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DNA methylation and integrity in aged seeds and regenerated plants

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Abstract

Seed longevity is a complex process of key ecological and agronomic importance. DNA damage is a significant factor affecting seed ageing. Likewise, epigenetic changes can control gene expression and, therefore, seed response to ageing. The aim of the present work was to investigate the effect of ageing on nucleic acid stability and to identify reliable molecular markers that might help to monitor epigenetic changes within plant genetic resources during conservation. DNA profiles, evaluated by RAPD (random amplified polymorphic DNA), and methylation patterns, obtained by MSAP (methylation-sensitive amplification polymorphism), were compared in non-aged and aged *Mentha aquatica* seeds and plants produced by them. Germination decreased to 50% by storing seeds at 35°C and 12% wc for 28 days. RAPD profiles were 99% similar in these aged seeds compared to non-aged seeds. However, seedlings produced from the aged seeds showed a 13% dissimilarity compared to seedlings produced from the non-aged seeds. About 8% difference in the MSAP epigenetic profile was detected in seeds after storage and 16% difference was detected in the seedlings produced from them. This indicates that stress from high temperature and humidity during storage induced changes on the methylation state of seeds, and that changes were also detectable in the regenerated plants. Our results suggest that DNA integrity was compromised in seeds during ageing, and on seedlings produced by aged seeds. Genotype screening techniques such as RAPD and MSAP have the potential as markers of nucleic acid stability during seed ageing.

Introduction

A universally acknowledged truth is that all biological organisms age with time. Maintaining seed quality despite the passage of time is the basis for the preservation of plant genetic resource as well as the dynamics within soil seed banks. Seeds can survive for years, and there are examples of extreme longevity (Steiner and Ruckenbauer, 1995; Daws et al., 2007; Sallon et al., 2008). However, all seeds eventually age, lose quality, and die. The decrease of seed quality during ageing, from seed vigour to DNA stability, is a significant impediment to efficient seed storage. However, changes in seed quality can be difficult to detect using standard germination tests during the initial phase of the ageing process or in seeds that are dormant. For example, some species show highly irregular germination during storage due to endogenous dormancy cycles (Froud-Williams et al., 1986; Rawat and Thapliyal, 2003). In those cases, germination assays under-represent the number of viable seeds and might also impose selection on the germplasm when it is regenerated (Pérez-García et al., 2007; Mira et al., 2011b; van Hintum and van Treuren, 2012). Thus, the evaluation of seed quality during storage would benefit from additional tests.

Though critical to ecological processes and conservation protocols, research to understand ageing mechanisms in seeds is limited. Seed deterioration may occur through different mechanisms depending on species and storage conditions, for example, low and high humidity, due to differences in molecular mobility within the cytoplasm (Walters, 1998, 2015; Ballesteros and Walters, 2019). Accumulation of reactive oxygen species and lipid peroxidation isgenerally considered as major contributors to seed deterioration (Merritt et al., 2003; Bailly, 2004; Mira et al., 2011a). Under humid conditions, such as in natural soil seed banks or seeds stored short term for agriculture purposes, ageing is also associated with controlled biochemical activities, such as programmed cell death (Kranner et al., 2011). Ageing processes result in changes in the structural and functional properties of membrane lipids, key proteins, and nucleic acids (McDonald, 1999; Kurek et al., 2019).

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One significant factor determining seed longevity is the level of DNA stability in the embryo, marked by DNA damage during ageing and repair response upon imbibition (Waterworth et al., 2015). Structural damage, such as single and double strand breaks of DNA, or point mutations accumulate in ageing seeds (Navashin, 1933; Roberts, 1978; McDonald, 1999; Tuteja et al., 2001). DNA fragmentation has been correlated with lower seed germination (Osborne, 2000; Kranner et al., 2006; Donà et al., 2013). While ageing processes occur, seeds might still be able to germinate. To do so, DNA repair mechanisms are activated in the embryo during early germination steps (Balestrazzi et al., 2011). It is unclear whether DNA alterations are retained within the seedling or subsequent plant. If there are lasting effects, our ability to provide germplasm with quality-assured genetic composition will be impacted.

