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Evaluation of DNA integrity as a potential marker to detect aging in soybean (*Glycine max*) seeds

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ABSTRACT. Seed aging is a complex process that includes degradation of macromolecules, such as DNA. Evaluation of DNA deterioration in asymptomatic stages of seed aging may help the development of improved methods for monitoring long-term conserved seeds. We examined DNA integrity in artificially aged soybean (*Glycine max*) seed lots using cytogenetics, quantitative polymerase chain reaction (qPCR), and random amplified polymorphic DNA (RAPD). Freshly harvested soybean cultivar BRS 7980 seeds were dried and kept at 10°C in a dry room until the analyses. Six lots of 450 seeds were artificially aged (42°C, 100% relative humidity) for 6, 12, 24, 48, 72 or 96 h to obtain lots with different germination capacities. One unaged seed lot (0 h) was used as a control. Germination of aged lots varied from 12 to 86% at 96 and 6 h of aging, respectively. Cytogenetic data did not characterized aged seed. DNA integrity among seed lots was also evaluated based on the comparison of frequencies of three fragments amplified using an anchored variable-length primer set. Fragments of 86 and 193 bp had similar frequencies, while the largest one (491 bp) showed greater variation among samples. Data suggested different fragment frequencies were due to variation in the number of intact templates

among lots, which might have been caused due to the fact that shorter DNA sequences have a smaller probability of developing random breaks than larger ones. RAPDs confirmed the differences in template amount detected using qPCR and variation in DNA repair capacity among aged lots. Though some seed lots were characterized using physiological parameters, most of them could not be characterized based on cytogenetic and molecular data, suggesting that these tests might not be ideal to detect aging in soybean seeds.

Key words: Long-term seed conservation; DNA integrity; Cytogenetics tests; qPCR, RAPD

INTRODUCTION

Gene banks are important sources of alleles to improve crops for many agronomical traits (Adeboye et al., 2015). Seed storage is the main way to conserve genetic resources of orthodox seed species since it is a low cost, efficient, reproducible and feasible method; it allows easy access to germplasm to be characterized, evaluated and used (Hawkes et al., 2000; Santos, 2006).

Production and germplasm preservation are seriously challenged by seed aging (Zhang et al., 2019). The monitoring of seeds in gene banks is conducted considering the initial germination power and the time the accession has been under long-term conservation conditions. Evaluation of germination power is done periodically to ensure that it remains high, which allows the genetic variability comprised in genetic resources to be accessed by breeding programs. The tetrazolium and germination tests are the methods most used to monitor long-term conserved seed germplasm (Deswal and Chand, 1997). Despite their wide use, they do not allow to clearly detect the distinct stages of aging of the conserved seeds, indicating only the final stages of this process, that is, when there is a lack of uniformity and delay in the germination process, loss of seedling vigor, formation of abnormal seedlings and seed death. The time under conservation is also an indicator of seed aging status, but it is known that seeds age at different speeds also depending on the different conditions to which they are given, such as post-harvest treatments or in sample preparation for conservation in the long-term (Zhao et al., 2020a). Low viability or non-germination makes the use of germplasm unfeasible when necessary and can lead to the loss of alleles or gene combinations involved in the expression of characteristics of great current or potential value for agriculture (Faleiro, 2013).

The transition between being alive or not, generally, occurs discreetly in a seed in conservation because the typical signs of life are only visible when water is supplied (Fleming et al., 2019). It is known that the loss of germination is preceded by a series of molecular, cellular, physiological and metabolic biochemical events in seeds, such as the disruption of the membrane system and the attack on its chemical constituents (proteins, lipids and nucleic acids) by free radicals (Sano et al., 2016).

Damage to nucleic acids may occur during seed aging, which is a major concern to maintenance of the seed viability for long-term conservation (Jyoti and Malik, 2013). Despite the importance of acid nucleic integrity for seed germination that issue has received relatively little attention in the literature (Kranner et al., 2011). The DNA integrity loss due

to aging has been in general evaluated using primary roots or seedlings and, in general, minor differences among different aged seed lots have been found (El-Maarouf-Bouteau et al. 2011; Donà et al., 2013; Menezes et al., 2014; Dantas et al., 2019).

Kranner et al. (2011) evaluated DNA degradation among artificially aged pea (*Pisum sativum*) seed samples using DNA laddering and observed different levels of degradation and DNA repair system efficiency among samples. Despite the remarkable results obtained in peas, DNA laddering requires considerable amounts of DNA (from 5 to 10 µg) (El-Maarouf-Bouteau et al., 2011) and it is not observed in all species (Radha et al., 2014; Vicente et al., 2016). Thus, it is important to identify techniques that requires low amounts of DNA that could be used to evaluate DNA integrity and repair system in long-term conserved seeds.

