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ORIGINAL RESEARCH

Inter-nucleosomal DNA fragmentation and loss of RNA integrity during seed ageing

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Abstract The germination of viable seeds is the basis for new plant growth and development. Seeds lose viability during storage, but the biochemical mechanisms of seed death are not fully understood. This study aimed to investigate degradation patterns of nucleic acids during seed ageing and subsequent water uptake. Seeds of *Pisum sativum* L. were artificially aged at 50°C and 12% seed water content (WC). Nucleic acids degradation was studied during ageing and during imbibition of four seed lots with differential viability from highly viable to dead. As seeds lost viability during ageing, DNA was gradually degraded into internucleosomal fragments, resulting in 'DNA laddering', in conjunction with disintegration of 18S and 28S rRNA bands. During imbibition, non-aged controls had high levels of DNA and RNA integrity through to radicle protrusion. In an aged seed lot with 85% total germination (TG) DNA fragmentation decreased upon imbibition probably due to nucleosome degradation, while rRNA integrity did not improve. In an aged seed lot with 44% TG, neither DNA nor rRNA integrity improved upon imbibition. Dead seeds showed DNA degradation as laddering throughout imbibition along with extensive degradation of rRNA. We present a model in which interlinked programmed and non-programmed events contribute to seed ageing, and suggest that protection of nucleic acids during ageing is key to seed longevity.

Keywords Ageing · DNA · *Pisum sativum* · Programmed cell death · RNA · Seed

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Introduction

The ability of desiccation tolerant 'orthodox' (Roberts 1973) seeds to survive at very low intracellular water content (WC) is the basis for their longevity, maintaining plant genetic resources over centuries (Daws et al. 2007) or even millennia (Sallon et al. 2008; Shen-Miller et al. 1995). In spite of this remarkable longevity, all seeds age and die eventually. The underlying mechanisms of seed ageing and death are less understood than the empirical description of seed longevity as a function of seed WC and temperature (Ellis and Roberts 1980; Pritchard and Dickie 2003; Roberts 1973; Walters 1998; Walters et al. 2005). Orthodox seeds can be dried to extremely low WC (e.g., <5%), inducing 'vitrification', which is the transition of the cytoplasm to the 'glassy' state (Sun and Leopold 1993; Williams and Leopold 1989). They can then be stored in gene banks at low temperatures (e.g., at −20°C, or in or above liquid nitrogen) where their DNA may be preserved long-term (Walters et al. 2004; 2006). Taken together, the glassy state and low temperatures will restrict molecular mobility, delaying degenerative processes (Walters 1998). Under such circumstances, seed death through a process that potentially involves gene expression, is less likely than cell death through random damage. For agricultural purposes, air dried seeds are stored at higher relative

humidities than in seed banks and their cytoplasm may be in a viscous rather than a glassy state. In natural soil seed banks, seed WC may fluctuate in response to environmental conditions (Mickelson and Grey 2006). Hence, due to differences in WC and variations in molecular mobility (Buitink and Leprince 2004; Walters 1998), seed death may follow different routes in 'dry' and 'wet' seeds (seeds in the glassy state vs. hydrated seeds).

In hydrated seeds, ageing is more likely associated with controlled biochemical activities, although it cannot be excluded that cell death follows similar routes as in the glassy state, but with faster kinetics. Programmed cell death (PCD) is a complex process of regulated cell suicide that has evolved in multicellular organisms to eliminate redundant cells (Hengartner 2000; Raff 1998; Samejima and Earnshaw 2005; Viannelo et al. 2007). PCD is mediated by a sequential cascade of events that activate enzymes targeted to destroy macromolecules including nucleic acids, proteins and lipids (Hengartner 2000; Raff 1998). Amongst other well-studied molecular pathways that contribute to PCD, the most common scenarios involve a variety of cell signalling events that trigger mitochondrial changes leading to the release of cytochrome *c* into the cytoplasm. Cytochrome *c* can then activate a caspase (in animals) or para- or metacaspase (in plants) cascade (Elbaz et al. 2002) that results in the systematic degradation of key structural proteins, nucleic acids and the cytoskeleton. (Meta)caspase-activated DNases cleave the genomic DNA between nucleosomes to generate DNA fragments with lengths corresponding to multiple integers of ~180 base pairs, the so-called 'DNA ladder' (Elbaz et al. 2002; Hengartner 2000; Hoeberichts and Woltering 2003). The presence of the DNA ladder has been extensively used as a marker of PCD in animal and plant cells. In addition, cleavage of rRNA was also observed during animal PCD (Crawford et al. 1997; Houge and Doskeland 1996). Cytosolic 28S and 18S rRNAs are integral components of 60S and 40S ribosomes, respectively, which associate into the 80S ribosomal complex, a crucial apparatus for protein synthesis. Hence, PCD-associated rRNA fragmentation may lead to a change in the higher structure of rRNA and ribosomes (Nadano and Sato 2000) subsequently impacting on the translational apparatus and protein synthesis during cell death (Bushell et al. 2004).

