Journal of Experimental Botany, Vol. 56, No. 418, pp. 2253–2265, August 2005
doi:10.1093/jxb/eri224 Advance Access publication 4 July, 2005

Journal of Experimental Botany

RESEARCH PAPER

In vivo ^{13}C NMR metabolite profiling: potential for understanding and assessing conifer seed quality

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Received 22 December 2004; Accepted 12 May 2005

Abstract

High-resolution ^{13}C MAS NMR spectroscopy was used to profile a range of primary and secondary metabolites *in vivo* in intact whole seeds of eight different conifer species native to North America, including six of the Pinaceae family and two of the Cupressaceae family. *In vivo* ^{13}C NMR provided information on the total seed oil content and fatty acid composition of the major storage lipids in a non-destructive manner. In addition, a number of monoterpenes were identified in the ^{13}C NMR spectra of conifer seeds containing oleoresin; these compounds showed marked variability in individual seeds of Pacific silver fir within the same seed lot. In imbibed conifer seeds, the ^{13}C NMR spectra showed the presence of considerable amounts of dissolved sucrose presumed to play a protective role in the desiccation-tolerance of seeds. The free amino acids arginine and asparagine, generated as a result of storage protein mobilization, were detected *in vivo* during seed germination and early seedling growth. The potential for NMR to profile metabolites in a non-destructive manner in single conifer seeds and seed populations is discussed. It is a powerful tool to evaluate seed quality because of its ability to assess reserve accumulation during seed development or at seed maturity; it can also be used to monitor reserve mobilization, which is critical for seedling emergence.

Key words: Amino acids, conifer seeds, germination, imbibition, *in vivo* ^{13}C NMR spectroscopy, metabolite profiling, monoterpenes, storage lipids, sucrose.

Introduction

In the United States and Canada the multi-billion dollar forest industry relies heavily on conifers, the cornerstone of softwood lumber production. Reforestation of natural stands and the development of manageable forest plantations are dependent on the availability of high quality seeds. Establishing optimal seed storage conditions to ensure that conifer seeds retain quality and longevity is particularly crucial due to the irregularity of tree seed production in both natural and cultivated stands.

One characteristic that can affect propagation is seed dormancy. Seeds of conifers that require little or no period to break dormancy, for example, Sitka spruce (*Picea sitchensis*), western hemlock (*Tsuga heterophylla*), and western redcedar (*Thuja plicata*), obviously have a distinct advantage in quickly occupying sites following logging. However, there are several conifer species which produce seeds that exhibit deep dormancy at dispersal – including yellow-cedar (*Chamaecyparis nootkatensis*), true fir (*Abies*) species, and western white pine (*Pinus monticola*). For these species, the seed numbers can potentially decline dramatically; during the prolonged period that they need to break dormancy after shedding, the seeds are subject to consumption by birds and rodents or deterioration caused by fungal attack (Pawuk, 1993).

Reforestation efforts are becoming increasingly important, yet the forest industry is greatly affected by problems associated with conifer seed dormancy and seed quality. Forest nursery operations must accommodate the lengthy treatments (generally 3–4 months in duration, and sometimes

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not optimized) to break dormancy. Poor germination rates and impaired seedling growth occur frequently. This causes a significant loss of valuable seed; increased operational costs to nurseries can also occur because of irregular seedling performance (reviewed in Kermode, 2004, 2005*a*).

The tree seed orchard industry accounts for a significant amount of stock for reforestation. Again, the success of this industry can be limited by problems associated with seed viability and germination capacity. Problems can occur when environmental conditions do not mimic those in natural stands, particularly factors such as elevation and temperature, which can have a negative impact on seed development. Tree seed orchards involved in the production of seeds of lodgepole pine (*Pinus contorta*), yellow-cedar and other species often yield seeds that are shrivelled (indicative of poor seed fill) and exhibit poor germination rates and impaired seedling growth. Insects may exacerbate problems with poor seed production because they attack the reproductive structures of conifers throughout their development from cone bud initiation to seed maturity, and even during subsequent cone collection and seed extraction (Bates *et al.*, 2001; Kolotelo *et al.*, 2001).

Seedling vigour and the potential for early growth following germination is ultimately dependent upon processes that occurred earlier (during conifer seed development) particularly the accumulation of reserve materials (primarily storage lipids and proteins) in both the embryo and megagametophyte (Kermode, 2003, 2004, 2005*a*). Efficient mobilization of these reserves during and following germination is critical for seedling establishment.

