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Morphology and germination of *Vigna unguiculata* (L.) Walp. seeds

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ABSTRACT: The occurrence of damage and empty space inside the seed can directly affect germination. The aim of this study was to evaluate the relationship between the occurrence of damage or empty space inside cowpea seeds and the physiological potential. In this study, seven lots of seeds of the cultivar 'BRS Guariba' were subjected to the X-ray test to identify damage by tissue deterioration, mechanical damage, damage by insects and malformed seeds and, later, to the germination test. The percentage of empty space between the embryonic area and the seed coat was determined from radiographic images in ImageJ software. Based on the results obtained, the seeds were classified into different categories, and the results were compared with the germination data. It was concluded that the analysis of radiographic images allowed the identification of damage and measurement of empty space in the internal cavity of the seeds, making it possible to establish a relationship between internal morphology and germination. The presence of damage, especially that caused by tissue deterioration or mechanical damage, depending on its location and intensity, has the potential to negatively affect the germination of cowpea seeds. Likewise, seeds that contain higher levels of empty space in their internal cavity tend not to germinate or to generate abnormal seedlings at the time of germination.

Index terms: cowpea, image analysis, X-ray.

RESUMO: A ocorrência de danos e espaço vazio no interior da semente podem afetar diretamente a germinação. O objetivo deste trabalho foi avaliar a relação entre a ocorrência de danos ou de espaço vazio no interior de sementes de feijão-caupi e o potencial fisiológico. Neste estudo foram utilizados sete lotes de sementes do cultivar BRS Guariba, que foram submetidas ao teste de raios X para identificação danos por deterioração de tecidos, danos mecânicos, danos por insetos e sementes malformadas e, posteriormente, ao teste de germinação. Na determinação dos percentuais de espaço vazio entre a área embrionária e o tegumento das sementes, foi utilizado o *software* ImageJ a partir de imagens radiográficas. Com base nos resultados obtidos, foi realizada a classificação das sementes em diferentes categorias, sendo os resultados confrontados com os dados de germinação. Concluiu-se que a análise de imagens radiográficas permitiu a identificação de danos e a mensuração espaço vazio na cavidade interna das sementes, possibilitando estabelecer relação entre a morfologia interna e a germinação. A presença de danos, sobretudo aqueles ocasionados por deterioração de tecidos ou danos mecânicos, a depender de sua localização e intensidade, tem potencial de afetar negativamente a germinação de sementes de feijão-caupi. Assim como, sementes que contém maiores níveis de espaço vazio em sua cavidade interna tendem a não germinar ou a gerar plântulas anormais por ocasião da germinação.

Termos para indexação: análise de imagens, feijão-caupi, raios X.

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INTRODUCTION

It is desirable that seed analysis techniques, existing or in development, be improved in order to facilitate the evaluation of seed quality, making decision-making faster and more efficient (Marcos-Filho et al., 2009). The use of image analysis associated with computational resources is a reality in the evaluation of seed quality, since it allows obtaining a great amount of information about the seeds from their characteristics, without the need for destroying the sample.

In the particular case of seed analysis by means of digital radiographic images, when the seed is subjected to an X-ray source it attenuates part of this radiation, due to its density, resulting in a region of light gray color in the image. In less dense or empty regions, there is no such attenuation of radiation, resulting in a dark gray image (ISTA, 2009; Crocker et al., 2014). From this behavior it is possible to identify the degree of seed formation and the occurrence of possible internal damage. In the literature, there are many studies demonstrating the efficiency of using radiographic images in the identification of internal damage in seeds of different species (Pinto et al., 2007; Brancalion et al., 2011).

Another morphological characteristic that can be considered is the embryonic area, defined based on the degree of embryo development and on the empty space in the internal cavity of the seed (Marcos-Filho et al., 2010). Dell'Aquila (2007) found that bell pepper seeds that had empty space greater than 2.7% of their total area showed a significant reduction in the number of normal seedlings. In this context, other studies have also attested to the effectiveness of this technique (Machado and Cicero, 2003; Gomes-Junior et al., 2012; Silva et al., 2012; Silva et al., 2013).

Cowpea (*Vigna unguiculata* (L.) Walp) is an expanding crop in Brazil, but it has low average yield, less than 500 $kg.ha^{-1}$ (Boukar et al., 2019). In addition to the edaphoclimatic factors, one of the causes of low cowpea yield is the use of cultivars with low production potential (Cardoso and Ribeiro, 2006), since the main accessions used are propagated and maintained by the producers themselves, in each crop season, causing the genetic degeneration of these cultivars (Santos et al., 2009).

A partnership between Embrapa, universities and private sector companies has stimulated the selection of cultivars suitable for the growing regions, associated with the use of seeds of high physiological potential (Freire-Filho, 2011). Although agricultural production cannot be higher than the genetic capacity of the seed used, the use of high-quality seeds is fundamental for increasing yield (Marcos-Filho, 2015).