DNA and RNA orchestrate gene activity implicated in life and cell death processes (Bushell et al. 2004; Hoeberichts and Woltering 2003). It is therefore surprising that the integrity of nucleic acids during seed ageing has received relatively little attention in the recent literature. Changes in nucleic acid content (Brockelhurst and Fraser 1980; Sen and Osborne 1974, 1977; Thompson et al. 1987) and more recently, DNA fragmentation (Kranner et al. 2006; Osborne 2000), have been reported in seeds in relation to maturation and germination rate. Single and double strand breaks of DNA accumulate in ageing seeds (Tuteja et al. 2001) and DNA fragmentation was correlated with seed death induced by drying in 'recalcitrant', i.e. desiccation intolerant, seeds (Faria et al. 2005; Kranner et al. 2006). Covalent modification of DNA, possibly as a result of Maillard (browning) reactions, has been observed after long-term desiccation of desiccation tolerant cyanobacteria (Shirkey et al. 2003). Maillard reactions include spontaneous reactions of reducing sugars with the primary amino groups of proteins and nucleic acids, finally producing 'advanced glycosylation end-products' (Papoulis et al. 1995). Such Maillard products have been found in aged wheat seeds (Strelec et al. 2008). In addition, seed viability has been correlated with the appearance of lesions in the processing of rRNA precursor species and in ribosomes in conjunction with a significant loss of RNA synthesising activity in the first hours of germination (Bray and Chow 1976; Bray and Dasgupta 1976). However, the relationship between DNA and RNA degradation during seed ageing and, perhaps more importantly upon subsequent imbibition, has not been studied. The major aim of this study was to investigate the overall patterns of nucleic acid integrity during seed ageing, visualised by DNA laddering and disintegration of 18S and 28S rRNA bands. *Pisum sativum* (garden pea) was chosen as a convenient model seed. Pea seeds do not display dormancy. Therefore, non-germination after ageing treatments cannot be confused with dormancy, allowing a clear correlation of nucleic acid degradation and viability loss.

Materials and methods

Materials

Organically grown seeds of *P. sativum* L. cv Alaska Early were purchased from the 'Abundant Life Seed Foundation', Port Townsend, Washington, USA. All chemicals used were of analytical grade (Sigma, St. Louis, MO, USA; Fisher, Loughborough, Leicestershire, UK or Roche (Burgess Hill, UK) and all solutions were made up with double-distilled, ultra-pure water.

Ageing and germination conditions

At −20°C, pea seeds in the glassy state with 5% WC (Sun 1997) have a projected longevity of 9932 years and 89 years at 12% WC (Ellis and Roberts 1980). To accelerate the ageing process, seeds of 12% WC were prepared and then aged at 50°C to produce seed lots with differential viability ranging from highly viable to dead. Seeds were first equilibrated for approximately 5 weeks in tightly sealed boxes over LiCl (30 g/100 ml) at 60% relative humidity (recorded with a Rotronic AWVC-D10 Hygropalm) in

a temperature-controlled room (20 ± 1°C). The relative humidity of the LiCl solution was monitored regularly and re-adjusted if required (Hay et al. 2008). Equilibrated seeds are referred to as 'non-aged controls'. Equilibrated seed lots were also subjected to artificial ageing in tightly closed bottles (without LiCl) at 50°C until viability loss, henceforth referred to as 'ageing'. At 50°C the relative humidity was found to shift to 75%. During ageing, the seed WC tended to decline, but this was not statistically significant ($n = 6$ replicates of 20 seeds each). Seed WC was expressed on a fresh weight (FW) basis. Dry weight (DW) was determined after heating to 103°C for 17 h and WC calculated: WC = (FW−DW)/FW × 100.

Fungal contamination has previously been observed during long-term storage of seeds (Walters et al. 2006). Therefore, care was taken to minimize the opportunity for fungi to grow, interfere with and potentially degrade, seed nucleic acids during artificial ageing. Surface sterilization of seeds alone may not suffice to avoid microbial growth because fungi may already be associated with internal seed tissues when shed from the mother plant (Mycock and Berjak 1992; Whitaker et al. 2008). Therefore, a constant seed WC of 12% was chosen that allowed relatively fast seed deterioration resulting in viability loss after c. 50 days, but was 'dry' enough to make fungal growth unlikely. Twelve % WC, corresponding to ~75% relative humidity (Vertucci and Roos 1993), is below the threshold that allows pea seeds to become measurably metabolically active. Activation of *P. sativum* seed metabolism was observed at 90% relative humidity (Vertucci and Roos 1990). However, some fungi are adapted to grow under very dry conditions. For example, Nielsen et al. (2004) reported that the lower limit for fungal growth, almost exclusively in species in the generea *Penicillium*, *Aspergillus* and *Eurotium*, on starch-containing materials was 78% RH at 20–25°C. Therefore fungal growth will unlikely occur in and on seeds at 75% relative humidity. Nonetheless, a second precaution was taken by testing for fungal contamination as described below.

Samples for nucleic acids extraction were taken at intervals up to 55 days of ageing. At each interval (0, 25, 31 and 55 days), germination tests were conducted using 1% water agar at 25°C under warm white fluorescent light at an irradiance of 15 µmol m^{-2} s^{-1} at a day/night cycle (8/16 h). Germination was defined as radicle emergence by at least 2 mm and scored until all seeds had either germinated or started disintegrating. Seed viability was expressed as percentage total germination (TG).