Cytogenetics, quantitative polymerase chain reaction (qPCR) and random amplified polymorphic DNA (RAPD) meet the above requirement. Cytogenetics is a low-cost method, which has been used for the characterization of aged seed lots of *Araucaria angustifolia* (Fontes et al., 2001), *P. sativum* (Khan et al., 2003), *Triticum aestivum* (Akhter et al., 1992; Menezes et al., 2014), *Hordeum vulgare* (Akhter et al., 1992) and *Oryza sativa* and *Phaseolus vulgaris* (Dantas et al., 2020). qPCR has been shown to be a useful tool in the quantification and evaluation of DNA degradation in soybean (*Glycine max*) based food products using the cycle threshold (C_t) variation detected using two sets of anchored primers that yielded four different length fragments of two soybean genes (Murray et al., 2009). RAPD also has been used for characterization of genetic integrity of artificially aged seeds of *Helianthus annuus* (El- Maarouf-Bouteau et al., 2011), *Silene vulgaris* and *Silene acaulis* (Donà et al., 2013).

The aim of this study was to compare the DNA integrity among samples of soybean seeds before and after artificial aging for different periods using cytogenetics, qPCR and RAPD. Besides, this study also aimed to contribute to the evaluation of the potential of the DNA integrity level as a marker of seed aging, especially for those asymptomatic stages with regard to germination power.

MATERIAL AND METHODS

Material

Fresh harvested seeds of soybean cultivar BRS 7980 were analyzed. One single accession was analyzed to avoid variations due to genotypic differences and pos-harvest treatment. All seeds were donated by Brazilian Agricultural Research Corporation (EMBRAPA). The analyses were performed in the Laboratory of Seed Physiology at Embrapa Genetic Resources and Biotechnology - Brasilia, Federal District, Brazil.

Methods

Artificial aging

Seeds were aged according to Marcos-Filho (1999) to obtain samples with distinct levels of germination. The mean BRS 7080 water content was 7.6% before accelerated aging treatment. The artificial aging was conducted in 11 × 11 × 3.5 cm transparent plastic

boxes in which 450 soybean seeds were uniformly distributed on a screen suspended inside the boxes containing 40 mL of distilled water. The boxes were convered and then placed inside the biochemical oxygen demand chamber, where they remained for different periods at 42°C and 100% relative humidity (RH). The aging times were 6, 12, 24, 48, 72 and 96 h. Control seeds (0 h), which did not undergo accelerated aging, were kept in a cold chamber (30% RH and 10°C) until the end of the accelerated aging treatment. After aging, a part of the aged seed lots and control were used in the germination and the electrical conductivity (EC) tests. The remaining seeds were dried in a drying chamber (22°C and 15% RH) until moisture values were between 4.7 to 6.5%. Next, they were stored at -20°C until the moment of molecular and cytogenetic analyses.

Germination potential and Germination Speed Index (GSI)

Four replicates of 50 seeds were sown on germitest paper moistened with distilled water in the proportion of 2.5 times the dry paper mass and kept at 25°C. The percentage of normal seedlings was evaluated after five and eight days to calculate the GSI, according to the formula described by Maguirre (1962), and to determine the germination potential (G%) as recommended by Rules for Seed Analysis (Brasil, 2009). The germination results were expressed as a mean percentage of the replicates.

Electric conductivity (EC)

The EC test was performed according to Vieira and Krzyzanowski (1999). Seeds remained overnight in at room temperature to get the water content uniform after accelerated aging treatment. Four replicates of 50 seeds were evaluated. Each replicate of each aging lot was weighed and immersed in 75 mL of distilled water, where they remained for 24 h at 25°C. EC was recorded from the water before and after the seeds were immersed. After the 24 h period, the EC was recorded using a conductivity meter (Digimed - DM31).

According to the literature, EC test results stabilize when soybean seeds have between 11 and 13% water content (Loeffler et al., 1988). After obtaining the EC results in the different aging treatments, the correction factors were figured out based on the 13% water content, using the following equation proposed by Vieira et al. (2002):

$$CE = \left[0.33227 + 0.05115(TA)\right] \text{x } CEo$$

Where:

- *CE* = electrical conductivity.
- *TA* = observed water content in seeds %;
- *CEo* = observed electrical conductivity.

This correction of water contents to 13% is to avoid errors from occurring due to different water contents (TA) in seed lots, which can influence the results of this test.

Cytogenetical analysis

Ten to 20 mm length roots from 20 to 30 soybean seeds were collected for each treatment. Roots were fixed in Carnoy (ethyl alcohol/acetic acid - 3:1, v / v) for 24 h in a fridge (4°C). After fixation, they were placed in 70% ethyl alcohol and stored at 4°C.

