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Review article

Towards a better monitoring of seed ageing under *ex situ* seed conservation

Yong-Bi Fu*, Zaheer Ahmed and Axel Diederichsen

Plant Genetic Resources of Canada, Saskatoon Research Centre, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, Canada S7N 0X2

***Corresponding author:** Plant Genetic Resources of Canada, Saskatoon Research Centre, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK, Canada S7N 0X2. Tel: +1 306 385 9298. Email: yong-bi.fu@agr.gc.ca

Long-term conservation of 7.4 million *ex situ* seed accessions held in agricultural genebanks and botanic gardens worldwide is a challenging mission for human food security and ecosystem services. Recent advances in seed biology and genomics may have opened new opportunities for effective management of seed germplasm under long-term storage. Here, we review the current development of tools for assessing seed ageing and research advances in seed biology and genomics, with a focus on exploring their potential as better tools for monitoring of seed ageing. Seed ageing is found to be associated with the changes reflected in reactive oxygen species and mitochondria-triggered programmed cell deaths, expression of antioxidative genes and DNA and protein repair genes, chromosome telomere lengths, epigenetic regulation of related genes (microRNA and methylation) and altered organelle and nuclear genomes. Among these changes, the signals from mitochondrial and nuclear genomes may show the most promise for use in the development of tools to predict seed ageing. Non-destructive and non-invasive analyses of stored seeds through calorimetry or imaging techniques are also promising. It is clear that research into developing advanced tools for monitoring seed ageing to supplement traditional germination tests will be fruitful for effective conservation of *ex situ* seed germplasm.

Key words: *Ex situ* conservation, seed ageing, seed storage, viability biomarkers, viability prediction

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Introduction

The last 100 years have seen increased concerns about the erosion of genetic diversity in agricultural crops and the loss of biodiversity in natural ecosystems (Baur, 1914; Harlan and Martini, 1936; National Research Council, 1972; Corvalan *et al.*, 2005), and large global efforts have been made to conserve plant genetic resources (Frankel and Bennett, 1970). Currently, there are more than 7.4 million accessions of seed germplasm conserved in 1750 genebanks around the world, and more than 130 genebanks have 10 000 or more accessions (FAO, 2010). More than 30 000 wild plant species are conserved in stored seeds in the Royal Botanic Gardens, Kew, and the Chinese Academy of Sciences' Kunming Institute of Botany (Li and Pritchard, 2009). *Ex situ* seed storage is among the most effective ways to conserve desiccation-tolerant seed germplasm (Smith *et al.*, 2003). However, seeds in long-term storage will eventually lose their viability; therefore assessment of seed deterioration over time is required (Walters *et al.*, 2005; Hay and Probert, 2013; van Treuren *et al.*, 2013). Given such a huge volume of *ex situ* genetic resources, the long-term conservation is a challenging mission, and yet critical for human food security and ecosystem services.

The maintenance of *ex situ* seed viability over long periods of time in genebanks is a key element in conservation of plant genetic resources. Standards aimed at maintaining seed viability have been developed and applied in genebank management

procedures, including drying and storage under low seed moisture content and temperature (FAO, 2014). However, seed longevity varies among species and genotypes, and stored seeds will lose their viability over time to a level at which seed regeneration is required (Walters *et al*., 2005; Nagel *et al*., 2009; Probert *et al*., 2009; van Treuren *et al*., 2013). Regeneration is a costly genebank operation and may also negatively affect the genetic integrity of an accession through exposure to the influence of genetic drift, selection, contamination and human error. Therefore, it is important to maximize seed longevity and keep operational costs and logistics manageable through monitoring of seed deterioration, an essential task for managing stored germplasm (Engels and Visser, 2003).

Seed ageing or seed deterioration is commonly described as the loss of seed quality or viability over time (Priestley, 1986; Coolbear, 1995). Currently, a germination test is the standard method used to assess viability of *ex situ* conserved seeds (Smith *et al*., 2003; FAO, 2014). However, it destroys germplasm; can be a time-consuming and labour-intensive operation, given the huge amount of conserved germplasm; and neither assesses underlying mechanisms of seed deterioration nor provides any early projection of seed longevity for regeneration timing. Therefore, new non-destructive, low-cost, quick, sensitive and equally reliable methods are being sought for seed ageing assessments (Kranner *et al*., 2010b; Colville *et al*., 2012; Donà *et al*., 2013). As a result, many new tools for assessing seed ageing have been developed (Corbineau, 2012) and, following recent advances in seed biology and genomics, more sensitive tools, such as genomic or biochemical markers, are anticipated. Much research has been directed toward understanding the fundamentals of seed ageing and the roles of programmed cell death (PCD), mitochondria and epigenetics in seed deterioration. These advances have given us a clearer sense of the complex process of seed deterioration (Priestley, 1986; Smith and Berjak, 1995; Walters, 1998; McDonald, 1999; Rajjou *et al*., 2012; Ventura *et al*., 2012). Application of next generation sequencing technologies (Metzker, 2010) may allow for better detection of genomic changes associated with seed ageing (Bräutigam and Gowik, 2010) and may have provided a new opportunity for effective management of *ex situ* seed germplasm (Kocsy, 2015).

Here, we present a literature review with the following aims: (i) to summarize existing tools for assessing seed deterioration; (ii) to explore sensitive signals of seed ageing from recent research in seed biology and genomics; and (iii) to discuss the perspectives for the development of new sensitive tools for predicting seed ageing under *ex situ* storage. It is our hope that this review will advance our understanding of seed ageing and help to stimulate research efforts towards better monitoring of seed ageing under *ex situ* seed conservation.

Tools for assessing seed deterioration

Seed deterioration in genebanks is normally predicted through assessments of seed viability, germination, vigour and integrity. There are many different methods available to assess seed ageing (ISTA, 2005). The simplest testing method is direct visual inspection of seeds, but such inspection is unreliable. The most commonly used alternative is the standard germination test. There are also other, more complicated, biochemical testing procedures and non-destructive or non-invasive methods available. Recent years have seen the development of many new tools for assessing seed deterioration. Here, we highlight some of the recent developments.