To study nucleic acids degradation during water uptake, samples of seeds that had been aged for 0, 25 or 55 days were assessed after 0, 6, 10, 15, 20, 50 and 80 h of imbibition on 1% water-agar. After each treatment (ageing and imbibition), seeds were immediately frozen in liquid nitrogen, freeze-dried for at least 5 days and ground to a fine powder in a hermetically closed, liquid nitrogen-cooled Teflon grinding capsule using a laboratory mill (Braun Microdismembrator). The powder was stored at −80°C in humidity-proof vials until use.

DNA extraction and analysis

Genomic DNA was isolated from approximately 50 mg freeze-dried seed powder using cethyltrimethylammonium bromide (CTAB) and quantified spectrophotometrically at 260 nm (Stewart and Via 1993). DNA quantity and quality were assessed spectrophotometrically at 260, 280 and 230 nm. Two microgram of DNA were separated on 1.5% agarose gel, stained with ethidium bromide (Sambrook et al. 1989), visualised using a UV transilluminator (Syngen) and the intensity of the 180 bp fragments quantified by image analysis, using the Syngen image analysis programme.

DNA extraction from microbial contaminants and PCR amplification of the ITS region

To investigate fungal contamination of seeds, it was tested if fungus-specific DNA regions could be amplified in DNA isolated from artificially aged seeds. To obtain fungal biomass, 55 days aged seeds that did not germinate and started to disintegrate approximately 80 h after the onset of imbibition, were incubated for another week. Typically, 4–5 days after the onset of imbibition, these dead seeds showed the first visual signs of microbial contamination and after 10 days were overgrown by fungi, yielding enough biomass for DNA extraction. DNA was extracted from fungal material as described above for seeds. For both seed and fungal DNA samples, PCR amplification was conducted of the Internal Transcribed Spacer (ITS) of the ribosomal DNA repeat cluster, which is typically sequenced for molecular classification of fungi. The universal primer pair ITS1-ITS4 was first tested (5′-TCCGTAGGTGAACCTGCGG-3′ for ITS1 and 5′-TCCTCCGCTTATTGATATGC-3′ for ITS4; White et al. 1990); it can co-amplify angiosperm DNA but did produce bands when seed DNA was used as a template (data not shown). For specific detection of fungal contamination the primer combination ITS1F-ITS4 was chosen that amplifies fungal DNA but excludes angiosperm DNA (5′-CTT GGT CAT TTA GAG GAA GTA A-3′ for ITS1F). To test if the seed DNA was a suitable template for gene expression experiments, PCR amplification of the 18S gene were conducted as a control.

RNA extraction and assessment of rRNA integrity

Seed RNA was extracted from approximately 50 mg freeze-dried seed powder as described by Birtić and Kranner

(2006). Briefly, RNA was extracted using hot borate buffer and purified by acidification and ethanol precipitation. DNA impurities were removed using DNase I and RNA quantity and quality were assessed spectrophotometrically at 260, 230, and 280 nm. To assess rRNA integrity, 1 μg RNA was run on 1.5% agarose gel electrophoresis, stained with EtBr (Sambrook et al. 1989), visualised and the intensity of the 28S and 18S rRNA bands quantified after image analysis, as above.

Replication and statistical analysis

For each ageing and imbibition treatment five independent biological replicates were used for nucleic acid analysis. For each replicate, seeds were aged in separate, tightly closed 500 ml bottles from which 20 seeds were taken randomly and ground to powder for nucleic acid analysis ($n = 5$); WC was determined using 40 seeds ($n = 5$). Germination tests were conducted with 20 seeds that were aged in the same bottle ($n = 5$). In addition, 120 seeds were used for nucleic acid analysis at six intervals during imbibition (20 seeds per interval, $n = 5$). Data were analysed for significance by one or two-way ANOVA in combination with LSD post hoc comparisons of means.

Results

Effect of artificial ageing on nucleic acid integrity

Non-aged control seeds were highly viable, showing 98% TG. During ageing seed viability was gradually lost. Seed lots in which the viability had dropped by 13% after 25 days of ageing are hereafter referred to as 'Aged (85% TG)' and those in which viability had dropped by 54% after 31 days of ageing are termed 'Aged (44% TG)'; in text that refers to both aged seed lots (85% TG and 44% TG), the term 'Aged' is used. Seeds with only 2% germination after 55 days of ageing are henceforth referred to as 'dead'. Artificial ageing did not significantly affect the content of total extractable DNA, but resulted in loss of total RNA (Table 1).

