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Weathering Damage in Soybean Seeds: Assessment, Seed Anatomy and Seed Physiological Potential

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ABSTRACT

Weathering damage in soybean (*Glycine max*) seeds wrinkles the cotyledons, cracks the seed coat and causes tissue death. The objective of this study was to assess weathering damage and relate damage severity with seedling symptoms and loss of viability, and show modifications of seed tissues with weathering damage using scanning electron microscopy (SEM). Two soybeans seeds lots, cv. 'TMG115-RR', were subjected to X-ray and tetrazolium (TZ) testing and seedling assessment, to assess weathering damage. Moreover, seeds with or without weathering damage were inspected using SEM. X-ray image analysis as well as TZ and seedling assessment tests confirmed that weathering damage interfered with seed physiological potential, varying according to the extent and location of the damage. SEM images showed that the seed coat hourglass cells occurred near the hilum region and gradually decreased until they disappeared opposite to the hilum region, a region where typical wrinkles caused by weathering damage are observed. On the cotyledons, the cells in damaged seeds appeared lengthened and compressed, probably accounting for cell death in the damaged region.

RESUMO

Danos por umidade em sementes de soja (*Glycine max*) causam enrugamento nos cotilédones, rachaduras no tegumento e pode causar a morte dos tecidos das sementes. O objetivo deste estudo foi avaliar danos por umidade em sementes de soja e relacionar a severidade do dano com sintomas em plântulas e perda de viabilidade, além de avaliar as modificações nos tecidos das sementes danificadas por meio da microscopia eletrônica de varredura (SEM). Dois lotes de sementes de soja, cultivar TMG115-RR, foram submetidos ao teste de raios X e de tetrazólio ou à avaliação de plântulas para avaliar os danos por umidade. Além disso, sementes com e sem danos por umidade foram submetidas ao SEM. Os resultados mostraram que a análise de imagens por meio de raios X, o teste de tetrazólio e a avaliação de plântulas confirmaram que o dano por umidade interfere no potencial fisiológico das sementes dependendo da extensão e localização destes. Por meio das imagens de microscopia eletrônica de varredura foi verificada a presença de "células em forma de ampulheta" na região próxima ao hilo a qual decresce até o total desaparecimento na região oposta ao hilo, região onde usualmente são observados sintomas típicos de enrugamento causados pelos danos por umidade. Nos cotilédones, as

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células de sementes danificadas se apresentam alongadas e comprimidas, o que provavelmente é a razão da morte celular na região danificada.

INTRODUCTION

At physiological maturity, the high moisture content of the soybean [*Glycine max* (L.) Merr.] seed, pod and plant prevent mechanical harvesting and seeds must stay in the field until they reach a moisture content suitable for harvesting. This time in the field can be considered as a period of “storage”, where climatic conditions are rarely favorable (França-Neto and Henning, 1984) and may cause weathering damage.

Weathering damage due to adverse weather conditions during the post-maturation and pre-harvest periods can lead to seed quality reduction. These adverse factors cause an increase in seed coat permeability, with the consequent rapid wetting and drying of the seed and tissue damage leading to deterioration, with a wrinkled or cracked seed coat as a probable indication of this process (Mesquita et al., 2006). During initial exposure to water, the seed coat resists rapid uptake of water, but with repeated exposure, permeability of the seed coat increases sharply and permits rapid wetting and drying of the seed in the pod. Stresses created in seed tissues by this process initiate accelerated deteriorative changes (Moore, 1971).

Weathering damage can also cause irregular cracks in the surface of the seed coat. This condition results from separation of epidermal (palisade cells) and hypodermal (hourglasses cells) tissues, exposing the underlying parenchyma tissue or the cotyledon. A cracked or wrinkled seed coat increases the possibility of splitting and seed damage during processing, conditioning and handling. It also increases pathogen infection (e.g., *Phomopsis* spp. and *Colletotrichum truncatum*), and decreases seed germination and emergence (Yaklich and Barla-Szabo, 1993; Prijic et al., 1998). The outer surface of the cotyledons just beneath the area of seed coat wrinkling becomes damaged, which may result in either abnormal seedlings or complete absence of germination.