Traditional tests

A germination test is the recommended method for testing seed deterioration in a genebank, because it is an accurate and reliable method. An accelerated ageing test (Delouche and Baskin, 1973) and electrical conductivity test (Thomas, 1960) are commonly used to assess seed vigour and facilitate seed ageing research. Available biochemical tests include the tetrazolium, vital colouring, enzyme activity, free fatty acid, hydrogen peroxide, indoxyl acetate, fast green, ferric chloride, sodium hypochlorite, excised embryo and X-ray tests. These methods are well described and their use is discussed in detail by Copeland and McDonald (1995). The use of these biochemical methods for seed quality testing is restricted specifically for seed viability, germinability, vigour or integrity under ISTA rules (http://www.ingentaconnect.com/content/ista/rules). Biochemical tests are useful when germination tests yield variable results and have the advantage of being quick. However, some tests have the weakness of low accuracy and also require special skills to conduct and specialized knowledge to interpret test outcomes. These features help to explain, at least partly, why these tests are not usually recommended for general use in assessing seed deterioration in genebanks (Ellis *et al*., 1985; FAO, 2014).

Non-destructive and non-invasive methods

Non-destructive and/or non-invasive evaluations of seed deterioration are desirable (Agelet *et al*., 2012; Ishimwe *et al*., 2014), particularly for seed collections of low amount or low viability, because assayed seeds may not be affected or destroyed and could be used for regeneration or other purposes. Efforts have been made to develop reliable non-destructive and/or non-invasive tests (e.g. Prat, 1952; Mourik and Bakri, 1991; Crane and Walters *et al*., 2009; Kim *et al*., 2014). Here, we highlight several developments in the last decade. Kranner *et al.* (2010b) presented a method using infrared thermography to diagnose the developmental stage of a germinating pea (*Pisum sativum*) seed, non-invasively and in real time. Likewise, Kim *et al.* (2014) described an infrared thermal signal measurement system and photo-thermal signal and image reconstruction techniques for viability estimation of pepper (*Capsicum annuum*) seeds. Xin *et al.* (2013) demonstrated a real-time, non-invasive, micro-optrode technique for detecting seed viability in several crops by using oxygen influx. Isothermal microcalorimetry was explored for use as a tool to predict seed longevity in *Ranunculus sceleratus* (Hay *et al*., 2006), and differential scanning calorimetry was applied

to measure seed deterioration in lettuce (*Lactuca sativa*; Crane and Walters, 2009). Measurement of volatile substance production from stored seeds has also been suggested as a valuable non-invasive alternative to predict the duration of the early, asymptomatic stage of seed deterioration (Hailstones and Smith, 1989; Mira *et al*., 2010; Colville *et al*., 2012). Min and Kang (2011) developed a simple, quick and non-destructive test method based on resazurin reagent, which was made by mixture of resazurin and yeast, for determining Brassicaceae seed viability. However, all of these methods have not been fully tested on stored seeds of different species and, consequently, are not yet widely applied in genebank operations.

Marker-based methods

Considerable research has also been conducted to develop biomarkers for evaluating seed quality (Corbineau, 2012). Table 1 summarizes the biomarkers available for assessment of seed ageing, which were developed largely from biochemical and physiological research. These biomarkers are largely associated with factors involved in processes such as electrolyte leakage and ethylene production during imbibition, the cell cycle (DNA replication, β-tubulin), soluble sugar metabolism (in particular, changes in the raffinose family of oligosaccharides), the efficiency of reactive oxygen species (ROS) scavenging through antioxidant defence systems (e.g. catalase activity) as well as various other proteins (11S globulin β-subunit, late embryogenesis abundant protein, heat-shock protein). One exciting example is the prediction tool used for detecting specific co-ordination during seed ageing mediated by common *cis*-elements and *trans*-factors, otherwise not detectable by conventional transcript analysis (Bassel *et al*., 2011). Recently, the half-cell reduction potentials of low molecular weight thiols, cysteine, cysteinyl-glycine and γ-glutamyl-cysteine, have been explored as biomarkers for seed ageing (Kranner *et al*., 2006; Birtic *et al*., 2011). These developments clearly illustrate the varied and exciting achievements in the search for informative biomarkers of seed viability, but no reports have been found so far on the applications of these biomarkers in genebank operations.

Some genetic markers have also been developed (El-Maarouf-Bouteau *et al*., 2011; Hu *et al*., 2012) for detection of DNA damage and mutational events, including point mutations, structural rearrangements, small insertions or deletions of DNA and other genetic changes (Shatters *et al*., 1995; Liu *et al*., 2005; Atienzar and Jha, 2006; Vijay *et al*., 2009). The last decade has also seen some research effort directed towards the inference of the genetic basis of seed deterioration through investigation of quantitative trait loci associated with seed longevity (e.g. Nagel *et al*., 2009; Arif *et al*., 2012; Han *et al*., 2014). These efforts have helped to identify favourable longevity alleles for better prediction of seed longevity in plant germplasm collections. For example, four genomic regions identified for wheat (*Triticum aestivum*) seed longevity are known to contain genes associated with spike traits or biotic and abiotic stress responses (Arif *et al*., 2012). These findings are encouraging, because an accurate prediction of seed ageing before storage would allow for better viability monitoring of stored seeds (Nagel *et al*., 2015).

