Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Deterioration of western redcedar (*Thuja plicata* Donn ex D. Don) seeds: protein oxidation and *in vivo* NMR monitoring of storage oils

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Abstract

Deterioration of conifer seeds during prolonged storage has a negative impact on reforestation and gene conservation efforts. Western redcedar (*Thuja plicata* Donn ex D. Don) is a species of tremendous value to the forest industry. The seeds of this species are particularly prone to viability losses during long-term storage. Reliable tools to assess losses in seed viability during storage and their underlying causes, as well as the development of methods to prevent storage-related deterioration of seeds are needed by the forest industry. In this work, various imaging methods and biochemical analyses were applied to study deterioration of western redcedar seeds. Seedlots that exhibited poor germination performance, i.e. those that had experienced the greatest losses of viability during prolonged storage, exhibited greater abundance of oxidized proteins, detected by protein oxidation assays, and more pronounced changes in their *in vivo* ^{13}C NMR spectra, most likely due to storage oil oxidation. The proportion of oxidized proteins also increased when seeds were subjected to accelerated ageing treatments. Detection of oxidized oils and proteins may constitute a reliable and useful tool for the forest industry.

Key words: Conifer seeds, *in vivo* NMR spectroscopy, MRI, oil peroxidation, protein carbonylation, seed deterioration, seed storage, storage lipids, western redcedar.

Introduction

Successful operation of tree seed nurseries relies on the supply of high quality conifer seeds. Conifer seeds, collected from cultivated or wild stands, are usually stored in tree seed banks for extended periods, and supplied to tree nurseries when requested. In British Columbia (BC), Canada, the Tree Seed Centre (Surrey, BC) is a central facility for seed storage for the entire province. Seeds of several conifer species generally exhibit good long-term storability at –20 °C; nevertheless, even under optimal storage conditions, conifer seeds will deteriorate with time reducing the quality of seedlots, and there are particular species which appear to be especially susceptible to deterioration. Efforts to minimize seed deterioration, are especially important when dealing with valuable or rare seedlots, and seedlots deposited in the seed banks for gene conservation purposes.

Lipid peroxidation is considered to be a major cause of seed deterioration during prolonged storage (reviewed in McDonald, 1999). The mechanism of lipid peroxidation is relatively well characterized (Hendry, 1993; Frankel, 2005); free radicals attack the unsaturated fatty acids of membrane phospholipids. A decline of seed viability may be related to the peroxidation of phospholipids and consequent membrane damage. Loss of membrane integrity is apparent when excessive electrolyte leakage accompanies seed imbibition (Bewley, 1986). The free radicals generated by membrane damage may subsequently attack other subcellular structures in seeds, including organellar membranes, proteins, and DNA.

Polyunsaturated fatty acids are more susceptible to peroxidation than are monounsaturated fatty acids, while saturated fatty acids are the most resistant. Thus preferential loss of polyunsaturated fatty acids in seeds during storage may serve as an indication of lipid peroxidation. For example, in pea (*Pisum sativum* L) seeds, both linoleic (18:2) and linolenic (18:3) fatty acids decrease during storage; saturated and monoenic acids show no changes (Harman and Mattic, 1976). Moreover, the rapid decrease in the quantity of linoleic and linolenic fatty acids is strongly correlated with ageing and viability loss. Similar findings have been reported for bean (*Phaseolus vulgaris*) (Lin and Pearce, 1990), and Norway maple (*Acer platanoides*) seeds (Pukacka, 1991).

Conifer seeds, with their abundant oil reserves (triglycerides) are rich in polyunsaturated fatty acids, and thus are particularly susceptible to uncontrolled and extensive peroxidation. Peroxidation of storage lipids may contribute to a progressive decline in seed viability, not only because of the depletion of nutrient reserves, but also because of the generation of toxic products of peroxidation—aldehydes, ketones, organic acids, and hydrocarbons. Oil rancidity was linked to the loss of viability of longleaf pine (*Pinus palustris* Miller) seeds (Kaloyereas, 1958). Slash pine (*Pinus Elliottii* Engelm) seeds subjected to accelerated ageing show a significant decrease in both linoleic and linolenic fatty acids (Millano *et al.*, 1991).

Fatty acid alteration in seeds during storage/ageing is usually detected via chemical analysis (i.e. iodine value), or via more complex total fatty acid profiling performed by gas chromatography (reviewed in Frankel, 2005). These and similar methods, including high-resolution NMR spectroscopy (Crookkel, 1997) require oil extraction, which is not only destructive, but also labour-intensive and cumbersome. Moreover, oil extraction itself can alter the characteristics of the fatty acids under investigation. Earlier, the use was demonstrated of *in vivo* ^{13}C NMR to profile several primary and secondary metabolites, including storage triglycerides (oils) of seeds of conifer species (Terskikh *et al.*, 2005*b*). In this work, a broader range of *in vivo* NMR techniques has been applied to study storage-related deterioration of seeds of western redcedar (*Thuja plicata* Donn ex D. Don), particularly the relationship between fatty acid peroxidation and deterioration. Storage of seed of this species for reforestation represents a considerable challenge; some of the seedlots deteriorate rapidly during storage, others remain viable for longer periods (Kolotelo, 1996). While the causes of deterioration in this species are probably numerous, oxidation of proteins may be another cause of losses of seed viability during prolonged storage. Protein oxidation can cause modification of amino acid side chains, backbone fragmentation, protein dimerization or aggregation, and the unfolding or altered conformation of proteins (Hawkins and Davies, 2001). The structural changes alter the functional activities of the modified proteins such as their ability to modulate gene expression, cell signalling, apoptosis, and necrosis. Reactive intermediates from protein peroxides can induce chain reactions that cause damage to other intracellular targets such as DNA, lipids, and other proteins (McDonald, 1999).

Protein modifications are often associated with ageing and diseases (Stadtman, 1992); however, protein oxidation (e.g. carbonylation) may provide a means by which reactive oxygen species are utilized or counteracted, for example, when metabolic activities are restored following imbibition of mature dry seeds (Job *et al.*, 2005).

In the present study, modifications to the oils and proteins of western redcedar seeds are examined in relation to long-term storage and accelerated ageing. The use of two markers that accurately reflect seed viability is proposed; these could be applied to assess seedlots having varying susceptibility to storage deterioration: (i) a low-resolution ^{13}C NMR method that provides a fast non-destructive screening tool, and (ii) a protein oxidation assay that detects the addition of carbonyl groups introduced onto amino acid side chains.

Materials and methods

Seedlots and germination capacity

Mature dry seeds of various seedlots of western redcedar were supplied by the Tree Seed Centre, British Columbia Ministry of Forests, Surrey, BC, Canada (Table 1). Seeds were collected in different locations in BC, and at different times; some seedlots had been stored for 30 years or longer. Prior to storage, the various seedlots exhibited high germination capacity (generally 80% or higher; Table 1). Seeds had been stored at –20 °C, at the Tree Seed Centre and the germination capacity of the different seedlots was tested on a regular basis. The moisture contents of the seedlots, were very similar and ranged from 5–7 wt.%. The different western redcedar seedlots showed different rates of deterioration, in some cases leading to a significant decline in germination capacity over time of storage (Kolotelo, 1996). The differences in germination capacity exhibited by the seedlots enabled comparisons with respect to viability losses and lipid/protein changes.

In addition to the regular germination testing performed at the Tree Seed Centre (generally every two years), germination tests were performed just prior to the NMR and protein oxidation studies conducted in 2005 at the Plant Biotechnology Institute (Table 1). Dry seeds were placed on a single layer of Whatman 3MM Chromatography paper (VWR Canlab, Mississauga, Ontario) and 14-ply germination paper (No. 87, Seedburo Equipment Company, Chicago, Illinois), previously moistened with 50 ml sterile double-deionized H$_2$O, in a clear plastic seed box (11.2×11.2×3.6 cm, Hoffman Manufacturing Inc., Albany, Oregon). Seeds were then transferred to germination conditions (at 30 °C for 8 h light, 20 °C for 16 h dark) and the germination percentage determined after a 21 d period. Germination percentages were based on three replicates of 50–60 seeds (Table 1; 2005 data).

