França-Silva, Carvalho, Carvalho, Andrade, Souza and Marques (2019).
Seed Science and Technology, **47**, 3, 249-260.
https://doi.org/10.15258/sst.2019.47.3.02

Radiographic analysis to test maize seeds for the presence of *Sitophilus zeamais* (Coleoptera: Curculionidae)

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(Submitted October 2018; Accepted May 2019; Published online September 2019)

Abstract

Examining seeds for insect infestation is one of the requirements established by the Brazilian standards for the release of seed lots. The traditional methodology prescribed in the Rules for Seed Analysis involves soaking and cutting of individual seeds which is time consuming, leads to visual fatigue and jeopardises the analyst's safety. Radiographic imaging analysis is an excellent method to detect insect infestation, both externally and internally to the seeds, and may be a viable alternative to the traditional methodology. The aim of this study was to evaluate the use of the radiographic analysis to test for infestation by the weevil *Sitophilus zeamais* (Coleoptera: Curculionidae) in maize seeds. The radiographic analyses were evaluated by 40 seed analysts, belonging to 10 laboratories, accredited by the Ministry of Agriculture, Livestock and Supply (MAPA), which received radiographs of maize seeds from four samples with 0, 1, 3 and 5% infestation. The data were submitted to statistical procedures for withdrawal of discrepant values and outliers in the variances; to evaluate the effects of laboratories and levels; to estimate repeatability and reproducibility; and to verify the accuracy and robustness. The methodology proposed showed accuracy, robustness and precision within the critical limits of 0.01 and 0.05%.

Keywords: corn weevil, damage, detection, image analysis, maize seeds, X-ray

Introduction

The production of maize seeds is the second most economically important activity in Brazilian agribusiness. With 835 thousand tons of seeds produced, a demand of 572 thousand tons is estimated to meet a production of 87.3 million tons of grain (Carvalho *et al*., 2017; Conab, 2018). To ensure the expansion of the productivity and quality of maize seeds, legal standards of quality are established, including the detection of damage by insect pests (Brasil, 2013; Frischtak *et al*., 2014).

The corn weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) penetrates the mass of seeds, where it performs oviposition. The larval stage develops into the adult insect inside the seed, and it provides a gateway for the development of fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium*, reducing the weight, germinationand commercial standard of seeds (Aquino *et al*., 2013; Vilarinho *et al*., 2016; Mateus *et al*., 2017). In the light of the direct and indirect losses, there is a need for detection of the damage caused by this insect in seeds.

The detection of insect-infested seeds is carried out according to the methodology of the "infested seeds test", following recommended standards of seed identity and quality for the release of lots and possible reanalysis (Brasil, 2004, 2013).

The detection of infested seeds is carried out with the aid of sharp objects and is based on the individual inspection of seeds to detect damage or signs of infestation in the seeds. It is time-consuming and can lead to eyestrain for the analyst. One of the alternative techniques for infested seed examination is radiographic image analysis, which has excelled in the evaluation of malformed and empty seeds, or seeds damaged by insects and fungi and mechanical damage (Carvalho *et al*., 1999, 2012; Huang *et al*., 2015).

Despite being simple, nondestructive, safe, practical and fast in the evaluation of seed quality, the methodology of radiographic analysis to quantify the degree of infestation by insects has not yet been validated. For this situation, the International Seed Testing Association (ISTA) recommends that the accuracy, precision (repeatability and reproducibility) and robustness of the proposed methodology should be checked (ISTA, 2007).

The goal of this study was to test the viability of radiographic analysis for the examination of *S. zeamais* infestation in maize seeds.

Material and methods

Obtaining the infestation levels and distribution of material to laboratories

Seeds infested with *S. zeamais* were obtained by the maintenance of sample composed of 1000 g hybrid maize seeds (semi-flint to semi-dent group with 13% moisture; variety name and company are confidential) with 10 pairs of adult insects, in 300 mL-volume glass containers, closed with voile fabric to ensure air circulation and survival of insects. The containers were kept in a climate chamber at 25 ± 2°C, 65% relative humidity in the dark for 35 days.