The tetrazolium (TZ) and X-ray tests can assess weathering damage in soybean seed lots. In the TZ test, stained seeds exhibit intense red or white color lesions on embryonic tissues adjacent to the wrinkles caused by the damage (Costa and Marcos Filho, 1994; França-Neto et al., 1998). According to these authors, weathering damage has a typical feature-lesion symmetry in both cotyledons. Arango et al. (2006) reported that weathering damage can in some cases be characterized by injured areas only within cotyledons or only in one of the cotyledons, as opposed to the symmetrical symptoms reported by França-Neto et al. (1998).

In X-ray testing, weathering damage is characterized by images showing visible wrinkles in the surface of the seed coat, cotyledons or embryonic axis. To emphasize the importance of weathering damage, Pinto et al. (2009), based on X-ray images of soybean seed lots, showed that seeds with severe damage on the cotyledons or embryonic axis were dead and, therefore, the germination of these lots was reduced.

Scanning electron microscopy (SEM) has been used to study the structural features of soybean seed coats (Pereira and Andrews, 1985; Yaklich et al., 1995)

as well as assess water absorption (Chachalis and Smith, 2001) and disease infection (Kulik, 1988; Kulik and Yaklich, 1991) in seeds and seedlings. Soybean seed coats possess characteristic layers — the cuticle and layers of palisade, hourglass and parenchyma cells (Pereira and Andrews, 1985). Some authors have pointed out that weathering damage was frequently observed in soybean seeds lacking hourglass cell layers of the hypodermis, in the region opposite to the hilum, so that a seed's expansion and contraction were not mitigated (Marcos Filho, 2005). Pereira and Andrews (1985), working with SEM to evaluate wrinkled and non-wrinkled soybean seed coats, also reported the absence of hourglass cells in the region opposite the hilum, where weathering damage was usually more severe and harmful.

The objective of the present study was to assess weathering damage in soybean seeds, and relate damage severity to seedling symptoms and loss of viability by different methods. Moreover, scanning electron microscopy (SEM) was used to examine modifications of the seed coat and cotyledon anatomy in seeds with weathering damage.

MATERIALS AND METHODS

Two soybean seed lots, cv. 'TMG115-RR', produced in Alto Garças (16°56'38" S and 53°31'41" W), Mato Grosso, Brazil, were used to assess weathering damage.

X-ray test

Four replications of 50 seeds from each lot were placed on a transparent sheet, fixed underneath with transparent adhesive tape so that the embryonic axis was positioned parallel to the sheet. The radiation intensity and exposure time used were 26 kV and 4.5 sec, respectively. To obtain radiographs, each transparent sheet with seeds was placed 28.6 cm from the radiation source. Images were then captured and saved for further image processing, which included amplification of size and a simultaneous seed assessment. Seed X-ray images were individually analyzed and each seed scored from 1 to 3, as described in Table 1.

Tetrazolium test

The same seeds analyzed first by the non-destructive X-ray test were then subjected to the TZ test, allowing comparisons of different tests on the same seeds. For the TZ test, four replicates of 50 seeds from both lots were preconditioned on paper towels moistened with an amount of water equivalent to 2.5 times the weight of the dry substrate, for a period of 16 h at 25 °C. Seeds were then placed for staining in a 0.075% (w/v) solution of 2,3,5-triphenyl-tetrazolium chloride, and incubated in darkness in an oven at a constant temperature of 40 °C for 150 to 180 min. Seeds were then rinsed with tap water several times to stop the staining reaction (Baalbaki et al., 2009). Weathering damage was recorded based on individual seed examinations. As outlined above, typical symptoms of damaged seeds included specific wrinkles on the cotyledonary region opposite the hilum or on the hypocotyl axis. For TZ-stained seeds, damage was manifested as dark-red and white lesions on embryonic tissues just beneath these wrinkles. The TZ test and subsequent assessments were carried out according

to the criteria established by França-Neto et al. (1998). Each seed was assigned a class of 1 to 5 if viable, and 6 to 8 if non-viable, as described in Table 1.

