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An assessment of mechanical and stink bug damage in soybean seed using X-ray analysis test

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Summary

The X-ray image analysis technique is a precise method that enables examination of regions that are damaged and/or altered in seed and their location and extension. It is a non-destructive method allowing the X-ray treated seed to be submitted to quality physiological tests. The objective of this study was to use X-ray image analysis technique to identify mechanical and stink bug damages in soybean seed. Seeds from different batches of the BRS184 cultivar were sorted by X-ray test and then were tested for germination with the aim to relate the damage with possible seed viability loss. Alternatively, the tetrazolium test was carried out to be compared with the X-ray test. Damage severity and location were considered for the interpretive analysis of X-ray images. The results showed that the X-ray image analysis technique was effective in detecting mechanical and stink bug damage in soybean seed.

Introduction

Establishing a crop with an adequate plant population is one of the factors that contribute mainly to ensure the basis for obtaining high yields and this aspect depends on the use of high quality seed. The use of low vigour soybean seed may result in the need for replanting and this practice causes losses to the producers in terms of reducing yields and increased production costs.

Soybean seed is very susceptible to mechanical damage since the vital parts of the embryonic axis (radicle, hypocotyl and cotyledon) lie under a thin seed coat that offers very little protection (França-Neto and Henning, 1984). Mechanical injury caused during the seed harvest and processing is therefore one of the main causes of reduction in quality (Paiva *et al*., 2000), and can lead to seed lot rejection.

Stink bug damage is also a major problem that affects the quality of soybean seed. When stink bugs feed on soybean seed, they inoculate them with the *Nematospora coryli Peglion* yeast (Sinclair and Backman, 1989), causing yeast spot (Ferreira *et al*., 1979). Colonization of the seed tissue by this yeast generates serious necrosis that result in

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germination and vigour losses (Bowling, 1980; Villas-Boas *et al*., 1990). The damaged seed may present typical spots and can be deformed or wrinkled, and their chemical composition may be altered, with an increase in protein content and a decrease in oil content. Stink bug attack during the grain formation phase causes grain or pod abortion, reduces productivity and can cause leaf retention or the presence of green stems at harvest (Gazzoni, 1998). The symptoms of seed with stink bug damage are typical and easily identified by the tetrazolium test (França-Neto *et al*., 1998). The seed tissue infection by *N. coryli* results in characteristic circular, sometimes shrunken and deep lesions. Tissues with lesions are dead and flaccid and are typically white, or sometimes greenish, yellowish, or greyish white. A distinct dark-red boundary commonly exists between damaged and sound tissues. Multiple lesions on a single seed might occur, and if they overlap, the typical circular wound will not be distinguishable. Frequently, a minor puncture caused by the insect can be noticed in the centre of the circular lesion. Deep punctures by the insect might result in inoculation of central seed tissues by *N. coryli*. Therefore, colonization of the tissues by the fungus will cause internal damage that is not always revealed on the outside of the seed.

Hence there is a need for the development of methods that allow quick and reliable assessment of the physiological potential of damaged seed and thus decisions regarding harvest, processing, storage and commercialization may be taken. The interest in X-ray test has been growing in seed technology because it analyses quickly internal seed morphology and provides the detection of possible embryo damage and abnormalities without seed destruction. In addition to these analysis characteristics, the X-ray test may be applied to the study of physiological changes during maturation, germination and conditioning processes (Simak, 1991; Foucat *et al*., 1993; Dell'Aquila, 2007a) and in breeding research (Bino *et al*., 1993). The viability of seed submitted to X-ray is not affected because non lethal radiation doses are generally used during the test (Bino *et al*., 1993; ISTA, 1995). The method has been adopted in the assessment of seed integrity in *Pinus* spp. (Kamra, 1971; Simak, 1984; Sahlen *et al*., 1995), corn (Smith and Grabe, 1985), some cucurbits and solanaceaes (Kamra, 1964, 1966; van der Burg *et al*., 1994) and various forest species (Swaminathan and Kamra, 1961; Kamra, 1976; Oliveira *et al*., 2003; Machado and Cicero, 2003). Moreover X-ray test has been also used to identify mechanical damage in corn (Cicero *et al*., 1998; Carvalho *et al*., 1999; Cicero and Banzatto Junior, 2003) and soybean (Obando-Flor *et al*., 2004) seed.

The objective of the present study was to assess the potential of the X-ray image analysis technique in identifying mechanical and stink bug damages in soybean seed and relate the severity of damage with loss of viability and vigour, as tested by germination and tetrazolium tests.

