.

Seed lot	Area	Perimeter	Width	Height	Circularity	Solidity	Relative density	Integrated density	Seed filling
	mm^2	mm	mm	mm			gray.pixel^{-1}	gray.mm^2.pixel^{-1}	%
1	6.80 b	19.82 c	2.11 a	5.00 c	0.243 a	0.930 b	115.31 a	784.87 a	95.29 a
2	7.12 a	22.50 a	2.11 a	5.26 a	0.186 c	0.921 c	111.16 a	793.35 a	95.34 a
3	6.66 b	20.54 b	2.05 b	5.11 b	0.216 b	0.919 c	101.70 b	677.66 b	93.5 b
4	6.70 b	20.87 b	2.05 b	5.11 b	0.214 b	0.920 c	98.88 b	664.23 b	92.51 c
5	7.33 a	22.15 a	2.14 a	5.30 a	0.202 c	0.924 c	88.28 c	647.49 b	91.43 d
6	7.12 a	20.47 b	2.11 a	5.14 b	0.228 a	0.935 a	117.41 a	836.93 a	95.57 a
7	7.19 a	22.62 a	2.13 a	5.25 a	0.191 c	0.922 c	114.11 a	821.08 a	95.61 a
Fc	18.59*	33.12*	9.58*	14.66*	15.56*	35.57*	27.36*	17.12*	60.47*
CV	1.75	1.83	1.13	1.05	4.88	0.21	3.84	5.2	0.46

Lowercase letter = clustering within each column for each evaluation by the Scott-Knott test ($p < 0.05$), * = significant by the F test ($p < 0.05$), Fc = calculated value of F, CV = coefficient of variation.

metabolomic, or genomic analyses (Jahnke et al. 2016).

For parameters related to tissue density, clustering of the seed lots were observed at three levels for relative density, two levels for integrated density, and four levels for seed filling. In general, lot 5 showed lower values for tissue density compared to the other lots, except for integrated density (which did not differ from lots 3 and 4); this is an indication of low-quality seeds.

The variables relative density, integrated density, and seed filling offer an idea of the integrity of the seed tissues, since tissues that are well-formed and without damage have greater resistance to the passage of X-ray photons through the seed, whereas damaged tissues and seeds offer less resistance. These parameters have not yet been greatly reported on in studies with seeds; however, in the few studies available, they provided promising results, often related to physiological attributes, such as seed germination and seedling length (Abud et al. 2018, Medeiros et al. 2018, Noronha et al. 2018, Silva et al. 2013).

Seed physiological analysis

Seeking to observe a possible association of the characteristics obtained from automated analysis of X-rays of brachiaria seeds with their physiological potential, the respective seeds used in the previous analysis underwent the germination test, in which some physiological parameters were obtained. Statistical differences were detected for most of the physiological variables, except for mean germination time (Table II).

For radicle emergence, greater performance was observed in lots 1, 2, 6, and 7; intermediate performance in lots 3 and 4; and low performance in lot 5. The same pattern was obtained for the germination data (normal seedlings). Nevertheless, only lots 1, 6, and 7 would be suitable for sale in Brazil according to official production and sale standards for forage grass seeds (Brasil 2008).

Table II. **Mean values obtained for the physiological parameters obtained by the germination test of seven lots of *Brachiaria ruziziensis* seeds.**

Seed lot	Radicle emergence	Germination	SRE	GSI	MTRE	MGT	SyncRE	SyncG
	%		index		days		index	
1	70 a	64 a	7.54 a	5.01 a	5 b	7	0.28 a	0.40 b
2	68 a	58 a	6.24 b	4.17 b	6 a	7	0.28 a	0.36 b
3	30 b	23 b	3.87 c	1.68 c	4 b	7	0.27 a	0.46 b
4	34 b	28 b	3.45 c	2.05 c	5 b	7	0.32 a	0.48 b
5	11 c	6 c	0.93 d	0.38 d	7 a	7	0.10 b	0.18 b
6	76 a	68 a	8.28 a	5.46 a	5 b	6	0.35 a	0.72 a
7	72 a	60 a	6.85 b	4.42 a	6 a	7	0.34 a	0.34 b
Fc	52.58*	46.42*	36.57*	45.02*	7.44*	0.89 ^{ns}	5.7*	4.23*
CV	14.05	16.43	16.44	17.36	11.11	11.17	25.6	37.75