In addition to the seedlots noted in Table 1A, which were stored under optimal conditions (–20 °C), four additional seedlots were subsequently stored for approximately 3 months at 2 °C (Table 1B), a treatment leading to a complete loss of germination capacity (Table 1B).

Table 1. *Seedlots of western redcedar used in this study and their storage history*

(A) Storage of seeds at –20 °C (optimal storage temperature)

Seed lot	Collection year	Location in BC	Elevation (m)	Latitude/Longitude	Initial germination (%)[a]	Germination in 1999–2000 (%)[a]	Germination in 2005 (%)[b]
27153	1983	Barriere Pass	660	51°00' N/119°00' W	78	80	86
60076	1997	Mt. Newton	219	50°08' N/125°06' W	89	87	82
34954	1990	Thunderbird	240	54°28' N/128°40' W	74	62	67
31459	1992	Kiwa Cr.	780	52°58' N/119°31' W	86	73	58
03392	1979	Erie Mt.	1200	49°12' N/117°23' W	88	31	39
09035	1974	North Creek	300	50°24' N/123°10' W	78	32	24

(B) Storage of seeds at –20 °C for years specified, then 2 °C for several weeks (suboptimal storage conditions)

Seed lot	Collection Year	Location in BC	Elevation (m)	Latitude/Longitude	Initial germination (%)[a]	Germination (%) in 2000 (storage at –20 °C)[a]	Germination (%) in 2005 (after storage at 2 °C)[c]
08484	1983	Edney Lake	845	52°28' N/121°33' W	77	75	0
20202	1985	Chilliwack[d]	225	49°04 N/121°46' W	87	63	0
04790	1975	Kitwanga	397	55°10' N/127°49' W	75	63	0
40768	1993	Cedar Creek	860	51°56' N/119°05' W	92	85	0

[a] All data on initial germination capacity of seeds and germination percentages in 1999–2000, after long-term storage at –20 °C, were determined at the Tree Seed Centre, BC, Ministry of Forests, Surrey, BC, Canada.
[b] Germination of seeds in 2005 (i.e. just prior to NMR studies) after long-term storage at –20 °C was determined at the Plant Biotechnology Institute, Saskatoon, Canada.
[c] Germination of seeds after storage at 2 °C was determined at the Plant Biotechnology Institute, Saskatoon, Canada.
[d] Chilliwack River Valley.

Oil and fatty acid composition

The content of extractable oil and the fatty acid composition was determined for three of the western redcedar seedlots (27153, 09035, and 20202). Analyses were based on three 50 mg replicates for each seedlot. Oil was extracted, converted to fatty acid methyl esters (FAME) and analysed by gas–liquid chromatography as described below.

The seeds were finely ground with a polytron and the oil was extracted with 3.0 ml of a chloroform/isopropanol (2:1, v/v) mixture containing 500 µg of 15:0 triacylglycerol (TAG) as an internal standard. After extraction, the mixture was centrifuged at 2500 rpm for 5 min. An aliquot of the clear supernatant (1 ml) was mixed with 0.5 ml of a chloroform/benzene/methanol (1:1:1 by vol.) mixture and evaporated to dryness under a stream of nitrogen gas.

To prepare FAME, the oil samples were transmethylated in tightly capped vials with 3 ml of a methanol/sulphuric acid (3 N) mixture for 1 h at 60 °C. The reaction mixture was then cooled to room temperature and 3 ml of an aqueous solution of NaCl (0.9 wt.%) was added. FAMEs were extracted three times with 1 ml hexane aliquots. The combined FAME/hexane extracts were mixed with 1 ml of a chloroform/benzene/methanol (1:1:1 by vol.) mixture and evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in 0.5 ml of hexane and analysed by gas–liquid chromatography.

GLC was performed on a Hewlett Packard model 6890 gas chromatograph equipped with a split/splitless injector using a 30 m DB 23 column×0.25 mm id and 0.25 µm film thickness (J&W Scientific, Folsom, CA). The temperature gradient was 160 °C for 1 min followed by a ramp of 4 °C min⁻¹ up to a final temperature of 240 °C, which was maintained for 8 min. The inlet pressure of the carrier gas (helium) was 14.20 psi. The flame-ionization detector was maintained at 250 °C and used 40.0 ml min⁻¹ of hydrogen.

A total of 16 individual FAME peaks were identified by comparison of their retention times with those in the standard sample containing a mixture of 12 known FAMEs. Identification of several FAME peaks was according to Wolff *et al.* (1997) and confirmed by mass spectroscopy. An internal 15:0 TAG standard was used to measure the total oil content of the samples.

NMR spectroscopy

All NMR experiments were performed at room temperature on a Bruker Avance 360 spectrometer at the NMR facility of the Plant Biotechnology Institute NRC (Saskatoon, SK, Canada). Two different NMR probes were used to obtain low and high resolution spectra.

Low-resolution static spectra of seeds were acquired with a liquid-state 10 mm ^{1}H/^{13}C Bruker NMR probe. A known amount of dry seeds without any additional treatment, typically ~ 0.1 g, were placed in a 10 mm NMR glass tube and ^{1}H and ^{13}C NMR spectra were recorded. For quantitative NMR measurements, three replicates of 0.1 g seeds were used. ^{1}H NMR spectra were recorded at a resonance frequency of 360.13 MHz with a spectral width of 50 kHz applying a single-pulse sequence with a 5 µs pulse and a 5 s relaxation delay between scans. Eight scans were accumulated for each ^{1}H NMR spectrum. Static ^{13}C NMR spectra were obtained at a resonance frequency of 90.56 MHz under low-power broadband proton decoupling and ^{13}C pulse width of 10 µs with a 5 s relaxation delay between scans. The number of accumulations was 1 K. NMR chemical shifts were referenced to external tetramethylsilane (TMS).

High-resolution NMR spectra of western redcedar seeds were recorded with a solid-state double-band Bruker BL4 magic angle spinning (MAS) probe. Dry seeds with seed coats removed were placed in a 4 mm o.d. ZrO$_2$ rotor and spun under the magic angle at a spinning speed of 3 kHz. ^{1}H MAS NMR spectra were recorded with a spectral width of 7 kHz applying a single-pulse sequence with a 5 µs r.f. pulse and a 5 s relaxation delay between scans. Time domain size of the spectra was 4 K with eight accumulations per spectrum. ^{13}C MAS NMR spectra were obtained with a spectral width of 22 kHz under low-power proton decoupling and ^{13}C pulse width of 3 µs and 1 s relaxation delay between scans. Time domain size of the spectra was 32 K. The number of accumulations was 4 K.

Magnetic resonance imaging (MRI) and X-ray radiography

MRI experiments were performed on the same Bruker Avance DRX 360 spectrometer equipped with a Bruker Micro 2.5 microimaging system.

Four randomly selected seeds from five different seedlots were assembled with a double-sided adhesive tape on a 1 mm thin acrylic plastic plate (25 mm×80 mm). The plastic plate had been cut to fit a 25 mm i.d. MRI probehead. Proton intensity MR images were recorded with a standard Hahn-echo pulse sequence. This pulse sequence consisted of 512 μs gauss pulses for excitation and refocusing with an echo time of 8.7 ms, a recycle delay of 1 s, and 128 accumulations per image. Each image consisted of a 256×256 data matrix with a field of view of 2.5×2.5 cm^2, resulting in a spatial pixel resolution of 98×98 μm^2. A slice thickness of 2 mm was chosen to ensure that all seeds on the plate appeared on the same MRI image simultaneously. Slices as thin as 0.25 mm, and higher spatial resolution were used to obtain images of individual seeds. Post-processing of MR images was done with the public domain Java-based image-processing program 'ImageJ'.

X-ray images of the same seed assembly were acquired at the Tree Seed Centre (Surrey, BC) with a Faxitron cabinet X-ray system, using 15 kV potential and 3 mA current. Exposure time was 1.5 s.