Six roots of each sample were randomly selected between the fixed roots. The slides were prepared according to the method proposed by Meneguetti et al. (2012) with the following modifications: freezing of the slides for 1 second in liquid nitrogen removing the coverslip quickly and the use of a Quick Color Kit (Instant Prov) that comprised 0.1% cyclohexadiene alcoholic solution, 0.1% aqueous azobenzene sulfonic solution and alcohol solution of 0.1% phenothiazines. A drop of Acrilex® colorless stained glass and a coverslip were placed on the slide that was left to dry overnight (Paiva et al., 2006). Four of six root tips were analyzed with 1,000 cells per root totaling 4,000 cells per treatment of aging. The slides were analyzed under a Nikon® Eclipse Ci microscope with a magnification of 1,000x.

The mitotic index (MI) was calculated dividing the total number of dividing cells by the total number of cells analyzed. The total number of cells with nuclear damage was calculated summing the number of nuclear buds, chromosomal alterations (CAs) and micronuclei (MN). The CAs analyzed were vagrant chromosome, chromosomal lagging and bridges.

The results were expressed as mean values and standard deviation. Normality was assessed by the Anderson-Darling test and the homogeneity of the variances by the Bartlett test, both at the significance level of $\alpha < 0.05$. For the comparisons of the means, the Tukey test was used for the data with normal distribution and Kruskal-Wallis for the data without abnormal distribution ($\alpha < 0.05$). All statistical tests were performed in Excel with supplementation of the ActionStat® statistical software (http://www.portalaction.com.br).

DNA extraction

DNA of each seed lot was obtained according to the method described by Ferreira and Grattapaglia (1996) using five soybean seeds embryos. Approximately 120 mg of tissue was macerated in liquid nitrogen in 1.5 mL microtubes. Samples were incubated per 30 min in a water bath at 60-65ºC with 700 µL of pre-warmed extraction buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; CTAB 2%; and 1% PVP) and 25 and 50 µL of β-mercaptoethanol. Tubes were gently agitated every 10 min for homogenization during the incubation. Next, the tubes were removed from the water bath and cooled to room temperature. 600 µL of CIA (chloroform/ isoamyl alcohol - 23:1, v / v) solution was added into each tube that were shaken manually for 10 min. The tubes were centrifuged at 13,000 rpm for 5 min and then the upper phase was carefully removed to a new tube. DNA was precipitated adding 2/3 of the volume of the cold isopropanol (-20 °C). The tubes were centrifuged again at 7,000 rpm for 5 min. The DNA pellets were washed twice in 1.0 mL of 70% ethanol and once in 1.0 mL of ethanol for 5 min. Afterwards, the pellets were dried, resuspended with 100 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) and stored in a freezer (-20ºC). DNA extractions were performed with three replicates. The DNA was quantified by Qubit® fluorimeter according to the manufacturer's recommendations and, when necessary, by droplets as an auxiliary method. DNAs were also analyzed using 1.5% agarose gel electrophoresis in 1X TBE buffer (90mM Tris base, 90mM boric acid, 2mM EDTA) at 5 V/cm for 60 min, which was stained with 0.2 µg/mL ethidium bromide. One to 3 µg of DNA was loaded on the agarose gel for the electrophoresis run.

qPCR

DNA integrities in unaged and aged lots were estimated based on the frequencies of three fragments amplified using an anchored single-sense primer (GmTDF Pfb, 5´CCTCTCACGCCTCGCCTCCCA3´) and three different antisense primers (GmTDF R86: 5´AGGATTGGATTTCGGACTCGAT3´; GmTDF R193: 5´CTGCGAGACACGGTAAATGGA3´; GmTDFR491: 5´ TATTCGGAATCTCACCGTTGCT3´) that yielded three different-sized amplicons (86, 193 and 491 bp) of the same region of a hypothetical leucine-zipper-like soybean gene *TDF5*, Genbank accession AB186918 (Murray et al., 2009). Fragments were amplified using qPCR in a StepOne Plus™ Real Time PCR Systems (Applied Biosystems) as previously described by Morgante et al. (2013). Two biological repetitions were analyzed and each of them had two technical repetitions. The negative control of the reactions was composed of all components of the reaction plus water instead of DNA.

The C_t values normality was evaluated using Anderson-Darling test and the homogeneity of the variances using Bartlett test, both at the significance level of $\alpha < 0.05$. Kruskal-Wallis non-parametric significance test ($\alpha < 0.05$) was used since data did not present normal distribution. All statistical tests were performed in Excel, with supplementation of the statistical software ActionStat® (http://www.portalaction.com.br).