Lowercase letter = clustering within each column for each evaluation by the Scott-Knott test ($p < 0.05$), $^{*, ns}$ = significant and non-significant by the F test ($p < 0.05$), Fc = calculated F value, CV = coefficient of variation. SRE = Speed of radicle emergence, GSI = Germination speed index, MTRE = Mean time for radicle emergence, MGT= Mean germination time, SyncRE = Sychrony of radicle emergence, SyncG= Sychrony of germination.

From the results obtained for speed of radicle emergence (SRE) and for the germination speed index (GSI), clustering of the lots is observed at four levels, which shows the greater sensitivity in relation to the germination test in differentiation of these seed lots. The mean time for radicle emergence (MTRE) ranged from four to seven days, and lots 2, 5, and 7 had the longest mean time for radicle emergence, whereas for mean germination time (MGT), differences were not observed among the lots, ranging from six to seven days. For synchrony of radicle emergence (SyncRE), lot 5 differed from the others, showing lower performance, whereas for synchrony of germination, lot 7 stood out with greater performance, differing from the other lots.

All these variables related to speed, synchrony, germination time, and radicle emergence are considered manifestations of seed vigor. In practice, seed lots with rapid and uniform germination bring greater advantages for the establishment of the crop in the field (Finch-Savage & Bassel 2016).

By means of seedling analysis, the differences observed among the lots in the tests already described were confirmed. Seedling length and the growth, uniformity, and vigor indices ranked the lots in three groups, of high (lot 1, 2, 6, and 7), intermediate (lot 3 and 4), and low (lot 5) vigor. The corrected vigor index, constituted by the product of the vigor index with the germination percentage (Medeiros & Pereira 2018), was the variable most sensitive in ranking the lots, differentiating them into four vigor levels, in which lots 1, 6, and 7 obtained greater performance (Table III).

In this study, all the indices showed excellent results in differentiation of the lots, including the new formula for the growth index adapted from Sako et al. (2001). The seedling performance indices used in this study were recommended in other studies by allowing access to useful information under aspects of initial growth and uniformity of seedling emergence, with good capacity for distinguishing lots in regard to their physiological potential (Castan et al. 2018, Medeiros & Pereira 2018, Sako et al. 2001).

Table III. **Mean values obtained for length and growth and vigor indices through analysis of seedling images coming from seven lots of *Brachiaria ruziziensis* seeds.**

Seed lot	Seedling length	Growth	Uniformity	Vigor	Corrected vigor
	cm	Index			
1	3.25 a	386 a	610 a	498 a	319 a
2	3.42 a	406 a	562 a	484 a	278 b
3	0.99 b	118 b	238 b	178 b	40 c
4	1.39 b	165 b	292 b	229 b	63 c
5	0.51 c	61c	59 c	60 c	4 d
6	3.62 a	430 a	613 a	522 a	352 a
7	3.62 a	429 a	612 a	521 a	312 a
Fc	44.02*	44.02*	49.34*	50.12*	119.15*
CV	17.23	17.23	15.17	15.43	14.26

Lowercase letter = clustering within each column for each evaluation by the Scott-Knott test ($p < 0.05$), * = significant by the F test ($p < 0.05$), Fc = calculated F value, CV = coefficient of variation.

Correlation between seed physiological and physical parameters

Given the results obtained for the physical characteristics (Table I), evaluated through automated X-ray analysis, and for the physiological characteristics, germination (Table II) and seedling growth (Table III), it is evident that the lots exhibit differences regarding physical and physiological parameters. Correlation analysis was performed in order to verify possible associations among the different parameters (Figure 4).