At each stage of *S. zeamais* development, random samples of 200 maize seeds, yellow, hard, ridged, malformed, flat and round, and measuring 19 to 24 mm, were removed from the glass pot, numbered one by one and distributed equally, with the side closest to the embryo turned upwards, in a single layer, on eight transparent acrylic slides with the capacity for 25 seeds each. These removal stages were specifically at 5, 18, 28 and 35 days, when there was, respectively: oviposition between the pericarp and the endosperm of the seeds; hatching of larvae and construction of galleries inside the seeds; pupal stage; and development of the adult insect inside the seeds. Each sample was subjected to radiographic analysis, without and with contrast, using the Faxitron HP MX-20 digital

device, at the Central Laboratory for the analysis of Seeds at the Universidade Federal de Lavras, calibrated with radiation level of 24 kv for one minute at a distance of 350 mm from the source. Contrast calibration was performed by placing the seed plates in contact with chloroform vapour for four hours in a sealed 1000 mL glass vial to validate the radiographic analysis without contrast, following Leite (2016).

After visually analysing the x-rays, the seeds were separated into two groups: symptom-free seeds and seeds with signs/presence of larvae, pupae and adult insects. For confirmation of the infestation by the traditional test, the seeds were removed from the plates and distributed in plastic boxes containing individual compartments, which allowed the immersion of the seeds in water for a period of 24 hours. After immersion, the seeds were cut open with a scalpel for internal analysis (Brasil, 2009). The seeds were divided into infested or not by the insect; they were compared with the x-rays and photographed with the aid of a stereoscopic microscope (40x).

After confirmation that the seeds were infested or not by larvae, pupae, adults and/or with damage caused by the insect, the selection of the x-rays was carried out using CorelDRAW® X8 software for elaboration of eight boards, with 25 seeds for each treatment, totaling 200 seeds per treatment, taken from the mean sample which possessed 1000 g of maize seeds. The adopted treatments were: control (0% infestation) and 1, 3 and 5% infestation, corresponding to the acceptable maximum limits of infestation for commercial lots of basic seeds C1 and C2 (3% infestation), and S1 and S2 (5%) (Brasil, 2013).

After the composition of the four treatments, corresponding to the four infestation levels (0, 1, 3 and 5%), 10 laboratories accredited by MAPA were selected, with different levels of experience in tests involving maize seeds infested by insects (table 1).

Each laboratory was sent a protocol which contained a guide for infestation identification using radiographic images, distinguishing the insect in the different developmental stages (figure 1), to familiarise and train the analysts.

Table 1. Laboratories accredited by the Ministry of the Agriculture, Livestock and Provisioning where the tests of corn seeds infested by *Sitophilus zeamais* were carried out.

Laboratories reponsible for the tests	City/State
APASEM – Paraná State Seed Producers' Association	Ponta Grossa-PR
Laboratory at Riber KWS	Patos de Minas-MG
Laboratory of Seed Analysis of the Federal University of Lavras	Lavras-MG
CATI - Central Seed Testing Laboratory	Campinas-SP
DuPont Pioneer - Seed Quality Laboratory	Itumbiara-GO
LASO – Minas Gerais State Official Laboratory for Seed Analysis	Belo Horizonte-MG
Genetic Purity Laboratory at Monsanto	Uberlândia-MG
Seed Physiological Analysis Laboratories at Monsanto	Uberlândia-MG
Qualiteste Agronomy Analyses	Uberlândia-MG
APROSMAT - Mato Grosso State Seed Producers' Association	Rondonópolis-MT