Table 1 Quantity of extractable total RNA and chromosomal DNA in relation to seed ageing in *Pisum sativum*

Treatment	RNA (μg mg^{-1} DW)	DNA (μg mg^{-1} DW)
Non-aged controls	0.50 ± 0.03	1.46 ± 0.23
Aged (85% TG)	0.34 ± 0.03*	1.54 ± 0.15
Aged (44% TG)	0.23 ± 0.09*	1.08 ± 0.20
Dead	0.20 ± 0.05*	1.18 ± 0.17

Data are means ± SE ($n = 5$). RNA data in Aged seeds annotated with an asterisk differ from non-aged controls at a significance level of $P < 0.01$. DNA data did not differ significantly from each other at $P < 0.05$

Non-aged control seeds showed only traces of DNA fragmentation and clear, distinct 18S and 28S rRNA bands, indicative of good quality of both DNA and RNA (Fig. 1a, c). These non-aged controls showed traces of DNA laddering, as would be expected because tissues will always contain a few cells that undergo PCD, particularly those of orthodox seeds that experience maturation drying, which will inevitably cause some damage to DNA upon desiccation. As seeds lost viability, DNA was degraded into internucleosomal fragments (Fig. 1a, b). The relative fluorescence intensity of the smallest fragment (~180 base pairs) significantly ($P < 0.01$; Fig. 1b) increased in Aged seeds. Aged (44% TG) and dead seeds showed very pronounced, typical DNA ladders of ~180 base pairs and multiples thereof. rRNA integrity was significantly ($P < 0.01$) impaired in Aged seeds, and in dead seeds rRNA appeared only as a smear on the gel while the 18S and 28S bands had disappeared (Fig. 1c, d). The relative fluorescence of the ~180 base pairs DNA fragments and intensities of the 18S and 28S bands correlated significantly ($R^2 = 0.762$ and 0.808, respectively) across all treatments.

To investigate if degradation of DNA on ageing may have been caused by fungal DNases, fungal contamination was assessed using PCR with the universal fungal ITS4-ITS1F primers. DNA extracted from fungal contaminants that grew on decaying seeds produced a clear band but no fungal contaminants were detected in any of the seed DNA samples (Fig. 2a). Some weak amplification may occur with highly concentrated pure DNA for some angiosperm species when ITS1F is used in combination with ITS4 (Gardes and Bruns 1993), but we did not observe any bands when seed DNA was used as a template (Fig. 2a). In contrast, the plant-specific 18S gene was not amplified when the fungal DNA was used as a template. Seed DNA produced a clear band for the 18S gene when isolated from non-aged control seeds and Aged seeds. DNA from dead seeds produced only a faint 18S band, indicating that after 55 days of ageing, the quality of the extracted seed DNA had deteriorated (Fig. 2b).

Effect of seed imbibition on nucleic acid integrity

Germination of non-aged control seeds was completed after 50 h of water uptake (Fig. 3a), assessed by radicle protrusion. Shoots were produced after 80 h (not shown). Following water uptake and germination, these highly viable seeds showed distinct bands of high-molecular-weight DNA with only traces of DNA fragmentation (Fig. 3a) and good rRNA integrity (Fig. 3e).

Aged (85% TG) seeds germinated slower than non-aged controls, indicative of 'vigour' loss (Fig. 3b). Germination was completed after 100 h rather than 50 h and no shoots