During seed ageing, DNA alterations could compromise epigenetic stability. Cytosine DNA methylation (^{m}C) is an epigenetic modification that is important for regulating gene expression in higher plants and other organisms (Gehring and Henikoff, 2007) and the molecular mechanism controlling gene expression by methylation–demethylation is shared among animal and plants systems (Law and Jacobsen, 2010). Methylation of cytosine in vertebrates occurs only in CG dinucleotides. In plants, methylation sites are predominantly at CG dinucleotides but can also occur in any sequence context (Vanyushin and Ashapkin, 2011). Cytosine methylation in DNA plays a key role in the regulation of gene expression, allowing cells to adapt to new conditions without affecting the DNA sequence, controlling growth, development, and responses to environmental stresses in plants (Qiao and Fan, 2011) and animals (Bird, 1993). Epigenetic mechanisms involved in longevity and ageing in animals have been studied in multiple systems (Sen et al., 2016). A comprehensive genome-wide study in human beings has shown that changes in the methylation level of cells highly correlate with chronological age, detecting a decrease in transcription activity with age due to hypermethylation in promotor regions, and hypomethylation in gene bodies (Åsa et al., 2013). In plants, DNA methylation changes between juvenile and mature plant stages, suggesting that the methylation status of genomic DNA can vary as plants mature (Yuan et al., 2014; Ogneva et al., 2016). The relationship between DNA integrity and epigenetic changes during seed ageing and, perhaps more importantly, upon subsequent plant generation is poorly understood.

The major aim of this study was to investigate the overall nucleic acid profile during seed ageing and the ultimate goal of the work is to identify reliable markers of seed deterioration. DNA profiles visualized by RAPDs and MSAPs analysis were compared between non-aged and aged seeds of *Mentha aquatica* and plants produced by germinating seeds from these seed lots. *M. aquatica* seeds are an interesting study system because it is difficult to evaluate longevity during storage because seeds display endogenous dormancy cycles that can mask viability results obtained by a standard germination test. Dormancy can be broken using gibberellic acid to prevent incorrect classifying of dormant and dead seeds, allowing a clear correlation between viability loss and nucleic acid stability. We hypothesized that ageing affects genetic stability and global methylation of genomic DNA. The work contributes to understanding the complex network of molecular events associated with seed ageing, which will benefit seed conservation efforts through improved detection of seed quality changes.

Materials and methods

Seed storage experiment

M. aquatica L. produces four-sectioned schizocarp fruits with one-seeded nutlets that are its dispersion and germination units (Morales, 2007). Lamiaceae nutlets possess mainly a schlerenchimatic pericarp, little endosperm, and a fully developed embryo, so embryo tissue proportion is bigger than both pericarp and endosperm (Martín Mosquero et al., 2003; Bonzani et al., 2011). Experiments were carried out with nutlets (hereafter called 'seeds') received from Semillas Cantueso S.L. (Córdoba, Spain). Seeds were stored at laboratory conditions (20°C, 35% RH in darkness) until use.

For the ageing treatment, seeds were placed over a saturated solution of NaCl (75% RH) at 25°C for 3 days (Vertucci and Roos, 1993) and then hermetically sealed in aluminium foil bags (S-156, Valsem Industries SAS, Lachelle, France) with 125 µm thickness of polyester, aluminium, and polyethylene layers. Seeds were then stored for 28 days at 35°C. The storage experiment was replicated in two aluminium bags. Water content (wc) was calculated by the low-constant-temperature-oven method (ISTA, 2017) using two replicates. Water contents were expressed as percentage of g H_2O/g dry weight.

Germination assays of non-aged and aged seeds were performed by placing eight replicates of 25 seeds in 9 cm Petri dishes on top of two discs of filter paper moistened with distilled water. Distilled water was added to the filter paper regularly when required. Incubation conditions were 25°C with a 16-h photoperiod provided by cool white fluorescent tubes with an irradiance of 35 µmol m^2/ s. The emergence of the radicle was the criterion for germination. Radicle length following 21 days of incubation and the number of days required to reach 50% of final germination (MGT) were calculated as vigour parameters. A solution of gibberellic acid (1000 mg/l^{-}) was added to the non-germinated seeds to stimulate germination of dormant seeds. Differences in total germination or vigour before and after storage were confirmed by a Chi-square test or *t*-test, respectively.

DNA extraction and analysis

Two replicates for DNA extractions were done for each treatment (non-aged and aged) of seeds and seedlings. Each seed DNA replicate was isolated from 30 mg (550 seeds per sample). The two replicates from aged seeds were taken from the two aluminium bags stored (one replicate per bag). Each seedling DNA replicate was isolated from 20 mg (50 seedlings per sample). Seedlings were bulked among the eight replicates of germination Petri dishes per treatment (non-aged and aged seeds) after 21 days of incubation. Plants with a similar developmental stage were used for DNA extraction.

Samples were pounded in liquid nitrogen and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen®, Netherlands), according to the manufacturer's instructions.