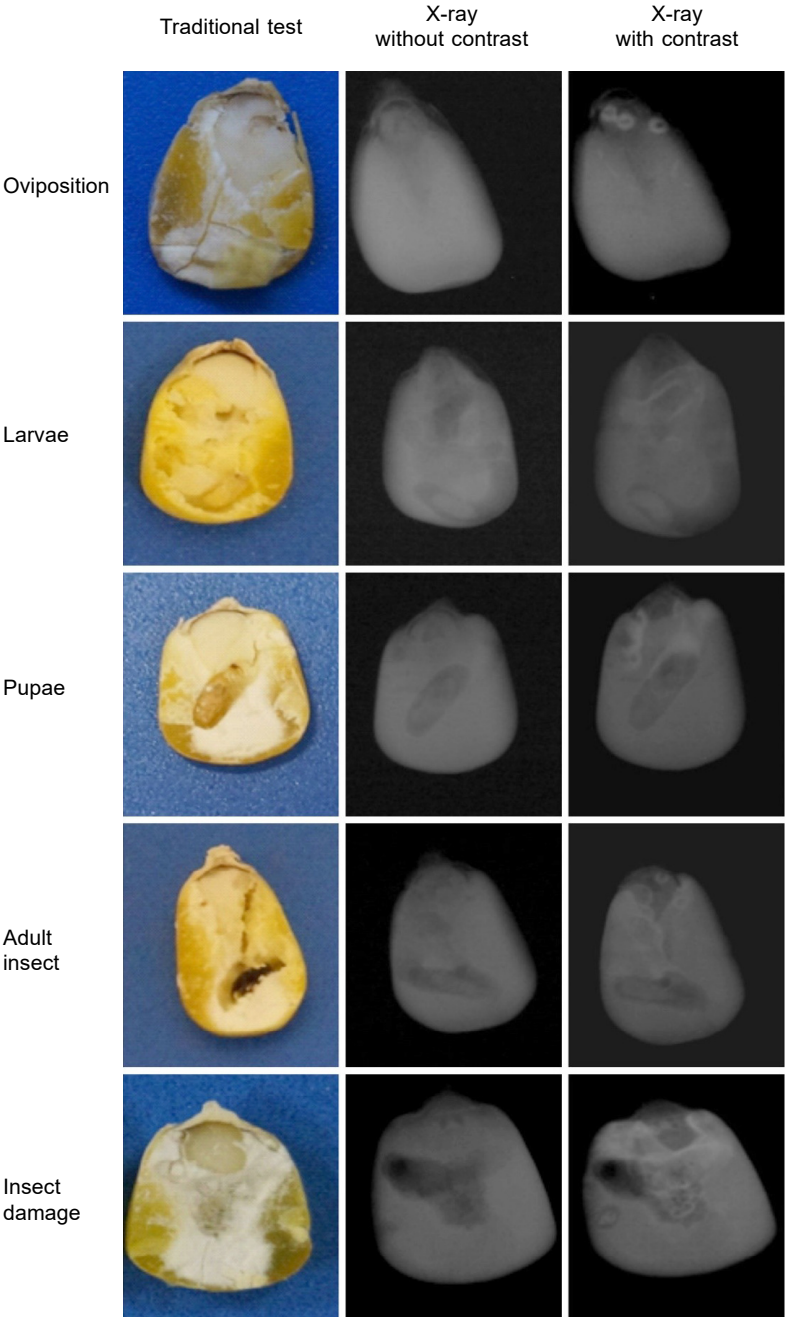

Figure 1. Guide for the identification of infestation by *Sitophilus zeamais* in maize seeds starting from being cut with a scalpel (left), x-rays without contrast (centre) and x-rays with contrast (right).

Seven days after sending the protocol, the 32 radiographs comprising eight plates of 25 seeds × 4 infestation levels were sent by e-mail, for appraisal by four analysts (repetitions) from each laboratory. The evaluation results were received by the organising laboratory to proceed with statistical analyses.

Statistical procedures

For the statistical procedures, the infestation sum obtained at each level (8 plates × 25 seeds) was considered, forming a repetition of 200 seeds per level. The variable was the level of weevil infestation observed in the seeds (Y). For the statistical analyses, specific statistical functions were used in R (R Development Core Team®, 2017).