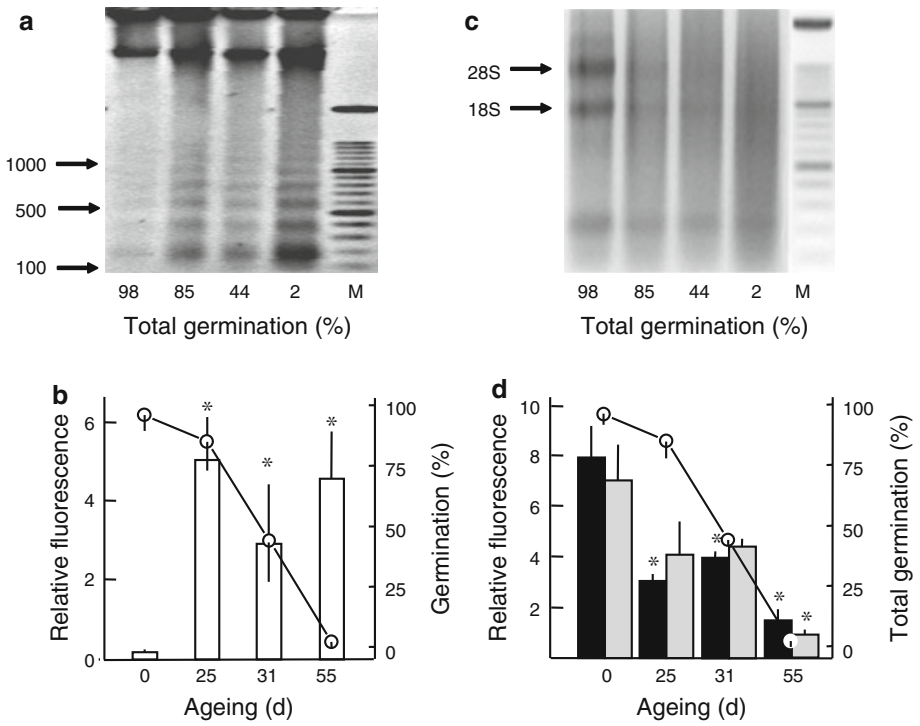

Fig. 1 Seed ageing and death correlates with internucleosomal fragmentation of chromosomal DNA and loss of rRNA integrity in *Pisum sativum* seeds. **a** Analysis of DNA from seeds of different ageing treatments. *Lane 'M'* represents a 100 bp molecular mass marker (Roche) and the further *lanes* represent one of five randomly taken independent biological replicates over the time course of ageing. **b** Semi-quantitative analysis of the 180 bp DNA fragments by image analysis (*white bars* show means ± SE; $n = 5$ independent biological replicates, consisting of 20 ground seeds each; *circles* represent total germination; $n = 5$ replicates of 20 seeds). **c** Total RNA analysis from seeds of different ageing treatments. *Lane 'M'* shows the 100 bp molecular mass marker and the further *lanes* show one of five randomly taken biological replicates. **d** Semi-quantitative analysis by image analysis of 28S and 18S rRNAs (*black* and *grey bars*, respectively; means ± SE; $n = 5$ biological replicates of 20 ground seeds). *Circles* denote total germination as in **b**. *Asterisks* in **b** and **d** indicate differences between non-aged control seeds and Aged or dead seeds at a level of $P < 0.01$ (one-way ANOVA)

were produced after 100 h. DNA fragmentation significantly decreased upon water uptake to the levels of non-aged controls after 50 h (Fig. 3b). In Aged (85% TG) seeds, the relative fluorescence intensity of the 18S and 28S rRNA bands had decreased by 38 ± 11% ($n = 5$) compared to those in non-aged controls ($P < 0.01$), and did not improve upon water uptake (Fig. 3f). Further ageing caused progressive viability loss. After 31 days of ageing, only 44% of all seeds germinated and none of them produced shoots 80 h after the onset of imbibition. The level of DNA laddering in non-imbibed Aged (44% TG) seeds did not significantly differ from that in non-imbibed Aged (85% TG) seeds. DNA laddering tended to decrease slightly up to 50 h after the onset of imbibition of Aged (44% TG) seeds, then increased again to pre-imbibition levels (Fig. 3c), while RNA integrity did not change upon imbibition (Fig. 3g).

Dead seeds showed intense DNA laddering and only faint bands of high-molecular-weight DNA, coinciding with loss of rRNA integrity prior to (Fig. 1) and upon imbibition (Fig. 3d, h). Throughout imbibition, the intensities of the 180 base pair DNA bands and the 18S and 28S rRNA bands in dead seeds were significantly different ($P < 0.05$) from those in highly viable controls and Aged seeds. Seed of all treatments did not show any statistically significant changes in the content of total extractable DNA or RNA (data not shown).

Discussion

Degradation of chromosomal DNA and rRNA in ageing seeds: programmed or non-programmed cell death?

Nucleolytic events such as DNA laddering and rRNA degradation are known characteristics of PCD that have been extensively studied in human and animal tissues (Chowdhury et al. 2006; Hengartner 2000; Raff 1998) and to a lesser extent in plants (Elbaz et al. 2002; Jones and Dangl 2006; Xu and Hanson 2000). Studies on PCD in relation to seed viability loss are rare (Cheah and Osborne 1978; Faria et al. 2005; Kranner et al. 2006; Osborne 2000; Spano et al. 2007) although damage to nucleic acids during prolonged dry storage has been implicated with seed death

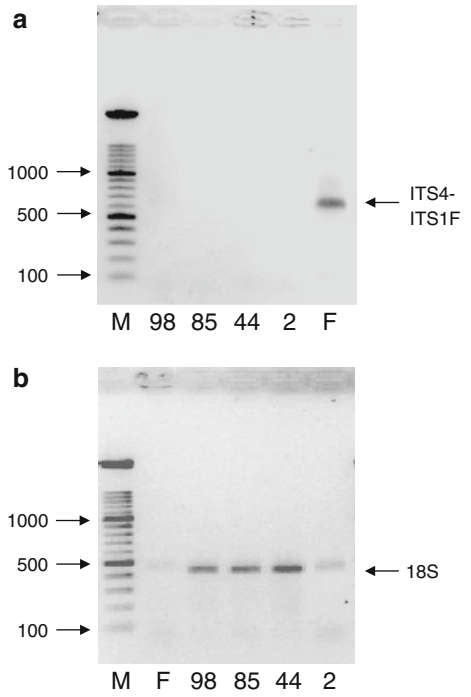

Fig. 2 Evaluation of fungal contamination in artificially aged seeds. The same DNA extracts were used to produce the gels shown in Fig. 1 and in this figure. *Lanes* '*M*' show molecular mass markers, *lanes* '*F*' show PCR products of fungal DNA, isolated from unidentified fungi growing on dead *Pisum sativum* seeds that were incubated on 1% water agar at 25°C for 1 week after completion of the germination test. All other *lanes* show PCR products of plant DNA isolated from *Pisum sativum* seeds after different ageing intervals; the total germination (in %) of the seed lots is indicated at the *bottom* of the *lanes*. **a** The primer combination ITS4-ITS1F (details in the text) amplifies fungal DNA rather than angiosperm DNA. The fungal sample produced a clear band, but seed samples did not, indicative of the absence of fungi in the seed samples. **b** As a control for the suitability of seed DNA for PCR amplification, the PCR product of the plant-specific 18S gene is shown