The size and shape descriptors (width, height, and perimeter) had little association with the physiological variables, shown by low correlations, which shows the limitation of these characteristics in prediction of germination data or seedling length for brachiaria seeds. In contrast, Abud et al. (2018) identified strong correlations between the area and the circularity of *Brassica oleraceae* seeds with seedling length, just as Medeiros et al. (2018), who observed correlations of the perimeter and circularity of *Leucaena leucocephala* seeds with germination. Thus, this response depends on the seed characteristics of each species, and for brachiaria, prediction of physiological quality of the seeds using shape descriptors does not seem to be viable.

However, positive, strong, and significant correlations were observed ($r > 0.94$, $p < 0.05$) of all the parameters related to tissue density (relative density, integrated density, and seed filling), obtained from automated X-ray

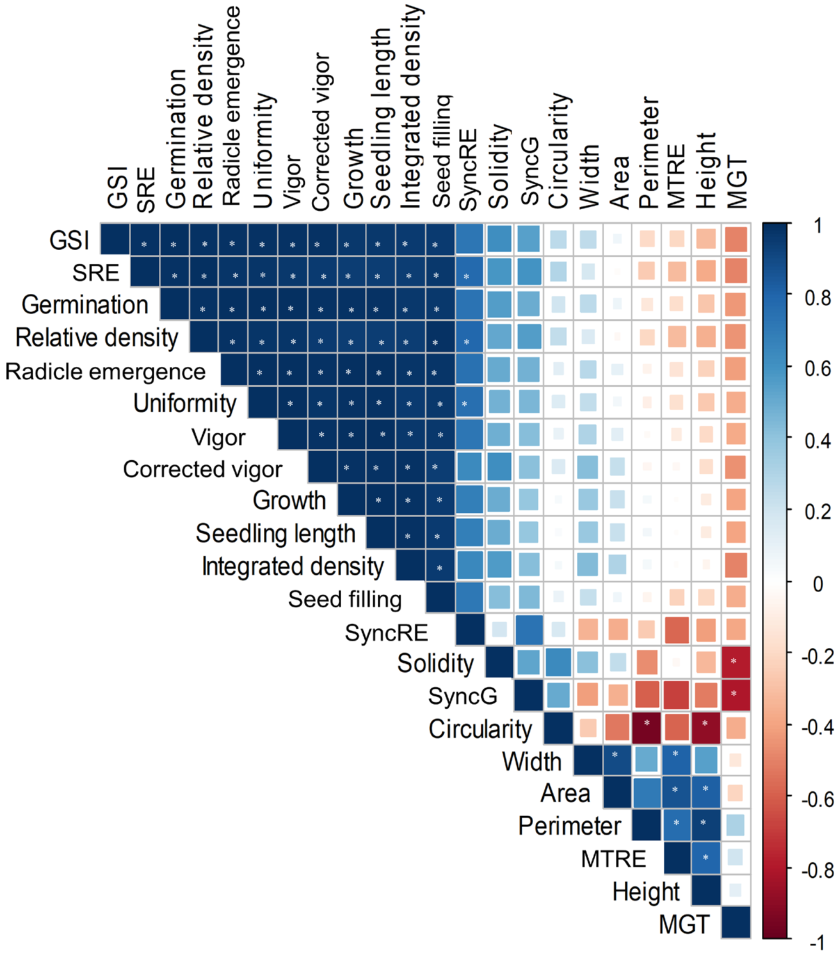


Figure 4. Correlation matrix obtained from physical and physiological data of seeds and characteristics of *Brachiaria ruziziensis* seedlings. * significant (p < 0.05) by the t test. SRE = Speed of radicle emergence, GSI = Germination speed index, MTRE = Mean time for radicle emergence, MGT = Mean germination time, SyncRE = Synchrony of radicle emergence, SyncG = Synchrony of germination.

analysis, with most of the physiological quality variables. This result shows the efficiency of the technique of automated analysis of X-ray images in estimating the physiological quality of brachiaria seeds.