Seed ageing signals

The causes of seed ageing and death are not fully understood, because seed ageing is a complex biological trait and involves a network of molecular, biochemical, physiological and metabolic processes. Large efforts have been made to understand these ageing processes from the aspects of seed development, vigour, viability, longevity and germination. In recent years, many reviews have been published attempting to explain various aspects around the progression of seed deterioration (e.g. Bove *et al*., 2001; Chaudhury *et al*., 2001; Weber *et al*., 2005; Le *et al*., 2007; Linkies *et al*., 2010; Nonogaki *et al*., 2010; Rajjou *et al*., 2012; Ventura *et al*., 2012; Diaz-Vivancos *et al*., 2013; Sreenivasulu and Wobus, 2013). Here, we focus only on those studies revealing detectable signals of potential use for seed ageing assessment. Also, we search only for those signals reflecting various stages of seed deterioration, rather than for the molecular or cellular changes associated with the regulation and development of a process.

Molecular signals

Research has shown that seed ageing is associated with chromosomal aberration, telomere length change, DNA damage, DNA methylation and abnormal gene expression. Chromosomal aberrations in aged seeds comprise fragmentation, fusion, bridges, ring formation of chromosomes and alterations in nuclear size (e.g. McDonald, 1999; Chwedorzewska *et al*., 2002a, b). Age-related DNA changes have been illustrated through the investigation of DNA profiles of differentially aged seeds of soybean (*Glycine max*) and safflower (*Carthamus tinctorius*) obtained using different DNA marker technologies (Vijay *et al*., 2009). These chromosomal changes affect the expression of genes essential for successful germination. Experiments with wheat and rye (*Secale cereale*) seeds have shown a negative correlation between telomere length and seed ageing (Bucholc and Buchowicz, 1992). Donà *et al*. (2013) also reported that dry and rehydrated seeds of *Silene vulgaris* and *Silene acaulis* exhibit significant differences in their average telomere length. The telomere length increased significantly upon rehydration, but decreased significantly when seeds of both species were subjected to artificial ageing. These findings point to the possibility of using telomere length as a reliable marker for seed ageing (Boubriak *et al*., 2007).

DNA damage in stored seeds can occur due to oxidative stress and needs to be repaired at the onset of imbibition for effective seed germination. Multiple DNA ligase genes and protein L-isoaspartyl methyltransferase (PIMT) are needed for DNA and protein repairs, respectively. In *Arabidopsis*, *atlig6* single and *atlig6 atlig4* double mutants exhibited significant hypersensitivity to controlled seed ageing and showed delayed germination and reduced viability when compared with the wild-type. These observations suggest that *atlig6* is a major determinant of *Arabidopsis* seed quality and longevity

Table 1: List of reported biomarkers associated with seed ageing of 17 plant species

Biomarker	Description	Signal[a]	Reference[b]
ATS	Aberrant tests shape	1; C	Clerkx *et al.*, (2004) [1]
DOG1	Delay of germination1	1; N	Bentsink *et al.* (2006) [1]
NIC2	Nicotinamidase enzyme	1, 2; N	Hunt *et al.* (2007) [1]
GAMT2	Gibberellic acid methyltransferase2	1, 2; N	Xing *et al.* (2007) [1]
PIMT1	L-Isoaspartyl methyltransferase	1, 2; C	Ogé *et al.* (2008) [1]
Atlig6	DNA ligase VI	1, 2; C	Waterworth *et al.* (2010) [1]
At3g08030	Cell-wall-associated protein	1; C	Garza-Caligaris *et al.* (2012) [1]
MT1	Metallothionein1	1; N	Revilla *et al.* (2009) [2]
eIFiso4E	Translation initiation factor	1; N	Dinkova *et al.* (2011) [2]
OGG1	8-Oxoguanine DNA glycosylase/lyase1	1, 2; N	Macovei *et al.* (2011) [5]
PIMT2	L-Isoaspartyl methyltransferase	1, 2; C	Verma *et al.* (2013) [6]
MT2	Type 2 metallothionein	1; C	Donà *et al.* (2013) [7,8]
Telomere length		1; C	Donà *et al.* (2013) [7,8]
HSFA9	Heat stress transcription factor	1; C	Prieto-Dapena *et al.* (2006) [10]
Genetic integrity	Chromosomal aberration, DNA and RNA oxidation, DNA laddering etc.	1; N, C	Cheah and Osborne (1978); Osborne *et al.* (1981); Vazquez-Ramos *et al.* (1988); Bednarek *et al.* (1998); Stein and Hansen (1999); Slupphaug *et al.* (2003); Corbineau (2012)
FPG	Formamidopyrimidine-DNA glycosylase	1,2; N	Macovei *et al.* (2011) [5]
TRX	Thioredoxin	1,2; N, C	Buchanan and Balmer (2005) [1]
DNA methylation		1,2,3; C	Michalak *et al.* (2013) [17]
MS	Methionine synthase	2; N	Gallardo *et al.* (2002a) [1]
MAT	*S*-Adenosyl-methionine synthetase	2; N	Gallardo *et al.* (2002a) [1]
AdoHcyase	*S*-Adenosyl-L-homocysteine hydrolase	2; N	Rocha *et al.* (2005) [1]
PLDα1	Phospholipase D-alpha1	2; C	Devaiah *et al.* (2007) [1]
PRT6	Proteolysis6	2; N	Holman *et al.* (2009) [1]
PP2C5	Protein phosphatase 2C5	2; N	Brock *et al.* (2010) [1]
LOX	Lipoxygenases	2; C	Li *et al.* (2007) [2]
ALDH	Aldehyde dehydrogenase	2; C	Shin *et al.* (2009) [3]
BiP	Immunoglobulin binding protein	2; C	Gurusinghe *et al.* (2002) [9]
Antioxidant activity		2,3; C	Sung and Jeng (1994) [13]
ABA/GA balance		2,3,4; N	Yamaguchi *et al.* (1998) [1]; Kushiro *et al.* (2004) [1]
Membrane integrity	Cell organelles, particularly mitochondrial membranes	2,3,5; N, C	McDonald (1999)
Methionine	An α-amino acid	2,4; N	Gallardo *et al.* (2002a) [1]
KAPA	7-Keto-8-aminopelargonic acid synthase	2,4; N	Hwang *et al.* (2010) [1]
SBP65	Seed biotinylated protein	2,4; N	Duval *et al.* (1994) [4]
Reactive oxygen species content		2,4; N	Puntarulo *et al.* (1991) [14]; Schopfer *et al.* (2001) [15]
Cysteine	α-Amino acid	2,4; C	Birtic *et al.* (2011) [16]