RAPD analysis

DNA integrity and repair system in unaged and aged (48, 72 and 96 h) soybean seeds imbibed in water for 0, 14 or 21 h were analyzed. Seeds were placed on germitest paper moistened with distilled water in the proportion of 2.5 times the mass of the dried paper at 25 °C. After the imbibition time the seeds were frozen at -80 °C until the DNA extraction.

RAPD reaction and cycles were described by Ferreira and Grattapaglia (1996), with minor modifications. DNA amplification reactions had the following reagents: 3.0 µL of genomic DNA (3 ng/µL), 3.76 µL of autoclaved deionized water, 1.30 µL of 10X buffer for *Taq* DNA Polymerase, 0.8 µL of $MgCl_2$ (50 mM), 0.6 µL dNTPs (10 mM), 1.04 µL BSA (10 mg/mL), 1.5 µL of primer (Operon Technologies) at 10 ng/µL and 1.0 µL of *Taq* DNA polymerase. The following five primers were used in the analysis: OPA-03, OPA-13, OPF-04, OPF-09, OPH-18. Amplifications were conducted in a Veriti® 96-well Thermal Cycle thermocycler (Applied Biosystems). A pre-denaturation at 94ºC for 5 min was performed, followed by 45 cycles of 94ºC for 1 min, 36°C for 1 min, 72ºC for 2 min, with a final extension of 15 min at 72ºC. The amplified fragments were separated in 1.5% agarose gel electrophoresis in 1X TBE buffer at a constant voltage of 5 V/cm for 2 h, stained with 0.2 µg/mL ethidium bromide and visualized under ultraviolet light. One kb molecular weight standard was used to estimate the size of the amplified fragments.

The markers were analyzed according to the presence (1) or absence (0) of a given band in each sample, generating a binary matrix, which was used to obtain a dendrogram through the hierarchical cluster analysis with Euclidean distance and Ward link in the R statistics program (https://www.r-project.org/).

RESULTS AND DISCUSSION

Physiological characterization

G%, GSI and EC of each sample are presented in Table 1. G% and GSI allowed the differentiation of three groups: a – 0, 6, 12 and 24 h; b - 48 and 72 h, c - 96 h of aging. Differentiation among samples was not possible based on EC test. Seed accelerated aging has been used in different studies to simulate natural aging during storage (El-Maarouf-Bouteau et al., 2011; Kranner et al., 2011; Gordin et al., 2015). In this study, accelerated aging was used to obtain the following two types of samples: 1 - samples that have same or similar physiological quality despite being aged for different hours (group a); 2 - samples that differed from each other based on physiological quality and aging times. Those two types are often found in seed gene bank and the first one is especially interesting to long-term seed conservation research since it includes different samples that cannot be distinguished based on G% despite being in different aging levels. The two types of samples were obtained to be analyzed using cytogenetics, qPCR and RAPDs.

Table 1. Physiological characterization of unaged and aged seed lots of soybean cultivar BRS 7980.

Aging time (h)	G (%)	GSI	EC ($\mu S.cm^{-1}.g^{-1}$)
0	89.0 ± 3.6^{a}	15.0 ± 0.9^{a}	36.4 ± 5.4^{a}
6	86.0 ± 8.6^{a}	14.5 ± 1.6^{a}	58.8 ± 8.2^{b}
12	87.0 ± 3.8^{a}	14.7 ± 1.1^{a}	46.8 ± 5.8^{ab}
24	85.0 ± 5.0^{a}	14.1 ± 0.7^{a}	43.6 ± 5.1^{ab}
48	59.0 ± 3.8^{b}	9.1 ± 0.8^{b}	52.6 ± 4.2^{bc}
72	52.0 ± 1.7^{b}	6.9 ± 0.3^{b}	59.6 ± 19.0^{bc}
96	12.0 ± 0.9^{c}	1.4 ± 0.0^{c}	70.1 ± 12.6^{c}

G% – germination percentage, GSI – germination speed index and EC – electrical conductivity. The data are represented by means ± SD ($n = 4$). Means followed by the same letter in the same column do not differ according to the Kruskal-Wallis test ($\alpha < 0.05$).

Cytogenetic characterization

Different types of nuclear damage were observed in soybean meristematic root cells of unaged and aged samples (Table 2). The vagrant chromosomes were the most frequent damages followed by nuclear buds. Nuclear damage also has been seen in artificially aged seeds of *A. angustifolia* (Fontes et al., 2001), *P. sativum* (Khan et al., 2003), *O. sativa* and *P. vulgaris* (Dantas et al., 2019).

Table 2. Mitotic indexes and nuclear damage frequencies of unaged and aged seeds of soybean cultivar BRS 7980.