Seedling assessment

Similar to the TZ test, the same seeds analyzed by the non-destructive X-ray test were subjected to the seedling assessment test. Individual seeds, previously identified in the X-ray test, were sorted and germinated on rolled paper towels moistened with an amount of water equivalent to 2.5 times the weight of the dry substrate, at 25 °C (ISTA, 1985). Seedling assessments were performed five days after sowing. Seedlings and dead seeds were individually photographed and images displayed on computer monitors. In this way, X-ray images of dry seeds and those of seedlings or dead seeds could be examined simultaneously, allowing for comparative diagnoses. Since weathering damage on seedlings resulted in cell death, typical symptoms on affected areas ex-

TABLE 1. Score and class descriptions for soybean seeds with or without weathering damage, using X-ray, tetrazolium and seedling assessment tests.

Test	Classification	Description
<i>X-ray</i>	Score	
	1	Seed without any weathering damage
	2	Seed with non-severe weathering damage affecting less than 50% of total surface, with small wrinkles on the seed coat over the embryonic axis and/or cotyledons
	3	Seed with severe weathering damage affecting more than 50% of total surface, with large wrinkles on the seed coat over the embryonic axis and/or cotyledons
<i>Tetrazolium</i>	Classes	
	1	Viable seed without any weathering damage
	2	Highly vigorous seed with non-severe and superficial weathering damage
	3	Non-severe damage located in non-critical areas of the seed
	4	Viable seed with severe damage that does not affect seed viability
	5	Seed with severe damage in critical areas, but still viable
	6	Non-viable seed with severe damage, more intensive than that observed in class 5
	7	Non-viable seed with severe damage in critical areas, or with more than 50% of the surface affected by weathering damage
	8	Unstained dead seed
<i>Seedling assessment</i>	Score	
	1	Seedling without any weathering damage symptoms
	2	Seedling with non-severe weathering damage symptoms, affecting less than 50% of the total cotyledon surface area
	3	Seedling with severe weathering damage symptoms, covering more than 50% of the total cotyledon surface area
	4	Dead seed

hibited dark stains or lesions. Similar to the X-ray analyses, seedlings were scored from 1 to 4, as described in Table 1.

Statistical analysis

In order to compare the tests, the means of X-ray and TZ tests for the same seeds and the means of X-ray and seedling assessment tests for the same seeds were compared using confidence intervals ($p \leq 0.01$).

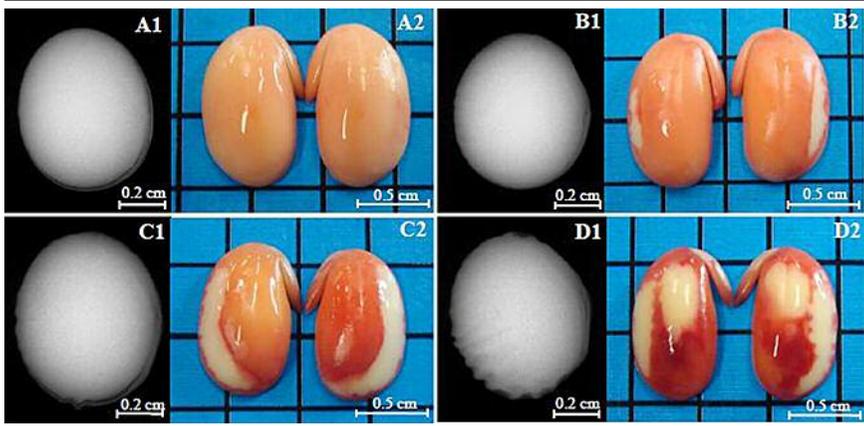
Seed anatomy using scanning electron microscopy (SEM)

Before SEM assessment, seeds were sorted by X-ray testing. Ten seeds from each class were then cross-sectioned transversely for seed coat observations, and longitudinally for cotyledon observations, using a single-edge razor blade, and mounted with the cut surface upwards on aluminum stubs. The samples were coated with gold in Bal-tec SCD 020 sputter and examined under a scanning electron microscope (LEO 435-VP) at 20 kV.