Rapid detection of vigor in cowpea seeds is essential to ensure the quality of the seeds marketed. Alternative methods of evaluation based on image analysis have already been assessed in some studies for the species in question, such as the use of X-rays in weevil-infested seeds (Melo et al., 2010) and computerized analysis of seedling images (Rego et al., 2021). Considering the advantages of using image analysis and that its applicability may constitute an important technique in cowpea seed quality control programs, the present study aimed to evaluate the occurrence of damage or empty spaces inside cowpea seeds and their relationship with physiological potential.

MATERIAL AND METHODS

The experiment was carried out at the Seed Analysis Laboratory and the Image Analysis Laboratory of the Plant Production Department of the "Luiz de Queiroz" College of Agriculture of *Universidade de São Paulo*, Campus of Piracicaba, SP, Brazil. For the experiment, certified seeds of cowpea, 'BRS Guariba' cultivar, from seven lots produced in the 2017/18 crop season, were used.

In order to verify whether the water content of the seeds was adequate for the evaluations related to the X-ray test, it was measured by the oven method, in which two replications with approximately 4.0 g of seeds for each lot were kept for 24 hours at 105 ± 3 °C, according to the Rules for Seed Testing (Brasil, 2009). The results were expressed in percentage (wet basis).

The internal morphology of the seeds was evaluated using four replications of 50 seeds of each lot. The seeds were fixed on a transparent double-sided adhesive tape, glued on an acetate sheet (210 mm x 297 mm) and numbered according to the position occupied on the sheet, for subsequent identification in the germination test. In order to obtain the radiographic images, the seeds were positioned at 28.6 cm from the X-ray emission source of the Faxitron X-Ray system, model MX-20 DC-12, connected to a Core 2 Duo computer (3.16 GHz, 3 GB RAM, 160 GB HD). The radiographic images were saved in JPEG (Joint Photographic Experts Group) format and stored in a specific folder on the computer's hard drive.

Subsequently, the seeds (in groups of 10) were placed to germinate in rolls of paper towel, distributed in the upper third of the substrate, so as to allow the individualized development of each seedling. After five days in germinator at 25 °C, in the dark, seedlings (normal and abnormal) and non-germinated seeds were analyzed individually by comparing the X-ray images of the seeds with morphological alterations (damage due to tissue deterioration, mechanical damage, insect damage and malformed seeds), relating them to possible abnormalities of seedlings and non-germinated seeds. Normal and abnormal seedlings and non-germinated seeds were photographed with a digital camera, and the results were expressed as a percentage of observed damage.

The radiographic images of the seeds without damage were analyzed by with ImageJ software, version 1.46r (Schneider et al., 2012), in order to obtain the area of the internal empty space, using the technique of ***fraction area***, which represents the internal free areas of the seeds, expressed in percentage, according to the methodology described by Silva et al. (2013). Although the seeds were analyzed individually, the mean value of each parameter was calculated for each lot in the sample of 200 seeds, in order to facilitate the discussion of the results. For the empty space area, the seeds were classified into three categories, described below, based on the mean (M) and standard deviation (SD) of the values obtained for all lots.

- Category I: seeds with empty space lower than the value of the mean minus the standard deviation (x < M-SD).

- Category II: seeds with empty space within the range of values between Category I and Category III (x |< M-SD and > M+SD|).

- Category III: seeds with empty space greater than the mean plus the standard deviation; (x > M+SD).

The data obtained from the X-ray test and evaluation of the empty space in the internal cavity of seeds were not subjected to statistical analysis, because they were analyzed comparatively; instead, the types of damage detected by image analysis (seed by seed) were related to the possible abnormalities of seedlings or non-germinated seeds. The total numbers of normal seedlings, abnormal seedlings and non-germinated seeds, as well as the occurrence of seed damage, were calculated as a percentage relative to the sample of seeds analyzed, being interpreted by comparison between radiographic images and photographic images of seedlings or non-germinated seeds. In addition, simple correspondence analysis (ANACOR) was performed to check the associations between the types of damage and/or category of empty space and germination.

RESULTS AND DISCUSSION

The water content of the seeds ranged from 8.9% to 9.5% and, in this situation, it was adequate for visualizing the internal parts of the seeds in radiographic images. According to Simak (1991), the water content of the seeds directly interferes with their optical density, which influences the visualization of their internal parts; hence, the lower the water content of the seed, the better the visualization of its parts.

Radiographic analysis allowed identifying the occurrence of damage to cowpea seeds (Table 1); different types of damage were detected, such as damage caused by tissue deterioration, mechanical damage, damage by malformation and damage caused by insect (true bug). The damage by tissue deterioration is the most observed among the lots; lot 2 had 47% of its seeds classified with this type of damage, followed by lot 7 (35%) and the other lots with values lower than 25%, on average.