Material and methods

The study was carried out in the Image Analysis and Seed Analysis Laboratories at the Crop Science Department at the "Luiz de Queiroz" Agricultural College, University of Sao Paulo (ESALQ/USP), Piracicaba, SP, Brazil and in the Seed Laboratory at Embrapa Soybean, Londrina, PR, Brazil.

Four soybean [*Glycine max* (L.) Merrill] seed lots of the same cultivar, BRS184 produced in Londrina in the 2005/2006 growing season were used in all the trials.

Seed water content
The test was carried out in an oven at 105±3°C/24h (Brasil, 1992), using two subsamples of 4.0 g of seed, for each lot. Results were expressed as mean percentages for each lot (wet basis).

Germination test
Four subsamples of 50 seed per lot were distributed on germination paper towel rolls, moistened with an amount of water equivalent to 2.5 times the weight of the dry substrate and placed to germinate at 25°C. The assessments were performed at four and eight days after sowing, according to criteria established by ISTA (1985); results were expressed as mean percentages of normal seedlings for each lot.

Accelerated aging
This test was performed according to the method known as "gerbox" using plastic boxes (11 cm × 11 cm × 3 cm), in a water jacket chamber (model 3015 VWR Scientific, USA); samples containing 8 grams of seeds were utilized, distributed so as to form a simple layer over the surface of the metallic screen suspended inside each plastic box (internal compartment), containing 40 mL water. The boxes, covered with lids, remained inside the chamber during 48 h at 41°C. Seeds were then tested for germination, according to methodology described for the germination test. The evaluation was performed at five days after sowing and the results were expressed as mean percentage of normal seedlings for each lot (Marcos-Filho, 1999).

X-ray test
Seeds were submitted to the X-ray analysis test to detect mechanical and stink bug damages using a Faxitron X-ray apparatus (model MX-20, USA). Five replications of 50 seed from each lot were placed in individual wells on an acrylic plate, fixed underneath with transparent adhesive tape that kept them placed so that the embryonic axis was positioned parallel to the plate. The radiation intensity and exposure time used were 25 kV and 40 seconds, respectively. To obtain the radiography, the acrylic plate with the seed was placed directly on radiographic film (Kodak MIN-R 2000, 18 × 24 cm in size) at a distance of 40 cm from the radiation source. The radiographic films were developed in a Hope X-ray automatic processor (model 319 Micro-Max, USA). The film images were captured with an Umax Scanner (model Power LooK 1100, USA) for a further computer image processing, which included amplification of image size and a simultaneous batch comparison.

Individual seeds were previously identified, sorted and subjected to the germination test (Brasil, 1992) to assess the relationship between the level of detected mechanical and stink bug damages and the germination response. All the normal and abnormal seedlings and the seed that did not germinate were photographed individually using a Nikon digital camera (model D1, Japan), and digital images were managed by a computer. In this way all the X-ray images of dry seed and those of seedlings or dead seed could be examined simultaneously on the monitor screen allowing a comparative diagnosis.

Stink bug and mechanical damages were identified and differentiated using image analysis, considering the visual analysis of the variations and intensity of grey colour in X-ray images of the seeds. Grey levels among the sound and damaged tissues and the ways in which the damages were presented in the seed were observed, as compared to a standard image (figure 1a), which illustrates a seed without any stains or cracks on the embryo. The variations observed on the grey levels between the cotyledons and embryo axis are due to the density difference of the seed tissues. The stink bug damage produced characteristic circular lesions on the seed (França-Neto *et al*., 1998, 1999), while the immediate mechanical damage caused cracks and cuts on the seed (Cicero *et al*., 1998). Mechanical and stink bug damages observed on the seed in the radiographic analysis were scored according to the criteria proposed by Cicero *et al.*, (1998): Score 1, seed without any damage: seed without cracks and/or stains; Score 2, seed with non-severe damages: seed showing stains on the cotyledons, with less than 50% of their total surface affected, or with cracks that do not affect reserve translocation to the embryonic axis; Score 3, seed with severe damages: seed showing stains on the cotyledons with more than 50% of their total surface affected (figure 1e) and/or cracks on the cotyledons on critical areas that may affect reserve translocation to the embryonic axis (figure 1e) and/or stains or cracks on the embryonic axis (figure 1c).