Fungal contamination can be a problem when working with seeds and seedlings, especially after high humidity storage. To discard samples that show fungal contamination on DNA extracts, we tested for PCR amplification products of fungus-specific DNA regions using the internal transcribed spacer (ITS) region of the ribosomal DNA repeat cluster, as well as markers for the specific detection of three fungi genera commonly found in seeds: *Fusarium*, *Penicillium*, and *Aspergillus* (Gálvez et al., 2016; Gil-Serna et al., 2016). The universal primer pair ITS1-ITS4 was

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Table 1. Primers and number of monomorphic and polymorphic PCR amplification products obtained from RAPD analysis of non-aged and aged *M. aquatica* seeds and plants produced by them

		Seeds		Plants	
Primer	Primer sequence (5′ to 3′)	Monomorphic markers	Polymorphic markers	Monomorphic markers	Polymorphic markers
OPA-1	CAG GCC CTT C	7	0	6	2
OPA-11	CAA TCG CCG T	5	0	5	4
OPB-7	GGT GAC GCA G	5	0	5	0
OPB-15	GGA GGG TGT T	8	0	8	0
OPD-11	AGC GCC ATT G	11	0	11	0
OPE-19	ACG GCG TAT G	10	0	10	1
OPF-1	ACG GAT CCT G	8	0	8	0
OPF-3	CCT GAT CAC C	8	1	8	0
OPF-4	GGT GAT CAG G	7	1	7	5
OPF-10	GGA AGC TTG G	12	0	12	0
OPO-5	CCC AGT CAC T	11	0	11	0
OPO-6	CCA CGG GAA G	8	0	8	1
OPO-7	CAG CAC TGA C	10	0	10	1
OPO-10	TCA GAG CGC C	10	1	11	1
OPO-15	TGG CGT CCT T	9	0	8	2
OPO-20	ACA CAC GCT G	7	1	7	5
total		136	4	135	22

tested (5′-TCCGTAGGTGAACCTGCGG-3′ for ITS1 and 5′TCCTCCGCTTATTGATATGC-3′) for ITS4 (White et al., 1990) and other markers specific included an elongation factor specific for *Fusarium* (5′-ATGGGTAAGGAGGACAAGAC-3′/5′-GGAAG TACCAGTGATCATGTT-3′) (O'Donnell et al., 1998) and betatubuline-specific for *Penicillium* and *Aspergillus* (5′-GGTA ACCAAATCGGTGCTGCTTTC-3′/5′-ACCCTCAGTGTAGTGAC CCTTGGC-3′ respectively) (Glass and Donaldson, 1995).

DNA integrity analysis

Random amplified polymorphic DNA (RAPD) analysis was carried out to assess DNA stability (Callow et al., 1997). A total of 16 random 10-base primers (sequences from Operon Technology, Spain) were used to screen variation in the amplicon product between non-aged and aged seeds and seedlings produced after 21 days of incubation (sequences are listed in Table 1).

DNA amplification reactions were performed in 25 µl tubes containing approximately 10 ng total DNA, 0.8 µM of a single decanucleotide, 5 µl of PCR buffer, and 1.25 U MyTaq DNA polymerase (Bioline, UK). The PCR amplifications were performed in a SimpliAmpl Thermal Cycler (Thermo Fischer Scientific, Waltham, Massachusetts, USA) using one cycle of 1 min at 95°C, followed by 35 cycles of 45 s at 92°C, 1 min at 37°C and 2 min at 72°C, and a final cycle of 10 min at 72°C. RAPD products were electrophoresed in 1.5% (w/v) agarose gels in 1× TBE buffer at 100 V for 2 h, stained with ethidium bromide (0.5 µg/ml) for 7–10 min and photographed under UV light. The size of the amplified bands was related by reference to the molecular size marker (100 Base- Pair Ladder, GE Healthcare, Chicago, Illinois, USA). All the reactions were repeated at least twice to monitor the reproducibility of banding patterns, and the polymorphism presented were repeatable.

The reproducible RAPD bands were scored in binary characters and a similarity matrix was constructed using the Jaccard coefficient, which was further subjected to clustering unweighted pair group method analysis (UPGMA) to eventually generate a dendrogram. The analysis was performed using the NTSYS-PC software package version 2.

Epigenetic stability

To study epigenetic stability during storage, we used a methylation-sensitive amplified polymorphism (MSAP) technique, which is a modification of the amplified fragment length polymorphism (AFLP) method that uses methylation-sensitive restriction endonucleases followed by an amplification of digested fragments. We selected the MSAP protocol involving methylation-sensitive isoschizomers *HpaII* and *MspI* (Reyna-Lopez et al., 1997). The adapter and primer for the enzyme *EcoRI* were the same as that used in standard AFLP analysis, while the *HpaII/MspI* adapter was designed according to Xiong et al. (1999) who modified it for the use in plant species.