The second most common type of damage found in the seeds was mechanical damage; lot 1 had the lowest percentage (8%) and lot 7 had the highest percentage (20%), while the other lots had 10% to 17% of mechanically

Table 1. Percentage of occurrence of seeds with tissue deterioration damage (TDD), mechanical damage (MD), malformation damage (MFD), insect damage (ID), total damage (TD), normal seedlings (NS), abnormal seedlings (AS) and dead seeds (DS) of seven lots of cowpea (*Vigna unguiculata* (L.) Walp), 'BRS Guariba' cultivar.

Lots	Seed damage					Germination		
	TDD	MD	MFD	ID	TD	NS	AS	DS
	------------------------ % ------------------------					----------------- % -----------------		
1	20	8	1	1	30	92	6	3
2	47	12	1	0	60	74	13	13
3	20	12	0	0	32	90	6	4
4	24	10	2	1	37	87	8	6
5	24	16	1	0	41	83	9	8
6	25	17	2	1	45	79	12	9
7	35	20	1	1	57	78	11	11

damaged seeds. It was also possible to observe that damage due to seed malformation and damage caused by insects also affected the seeds, but not exceeding 2%.

Also in Table 1, it was observed that lots 1, 3 and 4 had the lowest percentages of total damage (< 37%), while lots 2 and 7 had a percentage higher than 50% and lots 5 and 6 showed intermediate percentages. As for the performance in the germination test, it was found that, numerically, lots 1 and 3 had higher number of normal seedlings (92% and 90%, respectively) and, consequently, a lower number of abnormal seedlings and dead seeds. Lots 2 and 7 showed a higher number of abnormal seedlings and dead seeds (13% and 11% for both variables, respectively) and a lower number of normal seedlings (74% and 78%, respectively).

Lots 4, 5 and 6 showed an intermediate performance in the germination test, with 87%, 83% and 79% of normal seedlings, respectively, 8%, 9% and 12% of abnormal seedlings, respectively, and on average less than 10% of seeds that did not germinate. By comparing the percentage of seed damage evaluated by the X-ray test and the subsequent germination of the seeds, it is possible to affirm that lots with the highest percentage of damage to their seeds were also those that had a higher rate of abnormal seedlings or non-germinated (dead) seeds in the germination test.

Figure 1a shows a well-formed and undamaged cowpea seed, resulting in a normal seedling in the germination test (Figure 1g), as all essential structures were intact and well developed. However, the occurrence of seed damage does not always result in negative effects during germination, since the site of occurrence of the damage, its extent and depth can determine the real effect of the damage on the performance of the seed (Figures 1d and 1j). Even lots with lower percentage of damage (lots 1 and 3) had at least 30% of damaged seeds and these, when subjected to germination, resulted in more than 90% of normal seedlings, demonstrating that the site of occurrence and severity of the damage are crucial for a good performance of the seed in germination. The same applies to seeds that are not affected by some type of damage, because other factors may interfere in the germination process (Figures 1b, 1c, 1h and 1i).

Figure 2 shows seeds with damage by tissue deterioration; the main characteristic of this type of damage is the presence of darkened spots in the internal tissues of the seeds, due to the lower density, which facilitates the passage of radiation from X-ray beams, leading to the darkening of deteriorated areas. Figure 2a shows dark spots on the lower part of the embryonic axis and in the peripheral parts of the cotyledons and, when subjected to germination, the seed originated an abnormal seedling, with compromised root system (Figure 2g). Figure 2b shows the presence of a large, darkened spot covering much of the seed, in the region of the cotyledons and the embryonic axis, causing its death (Figure 2h).