RESULTS

The TZ test, performed after the seeds were submitted to the X-ray test, allowed visualization of different classes of weathering damage (Fig. 1). Seeds assessed by X-ray testing to be without damage (score 1; Fig. 1-A1) did not show lesions on the cotyledons in the TZ test (Fig. 1-A2). Weathering damage of cotyledons, classified as non-severe (score 2; Fig. 1-B1) or severe (score 3; Fig. 1-C1 and D1) based on X-ray images, was confirmed by TZ testing, wherein seeds with non-severe damage exhibited lesions as class 2 (Fig. 1-B2) and 3. Therefore, damage was present but did not affect seed germination. However, seeds

FIGURE 1. Soybean (cv. 'TMG115-RR') seed images from X-ray (A1, B1, C1 and D1) and tetrazolium (TZ; A2, B2, C2 and D2) tests. A1 and A2: seed with no weathering damage (score 1 in X-ray and class 1 in TZ tests); B1 and B2: seed with non-severe weathering damage on the cotyledons (score 2 in X-ray and class 2 in TZ tests); C1 and C2: seed with severe weathering damage on the cotyledons (score 3 in X-ray and class 4 in TZ tests); D1 and D2: seed with severe weathering damage on the cotyledons and embryonic axis (score 3 in X-ray and class 6 in TZ test). For each pair of pictures, the same seed is shown for both tests.



characterized by X-ray testing as severely damaged (score 3) were classified as class 4 (Fig. 1-C2) and 5 by the TZ test, indicating that seeds were viable but had decreased vigor. In some cases, when severe damage was observed in the embryonic axis and cotyledons by X-ray testing (Fig. 1-D1), the TZ test revealed extensive lesions (Fig. 1-D2), indicating death of most of the embryo tissue, rendering the seeds non-viable.

Using the seedling assessment test, it was possible to observe symptoms of weathering damage in the embryonic axis or cotyledons of seeds identified by the X-ray test as damaged (Fig. 2). Similar to the TZ test, seeds with scores of 1 and 2 based on the X-ray test (Fig. 2-A1 and B1) were viable and developed into normal seedlings with cotyledons without lesions (Fig. 2-A2 and A3), or with small lesions (less than 50% of the cotyledon area) that did not negatively impact seedling development (Fig. 2-B2 and B3). When seeds were given a score of 3 using the X-ray test (Fig. 2-C1 and D1), seedling assessment revealed that this damage reduced physiological potential, resulting in either abnormal seedlings (Fig. 2-C2 and C3) or dead seeds (Fig. 2-D2), with lesions covering more than 50% of the cotyledon area.