To identify epigenetic markers, two sets of restriction/ligation reactions were simultaneously carried out. To perform the digestion, approximately 500 ng of the extracted DNA was digested with 10 U of *EcoRI* and 10 U of *MspI* or 6 U of *HpaII*, in a final volume of 35 µl containing buffer R-L (10×). The mixture was incubated at 37°C for 3 h and the reaction stopped by incubation at 65°C for 5 min and then the samples were cooled for 30–45 min at room temperature. The ligation mixture was carried out by adding to the digested DNA 5 µl of ligation mix (5 pmol

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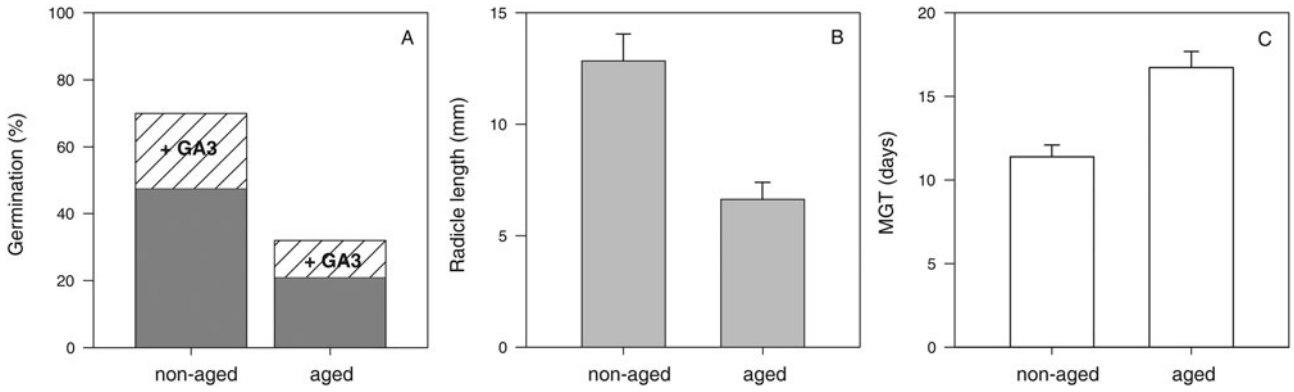

Fig. 1. Seed viability of *M. aquatica* before (non-aged) and after storage for 28 days at 35 °C and 12% wc (aged). Germination before and after gibberellic acid was added (A) and vigour parameters: radicle length growth (B) and MGT (number of days required to reach 50% of final germination) (C). Data are expressed as mean values ± standard error and were statistically significant ($P < 0.001$).

EcoRI adaptor, 50 pmol *MspI/HpaII* adaptor, 10 mM ATP, 1.2 U T4 DNA ligase (Boehringer, Germany), and 10× buffer R-L). The ligation was incubated for 3 h at 37°C and overnight at 4°C. After the incubation, the product of this double reaction was diluted 1:4 to be used as a template for the first amplification reaction.

The pre-selective PCR reaction was performed in 20 µl containing 30 ng of each primer *EcoRI* + A and *HpaII/Msp I* + A, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.4 U of Taq DNA polymerase (Biotaq, Bioline), and 3 µl of diluted fragments in the 1× PCR buffer provided by the manufacturer (Bioline).

The pre-selective amplification was performed with the following profile: 28 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C. Preamplified fragments were diluted tenfold to be used as the starting material for selective amplification. In this amplification, only the *EcoRI* primer was labelled with FAM fluorochrome; both the *EcoRI* and the *HpaII/MspI* primers contain the same sequences as those used in the preamplification but with three selective nucleotides at the 3′ end for *HpaII/MspI* primers and two nucleotides for *EcoRI* primers.

Selective amplification was carried out using a total of five primer combinations obtained with two *EcoRI* primers in combination with three *HpaII/MspI* primers (*EcoRI* + AC–*HpaII/MspI* + ATC; AC/ACT; AA/AAT; AA/ATC; AA/ACT). The PCR reaction was performed in a volume of 20 µl containing 6 ng of labelled *EcoRI* primer, 30 ng of *HpaII/MspI* primer, 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 0.4 U of Taq DNA polymerase, and 5 µl of diluted preamplified DNA in the 1× PCR buffer provided by the manufacturer (Ecogen, UK). Reaction was performed with the following profile: 1 cycle of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C, followed by 12 cycles in which the annealing temperature decreases 0.7°C per cycle, followed by 23 cycles of 1 min at 94°C, 30 s at 56°C and 1 min at 72°C.

Amplification products were analysed in an automated ABI3730 sequencer by the company SECUGEN S.L. (Madrid, Spain). The resulting electropherograms were analysed using GeneMarker® v1.90 software (SoftGenetics, LLC, PA, USA) and Peak Scanner V1.0 software.

MSAP markers were classified as type 1/1, type 1/0 and type 0/1 accordingly to the presence (1) or absence (0) of the corresponding amplified fragment with *HpaII* and *MspI*, respectively. Methylation sensitivity criteria are as follows (Bardini et al., 2003): *HpaII* only recognizes sites that are hemi-methylated at the external cytosine (^{m}CCGG) and *MspI* only recognizes sites being hemi- or fully methylated at the internal cytosine (C^{m}CGG). Sites that are fully methylated at the external cytosine (^{m}CCGG) or hemi- or fully methylated at both internal and external cytosines (^{m}C^{m}CGG) are not cut by either enzyme. However, CCGG-sequences being free of any methylation are digested by both. *HpaII/MspI* markers were scored as the presence (1) or the absence (0), and a matrix was arranged for further evaluation. Fisher's exact test was performed to verify the statistical significance of the results.