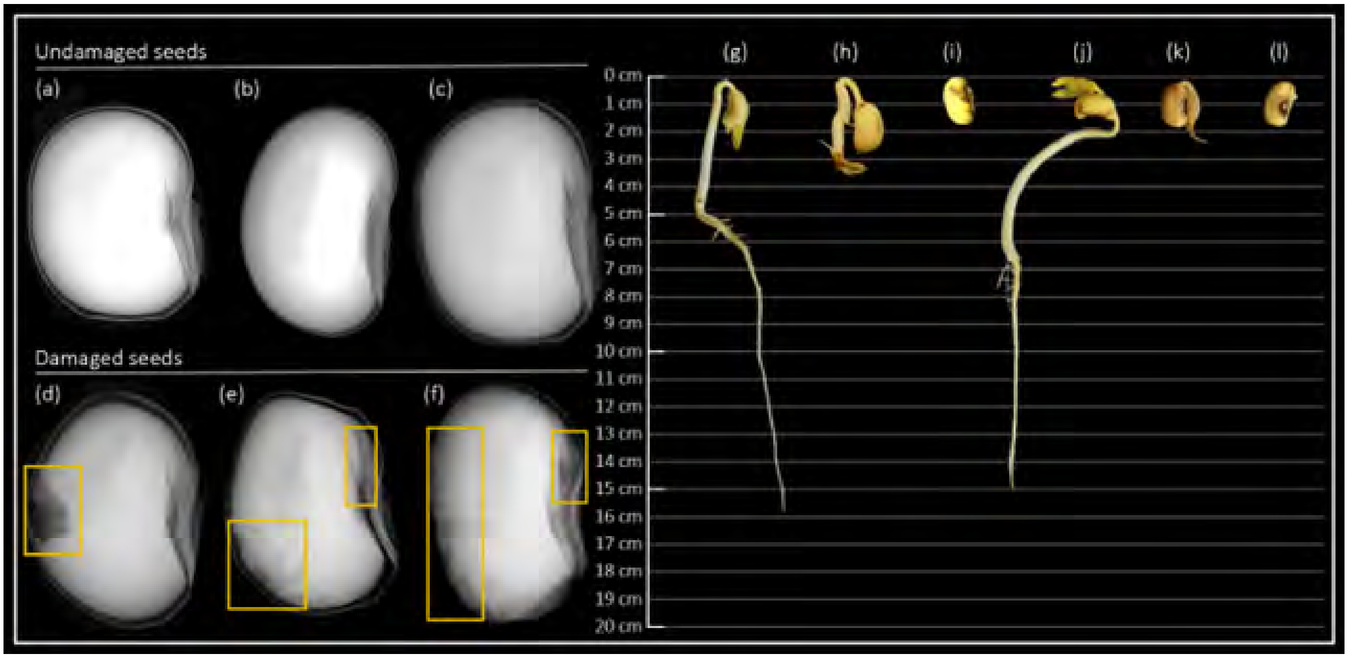

Figure 1. Radiographic images of cowpea (*Vigna unguiculata* (L.) Walp) seeds of the 'BRS Guariba' cultivar, undamaged (1a, 1b and 1c) and damaged (1d, 1and 1f), and their respective seedlings or dead seeds from the germination test (1g, 1h, 1i, 1j, 1k and 1l).