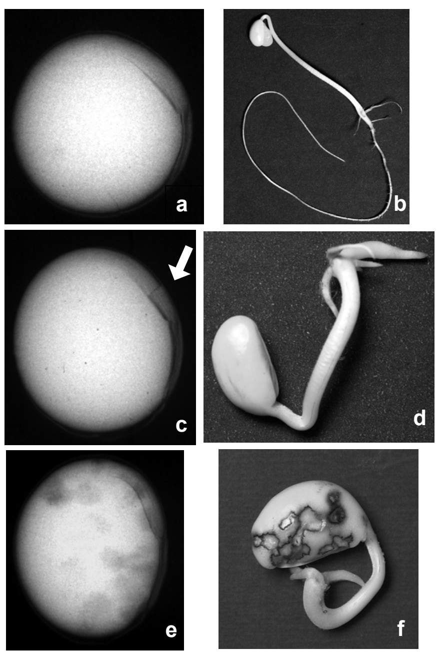

Figure 1. Soybean seed images obtained by X-ray test and related seedling: a), seed without mechanical or stink bug damages, resulting in a normal seedling (b); c), seed with a severe mechanical damage on the embryonic axis, resulting in an abnormal seedling (d); e), seed with severe stink bug damages on the embryonic axis and cotyledons, resulting in an abnormal seedling (f).

Tetrazolium test
The tetrazolium (TZ) test was carried out simultaneously for later comparison among the results. The test and assessment were carried out according to the criteria established by França-Neto *et al*. (1998). Fifty seed per replicate of every treatment were preconditioned on paper towel 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. Later, seeds were placed for staining in a 0.075% (w/v) solution of 2, 3, 5-triphenyl-tetrazolium chloride, and incubated in darkness in an oven with constant temperature of 40°C for 2.5 hours. After this period, seeds were rinsed with tap water several times to stop the staining reaction. Following the examination made on individual seed viability, vigour, stink bug and mechanical damages were recorded. Seed tissues damaged by stink bugs are dead and flaccid and are typically white, or sometimes greenish, yellowish, or greyish white, while mechanically damaged tissues can be characterized by cracks or by intensive red bruises (França Neto *et al*., 1998).

Seed viability and vigour were determined by the tetrazolium test according to methodology developed by Moore (1961, 1962, 1967) for corn and soybean seed, modified and described in detail for soybean by Franca-Neto *et al*. (1998). The presence, location and nature of staining and the physical condition of embryo structures were used in this classification. Each seed was assigned a soundness rating of 1 to 5, if viable, and of 6 to 8, if non-viable: Class 1 characterizes perfect seed without damage; Class 2 encompasses highly vigorous seed with minor and superficial damages; Class 3 seeds are those with minor damages located in non-critical areas of the seed; Class 4 seeds are viable but contain damages that do not hinder seed viability; Class 5 seeds have serious damages in critical areas, but are still viable; Class 6 characterizes non-viable seeds with severe damages more intensive than the ones in Class 5; Class 7 contains non-viable seeds with very serious damages occurring in critical areas of the seed, or with more than 50% of the seed surface affected by any type of damage; Class 8 are unstained dead seed. Seed viability was calculated by the summing the percentages of seeds as calculated in classes one through five. The vigour rating was determined by the summing the percentages of seeds within classes one through three. The percentage of seed mechanically damaged or injured by stink bugs for classes 1 through 8 and 6 through 8 were calculated and also recorded. Total percentage of seed injured by mechanical damage or stink bug was reported as level (1-8), while serious damage that hindered seed viability was reported as level (6-8). For more detailed explanation on this procedure, please refer to França-Neto *et al*. (1998).

Statistical analysis
The results were analyzed as comparison between the damage detected by X-ray image analysis in individual seed and the occurrence of seedling abnormalities or dead seed. A complete randomized block design was used with four quality tests per five replicates each. The data obtained by germination and accelerated ageing tests were transformed in arcsine of the square root of the percentage. The statistical analysis was carried out using the SANEST program software package (Zonta and Machado, 1984). The means were compared by the Tukey test with 5% probability level.

Results

The initial soybean seed quality assessment indicated that the moisture content, germination and accelerated ageing tests detected differences in the physiological quality of the seed (table 1). Seed from lots 3 and 4 presented a better quality performance than those from lots 1 and 2.

Table 1. Evaluation of initial moisture content (MC), germination (G) and accelerated aging (AA) test in four lots of soybean seeds, cv. BRS 184.

Treatments	MC (%)	G (%)	AA (%)
Lot 1	9.6	79 b*	65 b
Lot 2	9.9	81 b	64 b
Lot 3	9.6	95 a	84 a
Lot 4	10.0	94 a	82 a
CV (%)	-	4.1	7.2

*By column, means followed by same letter do not differ from each other by the Tukey test at the 5% probability level.