Results

Seed viability and vigour

A 54% decrease in *M. aquatica* seeds viability after 28 days of storage at 35°C and 12% wc was statistically significant ($P < 0.001$) (Fig. 1). About 48% and 70% of the non-aged seeds completed germination without and with, respectively, the application of gibberellic acid. The remaining 30% of non-germinated seeds were empty (i.e. no embryo), which is consistent with a previous report for the species (Martín Mosquero et al., 2003) and may be related to the high incidence of vegetative multiplication of the species. About 21 and 32% of the aged seeds completed germination without and with, respectively, the application of gibberellic acid.

Moreover, vigour was also affected by 28 days of storage ($P < 0.001$) (Fig. 1B, C). A 46% decrease in radicle length was noted after storage. The average radicle length in seedlings obtained from non-aged seeds was 13 ± 1 mm of the radicle, while those obtained from aged seeds was 7 ± 1 mm. Also, germination was faster in non-aged seeds compared to aged seeds, with MGT (time to achieve half of final germination, MGT) occurring in 11 ± 1 and 17 ± 1 days, respectively.

Considering these data, we used 28 days of storage as the time for germination or radicle length to decrease to 50% of maximum, P50.

DNA integrity analysis

DNA extracted from non-aged and aged seeds and resulting seedlings were tested for fungal contamination (Fig. 2). Control samples (material donated by Dr. Palmero) produced a clear amplification product for the ITS1-ITS4 fragment (Fig. 2A), the

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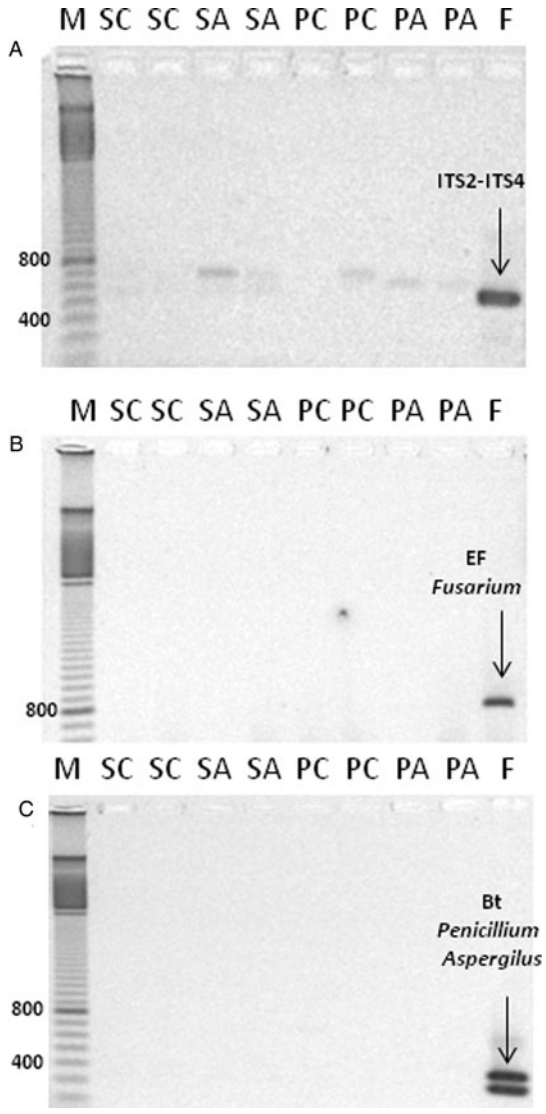

Fig. 2. Evaluation of fungal contamination by the presence of fungus-specific DNA markers in DNA extracted from seeds and plants of *M. aquatica*. PCR amplification products using the primer combination ITS1-ITS4 (A); elongation factor specific for detection of *Fusarium* (B); and betatubuline marker specific for detection of *Penicillium* and *Aspergillus* (C). *Lanes* 'M': bp marker; 'SC': seeds control; 'SA': seeds aged; 'PC': plants control; 'PA': plants aged; 'F' fungal DNA used as control.

transcription elongation factor specific for detection of *Fusarium* (Fig. 2B), and PCR amplification products specific to *Penicillium* and *Aspergillus* (Fig. 2C). In contrast, no amplification products corresponding to these primer sequences were observed for *M. aquatica* seed and seedling DNA extracts, suggesting that fungal contamination does not contribute to observed differences in DNA integrity.