Aging times (h)	Mitotic Index (%)	Relative frequency of cells with nuclear damage					
		Nuclear Buds	Vagrant chromosome	Chromosomal lagging	Chromosomal bridges	Micronuclei	Total
0	18.9 ± 0.8^{a}	1.0 ± 0.8^{abc}	1.0 ± 1.4^{a}	0.0^{a}	0.0^{a}	0.0^{a}	2.0 ± 0.8^{a}
6	16.4 ± 0.6^{b}	1.7 ± 1.2^{abd}	1.0 ± 0.0^{a}	1.0 ± 0.8^{ab}	0.0^{a}	0.0^{a}	3.7 ± 0.9^{ab}
12	16.0 ± 0.6^{b}	2.0 ± 0.0^{ad}	1.0 ± 0.8^{a}	0.0^{a}	0.3 ± 0.5^{ab}	0.0^{a}	3.3 ± 1.2^{ab}
24	14.2 ± 0.2^{c}	0.3 ± 0.5^{bc}	1.7 ± 0.9^{ab}	0.7 ± 0.5^{ab}	0.0^{a}	0.0^{a}	2.7 ± 1.2^{a}
48	13.7 ± 0.4^{c}	0.0^{c}	5.0 ± 2.2^{c}	1.0 ± 0.8^{ab}	0.0^{a}	0.0^{a}	6.0 ± 2.2^{bc}
72	12.1 ± 0.2^{d}	3.3 ± 1.2^{d}	3.3 ± 0.9^{bc}	0.3 ± 0.5^{a}	0.3 ± 0.5^{ab}	0.3 ± 0.5^{a}	7.7 ± 1.2^{c}
96	9.5 ± 1.1^{e}	3.0 ± 1.6^{ad}	5.3 ± 1.7^{c}	2.3 ± 1.2^{b}	0.7 ± 0.5^{b}	1.3 ± 0.5^{b}	12.7 ± 3.4^{c}

The data are represented by means ± SD ($n = 4$). Means followed by the same letter in the same column do not differ according to the Kruskal-Wallis test ($\alpha < 0.05$).

The most aged samples could be distinguished by a gradual increase of the total number of cells with nuclear damage (Table 2). However, the small distinction between samples suggested limited value of cytogenetic data in predicting the behavior of a seed lot in relation to its germination fall due to aging. The seeds used in the cytogenetics were the ones that had the roots first emerged, which suggested a higher vigor, probably due to a more efficient DNA repair system. In addition, sample size (six seeds per sample) used for cytogenetics analysis might have been too small to properly reflect the differences among germination power of the samples.

Although there was an increase in the number of cells with nuclear damage above 48 h of aging, a significant increase in MN frequency was only seen at 96 h (Table 2). CAs as vagrant chromosome, chromosomal lagging and bridge can lead to MN formation, but their effect on MN formation can be minimized by incorporation of vagrant chromosome into the nucleus, delay in cytokinesis and quick shrinking of chromosomal bridges toward nucleus (Rao et al., 2008). Therefore, only at the aging time of 96 h the number of CAs could have led to MN formation. The increase of MN frequency is associated with loss of viability and vigor in artificially aged seed of *A. angustifolia* (Fontes et al., 2001) and *P. sativum* (Khan et al., 2003).

MI was the most contrasting cytogenetic parameter to the aging, since it allowed distinguishing the seven samples analyzed into five groups (Table 2). In general, the longer the aging time the lower was the MI. Chauhan and Swaminathan (1984) also observed a significant reduction of MI soybean seeds in artificially aged up to 42 h. Studies in other species have also demonstrated that cell division is impaired by aging damage, showing a decline in older seed lots compared to the unaged ones (Menezes et al., 2014; Dantas et al., 2019). It has been also showed at gene level a correlation between MI and aging. Upregulation of cell cycle regulators and downregulation of mitosis promoters were observed in *Arabidopsis* mutants that lacked telomerase and had dysfunctional telomeres (Amiard et al., 2014). Increase of chromosome mis-segregation in aged mitotic cells was correlated with an early senescence-associated secretory phenotype and repression of Forkhead box M1, the transcription factor that drives G_2/M gene expression (Macedo et al., 2018). Thus, our and previous data suggested MI genes involved could be evaluated to find markers for seed aging.

qPCR

The use of qPCR technique to estimate DNA degradation among samples is based on the assumption that samples with different initial concentrations of intact DNA would reach thresholds after different reaction cycle numbers. A single DNA lesion is sufficient to block the progression of the polymerase, resulting in a reduction in template amplification (Sikorsky et al., 2004).