In this work, recent results on *in vivo* ^{13}C NMR metabolite profiling in seeds of several conifer species native to the Pacific Northwest are summarized as a means of establishing this technique to monitor or assess seed quality. NMR spectroscopy has become a well-established research tool in plant biology (Shachar-Hill and Pfeffer, 1996; Krishnan *et al.,* 2005) due to its adaptability to profile non-invasively different classes of metabolites *in vivo* in living plants (Ratcliffe *et al.*, 2001; Ratcliffe and Shachar-Hill, 2001; Shachar-Hill, 2002). By eliminating the need for extractions, *in vivo* NMR provides valuable information about plant metabolism without the complication of requiring an extraction process that can result in metabolite alteration. *In vivo* NMR can further generate kinetic measurements (e.g. the kinetics of water uptake) (Terskikh *et al.*, 2005) and examine metabolic responses on the same plant rather than on a set of similar plants; thus it is a more direct approach for correlating physiological processes and metabolism. The ability of NMR spectroscopy to monitor the metabolic status of seeds non-destructively, and at any stage of their development, may help in generating markers that can serve as predictors of seedling success.

In vivo NMR can readily detect several primary and secondary metabolites in plant seeds. For example, it has been used as a tool for the qualitative and quantitative non-destructive analysis of oils in a variety of angiosperm and gymnosperm seeds including those of canola (Wollenberg, 1991; Hutton *et al.*, 1999), soybean (Haw and Maciel, 1983), lettuce, *Arabidopsis*, garden pea (Bardet *et al.*, 2001; Bardet and Foray, 2003), sunflower (Rutar, 1989; Wollenberg, 1991), and silver fir seeds (*Abies alba* Mill.) (Rutar *et al.*, 1989). ^{13}C NMR has also been tested as a means of following the mobilization of oil reserves during seed germination and early seedling growth *in vivo*, for example, in soybean (Colnago and Seidl, 1983; Ishida *et al.*, 1990), lodgepole pine and Sitka spruce seeds (Sayer and Preston, 1996). ^{1}H and ^{13}C NMR may be viable tools for the analysis of protein and carbohydrate content in whole seeds (Schaefer and Stejskal, 1974; Haw and Maciel, 1983; Ridenour, 1992; Ridenour *et al.*, 1996), although certain limitations exist for this endeavour. The ^{31}P NMR spectra of mature and germinating seeds detects and identifies a number of phosphorus-containing compounds, including phytates, phospholipids, nucleic acids, and inorganic P compounds (Kime *et al.*, 1982; Ishida *et al.*, 1990; Ridenour, 1992; Barba *et al.*, 1997; Gambhir *et al.*, 1997).

Even though the information obtained with *in vivo* ^{13}C NMR has proven useful, such data, particularly for conifer seeds, are scarce, with only a few pilot studies reported to date. Using seeds of eight conifer species, including six of the Pinaceae family and two of the Cupressaceae family, a thorough and systematic investigation of primary and secondary metabolites by *in vivo* ^{13}C NMR is presented. The focus has primarily been on oils (and on the breakdown products of storage lipids and proteins), as these are the most significant reserves that support the early post-germinative growth of conifer seeds. The long-term goal is to develop markers using NMR-based techniques that are useful predictors of conifer seed quality as well as germination and growth potential. It is clear that similar approaches can be applied to assess the quality of seeds of non-coniferous species.

Materials and methods

Seeds and seed treatments

Mature seeds of seven conifer species were supplied by the Tree Seed Center, British Columbia Ministry of Forests, Surrey, BC, Canada (Table 1; Fig. 1s is part of the supplementary data and can be found at JXB online). One seed lot of loblolly pine (*Pinus taeda* L.) was obtained from the Louisiana Forest Seed Company (Lecompte, LA). Seeds were stored at −20 °C until needed. Mature dry seeds were studied without any additional treatment. To obtain fully imbibed seeds of western white pine (*Pinus monticola* Dougl. ex D. Don), dry seeds were soaked in running water at 23 °C for 12 d. Seeds of western white pine were soaked, moist chilled, and germinated as described in Feurtado *et al.* (2003).

GLC analysis to determine total oil content and fatty acid composition

Seeds of four *Pinus* species (Table 1) were used for the extraction of total lipids and for fatty acid methyl ester (FAME) analysis. Data are

Table 1. *Seeds of the conifer species studied in this work*[a]

Species	Code	Seed lot	Collection year	Location	Elevation (m)	Latitude/ Longitude	Seeds (g^{-1})	Germination capacity (%)
Western white pine *Pinus monticola* Dougl. ex D. Don	Pw	08006	1981	Harbour Lake	909	51°31′/119°12′	59	87
Ponderosa pine *Pinus ponderosa* P. & C. Lawson	Py	32000	1989	Camoo Junction	480	50°50′/122°07′	16	88
Interior lodgepole pine *Pinus contorta* var. *latifolia* Dougl. ex Loud.	Pli	34984	1991	Bob Quinn Lake	700	57°10′/130°25′	362	96
Western hemlock *Tsuga heterophylla* (Raf.) Sarg.	Hw	03901	1979	Wakeman	400	51°17′/126°17′	426	86
Pacific silver fir *Abies amabilis* (Dougl.) Forbes	Ba	24904	1985	Chipmunk Creek	1415	49°08′/122°45′	34	78
Yellow-cedar *Chamaecyparis nootkatensis* (D.Don) Spach	Yc	32839	1995	Doran Lake	750	49°19′/125°17′	227	71
Western redcedar *Thuja plicata* Donn ex D. Don	Cw	60076	1996	Mt. Newton	219	50°08′/125°06′	836	82

[a] One seed lot of loblolly pine (*Pinus taeda* L.) (GP-77-JP-TX) was also used.

based on six replicates of approximately 60 mg of seeds (3 seeds of western white pine and loblolly pine, 16 seeds of lodgepole pine, and 1 ponderosa pine seed). The seed coats were not removed. All six replicates were prepared at the same time and analysed the same day.