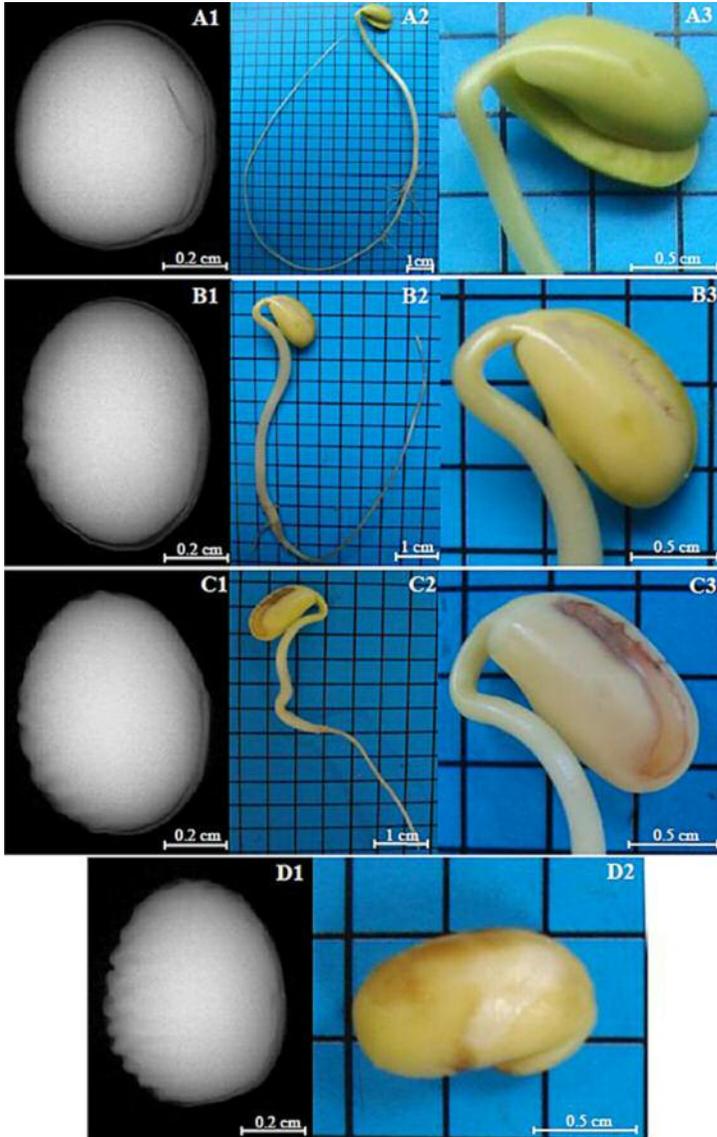
SEM seed coat images revealed the palisade, hourglass and the parenchyma cell layers at the region near the hilum (Fig. 3-A2, B2 and C2). The size of the hourglass cells gradually decreased until no cells were observed opposite the hilum region, where wrinkles caused by weathering damage are usually observed (Fig. 3-A3, B3 and C3). In addition, seeds with a score of 1 (Fig. 3-A1) and 2 (Fig. 3-B1) based on the X-ray test had expanded and undamaged hourglass cells (Fig. 3-A2 and B2), while in seeds with a score of 3 (Fig. 3-C1), hourglass cells were twisted, bent and flattened (Fig. 3-C2). SEM cotyledon images are only shown for seeds classified as having severe damage (Fig. 4). Longitudinal sections showed that wrinkles were not only present in the seed coat, but also in cotyledons (Fig. 4-A and B), with longer cells towards the "peak" (Fig. 4C), and compressed cells in "depressions".

TZ classes and seedling assessment scores were associated with X-ray scores (Table 2). Seeds from both lots with a score of 1 (seeds without weathering damage) in the X-ray test corresponded to class 1 in the TZ (seeds without damage) and a score of 1 in seedling assessment (seedlings without damage) tests. A score of 2 in the X-ray test (seed with non-severe weathering damage) corresponded to classes 2 and 3 in the TZ (weathering damage without affecting the germination and vigor) and score of 2 in seedling assessment (seedlings with non-severe weathering damage) tests. A score of 3 in the X-ray test (seeds with severe damage) corresponded to classes 4–8 in the TZ and scores of 3 and 4 in the seedling assessment (seedlings with severe damage and dead seeds) tests.

DISCUSSION

As a non-destructive method, the X-ray test made it possible to standardize all other analyses (TZ, seedling assessment test and SEM) of weathering damage, and helped identify the effects on seed physiological potential through seed tissue anatomy. The X-ray and TZ tests demonstrated that weathering

FIGURE 2. Soybean (cv. 'TMG115-RR') images from X-ray (A1, B1, C1 and D1) and seedling assessment (SA) tests (A2, A3, B2, B3, C2, C3 and D2). A1, A2 and A3: seed, seedling and cotyledons, respectively, with no weathering damage (score 1 in X-ray and SA tests); B1, B2 and B3: seed, seedling and cotyledons, respectively, with non-severe weathering damage on the cotyledons (score 2 in X-ray and SA tests); C1, C2 and C3: seed, seedling and cotyledons, respectively, with severe weathering damage on the cotyledons (score 3 in X-ray and SA tests); D1 and D2: seed with severe weathering damage on the cotyledons and embryonic axis (score 3 in X-ray and 4 in SA tests). For each set of pictures, the same seed that later developed into a seedling is shown for both tests.