C_t values obtained using TDF5-86 and TDF5-193 aged (Figure 1a and b) and positive control (Table 3, Figure 1d and e) DNAs were similar not allowing the distinguishment of the samples. The longest fragment (TDF5-491) was the only one that showed differences among some aged (Figure 1c) and positive DNA degradation control (Table 3 and Figure 1f) samples. Distinct capacities of DNA degradation detection were also seen when those primers were used to evaluate integrity of DNA extracted of soybean industrialized products (Murray et al., 2009). Those authors observed the DNA integrity

detection capacity increased with the length of the amplicons. They hypothesized that the larger the fragment the greater the probability of the template be randomly broken reducing the amount of DNA template that has both primers annealing sites what would increase consequently the C_t value. Those authors also estimated the amount of DNA in each sample using calibration curves obtained using amount standards of leaf soybean DNA. In our study, the potential of qPCR to estimate DNA degradation level in aged seed samples was evaluated only based on C_t value differences among samples that were aged for different periods (0, 6, 12, 24, 48, 72 or 96 h). Recently, C_ts value were used to evaluate mRNA integrity in naturally and artificially aged *Arabidopsis* (Zhao et al, 2020b), canola (*Brassica napus*) and wheat (*T. aestivum*) (Zhao et al., 2020a) seeds using qPCR. Both authors evaluated different DNA sequences using a pair of primers for amplification of a long fragment (1,000 to 2,000 bp) and another for amplification of a short fragment (~250bp). Since both authors analyzed stored mRNAs undergo degradation, an mRNA different from the one under analysis would not be a good reference for normalization, as usually performed in gene expression analysis. Thus, the authors used the short fragment as an internal reference to normalize the C_t of the larger fragment, as it was shown in our study and an earlier study (Fleming et al, 2018) that the frequencies of short fragments had low variation between samples. The authors concluded that: (1) there is a linear correlation between transcript degradation and aging time, which can be useful to characterize seed aging phases, at least in materials obtained by controlled aging; and (2) the number of mRNAs explored as molecular markers to assess the level of seed aging may be small, since different analyzed mRNAs were degraded at similar rates. A short fragment could also be used to normalize values of DNA degradation using genomic DNA and qPCR, since our and previous studies showed they have smaller variation among samples.

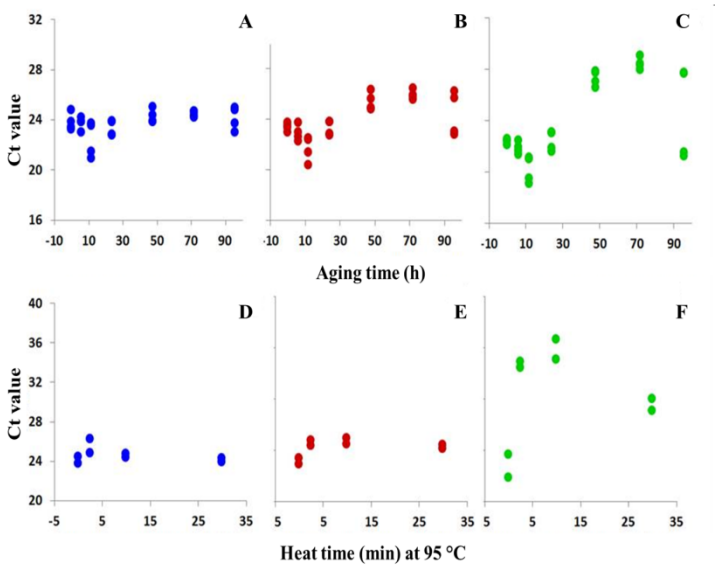

Figure 1. Dispersion of the C_t of each sample evaluated with the three pairs of primers, which allowed amplification of fragments of three different lengths (blue: fragment of 86 bp; red: 193 bp; green: 491 bp). The results with the three pair of primers and DNA of extracted from seed aged for 0, 6, 12, 24, 48, 72 and 96 h are presented in Figures 1 a, b and c with DNA degradation; controls are presented in Figures 1 d, e and f.

Table 3. Cycle thresholds mean variation for each amplicon obtained using the three pair of primers (86, 193 and 491 bp) and soybean positive degradation DNA control samples that were obtained by heating one soybean DNA sample at 95°C for 0, 2.5, 10 and 30 min.

Control samples	C_t (Cycle threshold)		
	86 bp	193 bp	491 bp
0 min	24.1 ± 0.3^{ab}	23.9 ± 0.3^{a}	23.4 ± 1.1^{a}
2.5 min	$25.5 \pm 0,7^{a}$	25.7 ± 0.3^{ab}	33.4 ± 0.3^{b}
10 min	24.6 ± 0.2^{ab}	25.9 ± 0.3^{b}	34.8 ± 1.0^{c}
30 min	24.1 ± 0.2^{b}	25.3 ± 0.2^{ab}	29.4 ± 0.6^{d}

The data are represented by means ± SD (n = 2). Means followed by the same letter in the same column do not differ according to the Kruskal-Wallis test ($\alpha < 0.05$).