When X-ray test was carried out in all four soybean seed lots, it was observed that the seed from lots 1 and 2 showed greater occurrence of mechanical damage classified as severe on the embryonic axes (table 2), but this type of damage was detected only on the cotyledons of seed of lot 1. In the embryonic axes of lot 1 it was found the highest value of non-severe damage class, while any difference between the seed lots was detected for non-severe damages occurring on the cotyledons.

Table 2. Mean values (%) of seed with different intensities of mechanical damage on the embryonic axis and cotyledons from four soybean seed lots, as determined by X-ray test.

Damage Location	Lot	Score[1]		
		1	2	3
Embryonic axis	1	91.6 b*	2.4 a	6.0 a
	2	94.0 ab	0.8 ab	5.2 a
	3	95.2 ab	0.4 b	4.4 b
	4	97.6 a	0.0 b	2.4 b
C.V. (%)		2.52	57.6	32.01
Cotyledons	1	90.8 a	4.8 a	4.4 a
	2	92.0 a	6.0 a	2.0 b
	3	91.2 a	7.6 a	1.2 b
	4	94.0 a	4.8 a	1.2 b
C.V. (%)		4.08	41.52	38.9

*By column and for each damage location, means followed by same letter do not differ from each other by the Tukey test at the 5% probability level.

Seed from lots 3 and 4 presented a lowest occurrence of stink bug damage, classified as severe in the embryonic axis (table 3). However, cotyledons from seed of lot 3 presented the lowest mean value of this damage as compared with lots 1 and 4. On the other hand, the damage classified as non-severe on the cotyledons was higher in seed of lot 2 than those deriving from lots 1 and 3.

Table 3. Mean values (%) of seeds with different intensities of stink bug damage on the embryonic axis and cotyledons from four soybean seed lots, as determined by X-ray test.

Damage Location	Lot	Score[1]		
		1[1]	2[2]	3[3]
Embryonic axis	1	94.4 b*	2.8 a	2.8 ab
	2	96.0 ab	0.4 b	3.6 a
	3	98.8 a	0.4 b	0.8 b
	4	97.2 ab	1.2 ab	1.6 b
C.V. (%)		2.49	55.7	34.59
Cotyledons	1	89.2 ab	7.6 b	3.2 a
	2	78.4 b	19.6 a	2.0 ab
	3	90.0 a	8.8 b	1.2 b
	4	82.4 ab	14.0 ab	3.4 a
C.V. (%)		7.04	30.14	33.53

*By column and for each damage location, means followed by same letter do not differ from each other by the Tukey test at the 5% probability level. [1] Score: 1 = not observed damage; 2 = non-severe damage; 3 = severe damage.

Regarding the percentages of normal and abnormal seedlings determined by the germination test in seed that presented X-ray visualised mechanical and stink bug damages, it was observed in the four lots that seeds with these damages resulting in abnormal seedlings were present in seed classes with severe damage that occurred in the regions of the cotyledon and/or embryonic axis. However, it was observed that mechanical damage accounted most for germination losses of the four soybean seed lots (table 4).

Most of the seed without mechanical and stink bug damage (figure 1a) resulted in normal seedlings (figure 1b). In figure 1c, a seed identified with severe mechanical damage on the embryonic axis with intact cotyledon (score 3) was able to give rise to an abnormal seedling (figure 1d). A seed with stink bug damage is shown in figure 1e. This type of damage localised on the embryonic axis and cotyledons was classified as severe (score 3), and resulted in the development of an abnormal seedling (figure 1f).

The tetrazolium test (table 5) confirmed differences in viability and vigour potential of each seed lot. The most common type of damage in classes 6-8, which resulted in non-viable seeds, was mechanical damage, especially for lots 1 and 2, which presented the highest level of this type of damage. This fact was confirmed by the X-ray evaluation, since lots 1 and 2 had the highest incidence of mechanical damage in class 3 (table 4), which resulted in the production of abnormal seedlings, confirming the severity of this type of damage. Lots 2 and 4 presented highest percentages for total stink bug damage (classes 1-8). However, lot 4 presented the greatest occurrence (3.6%) of non-viable seed for this type of damage as determined in classes 6-8. Nevertheless, the severity of stink bug damage to the seed in the four lots was within acceptable levels (less than 6%), as pointed out by Franca-Neto *et al.* (1998) and consequently, the recorded damage was not serious enough to reduce seed vigour (classes 1-3) and viability (classes 1-5) of the four lots. These findings were in agreement with the results obtained by the X-ray test, since the lots also had low occurrence of severe damage (score 3) caused by stink bug (table 2).