The seeds were finely ground with a polytron and the oil was extracted with 3 ml of a chloroform/isopropanol (2:1, v/v) mixture containg 500 μg of 15:0 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 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 peaks was done according to Wolff *et al.* (2000) and confirmed by mass spectroscopy. An internal 15:0 TAG (triacylglycerol) standard was used to measure the total oil content of the samples. FAME compositions of seeds of the four *Pinus* species are presented in Table 1s (which is part of the supplementary data and can be found at JXB online) and these are in close agreement with data reported previously for the same *Pinus* species (Wolff *et al.*, 2000).

NMR spectroscopy

All NMR experiments were performed on a Bruker Avance DRX 360 spectrometer at the NMR facility of the Plant Biotechnology Institute NRC (Saskatoon, SK, Canada). The spectrometer was equipped with a double-band Bruker BL7 magic angle spinning (MAS) probe. Seeds were placed in a 7 mm o.d. ZrO_2 rotor and additionally packed with 0.2 mm glass beads to achieve better balance. The rotor was spun under the magic angle at a spinning speed of 3 kHz. A single seed was used for species with larger seeds (western white pine, ponderosa pine, loblolly pine, and Pacific silver fir). Unless stated otherwise, determinations for medium- and smaller-sized seeds used four seeds (yellow-cedar) and 6–12 seeds (lodgepole pine, western hemlock, and western redcedar).

^{1}H NMR spectra were recorded at room temperature at a resonance frequency of 360.13 MHz with a spectral width of 5 kHz applying a single-pulse sequence with a 10 μs rf pulse and a 5 s relaxation delay between scans. Time domain size of the spectra was 4 K with a typical number of accumulations of 32.

^{13}C NMR spectra were obtained at a resonance frequency of 90.56 MHz with a spectral width of 30 kHz under low-power proton decoupling and ^{13}C pulse width of 3 μs and 1 s relaxation delay between scans. The time domain size of the spectra was 32 K (acquisition time of 0.55 s) with 32 K zero-filling and 0.5 Hz exponential multiplication before Fourier transformation. The typical number of accumulations was 8 K.

^{13}C NMR chemical shifts were referenced to the signal from the terminal methyl groups of the linoleic fatty acid at 14.00 ppm used as an internal chemical shift reference. Signals were assigned according to Wenkert *et al.* (1976), Fan (1996), or following assignments given in earlier works cited above. The free-domain online Integrated Spectral Data Base System for Organic Compounds was also used (Hayamizu, 2001). ^{13}C NMR data for monoterpenes are reported in a recent review by Ferreira *et al.* (1998).

Quantitative analysis of the ^{13}C NMR spectra

A detailed description of the quantitative analysis of the ^{1}H and ^{13}C MAS NMR spectra for the determination of the fatty acid compositions of oilseeds is found in Wollenberg (1991) and Hutton *et al.* (1999). In the present study, a simplified approach was used as