(*Continued*)

Table 1: Continued

Biomarker	Description	Signal[a]	Reference[b]
Ethylene	A natural plant hormone	2,4; N, C	Kepczynski and Kepczynska (1997)
Glutathione	Antioxidant	2,4,5; C	Birtic *et al.* (2011) [16]
CAT	Catalase	2,5; N	Revilla *et al.* (2009) [2]
SOD	Superoxidate dismutase	2,5; N	Revilla *et al.* (2009) [2]
APX	Ascorbate peroxidase	2,5; C	Yao *et al.* (2012) [4]
GR	Glutathione reductase	2,5; C	Yao *et al.* (2012) [4]
Lipid peroxidation		3; C	Sung and Jeng (1994) [13]
Testa		3,6; N	Debeaujon *et al.* (2000) [1]
Flavonoid	Polyphenolic compounds	4; N	Debeaujon *et al.* (2000) [1]
Tocopherol	Related to vitamin E	4; C	Sattler *et al.* (2004) [1]
Oligosaccharide/ total sugar ratio		4; C	Bernal-Lugo and Leopold (1992) [2]
Raffinose	Trisaccharide composed of galactose, glucose and fructose	4; C	Bernal-Lugo and Leopold (1992) [2]
Malondialdehyde	Reactive species	4; C	Shin *et al.* (2009) [3]
Thiols	Sulfur analogue of alcohols	4; C	Birtic *et al.* (2011) [16]
Cysteinyl-glycine	Intermediate metabolite in the glutathione metabolism	4; C	Birtic *et al.* (2011) [16]
γ-Glutamyl-cysteine	A precursor of glutathione	4; C	Birtic *et al.* (2011) [16]

[a]Ageing signals were roughly classified into six categories corresponding to those in the text as follows: 1, molecular signal; 2, biochemical signal; 3, physiological signal; 4, metabolic signal; 5, mitochondrial signal; and 6, morphological signal. The condition or treatment under which the signal was identified is shown with N for natural ageing and/or C for controlled ageing.
[b]The involved species presented in square brackets are as follows: [1] *Arabidopsis thaliana*; [2] *Zea mays*; [3] *Oryza sativa*; [4] *Pisum sativum*; [5] *Medicago truncatula*; [6] *Cicer arietinum*; [7] *Silene vulgaris*; [8] *Silene acaulis*; [9] *Lycopersicon esculentum*; [10] *Nicotiana tabacum*; [11] *Ceiba aesculifolia*; [12] *Wigandia urens*; [13] *Arachis hypogaea*; [14] *Glycine max*; [15] *Raphanus sativus*; [16] *Lathyrus pratensis*; and [17] *Pyrus communis*.

(Waterworth *et al.*, 2010). Altered expression levels of PIMT1 found in isolated *Arabidopsis* lines suggest that PIMT1 is a major endogenous factor that improves seed longevity and vigour (Ogé *et al.*, 2008). It is likely that the PIMT repair pathway works in concert with other anti-ageing pathways to eliminate deleterious protein products actively.

Epigenetic regulation can affect gene expression in stored seeds by DNA methylation, histone modifications, histone variants and chromatin remodelling (Ahmad *et al.*, 2010). Recently, Michalak *et al.* (2013) investigated the relationship between DNA methylation and desiccation in pear (*Pyrus communis*) seeds and seedlings and found that 1 year of seed storage induced a significant increase in the level of DNA methylation. This finding suggests that seed ageing may be coupled with increased DNA methylation. Also, several studies have shown that microRNA is involved in the germination process of *Arabidopsis* seeds (Martin *et al.*, 2005, 2006; Liu *et al.*, 2007; Reyes and Chua, 2007; Kim *et al.*, 2010). Recently, Li *et al.* (2013) discovered a diverse set of maize microRNAs and their regulatory functions in dry and imbibed seeds. However, little is known about the role of non-coding RNA in seed deterioration.

Seed germination is controlled by co-ordinated activities of various biological pathways, which in turn are regulated by spontaneous and differential expression of several gene families. Therefore, a characterization of gene expression level, enzyme activity, difference in signal transduction response and regulatory mechanisms in stored seeds could yield useful ageing signals (Ventura *et al.*, 2012). For example, genes involved in the glyoxylate cycle, sulfur amino acid pathway, starch mobilization pathway, ROS scavenging pathway, DNA and enzyme repair, and abscisic acid (ABA) and gibberellic acid (GA) signalling, may display differential expressions in stored seeds. The characterization of three genes (*NnMT2a*, *NnMT2b* and *NnMT3*) that encode metallothioneins (MT) from sacred lotus (*Nelumbo nucifera*) revealed that they were overexpressed during germination and upregulated in response to high salinity and oxidative stresses (Zhou *et al.*, 2012). Also, transgenic *Arabidopsis* seeds overexpressing *NnMT2a* and *NnMT3* exhibited remarkably increased resistance to accelerated ageing treatment and abiotic stresses. Garza-Caligaris *et al.* (2012) reported that *At3g08030* mRNA detection could serve as a molecular marker of seed ageing in a variety of plant species. However, much less attention has been paid to studying the role of stored mRNAs or long-lived

mRNAs (Dure and Waters, 1965; Nakabayashi *et al.*, 2005; Kimura and Nambara, 2010) in seed deterioration.

Biochemical signals

Biochemical changes associated with seed ageing include impairment of protein synthesis, protein inactivation, changes in enzyme activities, protein hydrolysis and post-translational modifications (Rajjou *et al.*, 2012). Based on these signals, many biomarkers have been developed to assess seed development, vigour, viability and germination (see Table 1). However, few studies have been conducted to evaluate and compare the effectiveness of these biomarkers in detecting ageing signals in different species and, consequently, they are rarely applied to assess seed ageing.