Outliers: for the detection of conflicting values in all of the laboratories, the Hampel method was used (Hampel, 1974). A graphical representation of the boxplot sketch (Tukey, 1977) was used to aid in the evaluation of analyst performance within each laboratory. Having detected outliers, these were removed from the database, before proceeding to the next analyses (ISTA, 2007).

Outlier identification in the variances: the identification of outliers in the variances from the doped averages at each infestation level was measured using the Levene test (1960). Laboratories that presented greater variances were withdrawn and the process repeated until only laboratories with homogeneous variances were present.

Evaluation of laboratory effects and levels of infestation: the data were submitted to analysis of variance and the effect of infestation levels, and their averages were grouped by the Scott-Kott test ($P < 0.05$). The theory of variance analysis was applied in a completely randomised factorial design (four levels of infestation × 10 laboratories × four replicates). There was no interaction between laboratories and significant levels, and so the interaction was included within the residues.

Mandel's repeatability, reproducibility and h and k statistics: the repeatability variance (S_r^2) represents the variability within the laboratories (ISO 5725-2, 1994). With its determined value, the critical limit (r) of repeatability, estimated by $r = Srj\ Dj$, was calculated in which Dj was obtained from the Tukey table, with degrees of freedom tending towards infinity and a confidence level of 99% (Banzatto and Konkra, 2006). The value of r was compared with the amplitude between the replicates of each laboratory at each level (*Lrjl*), in order to indicate laboratories with acceptable repeatability.

The value of r was compared to the amplitude between the replicates of each laboratory at each level (*Lrjl*), in order to indicate the laboratories with acceptable repeatability. The reproducibility variance (S_r^2) represents a measure of the variability between and within the laboratories (ISO 5725-2, 1994), and at its determined value, the critical reproducibility limit (R) was calculated for each level of infestation, estimated by $R = S_R D$. This R value was compared with the amplitude between laboratories for each replicate, determining the levels of infestation with acceptable reproducibility, with a confidence level of 99%.

Mandel's k-statistic was used to evaluate the accuracy of the results, while the h-statistic was used to graphically evaluate the bias estimation, and thus the accuracy of the results (ISO 5725-2, 1994). After the calculations, the graphs of the values of h and k were made for each level and laboratory, and compared with the critical values for $\alpha = 0.01$ and 0.05.

Results

There was significant variation in the mean of the observations of the analysts from each of the 10 laboratories, in relation to the weevil infestations in each of the four maize seed lots (figure 2). All analysts from laboratory-10 obtained identical responses regarding signs of infestation from radiographic images for lots 3 (3% infestation) and 4 (5% infestation). The outliers detected by the Hampel method, mostly present at the 0% infestation level, were withdrawn before the next statistical procedure.

Despite detecting variability among the laboratories for each infestation level, the Levene test did not indicate the acceptance of the hypothesis of heterogeneous variances ($P > 0.01$; table 2). When analysing the variance with the Shapiro-Wilk test, a highly significant effect was observed for both laboratories and infestation levels ($P < 0.0001$; table 3).

The amplitude results from each laboratory (Lr_j) (table 4) and from each level (Lr_{jk}) (table 5) associated with critical limits and confidence levels of 99% showed acceptable repeatability and reproducibility for all laboratories and infestation levels.