Specific and reproducible amplification products were obtained using 16 RAPD primers (Table 1), allowing a comparison of DNA profiles from non-aged and aged seeds and resulting seedlings. The size of RAPD-amplified DNA fragments ranged from 200 to 2000 base pairs (bp). Amplification produced between 5 (primer OPB-7) and 12 (primers OPF-4, OPO-10 and OPO-20) bands depending on the sample. A representative RAPD profile, obtained with the primer OPF-4, illustrates differences in the DNA band pattern from non-aged and aged seeds and resulting seedlings (Fig. 3A).

In seeds, 140 amplification fragments were detected, 136 of which were monomorphic. Only four primers (OPF-3, OPF-4, OPO-10 and OPO-20) produced PCR products with polymorphisms, resulting in only four polymorphic markers (one per primer). There were few differences detected in DNA extracted from non-aged and aged seeds, and the similarity coefficient was 0.99.

In plants, 157 amplification fragments were analysed, 135 of which were monomorphic. Ageing effects on RAPD profiles were detected by 9 out of the 16 random primers used, which produced 22 polymorphic bands (14% of the total); the other 7 primers resulted in a monomorphic product for all samples. The number of polymorphic bands ranged from 1 (primers OPE-19, OPO-6, OPO-7, and OPO-10) to 5 fragments (primers OPF-4 and OPO-20).

The UPGMA cluster analysis of seedlings revealed differences between non-aged and aged treatments, with a similarity coefficient of 0.87. While the two samples of aged seedlings were identical, the two samples of non-aged seedlings showed some differences, although presented a high percentage of similarity, 97% (Fig. 3B).

Epigenetic stability after ageing

MSAP profiles were generated from non-aged and aged seeds, and seedlings obtained by them. Analysis with the 5 selective-primer combinations revealed a total number of 147 MSAP markers in seeds and 158 in seedlings, with an average of 30 markers per primer set (Table 2).

Aged seed samples showed 92% similarity among MSAP markers, with 135 being identical to those observed in the non-aged seeds. Twelve polymorphic markers differed between aged and non-aged seeds, from which 2 markers showed a variation from type 1/0 to type 1/1, 1 marker showed a variation from type 1/1 to type 1/0, 3 markers from type 1/1 to type 0/1, and 1 marker showed a variation from type 1/1 to type 0/0. All variations indicate a *de novo* methylation (Bardini et al., 2003). Likewise, 2 variations from type 0/0 to type 1/0, 1 marker from type 0/1 to type 1/1, and 2 markers from type 0/1 to type 1/0 were considered as demethylation.

Higher variation was detected in seedlings compared to seeds. The number of monomorphic MSAP markers in aged seedlings decreased to 84% (133 markers). Twenty-five polymorphic markers differed between aged and non-aged treatments, from which 1 marker showed a variation from type 1/0 to type 1/1, 2 markers showed a variation from type 1/1 to type 1/0, 1 marker from type 1/1 to type 0/1, 6 markers showed a variation from type 1/1 to type 0/0, and 3 markers showed a variation from type 0/1 to type 0/0. All these changes were also considered as *de novo* methylation. Furthermore, 1 variation from type 0/0 to type 1/0, 4 markers from type 0/1 to type 1/1, 5 markers from type 0/0 to type 1/1, and 2 from type 0/0 to type 0/1 indicated demethylation.

In summary, percentages of stability and epigenetic changes observed by comparing samples from non-aged and aged *M. aquatica* seeds are shown in Fig. 4. The ageing treatment resulted in an 8% epigenetic change in seeds, detected by polymorphic markers, while a 16% epigenetic change was indicated by seedlings produced by non-aged and aged seeds ($P < 0.01$). The number of epigenetic changes corresponding to a *de novo* methylation or a demethylation was almost identical in both seeds and resulting seedlings.

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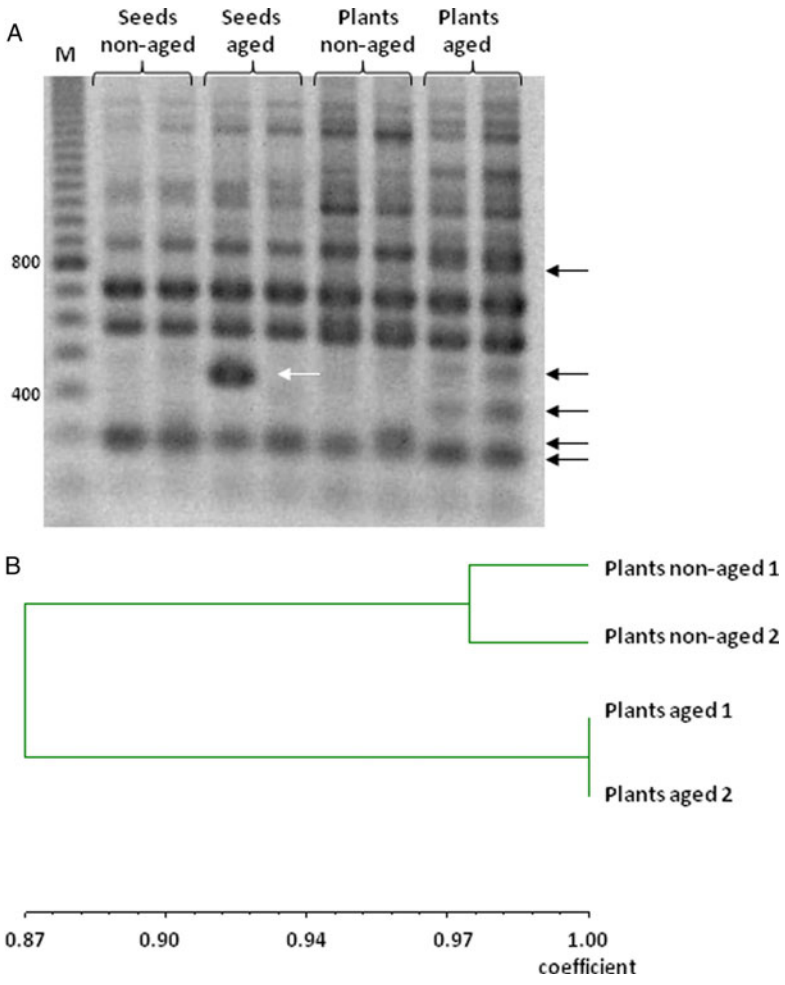