In aged seeds, the inability to synthesize proteins is associated with the loss of RNA synthesis ability (Bray and Dasgupta, 1976). However, protein synthesis can also be hampered at the translation level due to reduced activity of ribosomes as a consequence of severe structural modifications. Such modifications have been found in non-viable seeds (Roberts and Osborne, 1973). Proteins may also be structurally modified by non-enzymatic glycation through Amadori and Maillard reactions (Wettlaufer and Leopold, 1991). The non-enzymatic reactions are considered to be the most probable cause of protein inactivation during seed storage, because dry seeds lack active enzymatic metabolism. Previous studies suggest that Amadori and Maillard products were found in soybean (*Glycine max*) seeds subjected to accelerated ageing and formed most rapidly in seeds at high humidity (Wettlaufer and Leopold, 1991). Protein inactivation in stored seeds may occur from the gain or loss of certain functional groups, by oxidation of sulfhydryl groups or by conversion of amino acids within the protein structure. Protein damage, such as spontaneous deamidation, isomerization and racemization of normal L-aspartyl and L-asparaginyl forms to abnormal L-isoaspartyl and L-isoasparaginyl forms, was observed during cellular ageing (Galletti *et al.*, 1995). The inactivation of proteins would depress the metabolic capacity and reduce the ability of biological systems to repair cellular damage occurring during storage. Free radicals can also cause changes in protein structure. Soluble proteins are more susceptible to free radicals than membrane proteins. Certain amino acids, such as cysteine, histidine, tryptophan, methionine and phenylalanine, listed in typical order of sensitivity, are more susceptible to oxidative damage (Larson, 1997).

The activities of proteins in stored seeds may be altered, which in turn may affect metabolic processes. Alterations in protein activity could arise from conformational changes, including partial folding or unfolding, dissociation to monomers or subunits, and condensation to polymers. Various alterations in protein structure and function affect the ability of seeds to germinate, because various hydrolytic enzymes, including lipase, phospholipase, protease, DNase, phosphatase and amylase, are required for successful germination (Basavarajappa *et al.*, 1991). Likewise, the deleterious effects of ROS, which boost seed ageing and reduce seed viability, are also neutralized by the enzymatic antioxidative system, which consists of superoxide dismutase, catalase, ascorbate and glutathione reductase (Bailly, 2004). Various processes of post-translational modifications have been reported, including redox signalling, phosphorylation/dephosphorylation and nitrosylation. The roles of protein biotinylation, glycosylation, ubiquitination, farnesylation and acetylation in germination have also been demonstrated experimentally (Arc *et al.*, 2011). All of these post-translational modifications play crucial roles during germination by directly affecting the activities of various proteins and also by controlling the cascade of signal transduction between different components of a pathway. For example, a set of protein kinases and protein phosphatases has been shown to be involved in the control of germination through the modulation of ABA signalling by a regulatory mechanism of phosphorylation and dephosphorylation (Brock *et al.*, 2010; Hubbard *et al.*, 2010).

Accelerated ageing in pea seeds reduced seed viability, and this viability reduction was correlated with a substantial decrease in the transcriptional activation of prominent antioxidative genes (Yao *et al.*, 2012). Oxidative stress, due to an increase in lipid peroxidation and a decrease in the activities of antioxidative enzymes, is considered to play a critical role in seed ageing in various plant species (Bailly, 2004). In sunflower (*Helianthus annuus*) seeds, for example, the accumulation of hydrogen peroxide (H_2O_2), lipid peroxidation and a decreased activity of antioxidant enzymes were considered to be associated with loss of viability during accelerated ageing (Kibinza *et al.*, 2006). Catusse *et al.* (2011) demonstrated the utility of proteomics in developing biomarkers of seed vigour in sugarbeet (*Beta vulgaris*). However, many biomarkers based on biochemical signals were developed in controlled hot and humid ageing conditions (Table 1) and are not widely tested for their effectiveness in long-term cool storage conditions.

Physiological signals

Extensive research has been carried out to determine the association of seed ageing with many physiological processes (e.g. the reviews of McDonald, 1999; Ventura *et al.*, 2012; Diaz-Vivancos *et al.*, 2013). Physiological changes include lipid peroxidation, increase in ROS, imbalance in growth-regulating enzymes, impairment of metabolic transition, imbalance in growth-regulating hormones, loss of cytoplasmic glassy state, disruption of cellular membranes, and PCD. However, these physiological processes are significantly influenced by the seed moisture content and storage conditions. For example, lipid peroxidation, either autoxidation or enzymatic oxidation (lipoxygenases), is strongly influenced by moisture content. At lower seed moisture content (<6%), autoxidation is the primary cause of seed deterioration, while enzymatic oxidation increases when seed moisture content exceeds 14% for some species (Priestley *et al.*, 1985). During oxidation, free radicals are produced, and these radicals react with various cellular

components and cause damage to cellular organization (Diaz-Vivancos *et al*., 2013). Free radicals disrupt the cellular membrane, which causes movement of organic and inorganic solvents across the membrane, resulting in an imbalance, which leads to seed deterioration. Free radicals also disrupt genetic and enzymatic integrity, thus limiting the efficiencies of transcriptional and translational machineries. These observations suggest that various physiological processes are interdependent and, once the malfunctioning of one process starts, it triggers other events.

Early studies have established that the cytoplasm of a dry seed enters a vitreous or glassy state at a transitional moisture content that depends on temperature (Williams and Leopold, 1989; Maki *et al*., 1994). In a glassy state, the cytoplasm is so viscous that diffusional movement and many deterioration reactions are arrested (Williams and Leopold, 1989; Leopold *et al*., 1992). The acquisition of a glassy state depends upon moisture content, temperature and the amount of various sugars, such as sucrose, raffinose, stachyose and verbascose. Any physiological change that affects seed glassy state will lead to seed deterioration (Osborne, 2000). Recently, Walters *et al*. (2010) applied mechanical analysis techniques to the study of seed structure and transitions associated with changes in temperature and moisture and argued that relationships between seed structural properties and longevity would provide the necessary tool to predict seed ageing.