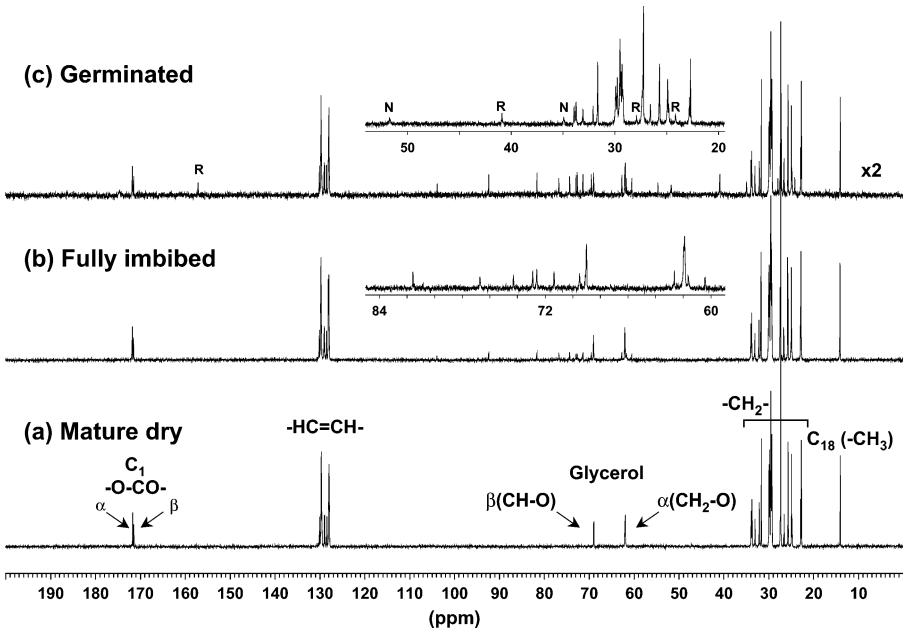


Fig. 1. ^{13}C MAS NMR spectra of single seeds of western white pine. (a) Dry seed. (b) Fully imbibed seed. Inset: expanded region of the spectrum showing signals from sucrose (see text). (c) Germinated/growing seed (the radicle length was *c*. 2 cm). Inset: expanded region of the spectrum showing signals from amino acids, asparagine (N) and arginine (R).

described below. The integrals from only three individual peaks in the whole ^{13}C NMR spectrum were used: (i) the peak at 26.5 ppm derived from C-4 carbons in pinolenic acid (I_1=P); (ii) the peak at 25.6 ppm, derived from C-11 carbons in pinolenic and linoleic acids (I_2=P+L), and (iii) the closely overlapping resonances at 24.8–24.9 ppm derived from C-3 carbons in the pinolenic, linoleic and oleic acids (I_3=P+L+O). Under the experimental conditions used in this study, these resonances had similar NMR properties (relaxation behaviour, nuclear Overhauser enhancement effects), thus allowing their use for quantitative analysis with a moderate degree of accuracy. Simple algebraic combinations of these three integral values gave a relative amount of each individual fatty acid in the mixture (P=I_1; L=I_2-I_1; O=I_3–I_2; P+L+O=100%). All results are based on the average ±SD of six independent NMR experiments (replicates). Each replicate contained a single seed for western white pine, ponderosa pine, and loblolly pine, and 12 seeds for lodgepole pine.

From analysis of the NMR spectra only the molar ratios of fatty acids in the oil can be found. If NMR results are to be compared with GLC data, the molar ratios have to be converted into weight ratios (wt.%).

To determine the total oil content in seeds by ^{13}C NMR a reference sample is needed with similar NMR properties and a known amount of oil. In the present study, seeds of western white pine were used as the intensity reference sample assuming an oil content measured by GLC of 35.4±0.5 wt.% fresh weight. NMR spectra for all other species were recorded under the same conditions and the integral intensities of the spectra were compared. All NMR results are based on the average of six replicates ±SD.

Results and discussion

Conifer species studied and typical spectra for western white pine seeds at different stages

In this work, the results of *in vivo* ^{13}C NMR metabolite profiling in seeds of several conifer species native to the Pacific Northwest (Table 1; Fig. 1s is part of the supplementary material and can be found at JXB online) are summarized. Western white pine seeds serve to illustrate typical high-resolution *in vivo* ^{13}C MAS NMR spectra at different stages; mature dry, imbibed, and germinated seeds (seedling growth phase) (Fig. 1). While these data are explained in more detail below, a few points are worth noting. The spectra were recorded by direct excitation of ^{13}C spins with low-power radio frequency pulses combined with low-power broadband proton decoupling (liquid-state NMR set-up). Under these conditions, only highly-mobile liquid-like components such as triglycerides (oils) can be observed. To detect ^{13}C NMR signals from solid constituents of the seed (e.g. the storage proteins and starch reserves of the embryo and megagametophyte and the polysaccharides/polyphenolics of the seed coat), solid-state ^{13}C MAS NMR techniques (MAS combined with cross-polarization transfer) are required (Fig. 2s, which is part of the supplementary material can be found at JXB online) (Haw and Maciel, 1983; Bardet *et al.*, 2001). In the present study, the focus has been on the mobile components of the seed (i.e. those metabolites present in liquid or highly mobile states). Because some of the materials used included imbibed and germinated seeds, the use of time-demanding NMR techniques involving slow repetition times and gated decoupling, as well as INEPT or DEPT pulse sequences, were avoided. Prolonged MAS experiments have notable adverse effects on seed quality due to water loss, which consequently leads to seed damage/metabolite changes. Nonetheless, the techniques used here generated accurate quantitative ^{13}C NMR measurements.