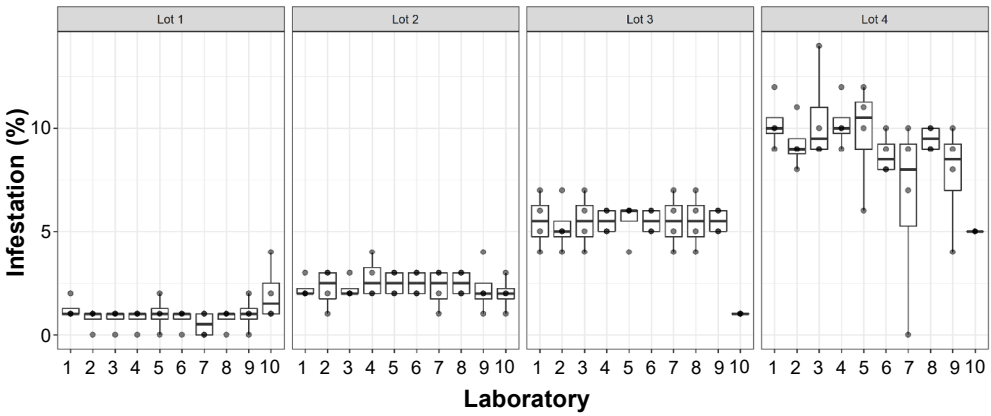

Figure 2. Infestation of *Sitophilus zeamais* in maize seeds observed via radiographic analysis by four analysts from each of ten laboratories. The dots represent the result of each respective laboratory analyst. The horizontal line in the center of the box indicates the median, the boxes represent the quartiles and the vertical lines are the tails.

Table 2. Results of the Levene test ($P < 0.01$), for outlier detection in the variances for each level of infestation of *Sitophilus zeamais* in maize seeds.

Levels (%)	*P*-value after removing outliers	Condition	Indication
0	–	Homogeneous variances	Continue the analysis
1	0.7687	Homogeneous variances	Continue the analysis
3	0.2424	Homogeneous variances	Continue the analysis
5	0.5983	Homogeneous variances	Continue the analysis

Table 3. Summary of variance analysis and Shapiro-Wilk test for the variable infestation of *Sitophilus zeamais* in maize seeds, with confounded interaction in the residue.

Variation source	D.F.	Mean square	F	*P*-value
Laboratory	9	10.7062	6.9041	< 0.0001
Levels	3	435.6060	280.9077	< 0.0001
Residual	132	1.5507		
C.V. (%)		26.91		

D.F. = degrees of freedom; C.V. = coefficient of variation.

Table 4. Width (Lr_{jl}), critical limit, standard deviation of repeatability and results of the determination of the repeatability (Re) condition or not (Nr) for the laboratories in each level of infestation of *Sitophilus zeamais* in maize seeds.

Laboratory		Mean infestation (%) observed by laboratories			
1	$Lr\ j_1$	0	1	3	3
	Condition	Re	Re	Re	Re
2	Lr_{j2}	0	2	3	3
	Condition	Re	Re	Re	Re
3	$Lr\ j_3$	0	1	3	5
	Condition	Re	Re	Re	Re
4	$Lr\ j_4$	0	2	1	3
	Condition	Re	Re	Re	Re
5	$Lr\ j_5$	0	1	2	6
	Condition	Re	Re	Re	Re
6	$Lr\ j_6$	0	1	1	2
	Condition	Re	Re	Re	Re
7	$Lr\ j_7$	0	2	3	3
	Condition	Re	Re	Re	Re
8	$Lr\ j_8$	0	1	3	1
	Condition	Re	Re	Re	Re
9	$Lr\ j_9$	0	3	1	6
	Condition	Re	Re	Re	Re
10	$Lr\ j_{10}$	0	2	0	0
	Condition	Re	Re	Re	Re
Critical limit*		0.00	3.75	4.71	7.69
S_r		0.00	0.81	1.01	1.67

(*) Critical limits calculated from the multiplication of the repeatability values by a numerical factor D, obtained in Tukey's table (1977), with degrees of freedom tending towards infinity.

Table 5. Width (*Lr jk*), critical limit, standard deviation of reproducibility and results of the determination of the reproducibility (Re) condition or not (Nr) for the levels of infestation of *Sitophilus zeamais* in maize seeds, in each repetition.