Seed sampling for detection of oxidized proteins

Seeds of two seedlots, 27153 (86% germination capacity in 2005) and seedlot 09035 (24% germination capacity in 2005) (Table 1), were used to compare the degree of protein oxidation. Seeds were incubated under germination conditions as stated above in the section 'Seedlots and germination capacity' and sampled at d0 (dry seeds), d2 (2 d in germination conditions), d4 (4 d in germination conditions), RE (radicle emergence), and R5 (seedlings with a radicle length of 5 mm). At each time point, seeds were dissected to separate embryos and megagametophytes. About 30–50 seeds were pooled together at each time point and the proteins analysed.

Ten of the dry seeds from each seedlot were also dissected and sampled individually. Embryos were put on half-strength MS plates to determine their germination capacity (elongation of the radicle) and the corresponding megagametophytes were used for analyses of oxidized proteins.

Accelerated ageing treatments

Seeds of seedlot 27153 (86% germination capacity) were placed on a mesh tray in a seed ageing box (11.2×11.2×4 cm) filled with 70 ml of water (the water level was below the mesh tray). These conditions created close to 100% humidity inside the ageing box. The seed box was wrapped with aluminium foil and incubated at 41 °C for 7 d. Seeds were then transferred to germination conditions and sampled at d0, d2, d4, and d8, dissecting them to isolate the embryos and the megagametophytes. Ten seeds were also dissected at the end of the ageing treatment (i.e. prior to transfer to germination conditions) and sampled individually. Embryos were placed in Petri dishes containing half-strength solid Murashige and Skoog medium for determination of germination capacity and the corresponding megagametophytes were used for analyses of oxidized proteins.

Protein extraction and detection of oxidized proteins by western blot analysis

Proteins were extracted from embryos and megagametophytes of western redcedar seeds by grinding tissues in a 1.5 ml micro-centrifuge tube in chilled buffer (50 mM TRIS–HCl, pH 7.3; 100 mM NaCl; 2% SDS, 10% glycerol, 1 mM dithiothreitol). Following centrifugation at 12 000 *g* for 10 min, protein concentration of the supernatant was determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin fraction V (Bio-Rad) as a standard.

Detection of oxidized proteins by western blot analysis was carried out using 'OxyBlot Protein Oxidation Detection Kit' (CHEMICON International Inc., Temecula, CA, USA) (Talent *et al.*, 1998). Proteins (10 μg) were denatured by adding 12% SDS to a final concentration of 6% SDS. The sample was derivatized by adding an equal volume of 1× DNPH (2,4-dinitrophenylhydrazine) solution and incubated at room temperature for 15 min. Neutraliza-tion solution (0.75 volume of that of the DNPH solution) and β-mercaptoethanol (5% v/v) were added. The treated sample was fractionated on a 10% SDS–PAGE gels and then electroblotted onto a Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). Following a brief rinse in PBST (phosphate buffered saline containing 0.05% Tween-20), membranes were blocked overnight with 5% skimmed milk powder in PBST and then incubated with the primary antibody (rabbit anti-DNP antibody) diluted 1:500 in PBST and 3% skimmed milk powder for 1 h at room temperature. The blots were then washed three times in PBST (each 20 min) and incubated 1 h in secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, 1:600) (Chemicon). Following three washes (20 min each) in PBST, immunodetection was achieved by incubating with chemilumines-cent reagent (ECL Advance Western Blotting Detection Kit, Amersham BioSciences) and exposing the membrane to an X-ray film (Hyperfilm ECL, Amersham Biosciences). All blots were done at the same time and exposed to the same X-ray film to ensure an identical exposure time allowing direct comparisons of the degree of protein oxidation in the different samples. The entire western blot procedure was repeated twice and same results were obtained.

Results and discussion

General characteristics of ^{13}C NMR spectra of dry western redcedar seeds

Seeds of conifers species contain storage oils (triacyl-glycerols) as a major reserve to support early seedling growth following germination. In western redcedar seeds the oil content is ~25% of the seed fresh weight (Table 2). During seed development, oils are accumulated in oil bodies within storage parenchyma cells of the embryo and megagametophyte (Bewley and Black, 1994; Huang, 1996; Kermode, 2003). It is noteworthy, that even at the low water content of mature dry seeds, the trigycerides within oil bodies remain liquid; the mechanisms that prevent oil body coalescence during seed drying may involve the integral oil body protein, oleosin. The unique characteristics of oils in dry seeds (i.e. numerous intra-cellular droplets of liquid enclosed by the rigid solid matrix of the seed coat) allows oilseeds to be studied by *in vivo* NMR spectroscopy.

High-resolution liquid-state NMR spectrometers are primarily designed to study liquid samples. A characteris-tic 'liquid-state' ^{13}C NMR spectrum for western redcedar seeds is shown in Fig. 1a. The static NMR spectra consist of several broad resonances originating from different types of carbon atoms in the seed's oils; only the liquid components of the seed are detected. Such spectra lack

Table 2. *Extractable oil*[a] *and fatty acid composition*[b] *in three seedlots of western redcedar as determined by gas-liquid chromatography*

Fatty acid	Seedlot		
	27153	09035	20202
16:0	5.42±0.58	6.09±0.06	11.12±0.95
16:1	0.11±0.01	0.12±0.01	0.24±0.03
18:0	1.73±0.14	1.87±0.01	3.89±0.45
9–18:1	11.68±1.45	13.17±0.22	21.93±1.67
11–18:1	0.42±0.06	0.48±0.01	0.80±0.05
5,9–18:2	0.10±0.01	0.08±0.01	0.17±0.13
9,12–18:2	28.18±0.17	27.81±0.22	25.41±0.62
9,12,15–18:3	29.88±1.77	28.44±0.52	15.13±1.31
20:0	0.27±0.02	0.26±0.02	0.51±0.07
11–20:1	0.89±0.03	0.89±0.03	1.44±0.14
5,11–20:2	0.72±0.06	0.76±0.02	1.24±0.10
11,14–20:2	2.09±0.01	2.05±0.07	2.03±0.03
5,11,14–20:3	4.78±0.11	4.77±0.10	4.97±0.05
11,14,17–20:3	0.61±0.05	0.81±0.03	0.45±0.04
5,11,14,17–20:4	12.30±1.03	11.31±0.21	6.21±0.64
22:0	0.17±0.06	0.21±0.02	0.51±0.06
24:0	0.26±0.02	0.26±0.02	0.79±0.04
26:0	0.16±0.04	0.20±0.02	0.81±0.10
28:0	0.09±0.10	0.25±0.03	0.65±0.08
Others	0.14	0.16	1.70
Saturated	8.10±0.89	9.14±0.12	18.28±1.62
Monounsaturated	13.10±1.56	14.66±0.24	24.41±1.89
Polyunsaturated	78.76±1.45	76.12±0.15	55.72±2.94
Oil content	26.23±2.01	20.19±1.00	7.49±1.91

[a] wt.% ±SD.
[b] wt.% of total fatty acids ±SD.

Fig. 1. Comparison of the static (a) and magic angle spinning (b) ^{13}C NMR spectra recorded for dry seeds of western redcedar (seedlot 60076).

resolution; the broadening of peaks is caused by magnetic susceptibility effects and residual dipolar interactions (Haw and Maciel, 1983; Rutar, 1989; Rutar *et al*., 1989; Bardet and Foray, 2003). Considerably improved spectral resolution of oilseed lipids can be achieved by 'solid-state' NMR spectra, i.e. under magic angle spinning (MAS) conditions (Wollenberg, 1991; Hutton *et al*., 1999; Sayer and Preston, 1996). Even moderate MAS eliminates undesirable line broadening and results in excellent spectral resolution (Fig. 1b), comparable with high-resolution NMR spectra of true liquids. The MAS NMR spectra of conifer seeds *in vivo* provides a wealth of information on a variety of primary and secondary metabolites (Terskikh *et al*., 2005*b*).