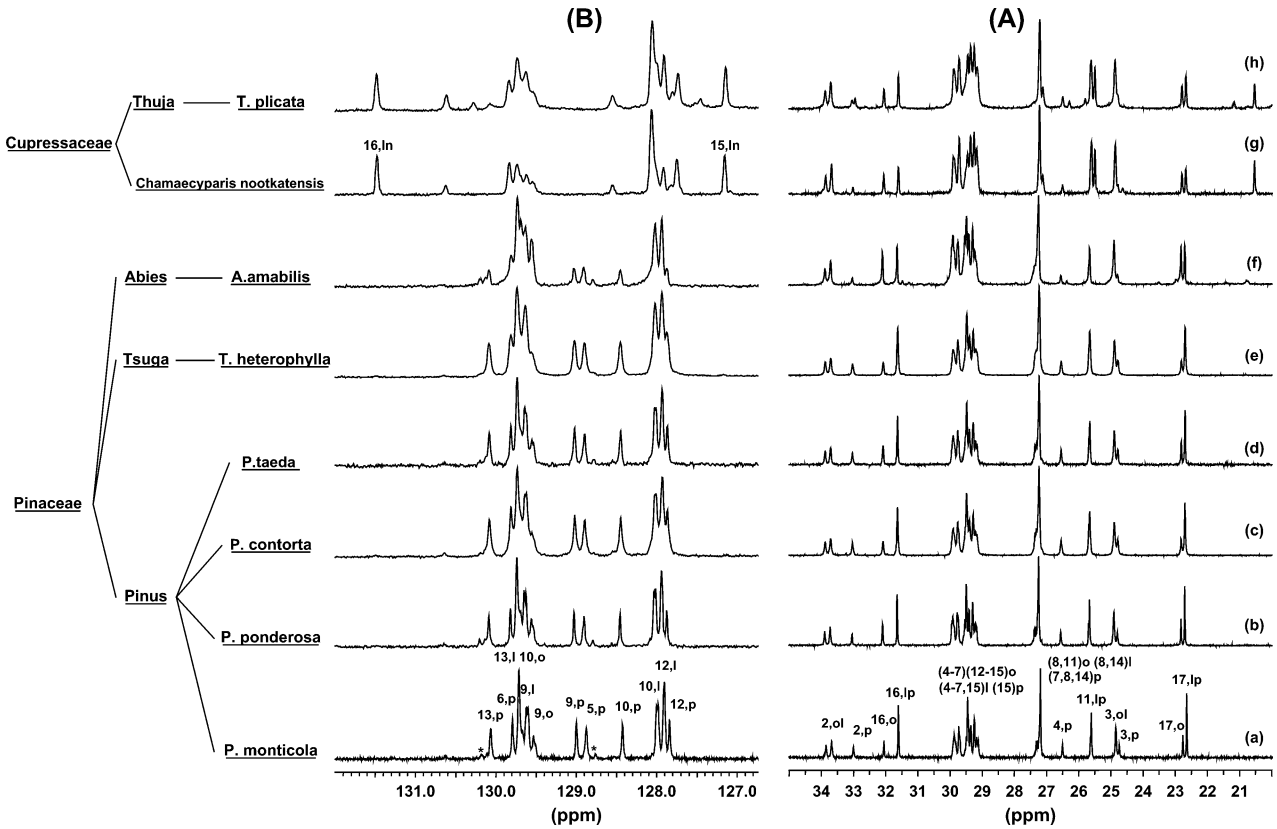


Fig. 2. Expansion of the methylene carbon region (A) and the olefin region (B) in the ^{13}C MAS NMR spectra of dry seeds of conifer species: (a) western white pine (the spectrum is given together with assignment of individual resonances, the chemical shift values are summarized in Table 2); (b) ponderosa pine; (c) lodgepole pine; (d) loblolly pine; (e) western hemlock; (f) Pacific silver fir; (g) yellow-cedar; (h) western redcedar. (Asterisks) Signals from 5,9–18:2 fatty acid. In the olefin region of the spectra for yellow-cedar and western redcedar seeds, two prominent resonances characteristic of α-linolenic acid (ln) are seen at δ 131.48 (C-16) and 127.15 (C-15). Two other signals at δ 130.74 (C-18) and 128.63 (C-17), are from 5,11,14,17–20:4 fatty acid. Other readily identifiable ^{13}C resonances from α-linolenic fatty acid are δ 14.19 (C-18), 20.51 (C-17), 25.48 (C-14), 127.74 (C-10) and 128.06 (C-13). The spectrum of western redcedar seed is additionally complicated by the presence of considerable amounts of oleoresin.

Although the results of Fig. 1 are discussed more fully below, the western white pine seed spectra show a general progression of changes in the major mobile metabolites of the conifer seed: in the dry seed all mobile macromolecules are oils (Fig. 1a); sucrose becomes detectable in the imbibed seed in which it probably confers an ability of these dormant seeds to remain desiccation tolerant; see later discussion (Fig. 1b); breakdown products of reserve mobilization become detectable in seedlings during early post-germinative growth (Fig. 1c).