(Bray and Chow 1976; Boubriak et al. 2000; Brockelhurst and Fraser 1980; Cheah and Osborne 1978; Osborne 2000). The final, or execution, phase of PCD is characterised by inter-nucelosomal DNA fragmentation (Elbaz et al. 2002; Hengartner 2000; Thomas and Franklin-Tong 2004) and can include RNA degradation (Hoat et al. 2006; Perrin et al. 2004). It has been reported occasionally for apoptotic animal cells that rRNA remained intact (Houge and Doskeland 1996) and conversely, rRNA fragmentation occurred without DNA laddering (Samali et al. 1997). Aged and dead *P. sativum* seeds showed both DNA laddering and loss of rRNA integrity (Fig. 1). Significant changes were already found in Aged seeds that had lost only 13% of their initial viability. A possible explanation for this finding is that molecular changes were triggered early in seed ageing, and occurred consistently in all seeds, when good total TG persisted, similar to that observed during pollination-induced petal senescence in *Petunia*, where RNA content also decreased at early stages of PCD (Xu and Hanson 2000). However, it is important to note that Figs. 1 and 3 show responses of seed populations that may not reflect the nucleic acid status in a single seed. For example, in a mixed population with 15% dead and 85% live seeds the partial rRNA degradation observed after 25 days of ageing may be accounted for by the dead seeds that had lost rRNA integrity entirely. Nonetheless, such changes could be used as a marker of early degradation of stored seed lots.

The experimental design in the present study used an ageing protocol at a constant WC of 12% and a constant temperature of 50°C. At 12% WC, pea seeds appear dry but their cytoplasm is in a viscous rather than a glassy state (Sun 1997), allowing considerable molecular mobility and relatively rapid ageing. In addition, 50°C will be close to the temperature optimum for many enzymes, enabling rapid chemical reactions including degradative processes. For example, the temperature optimum of barley nucleases is 55°C (Brown and Ho 1986). In the execution phase of PCD (para and meta)caspases inactivate DNase inhibitors, a step that can be mediated by cytochrome *c*, thus freeing DNases to cleave DNA into typical inter-nucleosomal fragments (Elbaz et al. 2002; Hengartner 2000; Jones and Dangl 2006; Thomas and Franklin-Tong 2004). Hence, the DNA laddering found in Aged and dead pea seeds (Fig. 1) suggests that degradative processes during seed ageing at 12% WC involve enzymatic activity. In addition, previous work using pea seeds with 12% WC showed that caspase inhibitor treatment of seeds prior to ageing reduced the ageing process, supporting the view that caspase-like proteins are involved in cell death during dry ageing (Kranner et al. 2006). The resulting enzyme-catalyzed DNA cleavage may be part of non-programmed cell death (Zangger et al. 2002) or PCD, involving more complex biochemical cascades (Hengartner 2000; Raff 1998).

As an alternative pathway that may occur in seeds at low WC, it was suggested that the 'effector phase' of PCD could be by-passed (Kranner et al. 2006). Instead of complex processes that may involve the MAPK cascade, gene expression and cytochrome *c* release to activate metacaspases, a series of nano-switches could activate metacaspases. Nano-switches are chemical reactions between adjacent molecules operating on a nanometer scale, such as the distance between two thiol groups in a protein with two intervening amino acids (Schafer and Buettner 2001). To by-pass the effector phase, a cascade of redox reactions, triggered by an ageing-induced increase in half-cell reduction potential of the antioxidant glutathione, could activate metacaspases directly through nano-switches based on thiol-disulphide conversions such as those described in ageing pea seeds (Kranner et al. 2006), and DNA laddering would be the result without gene expression, but with slow