damage could affect soybean seed viability and vigor, and this was directly dependent on the extent and location of the damage (França-Neto et al. 1998; Flor et al., 2004; Arango et al., 2006; Pinto et al., 2009). The TZ test is used to identify damaged areas and dead seeds. In damaged areas, tissue color upon staining is dark red as a result of intense tissue respiration, and in dead seeds the tissues are white (unstained) due to the absence of respiration (França-Neto et al., 1998; Baalbaki et al., 2009). When the wrinkled region, observed in X-ray images, was not extensive and only affected a small part of the cotyledon

FIGURE 3. Soybean (cv. 'TMG115-RR') seed X-ray images (A1, B1 and C1) and seed coat scanning electron microscopy (SEM) micrographs (A2, A3, B2, B3, C2 and C3). A1: seed with no weathering damage (score 1 in X-ray test); A2 and A3: SEM micrographs of seed coat at regions next to the hilum and opposite the hilum, respectively, in a seed with no weathering damage; B1: seed with non-severe weathering damage (score 2 in X-ray test); B2 and B3: SEM micrographs of seed coat at regions next to the hilum and opposite the hilum, respectively, in a seed with non-severe weathering damage; C1: seed with severe weathering damage (score 3 in X-ray test); C2 and C3: SEM micrographs of seed coat at regions next to the hilum and opposite the hilum, respectively, in a seed with severe weathering damage. pl = palisade cells, hg = hourglass cells, pa = parenchyma cells, co = cotyledon.

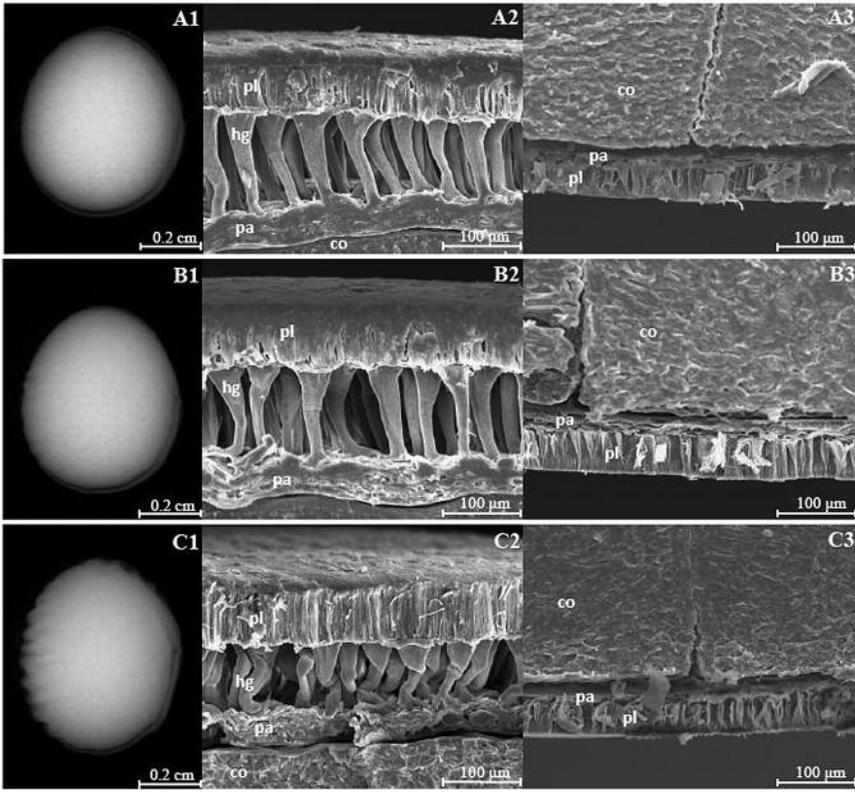


FIGURE 4. Soybean (cv. 'TMG115-RR') cotyledon scanning electron microscopy (SEM) micrographs of a seed with severe weathering damage. A: region of wrinkled cotyledons; B: detail of the "peak" of the wrinkle with visible cell compression; C: cotyledon tissue cells flattened and compressed in the region of the wrinkled "peak". sc = seed coat, co = cotyledon, cc = compressed cells.