^{13}C NMR for analysis of oil reserves in mature dry conifer seeds

Oils (storage triacylglycerols) are the most abundant storage reserve of coniferous seeds. During seed development they accumulate in oil bodies in the storage parenchyma cells of the embryo and megagametophyte (Bewley and Black, 1994; Huang, 1996; Kermode, 2003, 2005*a*); their breakdown during and following germination provides a source of energy and carbon to support early post-germinative seedling growth. Storage proteins and carbohydrates account for the remaining reserve compounds, with the latter comprising only 2–6%. In seeds of *Pinus* species, oil constitutes up to 40–60% of the fresh weight of the seed (Wolff and Bayard, 1995). Some seed oils of *Pinus* species contain rare fatty acids (e.g. Δ5-olefinic acids) that are important in the taxonomy and phylogeny of the genus; they are being assessed for their nutritional value and possible physiological and biochemical activity (Wolff *et al.*, 1996).

The storage triglycerides of oil bodies in conifer seeds are liquid at room temperature, i.e. they are genuine oils (not fats), allowing their detection by ^{13}C NMR spectra *in vivo*. An example of the spectra recorded for a single mature dry seed of western white pine is shown in Fig. 1a. Notably, the spectra resemble that recorded for common oilseeds, like sunflower (Rutar, 1989) and *Brassica napus* (Wollenberg, 1991; Hutton *et al.*, 1999), with the exception of the presence of characteristic signals from the pinolenic fatty acid (5,9,12–18:3), an isomer of linolenic acid, which is abundant in seeds of the Pinaceae including western white pine. ^{13}C NMR spectra of dry conifer seeds consist

primarily of two groups of lines from hydrocarbon chains of fatty acids: one group derives from olefin carbon atoms (125–132 ppm) and the other group derives from aliphatic carbon atoms (21–35 ppm). Solitary lines are from the fatty acid terminal methyl groups, triglyceride backbone carbons, and carboxyl carbons.

Figure 2 shows the spectra of the fatty acids of single dry seeds (western white pine in (a) and the other species examined in (b–h)) in more detail, i.e. expanded in the methylene carbon and olefin carbon regions. Individual carbon resonances are assigned within the spectrum for a single western white pine seed in Fig. 2a and the chemical shift values are summarized in Table 2. In many cases the spectral resolution was better than 0.01 ppm with a typical line width of 2–2.5 Hz comparable with the resolution in the liquid-state NMR spectroscopy. Indeed, the resolution was sufficient to resolve *sn-1,3* and *sn-2* positional isomers for oleic and linoleic fatty acids (Table 2). The spectral resolution further improves in fully imbibed seeds. 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 (Fig. 3).

Three major fatty acids were readily identified in ^{13}C NMR spectra of seeds of western white pine seeds and other *Pinus* species: oleic acid (9–18:1), linoleic acid (9,12–18:2), and pinolenic acid (5,9,12–18:3) (Fig. 2; Table 2). GLC analyses further revealed that these three fatty acids account for about 85 wt.% of the total seed oil content (see Table 1s of the supplementary material which can be found at JXB online). The less abundant fatty acids, each representing 2–4 wt.% or less of the total oil content, were undetectable with ^{13}C NMR under the experimental conditions employed here due to insufficient sensitivity and overlap with the dominant resonances. The ratios between different fatty acids (oil composition) can be determined by comparing the integral intensities of the individual resonances (see Materials and methods); this method yields reasonably accurate data. Regardless of whether ^{13}C NMR or GLC is used as the method for quantification, it is apparent that the oil compositions were similar in seeds of all four *Pinus* species studied, with only minor variations in the relative amounts of the individual fatty acids. Remarkably, ^{13}C NMR was successful in detecting these minor variations in the corresponding spectral patterns and the two data sets derived from GLC and NMR correlated almost perfectly (Table 3; Fig. 3s of the supplementary data which can be found at JXB online).

Table 2. *Assignment of lines in the ^{13}C NMR MAS spectra of tryglycerides (oils) in the seed of western white pine*

Region	δ^{13}C	Assignment, fatty acid[a]
	14.00[b]	C-18, LP
	14.04	C-18, O
Methylene carbons	22.66	C-17, LP
	22.78	C-17, O
	24.76	C-3, P
	24.86	C-3, OL
	25.60	C-11, LP
	26.51	C-4, P
	27.2–27.4	C-(8,11), O; C-(8,14), L; C-(7,8,14), P
	29.1–29.9	C-(4–7)(12–15), O; C-(4–7,15), L; C-15, P
	31.60	C-16, LP
	32.06	C-16, O
	33.00	C-2, P (*sn-1,3*)
	33.67	C-2, OL (*sn-1,3*)
	33.85	C-2, OL (*sn-2*)
Glycerol carbons	61.87	CH_2, OL (*sn-1,3*)
	61.92	CH_2, P (*sn-1,3*)
	68.96	CH, OL (*sn-2*)
Olefin carbons	127.87	C-12, P
	127.93	C-12, L
	128.00, 128.02	C-10, L (*sn-1,3; sn-2*)
	128.08	5,9–18:2 fatty acid
	128.44	C-10, P
	128.79	5,9–18:2 fatty acid
	128.90	C-5, P
	129.02	C-9, P
	129.52, 129.54	C-9, O (*sn-1,3; sn-2*)
	129.61, 129.63	C-9, L (*sn-1,3; sn-2*)
	129.68	C-10, O
	129.73	C-13, L
	129.81	C-6, P
	130.08	C-13, P
	130.12, 130.19	5,9–18:2 fatty acid
Carbonyl carbons	171.46	C-1, OL (*sn-2*)
	171.68	C-1, P (*sn-1,3*)
	171.72	C-1, OL (*sn-1,3*)

[a] Fatty acids: O, oleic, 9–18:1; L, linoleic, 9,12–18:2; P, pinolenic, 5,9,12–18:3.
[b] Used as an internal reference of the chemical shifts.