It should be emphasized that the methods currently in use for evaluation of seed quality in Brazil have been deeply refined over the years and are undoubtedly good tools. Tests of germination and seedling length, for example, are routinely used in decision-making processes of production companies to establish sales strategies for seed lots. Yet, these methods have limitations related to time spent for providing results, mistakes inherent to the subjectivity of the evaluations, and the destructive nature of the tests (Huang et al. 2015, Kotwaliwale et al. 2014, Rahman & Cho 2016). For brachiaria seeds, 21 days were necessary to carry out only the germination test conducted according to the RAS (Brasil 2009). Recently, Jeromini et al. (2018) proposed evaluation of brachiaria seed vigor in seven days through computerized seedling analysis; however, the results were not very promising.

With the use of the technique proposed in this study, only 10 seconds were needed to generate the X-ray image of 50 brachiaria seeds and 0.5 seconds more to process the image and obtain the results. Thus, it is evident that it is an extremely promising analysis and it can lead to advances in procedures of pre-selection of seed lots with higher levels of physiological quality during seed processing, although it is necessary further research using a wide range of commercial seed lots and using different X-ray equipment to validate the methodology. This is especially important for the large-scale seed industry in seeking to offer a quality product to farmers of more uniform seed lots with greater physical purity and high physiological potential.

Multivariate analysis of seed physiological and physical parameters

The multivariate principal component analysis (PCA) was performed using the dataset obtained for the seven lots studied and the fifteen traits evaluated (Figure 5). The first two components (PC1 and PC2) explained 84.5% of the total variability of the data, i.e., with this analysis, it was possible to reduce fifteen dimensions to only two, which explained a significant percentage of the observations.

In Figure 5, the first two components were presented in the cartesian plan. The individual parameters analyzed were showed as vectors, in which gray and green vectors represent physical and physiological variables, respectively. The diagram in the lower right-hand corner represents the circle of correlations indicating the representative quality of each variable for the PCA on a color scale, based on the cos2 values. The cos2 value shows the importance of a principal component for a given observation (vector of original variables) (Abdi & Williams, 2010). Each vector points in the direction in which the value of the characteristic has the maximum increase through this coordinate system. As the cosine of the angle between any two vectors is proportional to the linear correlation coefficient between these two vectors (and therefore also between the two seed traits) (Abdi & Williams 2010), it can be deduced that the characteristics of tissue density are highly correlated with physiological attributes such as germination, germination speed, and seedling uniformity and vigor, once more confirming what was observed for the correlation data (Figure 4). In contrast, weak correlations of vectors, as observed between the descriptor variables of shape and the physiological attributes, indicate that they are independent of each other and each one brings a substantial portion of new information that can be applied to other future studies.