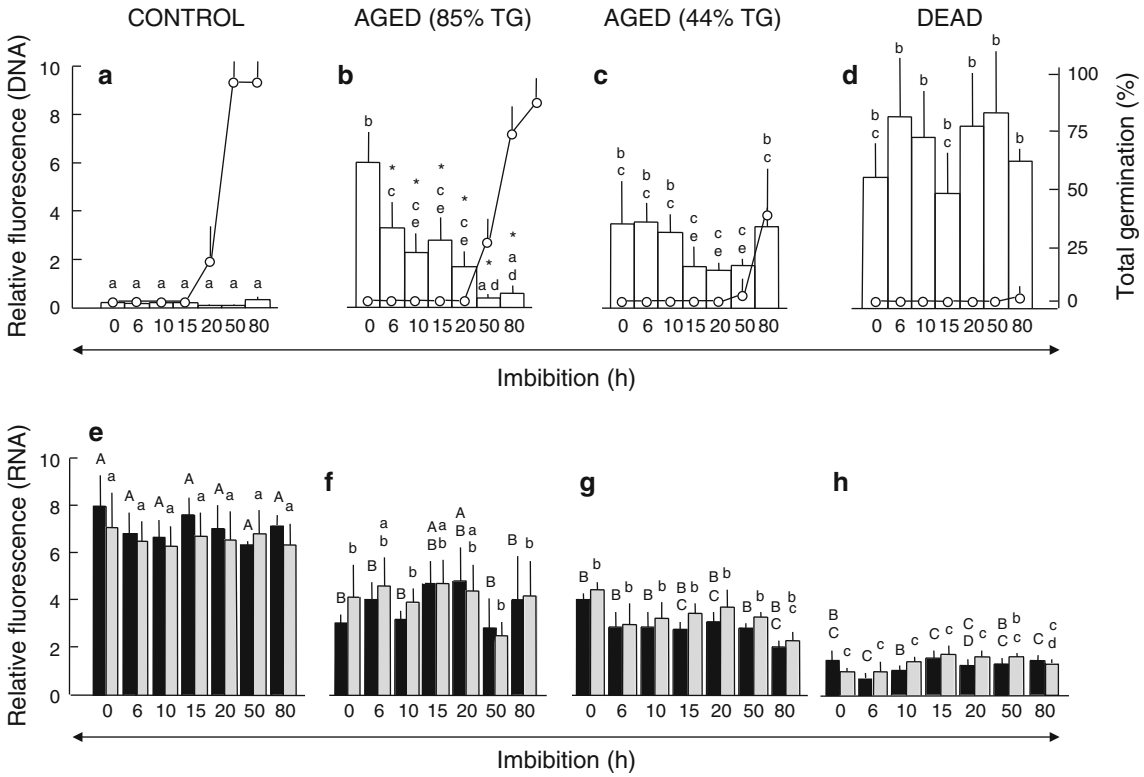

Fig. 3 DNA laddering and rRNA integrity during imbibition of *Pisum sativum* seeds with differential viability. *Panels a* to *d* show the semi-quantitative analysis of the 180 bp DNA fragments by image analysis after gel electrophoresis (means ± SE; $n = 5$ biological replicates of 20 ground seeds). Two-way ANOVA revealed significant differences ($P < 0.05$) between the intensities of the ~180 bp in seeds with differential viability upon imbibition, indicated by different *small letters*. In addition, differences between non-imbibed ('imbibition' 0) and imbibed seeds are symbolized by *asterisks* for a significance level of $P < 0.01$. *Open circles* indicate total germination (statistical differences not shown). *Panels e* to *h* show the semi-quantitative assessment of the 28 S (*black bars*) and 18 S (*white bars*) rRNA bands by image analysis following gel electrophoresis (means ± SE; $n = 5$ biological replicates of 20 ground seeds). Two-way ANOVA revealed significant differences ($P < 0.05$) between the band intensities at different intervals of imbibition following different ageing treatments, indicated by different *capital letters* for the 28S bands and *small letters* for the 18S bands

enzymatic activity. Such a redox-driven activation of caspase-like proteins could be part of PCD or necrotic death. In summary, the DNA fragmentation in ageing seeds (Fig. 1) may have followed PCD, involving the MAPK cascade, gene expression and cytochrome *c* release, but some of these PCD effector phase-specific reactions may have been by-passed. Both scenarios will have resulted in DNA laddering.

In addition to the DNA fragmentation found in Aged and dead seeds, ageing also induced rRNA degradation (Fig. 1). There is evidence that RNA breakdown during PCD is completed by RNases. Increasing RNase activity has been associated with plant PCD during petal senescence (Panavas et al. 1998; Xu and Hanson 2000) and has also been observed during seed ageing (Kalpana and Rao 1997). Therefore, ageing-induced rRNA degradation during seed ageing (Fig. 1) may have been the result of PCD that included RNase activation. Nonetheless, more research is needed to understand whether PCD or non-programmed cell death caused rRNA degradation.

Integrity of nucleic acids during dry ageing and subsequent imbibition: protection or repair?

Dead seeds neither germinated nor showed any improvement in DNA (Fig. 3d) and rRNA (Fig. 3h) integrity upon water uptake, suggesting that seed death was already triggered during ageing at 12% WC and unrecoverable through repair processes. Interestingly, upon imbibition of Aged (85% TG) seeds, DNA fragmentation appeared to be reversible (Fig. 3b). However, internucleosomal DNA fragmentation is considered to be irreversible (Hengartner 2000) and re-assemblage of nucleotides is extremely unlikely. More likely, nucleotides could have been further degraded during the first stages of imbibition of Aged (85% TG) seeds (Fig. 3b) through DNA-degradation pathways that exist in plants, animals and yeast (Lieber and Karanjawala 2004; Samejima and Earnshaw 2005). For an aged seed with partially degraded nucleic acids, such a controlled degradation of ~180 base pair fragments in the first stages of imbibition would make building blocks of nucleic

acids available. Ageing will also have affected other essential molecules including proteins and lipids (Bailly 2004; Bernal-Lugo and Leopold 1998). Hence, production of such new building blocks upon imbibition may have allowed Aged (85% TG) seeds to set downstream repair mechanisms in motion. This necessity for repair is consistent with reduced vigour in Aged (85% TG) seeds that required twice as long to germinate than non-aged seeds. Aged (44% TG) seeds did not show an improvement in rRNA integrity upon imbibition, nor was their rRNA further degraded (Fig. 3g). These low rRNA levels could have been due to complete loss of RNA integrity in 56% of the seed population. Alternatively, if the partial degradation was found on a single seed level, the remaining RNA copies may have sufficed for transcriptions required for germination.