Table 4. Percentages of total seed (T) and mean values of normal seedlings (NS), abnormal seedlings (AS) and dead sees (DS) observed in the germination test of four soybean seed lots with different scores as sorted by X-ray test.

Score*	Lot 1				Lot 2				Lot 3				Lot 4			
	T	NS	AS	DS	T	NS	AS	DS	T	NS	AS	DS	T	NS	AS	DS
	Mechanical damage															
1	80	72	8	0	83	78	5	0	87	82	5	0	92	88	4	0
2	6	6	0	0	6	6	0	0	7	7	0	0	4	4	0	0
3	14	0	14	0	11	0	11	0	5	0	5	0	3	1	2	0
Total	100	78	22	0	100	84	16	0	100	90	10	0	100	94	6	0
	Stink bug damage															
1	84	69	15	0	77	66	11	0	90	82	8	0	81	78	4	0
2	9	9	0	0	19	17	2	0	8	8	0	0	14	14	0	0
3	7	0	7	0	4	1	3	0	2	0	2	0	5	3	2	0
Total	100	78	22	0	100	84	16	0	100	90	10	0	100	94	6	0

* 1. Non-observed mechanical or stink bug damages; 2. Non-severe mechanical or stink bug damages; 3. Severe mechanical or stink bug damages.

Discussion

The X-ray image analysis technique and the tetrazolium test confirmed that mechanical damage interfered mainly with seed viability, as shown in the seed from lots 1 and 2. This type of damage has been considered as the most harmful to seed quality of soybean produced in Brazil (Carbonell *et al*., 1998). Mechanical damage with less than 5% occurrence in non-viable seed classes determined by the tetrazolium test (classes 6-8) did not lower seed viability. These results are in agreement with those of Costa *et al*. (2005) who stated that the occurrence of mechanical damage up to 5% in soybean seed (classes 6-8) did not result in seed quality reduction, but vigour decreased sharply when lesions where more than 6% (classes 6-8).

The X-ray test has the potential to amplify mechanical and stink bug damages, whose intensity may be associated with seed physiological performance. Since it is a non-destructive method, individual seed can be classified for their surface integrity characteristics and further subjected to physiological tests. The seed testing following the X-ray analysis permits the superficial visualization of mechanical and insect damage, such as cracks or fractures, resulting from other adverse pre- and post-harvest practices (ISTA, 1993; Paulsen *et al*., 1998).

Although the percentage of seed with total mechanical damage and stink bug damage assessed by the tetrazolium test (class 1-8, table 5) was higher than that obtained by the X-ray analysis technique (seed with scores 2 and 3, table 4), the effectiveness of this test was demonstrated by the easiness of this procedure to amplify visual imaging of different types of damage. The damage classified as severe by the X-ray test is related with the occurrence of abnormal seedlings and also with the occurrence of non-viable seed (classes 6-8) determined by the tetrazolium test. These findings confirmed those reported by Obando-Flor *et al*. (2004) who verified the efficiency of the X-ray test in detecting mechanical damage in soybean seed and its relationship with abnormal seedling and dead seed, as estimated by the tetrazolium test. In conclusion, it can be stated that the X-ray image analysis technique may be a non-destructive alternative method to assess mechanical and stink bug damage in soybean seed and to sort intact viable seed from those which present damaged coat. For further improvement of this test, which will provide more precise results, additional studies are needed in the area of software development for X-ray image analyses useful to identify and quantify the types of damage on the seed, as already reported by Dell'Aquila (2007b) for other evaluations.

Table 5. Mean values (%) of mechanical damage (MD), stink bug damage (SBD), vigour and viability (Viab.) determined by the tetrazolium salt test in four soybean seed lots.

Lot	MD		SBD		Vigour	Viab.
	1 – 8[1]	6 – 8[2]	1 – 8[1]	6 – 8[2]	(1 – 3)	(1 – 5)
	%					
1	37.0 a*	15.6 a	19.0 b	0.8 b	70.6 b	82.4 d
2	33.0 a	11.2 b	33.0 a	1.6 b	74.2 b	86.8 c
3	23.0 b	4.8 c	8.2 c	0.6 b	90.0 a	95.2 a
4	20.4 b	4.0 c	31.6 a	3.6 a	85.6 a	92.2 b
CV (%)	13.92	21.17	15.80	38.20	3.63	1.62

*By column, means followed by same letter do not differ from each other by the Tukey test at the 5% probability level. [1]Percentage of total damages, in classes 1 to 8, as determined by the tetrazolium test; [2]Percentage of serious damage in the classes 6 to 8, which resulted in loss of seed viability (França-Neto *et al.*, 1998).

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