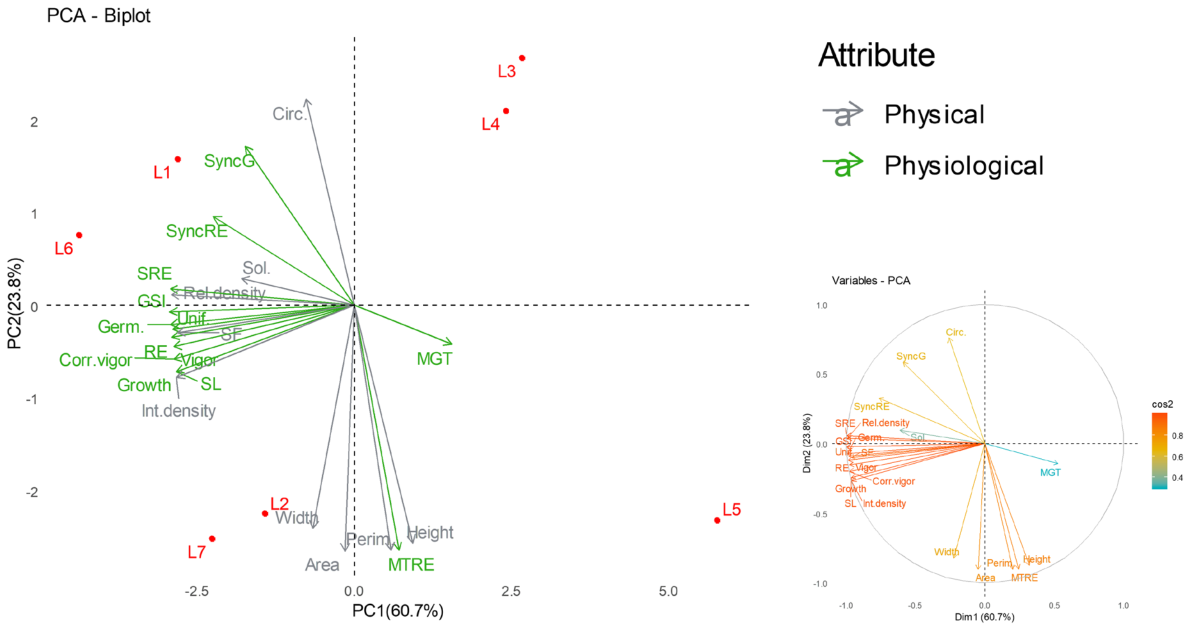


Figure 5. Biplot obtained by the linear combination of the variables related to the physical and physiological characteristics of seven lots of *Brachiaria ruziziensis* seeds. Germ. = Germination, RE = Radicle emergence, SRE = Speed of radicle emergence, GSI = Germination speed index, MTRE = Mean time for radicle emergence, MGT = Mean germination time, SyncRE = Synchrony of radicle emergence, SyncG = Synchrony of germination, SL = Seedling length, Unif. = Uniformity, Corr. Vigor = Corrected vigor, Perim. = Perimeter, Circ. = Circularity, Sol. = Solidity, Rel. density = Relative density, Int. density = Integrated density, SF = Seed filling.

In this study, the correlations between the physical and physiological characteristics were made from measurements obtained for the lots for all the characteristics, without a correlation on a unit level from seed to seed. Therefore, future studies are necessary that make this type of deeper association, aiming at establishing a possible direct relation of these physical characteristics with the physiological processes.

Furthermore, in PCA, clustering is clearly seen among the lots with similar characteristics. In general, lots that are nearer the quality vectors (e.g., germination, uniformity, relative density, and others), such as lots 1, 2, 6, and 7, exhibited better performance for those characteristics, i.e., they had higher mean values. In contrast, lots that were distant and opposed to the quality vectors can be considered having lower quality (lot 5).

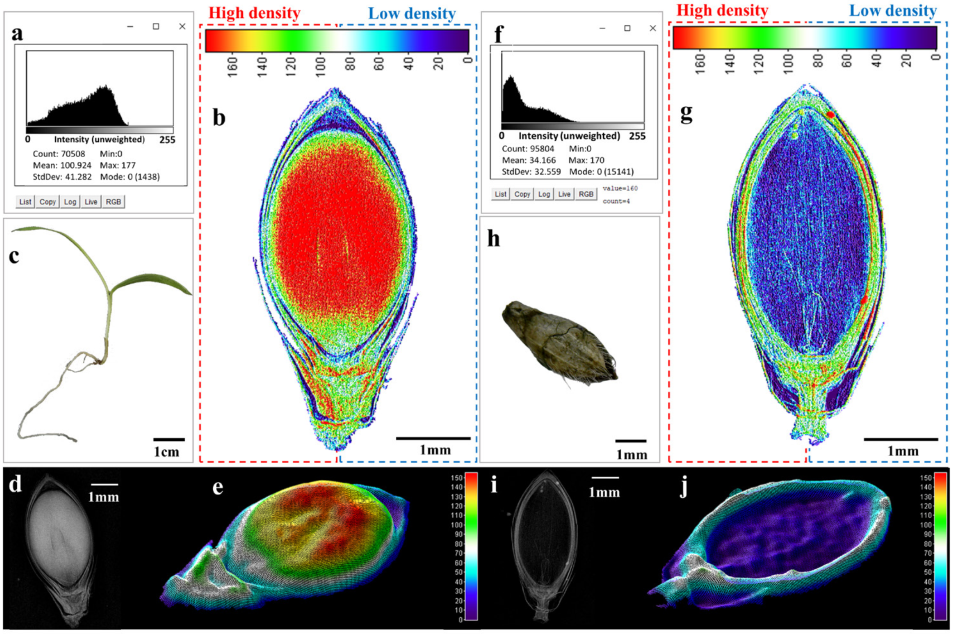
This type of multivariate approach on data of seed physiological quality is not yet widespread or widely used. However, as seen in this study, it can contribute to understanding and interpretation of results in a more objective and precise manner since there may be different responses when seed lots are compared using various vigor tests simultaneously. Other studies in the area of seeds have recommended this type of analysis through allowing identification of patterns in seed quality data and expression of them in such a way that similarities and differences can be observed, reducing dimensionality without losing much information (Abud et al. 2018, Medeiros et al. 2018).