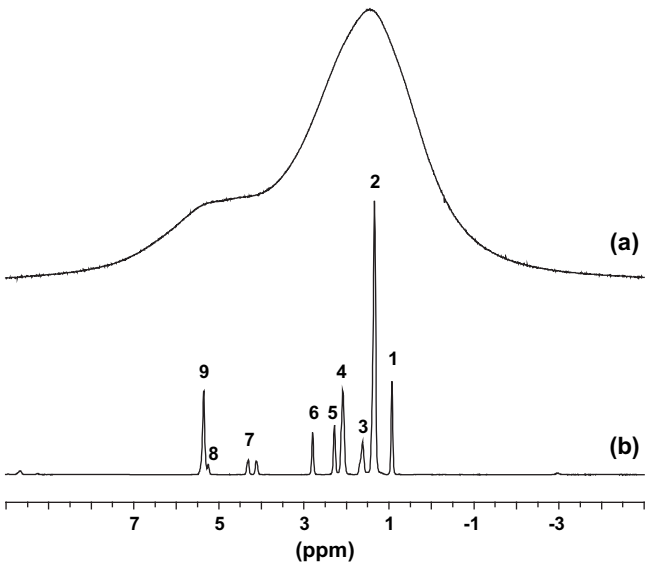


Fig. 3. ^{1}H NMR spectra of a single dry seed of western white pine: (a) static; (b) under moderate magic angle spinning (3 kHz). Individual resonances in the MAS spectrum (b) are assigned as follow: (1) -CH_3; (2) -$(CH_2)_n$-; (3) -$COCH_2CH_2$-; (4) -HC=$CHCH_2$-; (5) -$COCH_2$-; (6) -HC=CH-CH_2-HC=CH-; (7) -CH_2O-; (8) -CHO-; (9) -HC=CH-.

Table 3. *Oil content (mg seed^{-1} ±SD)[a] and composition of major fatty acids (wt.% ±SD)[b] in seeds of four pine species as determined by gas-liquid chromatography (GLC) and ^{13}C NMR spectroscopy*

		Western white pine	Ponderosa pine	Lodgepole pine	Loblolly pine
Oil content (mg seed^{-1})[c]	GLC	5.78[d]±0.08	17.63±3.13	1.10±0.04	3.02±1.31
	NMR	5.78[d]±0.50	15.78±2.21	0.83±0.05	5.14±0.18
9–18:1 oleic	GLC	19.3±0.9	24.7±0.7	17.0±0.2	21.7±1.2
	NMR	18.8±2.6	26.1±2.3	16.2±2.1	23.2±2.4
9,12–18:2 linoleic	GLC	57.8±0.7	54.5±0.8	55.0±0.2	55.2±1.5
	NMR	58.7±3.0	51.9±1.4	56.6±1.2	52.3±2.7
5,9,12–18:3 pinolenic	GLC	22.9±0.5	20.8±0.9	28.0±0.5	23.1±0.7
	NMR	22.5±0.8	22.0±1.5	27.2±1.9	24.5±1.4

[a] All the data are based on the average ±SD of six replicates. For GLC each replicate of western white pine and loblolly pine contained three seeds, one seed of ponderosa pine, and 16 seeds of lodgepole pine. For NMR experiments each replicate of western white pine, ponderosa pine, and loblolly pine contained a single seed, and 12 seeds of lodgepole pine. The correlation between GLC and NMR data is presented graphically in Fig. 3s of the supplementary materials which are available at JXB online.

[b] 9–18:1 + 9,12–18:2 + 5,9,12–18:3= 100%.

[c] The seed coat was not removed from the seeds.

[d] Used as an intensity standard in quantification of the ^{13}C NMR spectra.