Programmed cell death is a fundamental cellular process in plants and is involved in defence, development and response to stress (e.g. Reape and McCabe, 2008). The early studies of the role of PCD in seed viability were largely based on the observation of cereal seed germination coupled with aleurone autolysis. Plant hormones, particularly GA and ABA, were found to regulate this process tightly, and ABA is thought to slow down PCD (Fath *et al*., 2000). Several other reports also described the involvement of PCD in seed ageing (El-Maarouf-Bouteau *et al*., 2011; Hu *et al*., 2012). It is also believed that ROS trigger the primary events of PCD (Kranner *et al*., 2010a). Whether ROS-triggered PCD participates in the loss of seed viability during seed storage is largely unknown.

Metabolic signals

Many metabolic studies have been pursued to determine the role of metabolism in seed development and germination (Rajjou *et al*., 2012), but rarely in seed ageing *per se* (Bernal-Lugo and Leopold, 1992). Some research has been carried out to analyse metabolic changes in seeds through stress imaging techniques (e.g. Qiao *et al*., 2005) and to develop biomarkers based on metabolic changes in seeds (Table 1). Nevertheless, we still are far from understanding the metabolic changes during seed ageing (Shin *et al*., 2009; Wu *et al*., 2011). To facilitate the search for useful metabolic shifts in seeds stored over time, we highlight some advances in metabolic research below.

The transition from reserve accumulation to seed desiccation in *Arabidopsis* seeds is associated with a major metabolic shift, resulting in the accumulation of various sugars, nitrogen-rich amino acids, organic acids and other metabolites (Fait *et al*., 2006). However, seed priming is associated with decreased contents of several of these metabolic intermediates, reinforcing the idea that metabolic reorganization is required for seed germination. Likewise, the levels of other metabolites increase significantly during seed priming and are further elevated during germination (Rajjou *et al*., 2012). The close resemblance of gene expression patterns and metabolic signatures between the seed desiccation process at the time of maturity and seed germination implies that the preparation of seeds for germination begins during desiccation (Fait *et al*., 2006; Angelovici *et al*., 2010).

Among the essential amino acids synthesized by plants, methionine (Met) is a fundamental metabolite, which functions not only as a building block for protein synthesis but also as a precursor of polyamines, ethylene, biotin and other metabolites (Ravanel *et al*., 2008; Takahashi *et al*., 2011). During *Arabidopsis* seed germination, various enzymes involved in Met biosynthesis showed differential expression. Adenosylmethionine (AdoMet) synthetase is highly expressed at the stage of radicle protrusion to synthesize AdoMet, an intermediate during Met synthesis (Gallardo *et al*., 2001, 2002a, b; Bassel *et al*., 2008). Similar results were also reported in different plant species, such as tobacco (*Nicotiana tabacum*; Fulneček *et al*., 2011). Adenosylmethionine regulates a myriad of transmethylation reactions in plant cells, each of which is catalysed by a specific AdoMet-dependent methyltransferase, such as the repair methyltransferase, PIMT, mentioned earlier. Other AdoMet-dependent methyltransferases influence hormone signalling and homeostasis in plant tissues (Sawicki and Willows, 2010). Likewise, the requirement of biotin for seed germination was also reported in *Arabidopsis* (Hwang *et al*., 2010). Along with Met, other amino acids, such as cysteine (Cys) and lysine, and other compounds, such as biotin, ethylene and folate, can also play a major role in seed germination. For example, Cys is the precursor of the major antioxidant molecule glutathione, which is involved in several other processes required for successful seed germination (Bonsager *et al*., 2010). In dry pea seeds, the folate pool is present in very low concentration and increases considerably during germination (Jabrin *et al*., 2003). However, little is known about whether these metabolic processes and products during germination are associated with any ageing processes and/or conditions of stored seeds before germination.

It is possible to characterize major metabolic shifts in seeds stored over time for the development of useful biomarkers (Bernal-Lugo and Leopold, 1992; Shin *et al*., 2009; Wu *et al*., 2011). A good example is the exploratory research on the half-cell reduction potentials of low molecular weight thiols, cysteine, cysteinyl-glycine and γ-glutamyl-cysteine as biomarkers for seed ageing (Kranner *et al*., 2006; Birtic *et al*., 2011). More research is needed to search for informative biomarkers from metabolic profiling in stored seeds (Wu *et al*., 2011).

Mitochondrial signals

In seeds, the mitochondrion is the major organelle for energy supply, and its function is tightly coupled to many other cellular processes associated with seed germination, such as cell signalling, cell differentiation, cell death and cell proliferation (Bewley, 1997). Mitochondria are one of the targets for various forms of stress damage, probably due to the large turnover of ROS (Møller, 2001; Amirsadeghi *et al*., 2007; Macherel *et al*., 2007; Møller *et al*., 2007; Pastore *et al*., 2007). Reactive oxygen species have many deleterious effects on mitochondrial membranes, leading to release of cytochrome *c* into the cytosol to activate apoptotic cell death. Likewise, the mitochondrial DNA is more susceptible to ROS damage, because it lacks any protective membrane, and no histone proteins are associated with it. Damage to mitochondrial DNA can lead to dysfunction of mitochondria, which is considered to be a major component of seed ageing during prolonged storage. In animals, mitochondrial alterations are considered to be involved in, and possibly responsible for, regular or programmed cell death (Bras *et al*., 2005). They are often considered to be a central mechanism driving mammalian ageing (Kujoth *et al*., 2005).