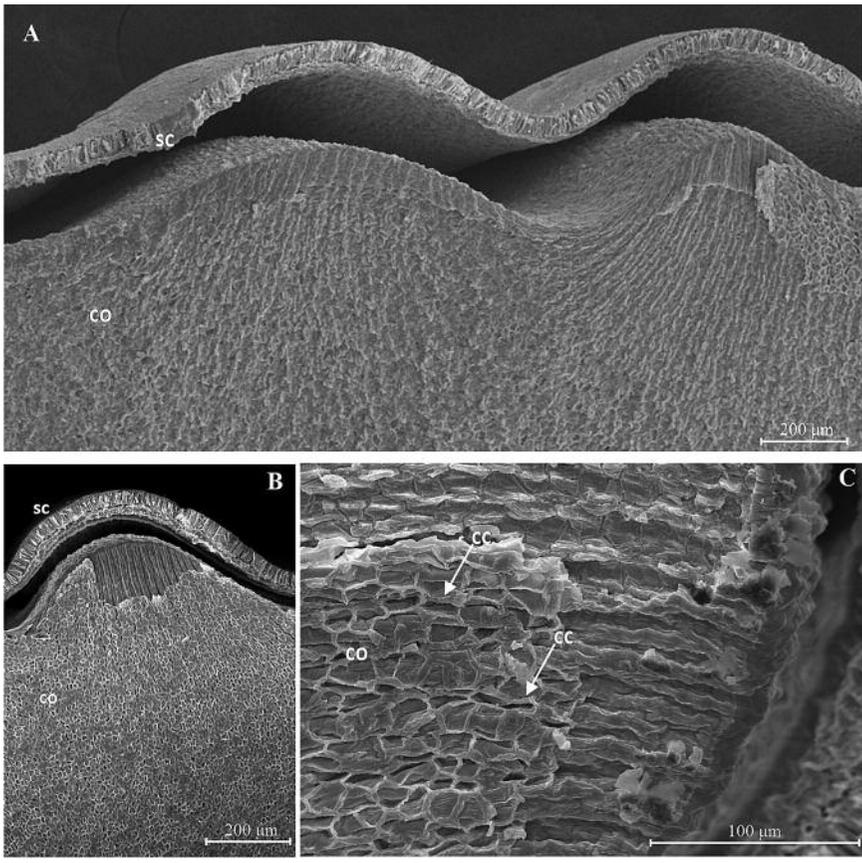


TABLE 2. Mean percentage weathering damage of soybean seeds, cv. 'TMG 115-RR', from two seed lots, determined by the tetrazolium (TZ), seedling assessment (SA) and X-ray tests.

		Lot 1	Lot 2			Lot 1	Lot 2
<i>X-ray (score)</i>	1	24 ± 2.10 [†]	35 ± 5.25	<i>X-ray (score)</i>	1	37 ± 8.76	37 ± 5.73
	2	54 ± 4.20	44 ± 3.15		2	40 ± 4.87	41 ± 2.46
	3	22 ± 3.15	21 ± 7.36		3	23 ± 5.71	22 ± 4.46
<i>TZ (classes)</i>	1	22 ± 2.73	42 ± 7.37	<i>SA (score)</i>	1	42 ± 7.72	42 ± 5.71
	2-3	58 ± 4.21	42 ± 4.20		2	36 ± 4.70	42 ± 4.70
	4-8	20 ± 1.58	16 ± 4.21		3 & 4	22 ± 2.46	16 ± 6.30

[†]Mean plus confidence interval ($p \leq 0.01$). Means within each column, for each seed lot, were used to compare X-ray and TZ tests for each lot, and X-ray and SA tests for each lot.

tissue, TZ testing verified that only this small part was dead. In many cases, limited damage that compromised a small portion of the cotyledons was not important because it did not reduce the efficiency of reserve hydrolysis and assimilation during germination. However, when damage was severe, affecting the embryonic axis or most of the cotyledons, it induced deterioration of these tissues (Pinto et al., 2009), leading to cell death and often rendering the seed non-viable.