C_t values obtained using primer pair TDF5-491 and 0, 6, 12 or 24 h aged sample DNAs were very similar (Figure 1c). These samples had high germination power (Table 1) and similar number of cells with nuclear damage (Table 2). Samples aged for 48, 72 or 96 h had higher C_ts and significant decrease on germination power (Table 1) and increase in number of cells with nuclear damage (Table 2). In general, most samples could not be distinguished from each other and those that were distinguished based on C_ts were also be distinguished based on germination test data, which is technically simpler and cheaper than qPCR. Ebone et al. (2020) did not observe degradation or oxidation in embryo DNA of artificially aged soybean seeds. On the other hand, significant differentiation was observed among all positive control samples, including the sample obtained by only 2.5 min heating (Table 3). We expected some aged samples analyzed using cytogenetics and qPCR showed more distinct C_ts since they were aged for different periods and had different numbers of alive and dead seeds. However, C_ts were similar suggesting the level of degradation on the DNA sequence analyzed among lots were more similar than expected. A possible cause was the sampling for cytogenetics and PCR analysis did not represent appropriately the number of alive and dead seeds of each aged lot, over representing the most frequent type. The control samples C_ts were more diverse than those of aged lots indicating qPCR allowed the detection of different levels of degradation in the evaluated DNA sequence. One difference between aged and control samples that could be a reason for the contrasting results is the samples used to obtain the controls were more homogeneous, which made their sampling more representative than those of the aged ones.

TDF5-491 C_t progressively increased, and the germination power dropped as the aging time was increased up to 72 h (Figure 2). However, the C_ts values of the longest treatment time samples (96 h of aging and 30 min of heating) decreased. That was not expected mainly for the positive control, since the results obtained using the other aged DNA samples suggested all samples had the same number of templates for the three pair of primers tested. This suggests a limitation of the technique that may be related to the lower amount of template available in these highly degraded samples.

Our results suggested that qPCR would be useful to characterize distinct levels of seed DNA degradation through the comparison of C_t values. However, more analyses should be carried out using larger samples and qPCR data normalization using small fragments.

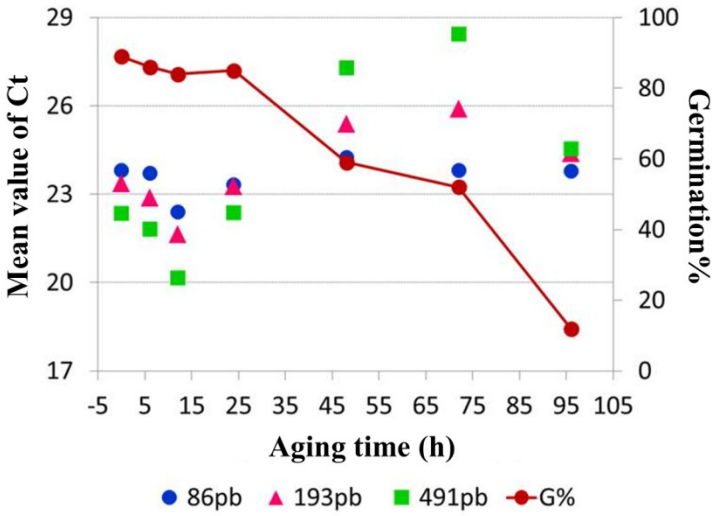

Figure 2. Relationship between seed germination rate, C_t values for different soybean seed lots and different amplicon lengths. An inversely proportional correlation was seen between germination and C_t value.

RAPD

A total of 34 fragments were detected and 20 were polymorphic (Table 4). OPH18 yielded the smallest number of fragments (4) and OPA13 the largest (9), being this last one the most informative since yielded five polymorphic bands. The patterns obtained using two primers are shown in Figure 3.

Table 4. Variation in the number of bands for soybean samples after aging with the five evaluated primers.

Fragment size	Disappeared bands	New bands
≤ 220 bp	0	5
221 to 300 bp	0	1
301 to 500 bp	7	0
501 to 900 bp	3	4

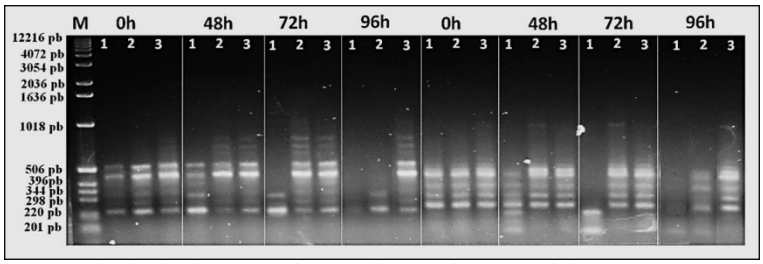

Figure 3. Band pattern obtained using DNA from soybean embryos aged for 0, 48, 72 or 96 h using primers OPA3 and OPA13. Each sample were analyzed individually after 0 (1), 14 (2) or 21 h of imbibition in water. The disappearance of bands larger than 300 bp with aging was observed, which were re-established after 14 h of imbibition in water. New bands smaller than 200 bp also appeared with aging, which disappeared after 14 h of imbibition in water.