Some research has revealed that energy metabolism and membrane integrity in mitochondria are closely associated with seed ageing (Benamar *et al*., 2003; Lo *et al*., 2011; Wang *et al*., 2012). One study (Lo *et al*., 2011) further reasoned that mitochondrial actin may be involved in mitochondrial DNA segregation and mitochondrial division. Law *et al.* (2012) suggested that the mitochondrial transition from a dormant to an active metabolic state was punctuated by an early molecular switch, characterized by a transient burst in the expression of genes encoding mitochondrial proteins. In artificially aged sunflower seeds with varying moisture contents, El-Maarouf-Bouteau *et al.* (2011) demonstrated that the effect of ageing on energy metabolism was related to moisture content, and mitochondrial dysfunction in aged seeds may be associated with the high moisture content. Recently, Wang *et al.* (2012) developed a method to monitor the structural alteration in mitochondrial membranes due to seed ageing, based on the early observation that mitochondrial alteration is associated with the damage or recovery of mitochondrial outer and inner membranes. However, insufficient studies have been carried out to characterize mitochondrial dysfunction under long-term storage and their associations with seed storage factors.

Integrating ageing signals for prediction of viability

Reviewing detectable ageing signals carries the hope that these signals can be used as seed viability biomarkers for prediction of viability loss over storage time, as illustrated in Fig. 1. It is clear that these ageing signals, even classified non-exclusively into five groups, are complex and interconnected. Some of these signals have been used to develop biomarkers (see Table 1), but no reports have been found so far on the use of these biomarkers for prediction of seed viability. This may reflect the fact that little effort has been made into the development and utilization of such viability prediction tools, and challenges exist in utilization of these ageing signals for prediction of viability.

The ideal monitoring tool for a genebank should provide an overall assessment of seed ageing status for a seed lot, such as generated by a germination test, allowing decisions to be made regarding the necessity of seed regeneration (Roberts, 1973; Ellis *et al*., 1985). An informative viability biomarker should allow for identification and quantification of seeds with viability loss through a bioassay of a seed lot. Thus, more effort is needed to develop such biomarkers from various ageing signals, with empirical validation of their ability to predict viability with traditional germination tests. However, given the complexity of these ageing signals, it is difficult to develop individual biomarkers capable of identifying clear-cut fingerprints of different ageing stages. Some signals may vary for different species even in the same storage conditions (Priestley, 1986; Probert *et al*., 2009), and related biomarkers may not always be effective. Some biomarkers were developed in controlled, not storage, ageing conditions, and their effectiveness in use for prediction of viability may vary with respect to ageing conditions. Different biomarkers developed from different signals may have variable weights in viability prediction. Thus, an integrative approach to using global ageing signals through multivariate modelling for viability prediction should be explored.

Our motivation to argue for a biomarker-based prediction of seed viability loss (Fig. 1) is based on the reasoning that biomarkers, such as genome alternations or telomere lengths, if available, should carry more accurate ageing information and are experimentally more reproducible, particularly for those genetically diverse seed collections of complex ageing kinetics (Walters, 1998), than traditional germination tests or other methods. Thus, the use of viability biomarkers can, at least theoretically, contribute to more accurate estimation of seed viability distribution spread (or standard deviation; σ_p) for given storage conditions (Fig. 1) and, consequently, more accurate prediction of seed viability loss (Ellis *et al*., 1985; Walters *et al*., 2005). However, whether such a biomarker-based approach is cost effective and if it can address other challenges currently faced with seed viability predictions (Pritchard and Dickie, 2003; Walters *et al*., 2005) remains to be seen. Among those challenges is the early biomarker-based projection of seed longevity for a seed collection in given storage conditions for the beneficial timing of seed regeneration.

Perspectives for viability prediction tools

Our review, although not exhaustive, shows that many tools are available for assessing seed deterioration. However, no comprehensive research has been done to evaluate, compare and standardize these tools and to make recommendations for use in *ex situ* genebanks. Clearly, further research is needed to assess the effectiveness and applicability of existing