Fig. 3. (A) Representative RAPD profile of amplification products using primer OPF-4 and DNA extracted from non-aged and aged *M. aquatica* seeds and the plants produced by them. (B) Dendrogram for *M. aquatica* plants from aged and non-aged seeds generated by the UPGMA method using Jaccard's similarity coefficient using RAPD markers in (A).

Table 2. Comparison of detected changes in MSAP in DNA extracted from non-aged and aged *M. aquatica* seeds and the plants produced by these seeds

		Number of changes	
Type of change		Seeds	Plants
1/0 → 1/1	*de novo* Methylation	2	1
1/1 → 1/0		1	2
1/1 → 0/1		3	1
1/1 → 0/0		1	6
0/1 → 0/0		–	3
0/1 → 1/1	Demethylation	1	4
0/1 → 1/0		2	–
0/0 → 1/0		2	1
0/0 → 1/1		–	5
0/0 → 0/1		–	2

Binary code corresponds to the selective amplifications of the digested DNA with *HpaII/MspI*.

Discussion

The effect of storage and consequent ageing on DNA stability were studied in *M. aquatica* seeds and seedlings produced from these seeds. Aged seeds were studied at P50, the time when 50% of germination was lost. A 50% loss of viability is an important parameter to evaluate a seed lot storage performance (Priestley, 1986), and it was a key point for DNA alteration identified by RAPD analysis in sunflower (El-Maarouf-Bouteau et al., 2011). In *M. aquatica*, non-aged and aged seeds at P50 shared 99% similarity in RAPD profiles, but when seedlings were compared the percentage of variation in RAPD and MSAP markers was higher. We hypothesize that alterations of nucleic acids might occur in seeds during ageing, and those changes could not be detected in *M. aquatica* seeds but were detectable in the seedling produced by them. Alterations produced on the embryo, particularly on their meristematic cells from which the plant will develop, could have been masked in the analysis of a pool of seeds. DNA extracts from pooled seeds are a mix of genotypes, physiological stages (dead, alive and empty seeds) and also a pool of tissues, which may have different susceptibilities to ageing as well as different importance in PCR amplification. Marcos-Filho et al. (1997) found that RAPD profiles were similar between non-aged and aged soybean seeds. On the other hand, working with the same species and technique, Shatters et al. (1995) found differences between non-aged and aged treatments when analyzing individual seeds, and also detected polymorphic fragments in plants that were not observed in seeds.

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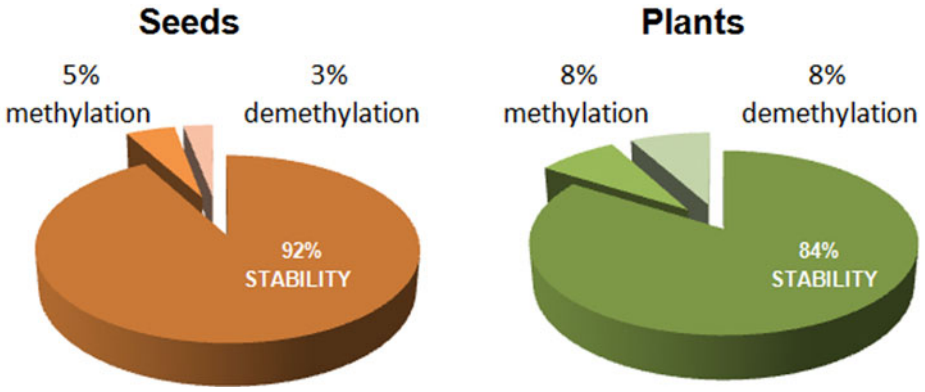

Fig. 4. Percentage of stability and epigenetic changes detected by MSAP markers between non-aged and aged *M. aquatica* seeds and the plants produced by them.