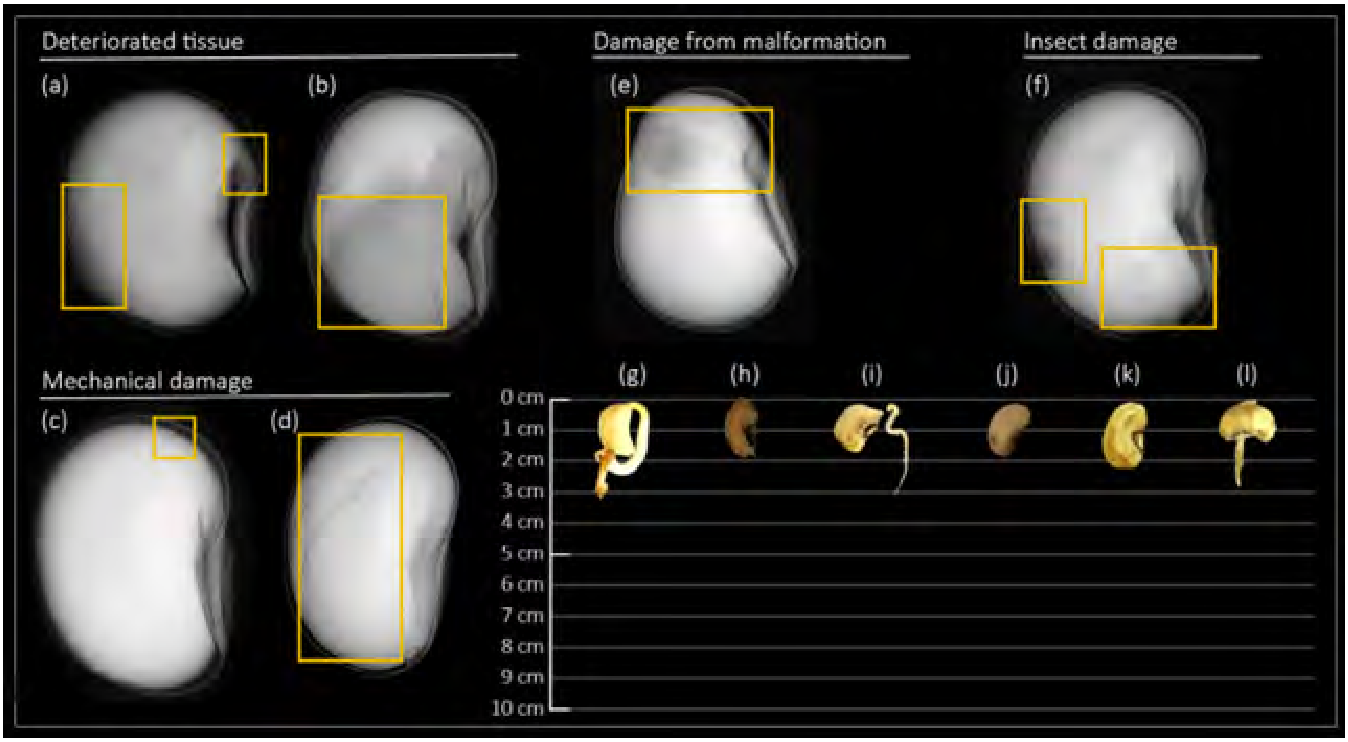

Figure 2. Radiographic images of cowpea (*Vigna unguiculata* (L.) Walp) seeds of the 'BRS Guariba' cultivar, with damage due to tissue deterioration (2a and 2b), mechanical damage to the embryonic axis (2c) and cotyledons (2d), damage due to malformation (2e) and damage caused by insect (2f) and their respective seedlings or dead seeds from the germination test (2g, 2h, 2i, 2j, 2k and 2l).

Seed deterioration involves any degenerative transformation of biochemical, physical, physiological or genetic nature, which culminates in the loss of vigor and viability (Marcos-Filho, 2015). The occurrence of this type of damage is related to unfavorable environmental conditions in the field after the seed reaches physiological maturity; in addition, the occurrence of fungi in seeds can accentuate the occurrence of this type of damage.

Figure 2 shows the occurrence of mechanical damage, characterized by the presence of cracks on the parts that make up the seed. The severity of this type of damage is based on its location, extent, and depth. When this type of damage is opposed to the embryonic axis or superficial in the seeds, it tends not to be so harmful to seed germination; conversely, when it affects the embryonic axis, it can result in the death of the seed. Figure 2c shows the mechanical damage affecting the plumule region, resulting in an abnormal seedling, with rupture of the cotyledons and the hypocotyl-radicle axis (Figure 2i). As observed in Figure 2d, damage that affected from cotyledons to the plumule region resulted in the death of the seed (Figure 2j).

Seeds with cracks, ruptures or losses of part of the cotyledons are still able to germinate, but may originate seedlings with smaller size and, depending on the site of rupture, germination may not occur. Cicero et al. (1998), when using X-rays in the identification of mechanical damage and its effect on the germination of maize seeds, found that ruptures transverse to the endosperm compromise the translocation of seed reserves to the embryonic axis or, when they occurred directly on the embryonic axis, seed germination was negatively affected.

Figure 2 also shows damage by malformation of the embryonic axis and damage due to tissue deterioration in the cotyledons and in the embryonic axis (Figure 2e). As the damage due to tissue deterioration directly affected the embryonic axis, it resulted in the death of the seed (Figure 2k). In addition, it is possible to observe that the seed has a reniform shape, differing from the others.

Figure 2f shows damage caused by insect (true bug) on cotyledons; however, it affects the region opposite to and distant from the embryonic axis, resulting in the formation of an abnormal seedling (Figure 2l). The damage caused by the true bug bite can negatively affect the performance of the seed, depending on the location and extent of the injured area, being more severe when it directly affects the embryonic axis (França-Neto and Krzyzanowski, 2018).

To determine the relationship between types of damage and the physiological potential of the seeds, a simple correspondence analysis was performed between the types of damage and the performance of the seeds in the germination test. The correspondence analysis presents the relationships between two or more categorical variables from the frequencies of a contingency table, which are transformed into chi-square distances (χ^2) and used to establish a perceptual map of the relationship between the variables.

Figure 3a shows the first two axes that express 90.51% (Dim. 1) and 9.49% (Dim. 2) of the total variation of the data. Figure 3b contains the adjusted standardized residuals obtained from the chi-square test, and its results indicate that the occurrence of damage caused by tissue deterioration and mechanical damage is strongly associated with seeds that did not germinate or that originate abnormal seedlings in the germination test, whereas undamaged seeds are strongly related to normal seedlings in the germination test. There was also a weak association of damage caused by insects or malformed seeds with the categories related to germination, since they had a low percentage of occurrence in the seed lots analyzed.

The analysis of radiographic images through ImageJ software allowed the measurement of empty spaces inside undamaged seeds, making it possible to separate them into different categories. Table 2 contains the data referring to the average percentage of empty space and the standard deviation of the different seed lots; these values served as a basis to determine the different categories proposed in this study. In Table 2, it is also possible to observe that, on average, there was a variation of 4.48% and 6.08% in the empty space of the seeds.