Mechanisms of cell death: cause and effect

The appearance of distinct DNA ladders and the loss of rRNA integrity that occurred during ageing suggest that mechanisms of cell death were already initiated and executed at 12% WC. Using seeds stored long-term at ambient conditions, Walters et al. (2006) recently also demonstrated that DNA could be extracted from non-viable seeds. Although 90-year-old seeds displayed only a faint band of high molecular weight DNA, indicative of diminished DNA quality, DNA extracted from 135-year old seeds was hardly detected. Importantly however, it was shown that such 'old' DNA still provided a suitable template for PCR amplification. While such partially degraded DNA is probably not functional in non-viable seeds, it is worthy of note that seeds aged for about 100 years at sub-optimal conditions contain enough DNA for sequencing and identification. However, Walters et al. (2006) pointed out that some of the seeds studied were heavily infested by fungi, and also detected ITS DNA regions of fungal origin. Therefore, in the present study, care was taken to age seeds at WCs below those that promote rapid fungal growth (see "Methods") and the seed material was carefully investigated for the presence of fungal contamination. In contrast to the report by Walters et al. (2006), artificially aged and dead seeds in this study did not show visual fungal contamination. Moreover, Fig. 2 shows that fungal DNA was not detected in Aged and dead seeds.

In the absence of fungi that could have potentially contributed to the degradation of nucleic acids, the mechanisms of cell death during seed ageing are likely associated with the programmed and non-programmed events discussed above. The question remains whether the observed degradation of nucleic acids is a cause or an effect of ageing. However, we suggest that there is no simple relationship regarding the dependency of cause and effect in processes that involve autocatalytic cascades (Fig. 4). Such cascades can produce second toxic messengers, inducing other, or the

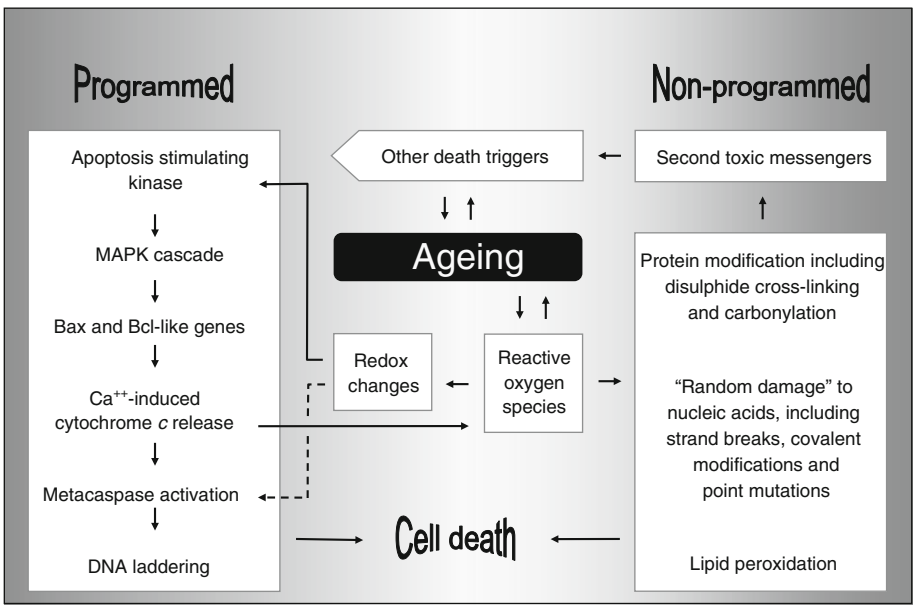

Fig. 4 Simplified scheme of mechanisms that contribute to cell death during seed ageing. In this scheme, the process of ageing is envisaged as an elicitor of a chain of interlinked proximate causes and effects, with cell death being the ultimate effect. For example, reactive oxygen species and other 'death triggers' can be formed as a result of ageing, but they will also be the cause for more ageing. Importantly, it seems unlikely that seed death results from only programmed or only non-programmed cell death as links between the two processes exists, here exemplified for second toxic messengers (such as the lipid peroxidation by-product 4-hydroxy-nonenal) that may be produced through non-programmed events, but can trigger programmed cell death. The non-exclusive scheme for programmed cell death in relation to intracellular redox changes on the left is a simplification of the model published by Kranner et al. (2006)

same cascades again. For example, lipid peroxidation by-products such as 4-hydroxy-nonenal that result from oxidative stress could be viewed as products of non-programmed events, but they can become second toxic messengers that induce PCD. Hence, compounds formed as a consequence of an initial trigger (the cause) can become a cause for the subsequent reaction, resulting in a chain of causes and effects, a hallmark of oxidative stress pathways. Hence, the ageing treatment could be viewed as the initial cause for cascades of biochemical reactions that result in loss of nucleic acid integrity as the final effect. However, the loss of nucleic acid integrity together with other PCD phenomena may also be interpreted as the cause for further ageing and eventually death. In addition, deteriorative processes will also proceed *post mortem*, such as observed in archaeological material (Evershed et al. 1997; VanBergen et al. 1997). Again, in such materials, deteriorative processes, such as Maillard reactions, could have contributed to cell death (as a 'cause'), but could also have proceeded in decaying material (as an 'effect'). In other words, we envisage the observed degradation of nucleic acids as a central part of the ageing process that most probably contains elements of both, cause and effect. In conclusion, we suggest that artificial ageing induces a complex process of interlinked programmed and non-programmed events that lead to cell death.

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