Low-resolution static ^{13}C NMR spectra for western redcedar seeds

A typical static ^{13}C NMR spectrum recorded *in vivo* for western redcedar seeds is shown in Fig. 1a. This spectrum originates from liquid oils and consists of several broad resonances: (i) the several overlapping lines at 15–35 ppm are from saturated (methyl/methylene) carbon atoms in the hydrocarbon chains of fatty acids; (ii) two resonances at 60–70 ppm are from the triglyceride moiety of the backbone; (3) a signal at ~130 ppm is from unsaturated carbon atoms; and (iv) the signal at ~170 ppm is from the
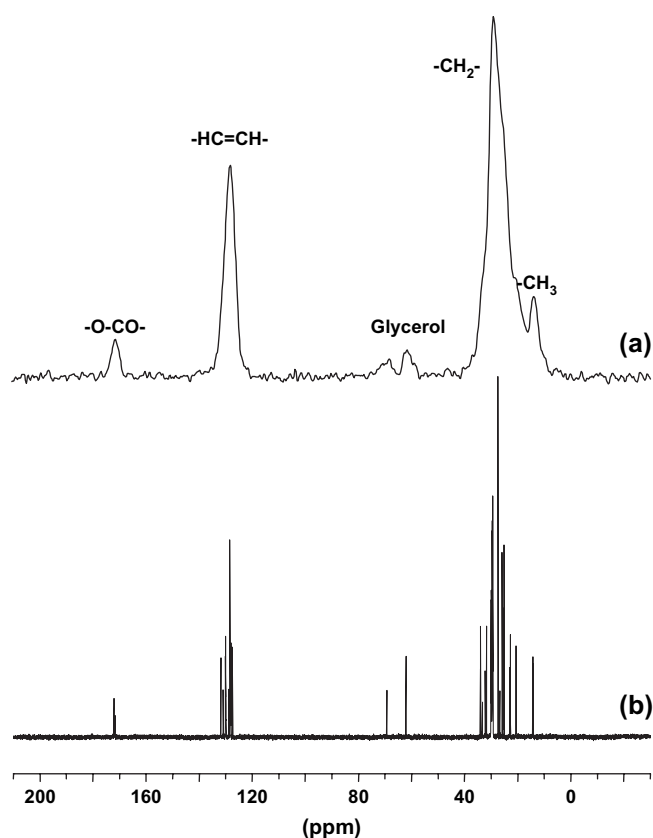
carboxyl atoms of the fatty acid. Even though static ^{13}C NMR spectra give only moderate resolution, they have the advantages of requiring only a standard liquid-state NMR set-up and being very straightforward to perform. No special treatment of seeds is needed; after the NMR analysis is completed, seeds can be returned to storage or used for germination. Static ^{13}C NMR spectra taken alone or together with static ^{1}H NMR spectra can be useful for measuring total oil content of seeds, while the relative integral intensities of ^{13}C NMR resonances from unsaturated and saturated carbon atoms give a reasonably accurate estimate of the saturation ratio of seed triglycerides and in a non-destructive manner (Terskikh *et al*., 2005*b*).

Are changes in the ^{13}C NMR spectra correlated with different rates of seed deterioration?

In this work, western redcedar seeds of different origin, collected in different places and at different times (Table 1) were studied. For our analysis of storage oils, it was assumed that seeds would be of similar oil contents and fatty acid compositions at the time of their collection. This assumption is not unreasonable: our experiments show that high-performing western redcedar seedlots indeed have very similar storage oil content and composition.

The different seedlots all exhibited high germination capacity at the time of their collection. It is only in compromised/deteriorated seedlots that these parameters deviate considerably from the norm. Furthermore, an independent study of oils extracted from western redcedar seeds showed that the fatty acid composition is almost identical to what is found in viable seedlots (Wolff *et al.*, 1997). It was not feasible to follow the same seedlot over the 10–30 year storage period. Two approaches were used: (i) a comparative analysis of spectra derived from seeds of different seedlots exhibiting different germination percentages and rates of deterioration during storage; and (ii) an analysis of the effects of an accelerated ageing treatment, with the recognition that accelerated-aged seeds do not necessarily model naturally-aged seeds because the former involves moist conditions not typical for long-term seed storage (Bewley, 1986).

Static ^{13}C NMR spectra for western redcedar seedlots with different germination capacities and rates of germination decline over storage (Table 1) are shown in Fig. 2. To enable direct comparisons, the spectra were obtained under the same experimental conditions and with the same amounts of seeds. The most noticeable change that seemed to correlate with a decline in germination capacity was a gradual decrease in intensity and broadening of all the resonances (15–35, 60–70, 130, and 170 ppm). In dormant conifer seeds, the intensity of the ^{13}C spectra is known to be proportional to the amount of liquid oils (Terskikh *et al.*, 2005*b*); thus in seedlots with compromised germination, the triglyceride reserves are apparently considerably reduced as compared with the seedlots exhibiting high germination performance and little storage-related deterioration. The decreased amount of mobile lipids may be associated with seed deterioration during storage. Line broadening and decreased spectral intensity were also observed in the static ^{1}H NMR spectra (see Fig. 1s of the Supplementary data at *JXB* online).

Free-radical mediated lipid peroxidation is proposed to be one of the main causes of seed deterioration. Among other negative effects, this peroxidation of lipids leads to oxidative polymerization (Bernstein, 1946). Vegetable oils with high content of polyunsaturated fatty acids are especially susceptible to oxidative polymerization. At high levels of polymerization, multiple cross-linkages form between individual fatty acid chains leading to the formation of extended three-dimensional polymer networks, i.e. solidification of oils (Wolman, 1975; Kamal-Eldin *et al.*, 2003); at the same time, the amount of extractable oil decreases. Segments of the hydrocarbon chains of fatty acids involved in cross-linkage become rigid and thus are rendered invisible by liquid-state ^{13}C NMR (Maunu, 2002; Reichert, 2005; Chen and Kurosu, 2007).

In a similar manner, the observed changes in the ^{13}C NMR spectra of the deteriorated seedlots are most probably caused by solidification of storage oils. To
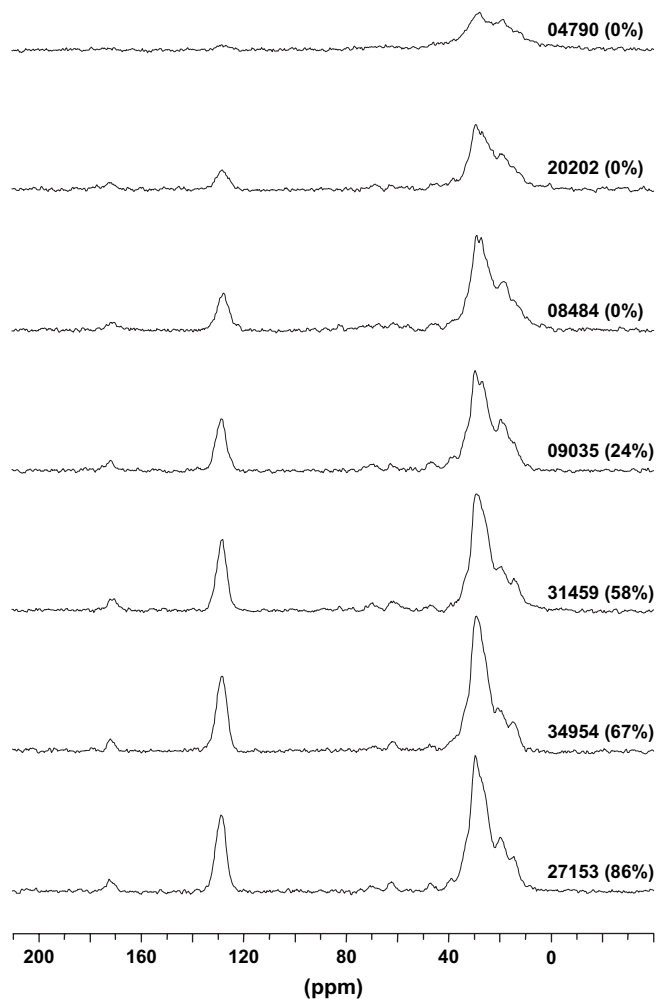

Fig. 2. Static ^{13}C NMR spectra for different seedlots of western redcedar. Seedlot number is shown with each spectrum. The same amount of seeds (by weight) was used for each experiment under the same experimental conditions. The relative vertical intensities of the spectra are preserved. Germination percentages are given for each seedlot.

confirm this, the amount of extractable oils of three different seedlots was determined via chloroform/isopropanol extraction (Table 2). In a poorly performing seedlot (09035), in which germination capacity in 2005 was only 24%, the content of extractable oils was diminished as compared to a high performing seedlot (27153); in a seedlot with no viable seeds (20202), the amount of extractable oils was markedly decreased. The diminished oil content in compromised seedlots is most probably caused by oxidative polymerization as a result of lipid peroxidation.