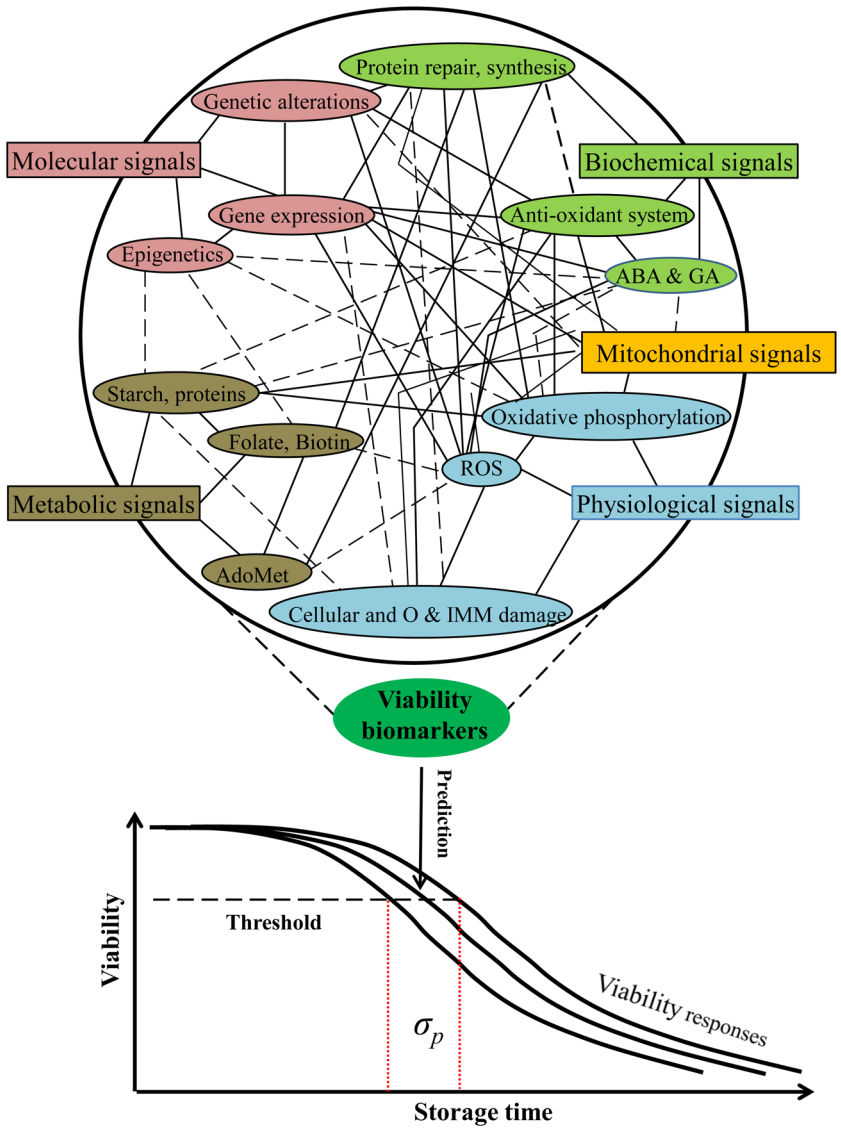

Figure 1: Illustration of complex and interconnected ageing signals and their potential use as viability biomarkers for early predictions of seed viability loss over storage time. A continuous or dashed line represents known or assumed relationships among ageing signals, respectively. Abbreviations: ABA, abscisic acid; AdoMet, *S*-adenosylmethionine; GA, gibberellic acid; O & IMM, outer and inner mitochondrial membrane; ROS, reactive oxygen species; and σ_p, the spread (or standard deviation) of seed viability distribution in the improved equation of seed viability prediction (Ellis and Roberts, 1980).

tools in genebank operations. More importantly, more efforts should be directed towards the development of advanced viability prediction tools for assessing seed ageing under *ex situ* storage.

The exciting fact obtained from this review is that many opportunities exist for the exploration and development of more accurate tools for monitoring seed ageing under *ex situ* seed conservation. Several interesting ageing signals have emerged from this literature search. They are the changes reflected in ROS and mitochondrial triggered PCDs, expression of antioxidative genes and DNA and protein repair genes, seed telomere lengths, epigenetic regulation of related genes (microRNA and methylation), and altered organelle and nuclear genomes. Although challenges exist in the use of ageing signals for viability prediction, as discussed above, it is our hope that these ageing signals should be better explored and used as biomarkers to play a role in seed viability monitoring for *ex situ* seed conservation.

Here, we promote two lines of research with great potential to take advantage of recent developments in next generation

sequencing, calorimetry and imaging technologies. Recent advances in next generation sequencing technologies have made the acquisition of global ageing signals through genomic, transcriptomic, proteomic or metabolic analyses feasible and practical (Le *et al*., 2007; Rajjou *et al*., 2008; Chen *et al*., 2013), and new sensitive and effective biomarkers can be developed to identify ageing signals and assess ageing status (Koboldt *et al*., 2013; Nagel *et al*., 2015). Much could be learnt from next generation sequencing as applied to the development of biomarkers for human diseases (e.g. Schwarzenbach *et al*., 2011; Krock *et al*., 2014; Zhou *et al*., 2014). Among next generation sequencing applications, searching for ageing signals from related DNA alteration or mitochondrial dysfunction may be fruitful and informative (Chen *et al*., 2013), because these processes or molecules may be critical to seed ageing and/or represent the fingerprints of seed deterioration. Specific effort may be made on the genomic analysis of mitochondrial dysfunction, transcriptome alteration, microRNA expression alteration and abnormal methylations in response to different seed storage conditions present in genebanks. More focus should be placed on how variable ageing signals are integrated into a predictive tool for an overall ageing assessment of a seed lot (Ellis and Roberts, 1980; Walters *et al*., 2005).

The non-destructive or non-invasive analyses of stored seeds through microcalorimetry and/or stress imaging techniques may also hold the potential to provide new methods for assessment of seed ageing. Studies using microcalorimetry (Criddle *et al*., 1991; Wadso, 2000) have shown that metabolic heat flows can be used to assess gross metabolism associated with germination processes and have demonstrated the potential of extracting ageing signals (Prat, 1952; Mourik and Bakri, 1991; Hageseth and Cody, 1993; Sigstad and Prado, 1999; Edelstein *et al*., 2001; Qiao *et al*., 2005; Hay *et al*., 2006). Some studies have demonstrated good potential, such as the applications of isothermal microcalorimetry to predict seed longevity in *R. sceleratus* (Hay *et al*., 2006), the image reconstruction technique to estimate pepper seed viability (Kim *et al*., 2014) and mechanical analysis techniques to quantify differences among seed structures associated with ageing (Walters *et al*., 2010). More research is needed to assess the accuracy of prediction of viability for seeds of different species under long-term storage (Walters *et al*., 2005; Hay and Probert, 2013). The prediction tools developed should be tested for their effectiveness and applicability in genebank operations (FAO, 2014).

Nonetheless, the traditional germination test will continue to play a central role in seed viability monitoring. With advances in new technologies for detecting ageing signals, however, it is possible to explore and develop innovative viability prediction tools that are more accurate, sensitive, quick and cost effective (Kocsy, 2015). By combining all of these approaches, seed viability under storage can be monitored better for long-term management and conservation of *ex situ* seed germplasm.

Concluding remarks

Seed ageing is a complex biological trait and difficult to monitor. This review summarizes the recent development of tools for assessing seed ageing and reveals several biological signals that could be used to assess seed deterioration. Two lines of research are promoted that have great potential to take advantage of recent developments in next generation sequencing, calorimetry and imaging technologies for the development of biomarker-based seed viability prediction tools. These research efforts will provide useful methods to supplement traditional germination tests, enhancing the monitoring of seed deterioration for long-term conservation of *ex situ* seed germplasm.

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