Levels (%) of infestation		Repetition				Critical limit*	S_R
		1	2	3	4		
0	$LR\ j_1$ Condition	0 Re	0 Re	0 Re	0 Re	0.00	0.00
1	$LR\ j_2$ Condition	2 Re	3 Re	2 Re	3 Re	3.39	0.73
3	LRj_3 Condition	6 Re	6 Re	6 Re	5 Re	7.75	1.67
5	$LR\ j_4$ Condition	8 Re	6 Re	9 Re	6 Re	10.08	2.19

(*) Critical limits calculated from the multiplication of the repeatability values by a numerical factor D, obtained in Tukey's table (1977), with degrees of freedom tending towards infinity.

With the graphing of the values of k, different degrees of variability between laboratories are apparent, with laboratory-5 and laboratory-9 tending to reach a critical limit of 5% in relation to level 5 of infestation (figure 3). A random pattern was observed regarding overestimation and underestimation of results for the h-statistic of Mandel, where laboratory-4 overestimated level 1 infestation, and laboratory-10 underestimated all levels, exceeding the critical limits of 1 and 5%.

Discussion

Variation in the responses of the analysts regarding observations of infestation levels may be related to their experience regarding insect identification, training or prior preparation for the analysis of radiographs, distinct visual acuity, unexpected events or different evaluation criteria among the analysts (Behrens, 1997). As very different responses among analysts lead to incoherent results, it is emphasised that although variation was found between the distinct groups (analysts and laboratories) in the Levene test, homoscedasticity was achieved, which means that laboratory variances, in relation to infestation levels, are acceptable (Kataoka *et al*., 2011).

Although laboratory-4 and laboratory-10 exceeded the critical limits of repeatability and reproducibility, they were kept in the study, because the precision monitoring in these laboratories functions as an indicator for the improvement in quality of these laboratories (ISO 5725-2, 1994; Chui *et al*., 2004). As the radiographic images are generated in black and white, the analysts must have enough experience to avoid ambiguous interpretation of the signs of infestation (Schmidt, 2000; Dogan *et al*., 2010).