High-resolution ^{13}C MAS NMR spectra of western redcedar seeds

Additional insight into possible oil oxidation in deteriorated redcedar seeds can be obtained by *in vivo* high-resolution ^{13}C MAS NMR spectra. One such spectrum

recorded for the highly viable seedlot 60076 is shown in Fig. 3. Many individual ^{13}C NMR resonances can be assigned to individual carbon atoms in the hydrocarbon chains of fatty acids (Terskikh *et al.*, 2005*b*). In this spectrum only resonances from four major fatty acids are indicated, oleic (9–18:1), linoleic (9,12–18:2), α-linolenic (9,12,15–18:3), and juniperonic (5,11,14,17–20:4) (Table 2). Total oil and individual fatty acids can be quantified via integration of the spectra; thus ^{13}C MAS NMR provides a valuable tool for *in vivo* monitoring of the composition of storage oils. The corresponding ^{1}H MAS NMR spectra are also well resolved, but the narrow range of the proton chemical shifts prevents identification of individual fatty acids (see Fig. 2s of the Supplementary data at *JXB* online).

^{13}C MAS NMR spectra recorded for different western redcedar seedlots show dramatic changes in storage oils that can be correlated with decline in germination capacity (Fig. 4; see Fig. 3s of the supplementary data at *JXB* online). In the spectra of seedlots that showed little decline in their germination capacity during storage, individual ^{13}C NMR resonances are very narrow, which indicates very high mobility of fatty acid hydrocarbon chains, i.e. a low viscosity of oils. In deteriorated seedlots, the resonances are broader, most likely because the mobility of the fatty acids becomes more restricted due to cross-linkages caused by oxidative polymerization. Both the total liquid oil content and the ratio between individual fatty acids, i.e. the composition of triglyceride fatty acids in the mixture, change with deterioration (Fig. 4). Gradual line broadening and disappearance of spectral

peaks are also seen in the corresponding ^{1}H MAS NMR spectra (see Fig. 2s of the supplementary data at *JXB* online).

The results of integration of the ^{13}C MAS NMR spectra are summarized in Fig. 4. As compared to more vigorous seedlots, the concentrations of polyunsaturated fatty acids α-linolenic (18:3) and juniperonic (20:4) decreased sharply in seedlots exhibiting low germination capacity. This preferential loss of polyunsaturated fatty acids in oils of compromised seedlots further supports our contention that deterioration in western redcedar seeds is most likely caused, in part, by lipid peroxidation.

Similar conclusions can be drawn based on the GC analysis of oil extracts (Table 2). In the seedlot exhibiting no decline in germination capacity (27153), the fatty acid composition was very similar to that previously reported for western redcedar seed (Wolff *et al.*, 1997). The very high concentration of polyunsaturated fatty acids may render the oils of western redcedar seeds particularly susceptible to oxidation. In the poor performing seedlot 09035, the content of polyunsaturated fatty acids decreased; polyunsaturated fatty acids were drastically diminished in the non-viable seedlot. The fatty acid profile changed sharply in a dead seedlot 20202. A preferential loss of polyunsaturated fatty acids is indicative of lipid peroxidation; the decreased amount of extractable oil may be linked to oxidative polymerization, which also supports the occurrence of lipid peroxidation. Similar results have been reported for seeds of slush pine (*Pinus Elliottii* Engelm) subjected to accelerated ageing (Millano *et al.*, 1991), in which the contributing factor was attributed to
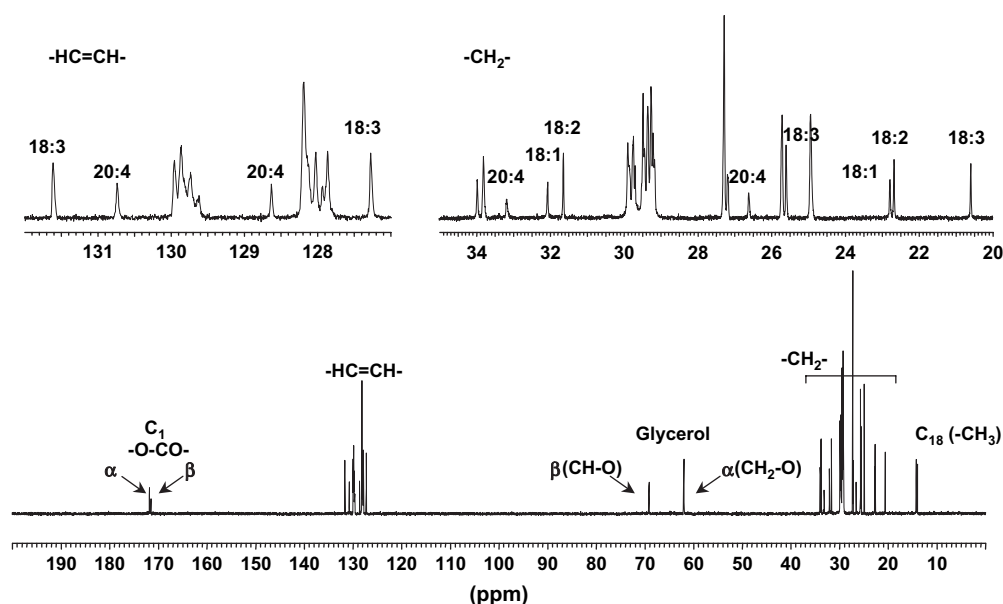

Fig. 3. Full ^{13}C MAS NMR spectrum of dry western redcedar seeds (seedlot 60076) shown with two expanded regions with signals from olefin and methylene carbon atoms. Several solitary resonances attributed to individual fatty acids are marked: 9–18:1 (oleic); 9,12–18:2 (linoleic); 9,12,15–18:3 (α-linolenic); 5,11,14,17–20:4 (juniperonic).
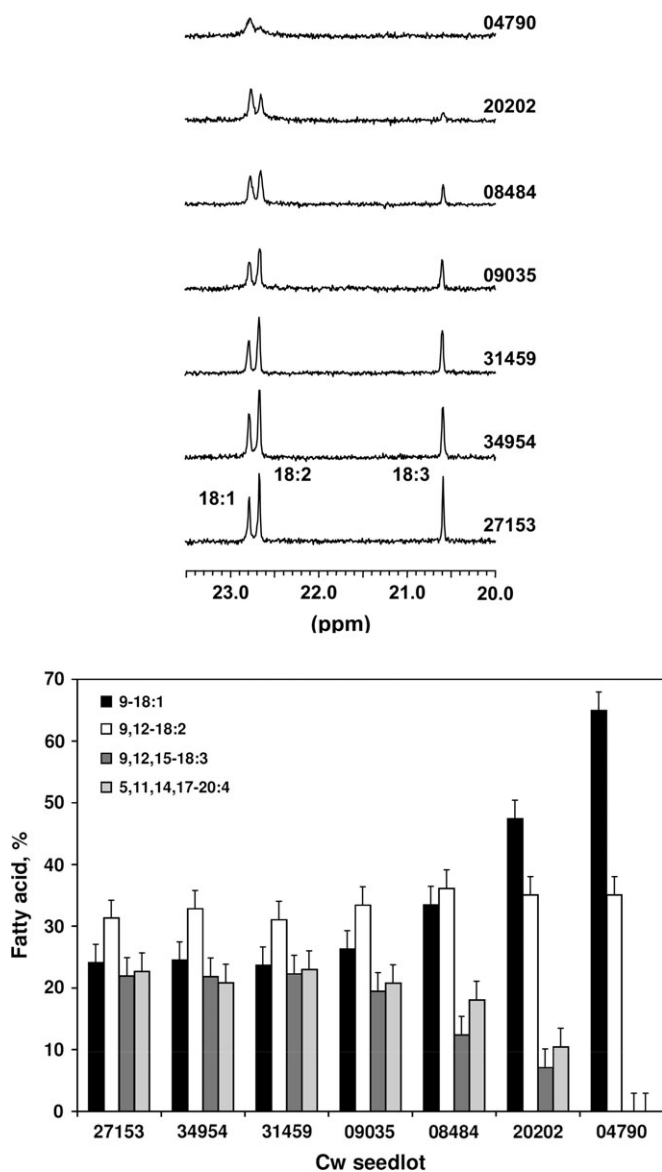
Fig. 4. (Above) ^{13}C NMR MAS spectra (the region of ω_2 CH$_2$ carbon atoms, 18:1, oleic; 18:2, linoleic; 18:3, α-linolenic) recorded for different seedlots of western redcedar demonstrating preferential oxidation of poly-unsaturated fatty acids. Seedlot number is shown with each spectrum. (Below) The ratio between individual fatty acids in different seedlots of western redcedar as determined by integration of the ^{13}C NMR MAS spectra (mol.% of the total four): 9–18:1 (oleic), 9,12–18:2 (linoleic), 9,12,15–18:3 (α-linolenic), and 5,11,14,17–20:4 (juniperonic). A total of 10–12 seeds were analysed for each seedlot. The average error due to NMR integration is shown.