Figure 4 contains examples of cowpea seeds distributed in the three categories according to the different levels of empty space; it is possible to observe that the seeds in Figure 4a and 4c have a larger embryonic area than the seed shown in Figure 4e. However, visual estimates can lead to inaccurate quantification of the actual dimension of empty spaces inside the seeds; in the determination of empty space through the ImageJ software (Figures 4b, 4d and 4f),

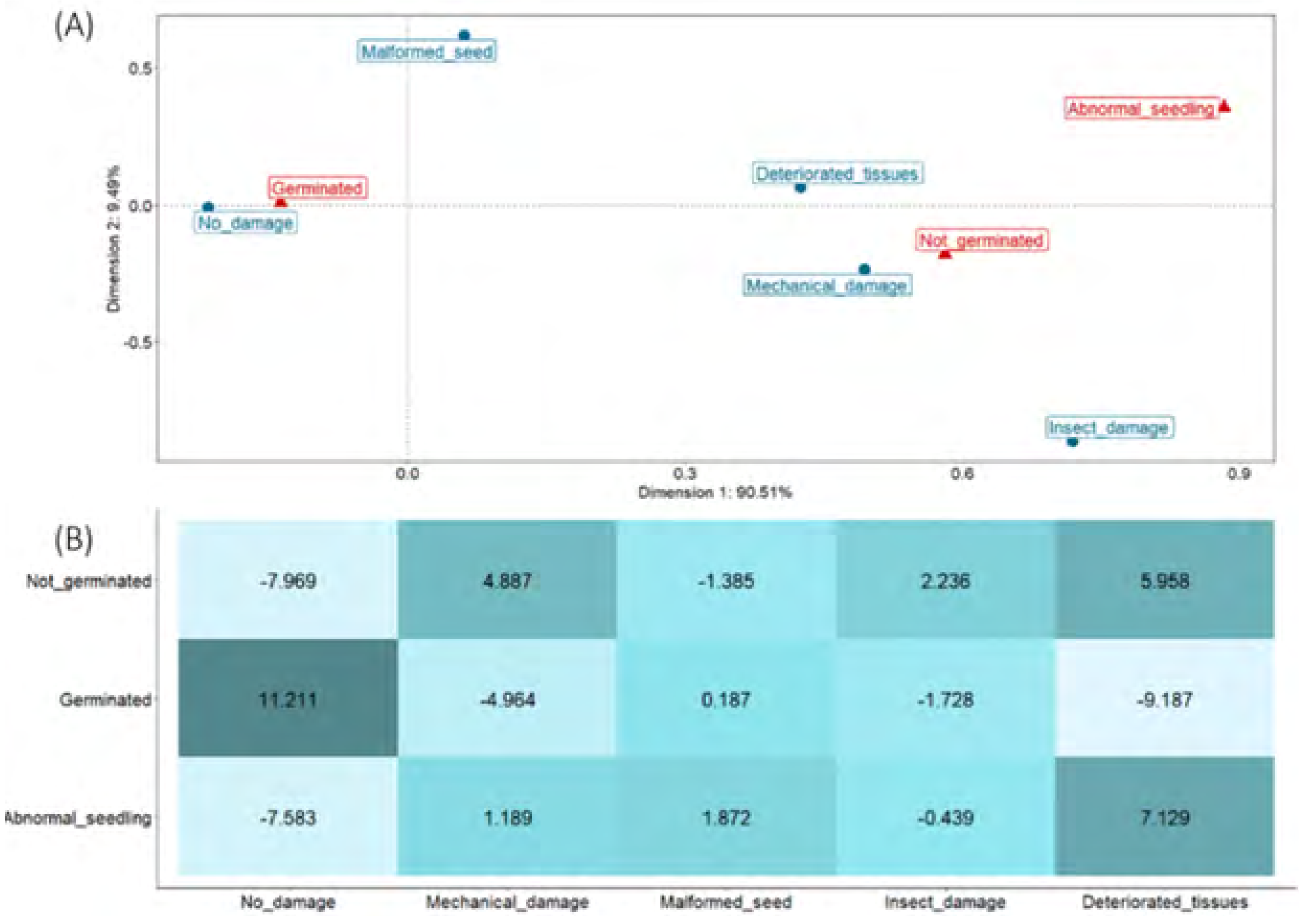

Figure 3. Biplot of the first two axes of the correspondence analysis (ANACOR) illustrating the different types of damage in cowpea (*Vigna unguiculata* (L.) Walp) seeds and their associations with seed performance in the germination test (3a); heat map of adjusted standardized residuals from the chi-square test - x^2 (3b).

Table 2. Means of empty spaces (%) in the internal cavity of cowpea (*Vigna unguiculata* (L.) Walp) seeds and standard deviation, evaluated with ImageJ software, used to define the categories.

Lots	Empty space (%)	Standard deviation
1	4.75	0.94
2	5.85	0.98
3	4.48	0.95
4	4.72	0.84
5	5.49	1.09
6	6.08	0.93
7	6.08	1.36
Mean	5.35	1.01
Category I	Category II	Category III
≤ 4.34%	> 4.34% and < 6.36%	≥ 6.36%