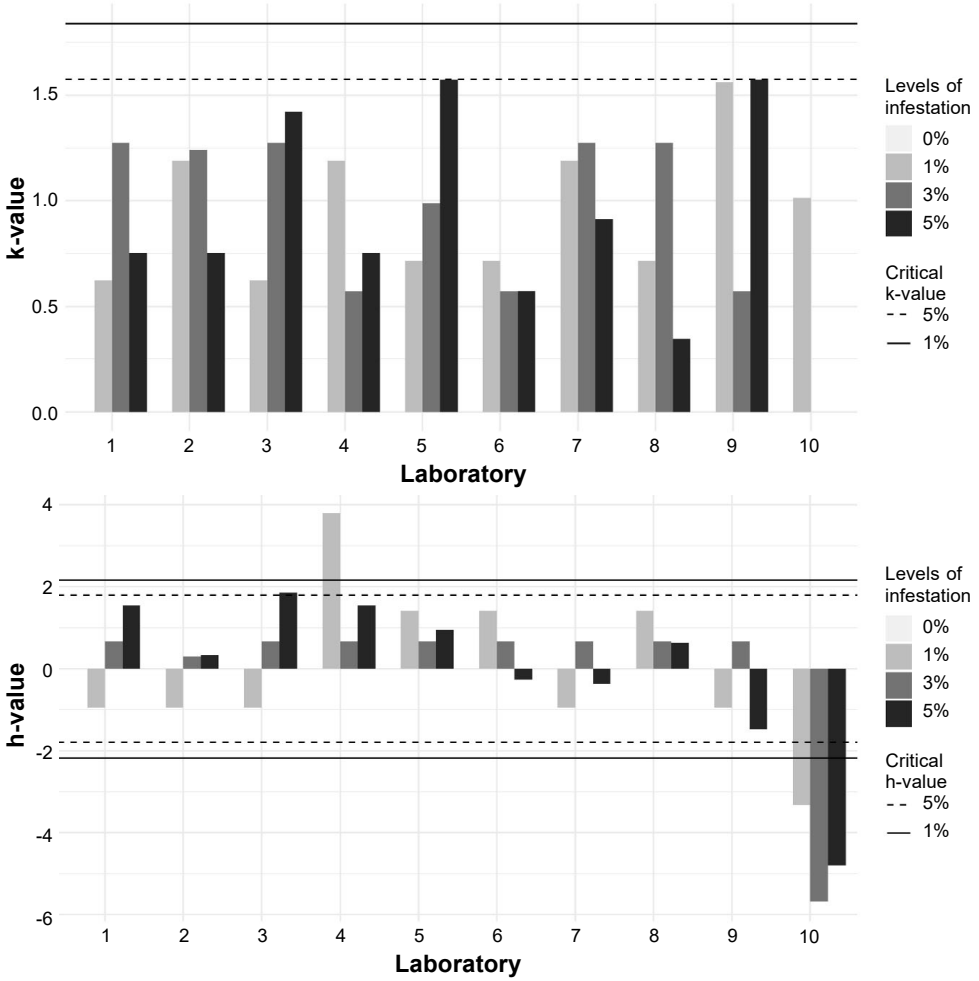

Figure 3. Graphs of k and h values for the examination of infestation of *Sitophilus zeamais* in maize seeds performed from radiographic analysis.

As the radiographic analysis methodology was demonstrated to be repeatable and reproducible in 80% of the laboratories, the analysts from these laboratories considered the methodology to be viable for routine use, because it is rapid, practical and easy to execute when compared with the traditional methodology using a slide, which is tiring and worse for body posture. However, the laboratories requested additional training in visual acuity to avoid confusion between mechanical damage, seed structures and signs of infestation. They also suggested improvement in the radiographic procedure to improve the clarity of the radiographs.

The efficiency of the use of radiographic analysis for the identification of insects was confirmed in studies on the detection and classification of insect damage in cotyledons of *Crotalaria juncea* L. (Arruda *et al*., 2016); by cowpea weevil, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae) in soybean seeds (Chelladurai *et al*., 2014); in wheat grains infested or not by *Cryptolestes ferrugineus* (Stephens, 1831) (Coleoptera: Laemophloeidae) (Karunakaran *et al*., 2004); and also in the classification of stages of development of the maize weevil in popcorn seeds (Brabec *et al*., 2017). In those studies, the results can be explained by the capacity of X-rays to form different levels of attenuation between the seeds and the signs of infestation, especially in terms of density or composition that differ between objects (Crocker *et al*., 2014).

The method of radiographic analysis for detecting levels of infestation is useful when the damage or signs of infestation by the insect are not visible to the naked eye on the outer and inner surfaces of the seeds. This justifies the approval in Europe of this method for detecting infestation and determining seed quality levels (Dogan *et al*., 2010). AOSA (1979) and Simak *et al*. (1989) attribute the success of radiographic analysis in the detection of infestation to its greater precision, speed of analysis and the fact that it is non-destructive, as seeds are not cut. It can also indicate precisely which factors interfere in quality, whether it is due to empty spaces, morphological deficiencies or mechanical damage.

In conclusion, the statistical results for repeatability, reproducibility and k and h statistics of the Mandel test presented exactitude and precision that are within the critical limits of 1 and 5% for infestation by *S. zeamais* in maize seeds. The radiographic analysis method used by analysts for the *S. zeamais* infestation test in maize seeds is non-destructive and direct, making it possible to examine individual seeds in expanded digital images, with more details on the extent and location of damage caused by the insect. Consequently, under the conditions in which it took place, the proposed method can be used routinely for testing maize seeds infested by insects.

Acknowledgements

The authors are grateful to FAPEMIG (Minas Gerais State Research Foundation) and CNPq (National Council for Scientific and Technological Development) for financial support and to MAPA accredited laboratories (Ministry of Agriculture, Livestock and Supply), for collaboration in the proposed validation.

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