oil oxidation. Harman and Mattick (1976) and several other research groups (see above), have also directly linked lipid oxidation with seed ageing and death.

Distribution of storage oils in western redcedar seeds: MRI and X-ray radiography

High-resolution ^{1}H Magnetic Resonance Imaging (MRI) has been used to assess the internal anatomy of conifer seeds in a non-destructive manner (Terskikh *et al.*, 2005*a*, 2006). In mature dry conifer seeds, the MRI image originates from liquid oils; it is the variations in oil content of the different seed tissues that allow for the contrast required to differentiate the internal seed structures in detail. This suggests that MRI may be a useful tool for the rapid screening of conifer seeds, providing information on the proportion of filled versus empty or partially emptied seeds, the latter arising due to insect or mechanical damage, or to developmental problems. Similar information can be obtained with X-ray radiography (Keagy and Schatzki, 1991; Kolotelo *et al.*, 2001; Karunakaran *et al.*, 2004). The two techniques supplement each other in the non-invasive/non-destructive assessment of the internal anatomy of seeds; X-ray radiography shows the greatest contrast of the hard tissues of the seed, while the MR images can differentiate the soft tissues and liquid-like components.

The typical high-resolution MR images of a mature dry western redcedar seed are shown in Fig. 5. In the coronal and axial MR images, the embryo with its hypocotyl (HP) and two cotyledons (C), as well as the surrounding megagametophyte (ME) tissues contain a significant amount of oil. There is no signal in the seed coat of the dry seed (because of its lack of oils and other mobile liquid-like components); however, the seed coat becomes visible when seeds are imbibed. The intense MRI signals in the area adjacent to the megagametophyte originate from oleoresin stored in specialized resin vesicles; these are found in seeds of western redcedar and several other conifers. In western redcedar seeds, the resin vesicles form within the middle or outer layer of the seed coat (Kolotelo, 1997). The oleoresin of resin vesicles in redcedar seeds is a low-viscous liquid, mostly composed of monoterpene thujone; thus it can also be detected with MRI, as well as with ^{13}C MAS NMR (Terskikh *et al.*, 2005*b*).

To examine the distribution of storage oils in western redcedar seeds, four randomly selected seeds from five different seedlots were assembled on a thin plastic plate (Fig. 6). There were no visible differences in the outward appearance of seeds from the different seedlots. Similarly, X-ray radiography indicated that seeds from all five seedlots were filled, and had sustained no internal damage or visible physical degradation. The MR images, however, revealed significant differences in the distribution of liquid oils in the seeds, especially between seeds of the viable and non-viable seedlots. In seedlots 27153 and 09035 the MR images were similar, but certainly not identical, with seed from the latter lot containing a lower oil content. On the other hand, the non-viable seedlots had a greatly reduced content of liquid oils, both in the embryo and in the megagametophyte. No MR signals were detectable for seeds of seedlot 04790. MRI experiments were repeated for three different seed assemblies with nearly identical

results. The absence or paucity of MR signals in compromised seedlots is due to the lack of liquid oils in seed tissues (Alberti *et al.*, 2002). Diminished content of liquid oils is most likely caused by oxidative polymerization of lipids during prolonged storage, as has been previously discussed. Thus, solidified oils are MRI-invisible, even though seeds look normal both from their external appearance and in X-ray images.

Static ^{13}C NMR spectra to monitor viability of western redcedar seeds *in vivo*

In the static ^{13}C NMR spectra of deteriorated seedlots all the signals, including those from saturated and unsaturated carbon atoms of the hydrocarbon chains of fatty acids, decrease in intensity, most probably due to lipid peroxidation and associated peroxidative polymerization (Fig. 2). Because the mecha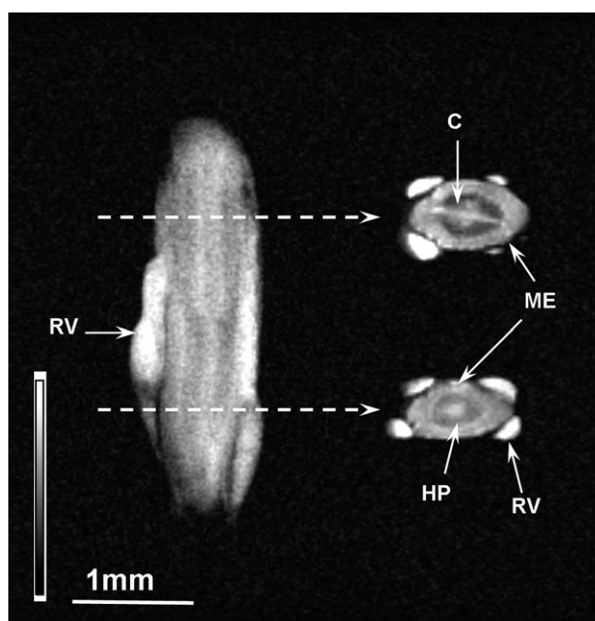nism of both processes directly involves carbon atoms at double bonds, the ^{13}C signals from unsaturated carbon atoms will be the most sensitive to peroxidation. The integral intensity of the corresponding line in the static ^{13}C NMR spectra recorded for ten different western redcedar seedlots as a function of their germination capacity is presented in Fig. 7. Most of the seedlots showed a clear trend of a gradually declining signal intensity as the germination capacity decreases. This potential correlation can be fitted with a hyperbolic correlation curve as

$$I/I_o = \frac{a}{1 + \frac{100}{x+b}} \qquad (1)$$

where I/I_o is the normalized signal intensity, x is the germination capacity (%), and a and b are the correlation parameters. Fitting of the data using the least square deviation method gives the correlation parameters $a=1.68\pm0.05$; $b=35.7\pm4.0$ ($R^2=0.974$). One non-viable seedlot (40768) did not fit the correlation by statistical criteria. High resolution ^{13}C MAS NMR spectra for this seedlot did not show any anomalies, i.e. the oil content and the fatty acid composition were similar to those found in highly viable seedlots.

Two other seedlots, 04790 and 20202, were also non-viable, and exhibitied broadened static ^{13}C NMR spectra that were greatly reduced in intensity (Fig. 2). The extent to which peroxidation of oils in seed storage tissues continues in non-germinable, non-viable (dead) seeds is not known. To accommodate for continued oil oxidation after seed death the correlation curve can be extended beyond zero germination (Fig. 7). Even though seedlots, 04790 and 20202, were not used in the fit (equation 1), their assumed positions on the extended correlation curve may be predicted (Fig. 7).