Comparison between control (Figure 3; 0h-1) and non-imbibed aged samples showed, in ten control bands (Figure 3; 48h-1, 72h-1, 96h-1), the disappearance of bands above 300 bp and the appearance of new bands below 300 bp. The most frequent change in RAPD patterns among aged sunflower (El-Maarouf-Bouteau et al., 2011) and soybean (Shatters et al., 1995) seeds was the appearance of new bands. One explanation for this would be that the probability of some mutation occurring between the annealing sites of the primers, leading to non-amplification, increases with the increasing of fragments lengths, as seen with those analyzed using qPCR.

The non-imbibed samples patterns showed the degradation levels between 0 and 48 h (Figure 3; 0h-1h and 48h-1) were similar while at 72 and 96 h (Figure 3; 72-0h and 1-48h) distinct patterns were observed, suggesting similar DNA amount and level of degradation between those two sets of samples. That also corroborated qPCR data that showed better differentiation among samples aged for 72 and 96 h using 491 bp primer pair.

The soybean seed DNA repair system was evaluated by RAPD using seeds aged for different times (0, 48, 72 or 96 h) and three imbibition times (0, 14 or 21 h). As can be seen in Figure 4, the samples were divided into three clusters: the first one included the non-aged samples (0hEv0Eb, 0hEv14Eb, 0hEv21Eb), 48hEv14Eb and 48hEv21Eb); the second cluster comprised 72hEv14Eb, 72hEv21Eb and 96hEv0Eb samples; and the third comprised 48hEv0Eb, 72hEv0Eb, 96hEv14Eb and 96hEv21Eb samples. The grouping of non and 48 h aged samples showed that the repair has occurred, and it could be detected using RAPDs. The existence of the clusters suggested the variation on the DNA repair system efficiency among the samples. Kranner et al. (2011) also observed that DNA repair system efficiency in pea samples aged for different times was gradually reduced as the aging time increased and that a certain level of repair was observed in the different samples earlier after imbibition in the less aged samples than in the older ones.

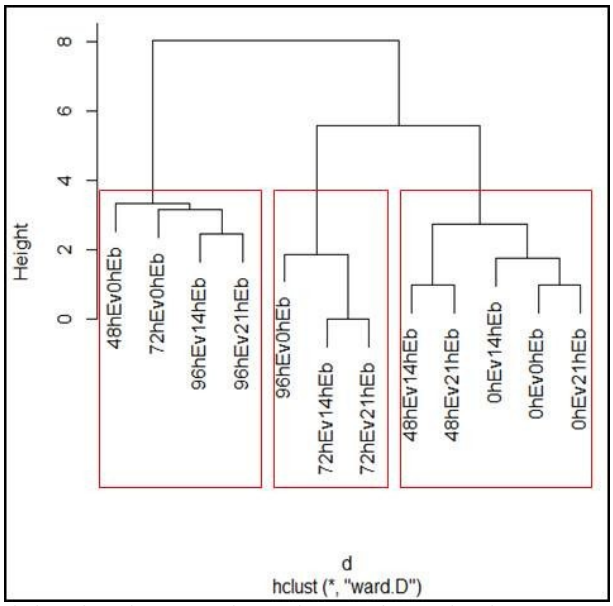

Figure 4. Cluster analysis based on the RAPD data and DNA of control and 48, 72 or 96 h of aging imbibed in water for 0, 14 or 21 h. Three main clusters were seen, suggesting different levels of efficiency in the DNA repair system.

The variations observed among the samples in this cluster, especially the controls, suggested that the repair was not 100% efficient to the point that the samples have the same pattern as the control and/or that the patterns obtained with RAPD can be variable even between 100% genetically identical samples, due to different factors, such as quantity and quality of DNA between samples.

CONCLUSIONS

The results suggest that DNA deterioration has low potential for use as a marker to identify the different stages of aging of long-term preserved soybean seeds, including those stages that are asymptomatic in terms of germination power. Part of this may have been the fact that the DNA undergoes repair as soon as water enters the seed. Larger samples for cytogenetic and molecular analysis may better reflect the germination power of each sample.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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