Plants produced from aged seeds showed lower vigour, but phenotypes at the first stage of plantlet development were normal. Aberrant phenotypes such as chlorophyll mutants, anomalous leaf shapes, or chlorotic spots, which could indicate major DNA modifications during ageing were not detected. However, DNA variation between plants produced from non-aged or aged seeds was detected through the RAPD analysis. Our results indicate that DNA integrity was compromised during ageing and was not repaired upon imbibition, being detectable by RAPD analysis on the plants produced by aged seeds (13% dissimilarity). Both the disappearance and the appearance of new amplification products on RAPD profiles of aged plants can be due to structural changes in the DNA (extensive rearrangements of DNA) to point mutations (Karp et al., 1996). Different responses have been reported concerning DNA lesions during seed storage and ageing. At 12% wc, cytoplasm mobility would allow both enzymatic and non-enzymatic mechanisms related to ageing (Walters et al., 2005), such as programmed cell death reactions, nuclease activity, and oxidative degradation of macromolecules by volatile aldehydes or free radicals (Mira et al., 2010, 2016; Kranner et al., 2011; Morscher et al., 2015). Damage to chromatin nitrogen bases, sugars, and histones may lead to structural changes (strand breakage, chromosomal aberrations, rearrangements, small inserts, or deletions) and point mutations (Navashin, 1933; Cheah and Osborne 1978; Waterworth et al., 2015). Upon imbibition during early germination, the DNA repair mechanism will be activated, and while some DNA alterations appear to be reversible, most are considered irreversible (Waterworth et al., 2010, 2015). A simple point mutation within the primer site can generate significant changes in RAPD patterns (Williams et al., 1990; Vijay et al., 2009). RAPD primers scan the entire genome at random, so it is possible that most of the polymorphisms observed are located in regions that are non-expressed, not highly conserved and more likely to be altered and, therefore, allow seed germination and plant early development processes. According to the data of *M. aquatica* genome size (Bennett and Smith, 1991), our RAPD analysis has screened approximately 0.005% of the whole genome; therefore, it may be possible that other DNA sequences were not detected and could have been modified.

Epigenetic regulation, by DNA methylation, of gene expression is known to be involved in plant responses to environmental stresses (Kim et al., 2015). Methylation in seeds affected by storage and ageing has barely been studied. Our data suggest that the DNA methylation profile changed during seed storage, which is consistent with other work using orthodox and recalcitrant seeds (Michalak et al., 2013, 2015). Of the epigenetic changes detected in aged *M. aquatica* seeds and plants, half corresponded with new methylations and half with demethylations, so no clear trend was detected, but variations in the methylation state could result in changes in gene transcription, some of them responsible for age-related plant deterioration.

We found that changes on the cytosine methylation profile could be detected in seeds after storage (8%) and, in a higher proportion (13%), in plants produced from aged seeds. This indicates that stress due to high temperature and humidity of storage induced changes on the methylation state of seeds and that changes were also detectable in the regenerated plants. In pear, an increase from 3 to 5% in DNA methylation level of seeds was found in response to storage at 3°C, and seedlings derived from those seeds maintained an altered methylation profile (Michalak et al., 2013). It is known that most of the stress-induced epigenetic modification of DNA are reset upon relief from the stress, but some of the alterations might be stable and be carried forward in the form of a 'stress memory' that is inherited across mitotic or even meiotic cell divisions in plants (Moliner et al., 2006; Verhoeven et al., 2010). Therefore, changes in the DNA methylation level triggered by environmental stresses may be inherited by successive generations, being a potential factor in the adaptative and evolutionary mechanism in plants (Avramova, 2015).

We show that genetic screening methods detected differences in plants produced by aged seeds and these epigenetic changes might have important, but unknown consequences for their optimum conservation. Mutation accumulation during storage could alter the genetic profile of the conserved material during long-term storage (Schoen et al., 1998; Schoen and Brown, 2001; Chwedorzewska et al., 2002a, 2002b, 2006). Also, epigenetic changes are known to be the first step towards phenotypic changes that could have an unknown effect on mature plants (Bird, 2007) and perhaps emerge as a new source of phenotypic variation to improve adaptation to changing environments and drive breeding strategies (Gallusci et al., 2017). Efficient methods to establish the quality of a seed lot is a critical step for *ex situ* conservation management of plant germplasm, and our data indicate that quality should be evaluated both phenotypically and genetically in stored seeds and the plants obtained after regeneration. Genotype screening techniques such as RAPD and MSAP, independent of genome sequence procedures for detecting DNA polymorphisms, have a strong potential as molecular indicators of nucleic acid stability during seed storage and ageing.

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