It is noted that the intensity of the ^{13}C NMR line from *unsaturated* carbon atoms, which are directly affected by peroxidation and oxidative polymerization, shows high sensitivity to the level of oxidation; the signal is greatly diminished in deteriorated seedlots (Fig. 2). This makes this signal useful for monitoring deterioration in oil seeds *in vivo*. At the same time, the saturated fragments of the hydrocarbon chains of fatty acids remain largely intact
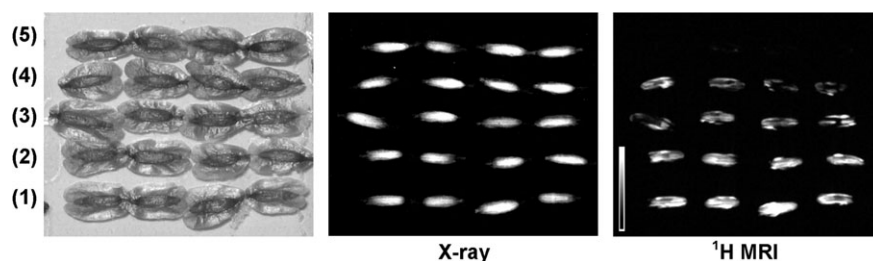

Fig. 5. High-resolution ^{1}H MRI images of a dry western redcedar seed in a coronal plane and in two axial planes. Images were obtained with a 250 μm slice thickness and in-plane resolution of 20×20 μm^2. ME, megagametophyte; C, cotyledons; HP, hypocotyl; RV, resin vesicles.

Fig. 6. Comparison of X-ray images and ^{1}H MRI images of western redcedar seeds. Left: Seeds from five seedlots of western redcedar assembled on plastic plate as follows: (1) 27153; (2) 09035; (3) 08484; (4) 20202; (5) 04790. Middle: X-ray image of the same assembly. Right: ^{1}H MRI image.
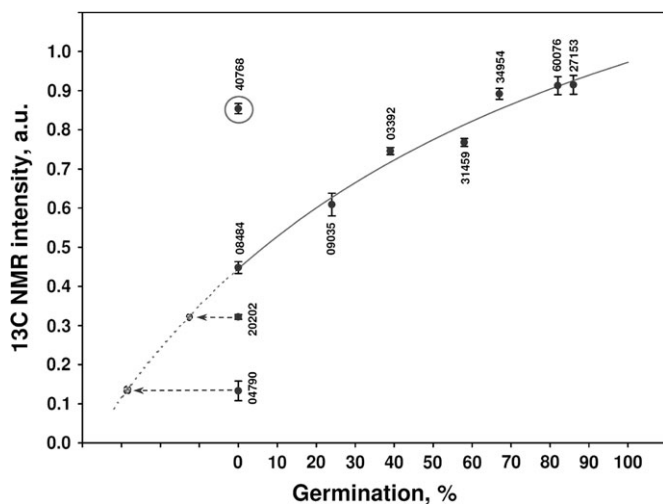
Fig. 7. Integral intensity of the line from unsaturated carbon atoms in the static ^{13}C NMR spectra recorded for different seedlots of western redcedar (Fig. 2) as a function of germination capacity. Data are based on the average of three replicates of 0.1 g of seeds each ($\pm$SD). The correlation curve is a hyperbolic dependence $I/I_o = a/(1+100/(x+b))$, where I/I_o is the normalized signal intensity, x is the germination capacity (%), a and b are the correlation parameters: $a=1.68\pm0.05$; $b=35.7\pm4.0$ ($R^2=0.974$). One non-viable seedlot (40768) does not fit the correlation by statistical criteria and is therefore omitted. The correlation curve is extended beyond zero germination to accommodate for continued oil oxidation after death of the seed. For two seedlots (04790 and 20202, not used in the fit) assumed positions on the extended correlation curve are indicated.

during peroxidation (Miakar and Spiteller, 1994; Frankel, 2005). As a result, the ^{13}C NMR line from *saturated* carbon atoms is not affected to a great extent as a result of deterioration (see Fig. 4s of the Supplementary data at *JXB* online). The saturated fragments of the hydrocarbon chains would also maintain some degree of mobility even in heavily polymerized samples, i.e. the static ^{13}C NMR line would be broadened, but not beyond detection. Similarly, even though static ^{1}H NMR spectra did show some broadening and decline in the total integral intensity (see Fig. 1s of the Supplementary data at *JXB* online), the response to peroxidation in such spectra was rather indistinct and difficult to interpret (see Fig. 5s of the Supplementary data at *JXB* online).

In addition, in the *in vivo* static NMR spectra from western redcedar seeds there will be a certain unknown contribution from signals derived from oleoresin, since seed coats were not removed in these experiments. In western redcedar seeds the oleoresin is mostly composed of the monoterpene thujone. The high-resolution ^{13}C MAS NMR spectrum of thujone in western redcedar seed consists of nine resonances with chemical shifts from 18 ppm to 47 ppm and a single resonance at 220 ppm (Terskikh *et al.*, 2005b). Even though the content of oleoresin in seeds is low compared with the oil content, for heavily oxidized seedlots this may introduce significant errors in measuring the intensity from the ^{13}C static

line from *saturated* carbon atoms (at 15–35 ppm). However, there is no contribution from thujone in the intensity of the ^{13}C static line from *unsaturated* carbon atoms at around 130 ppm, which makes this line a more reliable marker to measure the oxidation level in seeds *in vivo*.

Deteriorated seedlots exhibit a greater proportion of oxidized proteins

Protein oxidation can be caused by many factors such as high energy radiation (X-rays, UV), and metal ions (ascorbate/peroxide systems). Reactive intermediates from protein peroxides can induce chain reactions and damage to other targets such as DNA, lipids, and other proteins. The status of protein oxidation may be used as a marker for seed quality; therefore the different seedlots of western redcedar showing different rates of deterioration were analysed for this parameter using Chemicon's OxyBlot Oxidized Protein Detection Kit. As a consequence of free radical-induced protein damage, carbonyl groups are introduced into protein side chains. By using the kit protocol, the carbonyl groups are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH), and then detected by antibodies specific to the attached DNP moiety of the proteins. This method can detect as little as 5 fmol of carbonyl residues and its sensitivity is at least 100 times greater than that obtained from other procedures such as radioisotope labeling with ^{3}H-NaBH$_4$ (Talent *et al.*, 1998).