The effect of weathering damage on seed physiological potential was confirmed by the seedling assessment test following X-ray testing. Seeds with no or non-severe weathering damage developed into normal seedlings since the damage did not interfere with germination. Severe damage affected germination and frequently resulted in either abnormal seedlings or dead seeds. In addition, damage caused tissue necrosis and darkened lesions on cotyledons, and when it was over 50% (ISTA, 2009), interfered in the hydrolysis and assimilation processes of compounds necessary for germination and seedling development. Moreover, damage occurring at the cotyledon junctures or near the embryonic axis impaired translocation of hydrolyzed substances from cotyledons, resulting in abnormal seedlings.

Based on SEM results, conspicuous hourglass cells of the sub-hilar region probably acted as a “cushion”, preventing the occurrence of wrinkles as a consequence of successive cycles of hydration and dehydration. Since there were no hourglass cells in the region opposite the hilum, the expansion and contraction of the seed coat could not be “cushioned”, and was forced to wrinkle (Pereira and Andrews, 1985; Yaklinch and Barla-Szabo, 1993). Consequently, deterioration was initiated, eventually spreading to the cotyledons or embryo axis below the wrinkled or cracked areas (Forti et al., 2010). This deterioration probably occurred because, in this damaged region, the underlying tissues were exposed to adverse weather conditions reducing seed quality. In addition, cotyledon cell compression observed in seeds with weathering damage could cause cell death. Therefore, it could be inferred that cell death of cotyledon or embryonic axis tissues exhibiting weathering damage symptoms occurred due to their exposure to adverse weather conditions, or because cotyledonary cells underneath the wrinkles were subject to unequal pressure, causing them to bruise or even die (Pereira and Andrews, 1985), affecting seed lot viability.

When heat stress and air RH fluctuations occur after physiological maturity, death of cells adjacent to weathering-damaged tissues can occur due to oxidative damage. Previous studies revealed that heat stress could damage cell membranes (Scandalios, 1993; Marcum, 1998; Galleschi et al., 2002) leading to cell death (Abernethy et al., 1989; Scandalios, 1993). In aerobic biological systems, superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH\cdot$), and singlet oxygen (O_2^*) are species of active oxygen derivatives that may attack and damage macromolecules in living cells (Foyer et al., 1994). Plants have developed enzymatic and non-enzymatic scavenging systems to quench active oxygen. When plants are subjected to adverse conditions such as high temperature and drought, the scavenging system may lose its function and the balance between producing and quenching active oxygen species can be disturbed,

resulting in oxidative damage (Scandalios, 1993; Zhang and Kirkham, 1994; Zhang and Han, 1997).

The percentage of seed with weathering damage, based on TZ and seedling assessment tests, was sometimes higher than that obtained by X-ray analysis. This was probably because small damage could not always be detected by the X-ray testing (Pinto et al., 2009). Considering other scores, we observed a similarity between the percentages of seeds classified with a score of 3 by the X-ray test and those with scores of 4–8 by the TZ test, and classes 3 and 4 based on seedling assessment. Such results indicated that severe weathering damage influenced soybean seed viability and vigor, affecting physiological potential. Results also demonstrated the effectiveness of the three testing procedures in evaluating weathering damage.

In conclusion, results from this study showed that X-ray image analysis as well as TZ and seedling assessment tests could be used to analyze weathering damage. Results also confirmed that weathering damage reduced seed physiological potential, and could be one of the most harmful factors affecting soybean seed quality. Use of SEM allowed for observing anatomical modifications in seed coat and cotyledon tissues of seeds with weathering damage.

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