Relationship between gray density and seed quality

The X-ray images of two brachiaria seeds that contrast regarding their tissue density are shown in Figure 6. The seed to the left in the image, apparently well-formed (Figure 6d), shows distribution of its pixels in higher gray level regions of the histogram (Figure 6a). This is an indication of the high tissue integrity of the seed, which may have contributed to development of a well-formed seedling (Figure 6c). In contrast, the seed to the right in the image is lacking embryo and endosperm, as shown in Figure 6i, in which it is possible to observe a darker central region. In this seed, most of the peaks of the histogram are in low gray level zones (Figure 6f), showing the low relative density of the seed (< 35) (Figure 6g and 6j).

Recent studies with the use of other types of optical technologies based on electromagnetic waves for seed analysis, such as nuclear magnetic resonance, optical projection tomography, and tomography by X-ray absorption (Rolletschek et al. 2015, Rousseau et al. 2015) have also shown excellent results in accurate determination of seed physiological attributes. Since these methods are not destructive, they become very valuable. Destructive trials are inadequate if there is the need for expanding post analysis of the material, and they are especially undesirable in situations in which the quantity of seed available is limited (Rolletschek et al. 2015) or the seeds are of high cost, as in hybrids. Thus, the non-destructive nature of the X-ray test is an important additional feature in the context of the functional technologies of seed analysis. Nevertheless, for the test to achieve maximum efficiency, it is necessary that analysis of the X-rays generated is adequate (Abud et al. 2018).

While the methods currently used for accessing information in X-ray images are slow or inefficient on a large scale, the macro *PhenoXray*, developed in this study, allowed high-throughput phenotyping of *Brachiaria ruziziensis* seeds, generating measurements of morphometric parameters and parameters related to the tissue density of the seeds in a rapid and totally automated manner. In addition, the macro is personalized and may be adapted to any other type of seed. With the aim



```
Count: 70508    Min:0
Mean: 100.924   Max: 177
StdDev: 41.282  Mode: 0 (1438)
```

```
Count: 95804    Min:0
Mean: 34.166    Max: 170
StdDev: 32.559  Mode: 0 (15141)
value=160
count=4
```

Figure 6. Images representative of two *Brachiaria ruziziensis* seeds with gray level histograms (a and f), 2D (b and g) and 3D (e and j) representations in density color gradient across the seed, original X-ray image (d and i), and their respective seedlings or non-germinated seed (c and h).

of assisting researchers incorporate *PhenoXray* in their phenotyping strategies and genetic and functional analyses, it was developed as an easily used tool that can be freely downloaded by the entire scientific community at the dedicated site (https://seedsciencebrazil.wixsite.com/seedscience).

From the results presented, it was shown that the proposed method provides a fast, inexpensive, and reliable solution for evaluation of X-rays of brachiaria seeds from digital images. It is a highly applicable tool that furthermore allows fast prediction of physiological attributes linked to seed viability and vigor, through the parameters of relative density, integrated density, and seed filling.

CONCLUSIONS

Automated analysis of *Brachiaria ruziziensis* seeds is efficient for obtaining morphometric data and data on the integrity of internal tissues. Parameters such as relative density, integrated density, and seed filling show a close relationship to physiological attributes of seed quality. The use of the macro *PhenoXray* allows large-scale phenotyping of seed X-rays in a simple, fast, robust, and totally free manner.

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