This method was employed to test the two seedlots with a marked difference in their germination performance after more than 20 years of storage; seedlot 27153 (86% germination capacity) and seedlot 09035 (24% germination capacity) (Table 1). Protein oxidation status correlated very well with seed viability (Fig. 8); generally higher contents of oxidized proteins were associated with poor seed quality and low germinability. The level of oxidized proteins in the dry seeds of seedlot 27153 was significantly lower than that in dry seeds of seedlot 09035 (Fig. 8 A, C, G-d0). Oxidized proteins were gradually degraded during germination; this was more evident in seeds of seedlot 09035 (Fig. 8C, G-d2, G-d4, and RE). Interestingly, many proteins in the embryo become oxidized during post-germinative growth (Fig. 8A, C, R5mm of embryos). This is evidently related to growth because in megagametophytes the oxidized proteins are at the lowest levels at the R5mm stage (Fig. 8A, C, R5mm of megagametophytes). In a number of plant species, the production of H$_2$O$_2$, nitric oxide, hydroxyl radicals, and superoxide radicals occurs during germination and early growth (Bailly, 2004). New carbonylated proteins are also found in *Arabidopsis* seeds during germination and several of them are involved in glycolysis/gluconeogenesis pathways (Job *et al.*, 2005).
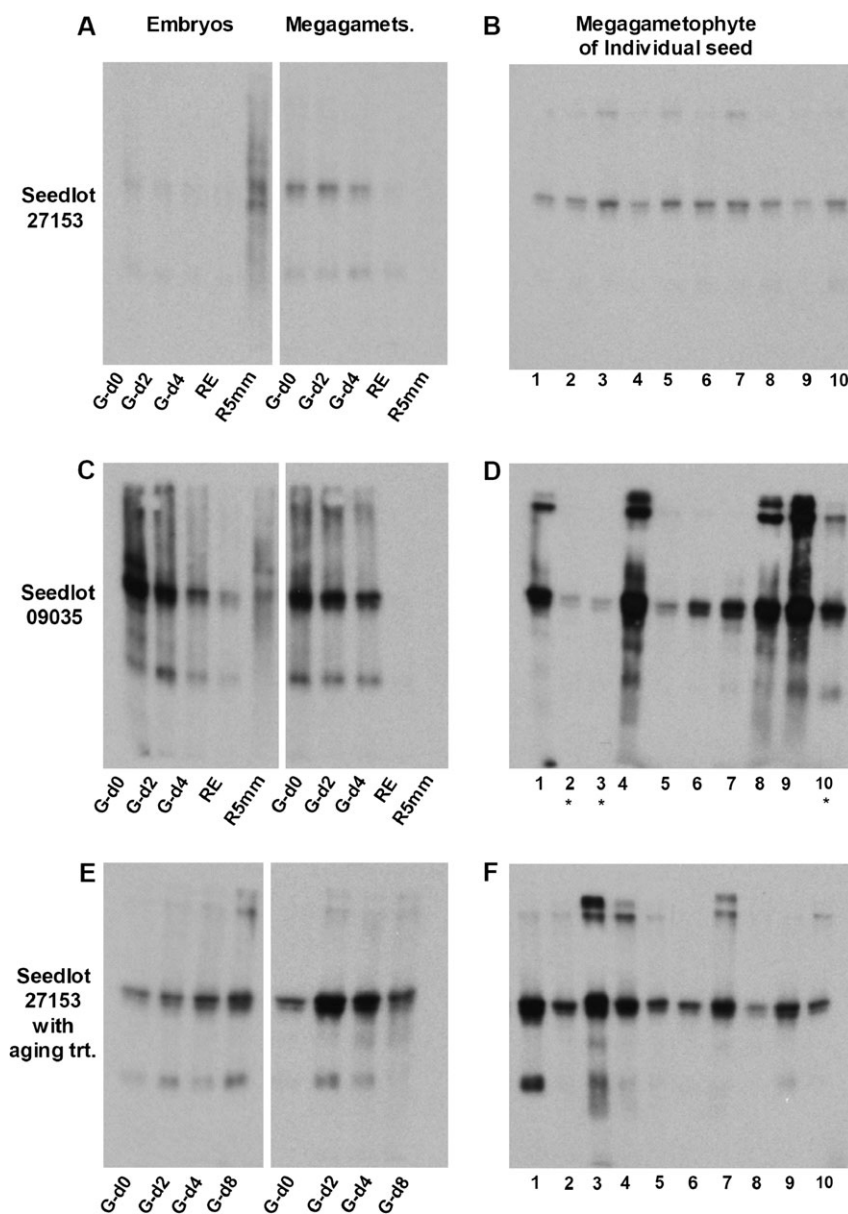
Fig. 8. Western blots showing oxidized proteins in western redcedar seeds in the dry state (G-d0), during germination (G-d2, G-d4, and G-d8, seeds incubated for 2, 4, and 8 d in germination conditions, respectively), at the completion of germination (RE, radicle emergence), and during early growth (R5mm, radicle 5 mm long). Each lane of the left panel blots (A, C, and E) contains 10 μg total proteins extracted from 30–50 seeds of the same stage. Each lane in the right panel blots (B, D, F) contains 10 μg total proteins from a single megagametophyte. The corresponding embryos in (B), (D), and (F) were incubated under germination conditions to determine their germinability. All embryos in (B) germinated while none germinated in (F). Embryos of 2, 3, and 10 in (D) germinated. Samples of (E) and (F) are seeds of seedlot 27153 following a 7 d accelerated ageing treatment (prior their transfer to germination conditions). All blots were exposed to the same X-ray film to ensure identical exposure times.

When seeds are examined individually, the results also show that proteins of the megagametophyte have much less oxidative modification if the corresponding embryo is able to germinate. All of the 10 embryos of seedlot 27153 germinated and their respective megagametophytes exhibited a low proportion of oxidized proteins (Fig. 8B) compared to those of the seedlot 09035 (Fig. 8D). For seedlot 09035, embryos of 2, 3, and 10 in Fig. 8D germinated and the proportion of proteins in their megagametophytes that are oxidized are much lower, especially of 2 and 3.

Seeds of seedlot 27153 were subjected to an accelerated ageing treatment at 41 °C for 7 d with 100% humidity. These seeds lost their ability to germinate (in a population of ~200 seeds); this was accompanied by a marked increase in the proportion of proteins that were oxidized (Fig. 8E, F versus Fig. 8A, B).

It is evident that protein oxidation status is a useful marker for seed quality and viability. It is especially useful when comparing seeds of different seedlots that show variations in their germination capacity due to storage-related deterioration.

Conclusions

In this study the possible correlation between the intensity of the static ^{13}C NMR line from unsaturated carbon atoms of storage oils and the germination capacity of western redcedar seeds is reported. This correlation, if further confirmed on a larger set of seeds, may represent a reliable means of monitoring and predicting germination in western redcedar seedlots in a quick and non-destructive manner. The approach may well be extended to screen seeds of other conifer species rapidly, particularly those with deep dormancy at maturity. For example, routine germination tests for seeds of yellow-cedar (*Chamaecyparis nootkatensis*) and western white pine (*Pinus monticola*) requires 3–4 months of a dormancy-breaking treatment (Feurtado *et al.*, 2003) and an additional 30 d to monitor germination. More advanced *in vivo* spectroscopic research tools, such as high-resolution ^{13}C MAS NMR spectroscopy and ^{1}H MR imaging provide additional information on the possible causes of seed deterioration.

A remaining question concerns the causes of the wide variability in deterioration rates in different seedlots of western redcedar. While some seedlots retain their full germination capacity during prolonged storage, others deteriorate very rapidly, often with a characteristic abrupt drop in the germination capacity (Kolotelo, 1996; Kolotelo *et al.*, 2001). A major cause of deterioration in these seeds is probably due to the oxidation of intracellular macromolecules (lipids and proteins). However, with respect to understanding some of the mechanistic features underlying seed deterioration, it is very difficult to prove cause and effect. There is an extremely complex cascade involved in cellular and subcellular deterioration changes; some early changes may be associated with the generation of reactive oxygen species, and a compromised capacity of antioxidant-related protective or restorative processes. Later, changes to subcellular constituents will be more associated with cell death per se, particularly the execution phase of cell death. It is possible that lipid peroxidation in seeds is both an early, and potentially signal-triggering event involved in seed deterioration. In addition, this process could contribute to the 'cascade' of changes associated with the execution phase of cell death. Further experiments are needed to prove the hypothesis.

A certain proportion of seeds from trees of natural stands or grown in tree seed orchards may not complete important maturation processes including the accumulation of protective molecules. Some of the protective mechanisms that prevent seeds from undergoing excessive oxidative damage include antioxidants, for example, tocopherols, that must be accumulated during seed development in order to extend the longevity of mature dispersed or stored seeds (Pukacka, 1991; McDonald, 1999; Sattler *et al.*, 2004). Seeds collected from different natural stands probably have different contents of stored antioxidants and the seedlots deficient in antioxidants would deteriorate more rapidly in storage. Pretreatment of seeds with antioxidants may be successful in protecting them from deterioration (Basu and Dasgupta, 1978; Vichnevetskaia and Roy, 1999; Blake and Smith, 2004). The oxidation of proteins, while a part of normal germination and post-germinative growth must be kept under strict control; it is quite conceivable that excessive protein oxidation is a hallmark of susceptibility to deterioration. The assay to detect oxidized proteins is a relatively simple one and may be amenable to seed screening.

Supplementary data

Additional static and MAS ^{1}H and ^{13}C NMR spectra recorded for western redcedar seeds, and results of the integration of the static ^{1}H and ^{13}C NMR spectra are provided as supplementary data in Figs s1–s5 and can be found at *JXB* online.

Acknowledgements

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