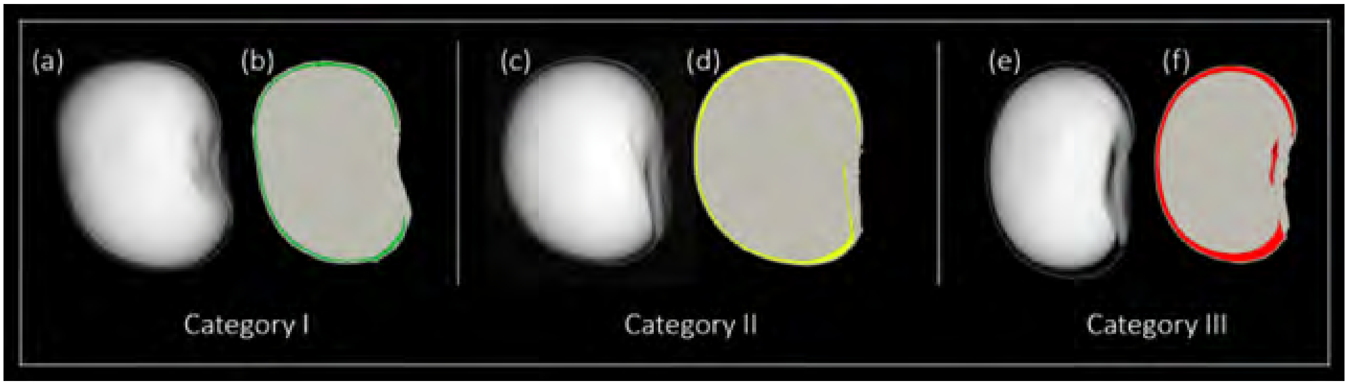

Figure 4. Cowpea (*Vigna unguiculata* (L.) Walp) seeds distributed in three categories according to their empty space: X-ray images (4a, 4c and 4e) and X-ray images after processing with ImageJ software (4b, 4d and 4f).

Table 3. Percentage of cowpea (*Vigna unguiculata* (L.) Walp) seeds of the 'BRS Guariba' cultivar, distributed in three categories based on the mean values of empty space inside them.

Lots	Category I	Category II	Category III	Germination		
				NS	AS	DS
		(% of seeds)		---------------- % ----------------		
1	34	61	5	98	1	1
2	8	59	33	84	2	14
3	44	51	4	98	1	1
4	37	59	4	99	0	1
5	11	66	23	87	2	11
6	2	57	41	84	4	12
7	10	47	43	89	2	9

the quantification of the actual extent of embryonic development is more accurate. In addition to ImageJ software, the literature indicates *Tomato analyzer*® (Marcos-Filho et al., 2010) and *Image ProPlus*® (Dell'Aquila, 2007; Silva et al., 2012).

Table 3 shows the percentages of seeds distributed in the three established categories of empty spaces; it is possible to observe that in Category I, that is, seeds with smaller empty spaces in the internal cavity, lots 1, 3 and 4 were those with the highest percentages of seeds (34-47%), while lots 2, 5, 6 and 7 had the lowest percentages (2-11%). In Category II, all lots behaved similarly, with a large number of seeds within this range of empty space (51-66%), while in Category III, lots 2, 5, 6 and 7 were those with the largest empty space inside their seeds; in parallel, lots 3, 4 and 1 had 5% less of seeds in this class.

In Table 3, there seems to be a relationship between the area occupied by the embryo inside the seed and the seed performance in germination; indeed, lots with seeds with larger empty space (Category III) had higher numbers of abnormal seedlings (2-4%) and dead seeds (9-14%) and lower number of normal seedlings (84-89%). Lots 1, 3 and 4 had the highest number of normal seedlings in the germination test (98-99%) and the lowest number of abnormal seedlings (0-1%), with 1% of dead seeds, and these lots were the ones with seeds mostly classified in Category I.

The differences of empty space in the seeds within each lot may occur due to the uneven maturation of seeds, common in cultivars of indeterminate growth habit, as is the case of the studied cultivar (Gonçalves et al., 2009).

It is possible to affirm that the physiological performance of seeds without empty space is favored by the greater availability of stored reserves, since these components stored in the seeds during the maturation process are essential for the initial germination processes, as protein synthesis and initial respiratory activity use the components stored during seed maturation as substrate (Marcos-Filho, 2015).

Several studies report that seeds with higher percentages of internal empty spaces were not able to originate normal seedlings in the germination test, for instance in bell pepper (Dell'Aquila, 2007; Gagliardi and Marcos-Filho, 2011), whose seeds with the largest empty space had their germination compromised. In studies with other species, similar results have also been observed (Machado and Cicero, 2003; Silva et al., 2013). On the other hand, the presence of a larger area occupied by the embryo and endosperm did not favor germination in eggplant seeds (Silva et al., 2012).

In the present research, the relationships between the categories of empty space in the seeds and their behavior in the germination test were also determined by multivariate analysis. In Figure 5a, the first two axes of ANACOR express 98.7% (Dim.1) and 1.3% (Dim.2) of the total variation of the data. The adjusted standardized residuals, presented in Figure 5b, indicate a strong association of the categories of smaller empty space (I and II) with the category "Germinated" and a negative association with abnormal seedlings and seeds that did not germinate, whereas the seeds with greater empty space were strongly associated with these categories.

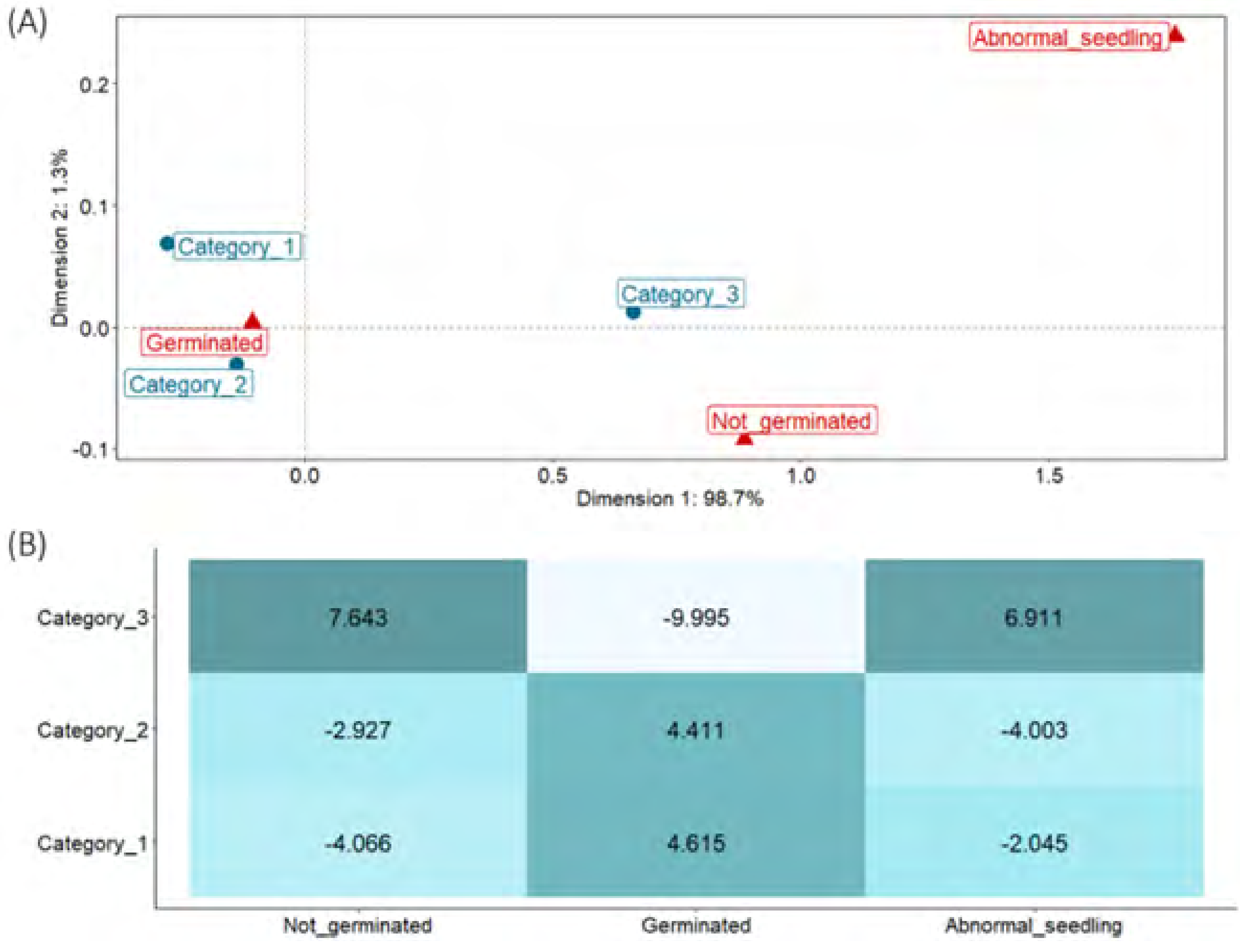

Figure 5. Biplot of the first two axes of the correspondence analysis (ANACOR) illustrating the different types of damage in cowpea (*Vigna unguiculata* (L.) Walp) seeds and their associations with the different categories of empty space with the performance of seeds in the germination test (3a); heat map of adjusted standardized residuals from the chi-square test - x^2 (3b).

In general, the use of X-rays was effective in detecting damage and measuring empty space with the aid of ImageJ software in cowpea seeds, being able to provide comprehensive information on the association of internal seed morphology and performance during the germination process, so it can be used as a complementary procedure in the evaluation of the physiological potential of seeds. Indeed, the presence of damage, or the increase in empty space inside the seeds, is associated with a higher incidence of abnormal seedlings and non-germinated seeds.

CONCLUSIONS

Internal damage in cowpea seeds, identified by radiographic images, depending on its location and intensity, affects their germination. Moreover, computerized measurement of the empty space inside the seed, through ImageJ software, makes it possible to establish its relationship with germination.

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