Similar spectra for other representatives of the Pinaceae family were generated using single mature dry seeds of western hemlock (*Tsuga heterophylla*) and Pacific silver fir (*Abies amabilis*) (Fig. 2) and using Siberian stone pine (*Pinus sibirica* Du Tour) and Interior white spruce (*Picea glauca* (Moench) Voss) seeds (data not shown). In the spectrum for Pacific silver fir seeds, the relative amounts of oleic and linoleic fatty acids were somewhat increased at the expense of pinolenic acid (Fig. 2f). Differences in the ^{13}C NMR spectra were much more pronounced in seeds of the non-Pinaceae family, i.e. in seeds of the two species of the Cupressaceae family, yellow-cedar (*Chamaecyparis nootkatensis*) and western redcedar (*Thuja plicata*). In particular, α-linolenic acid (9,12,15–18:3) was far more abundant than in seeds of the Pinaceae family, while pinolenic acid (5,9,12–18:3) was a minor component (Fig. 2). Fatty acid compositions of conifer seeds have been used for chemotaxonomic grouping of the major families and to differentiate further between several genera of the Pinaceae (Wolff *et al.*, 1997). ^{13}C NMR spectroscopy could serve a similar function (Fig. 4), and may further aid in the non-destructive identification of seeds of questionable and/or unknown origin.

Because the intensity of the ^{1}H and ^{13}C NMR spectra is proportional to the amount of the oil in the sample, NMR is a convenient tool to measure the total oil content in seeds *in vivo* and low-resolution ^{1}H NMR has been approved by the American Oil Chemists' Society for this purpose (AOCS methods Ak3-94, Ak4-95). The close correlation between ^{13}C NMR and GLC data on the oil content of *Pinus* seeds (Table 3) suggests that ^{13}C NMR is a convenient and accurate method for oil determinations in conifer seeds, but has the additional advantages of being non-destructive and less time-consuming. The spatial distribution of oils in conifer seeds can also be assessed non-invasively via ^{1}H magnetic resonance microimaging (MRI) (Terskikh *et al.*, 2005).

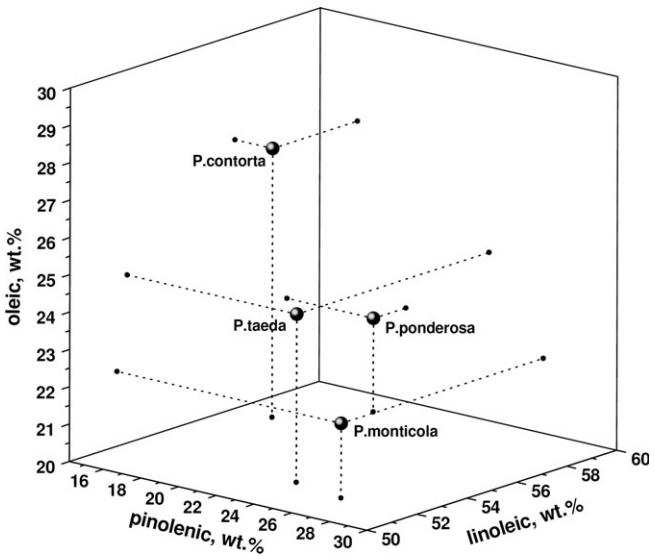


Fig. 4. Chemotaxonomic differentiation using ^{13}C NMR fatty acid composition data for four *Pinus* species.

^{13}C NMR for detection of breakdown products of reserve mobilization in germinated conifer seeds

In most conifer seeds, mobilization of the major protein and lipid storage reserves commences within the embryo during germination and is probably important for early seedling establishment. In the megagametophyte (which generally comprises the bulk of the seed), it commences after radicle elongation, i.e. it is a post-germinative event. The breakdown products are exported by the megagametophyte and are taken up by the developing seedling where they are used as a nutritive source. Free amino acids, especially arginine, derived from storage protein mobilization, accumulate in both the megagametophyte and seedling and this is accompanied by a marked increase in arginase activity within the cotyledons and epicotyl of the seedling (King and Gifford, 1997; Todd *et al.*, 2001). Cytosolic NADP^{+}-linked

isocitrate dehydrogenase generates 2-oxoglutarate and thus provides the carbon skeleton for ammonia assimilation and glutamate synthesis through the GS/GOGAT (glutamine synthase/glutamate synthase) cycle (Palomo *et al.*, 1998). Storage lipids (triacylglycerols) are metabolized by lipases to yield free fatty acids and glycerol. Most of the glycerol and free fatty acids are metabolized to sucrose which is exported to the growing axis (Stone and Gifford, 1999).

Reserve mobilization during early post-germinative growth is critical for seedling emergence. ^{13}C NMR readily detects some of the products of reserve mobilization; hence the rate of appearance of these breakdown products following germination (detected by this method) could serve as a marker or index of the capacity for seedling emergence/vigour. Developing markers for seedling vigour is important for different conifer seedlots that exhibit marked variation in quality.

Sucrose signals in the ^{13}C MAS NMR spectra are first detected during imbibition of western white pine seeds (Fig. 1b); signals corresponding to sucrose are also present during early post-germinative growth (Fig. 1c). Sucrose was identified by the presence of 12 new lines (close to the region corresponding to glycerol) (δ 60.4 (C-6), 61.6 (C-1′), 62.6 (C-6′), 69.5 (C-4), 71.3 (C-2), 72.6 (C-5), 72.9 (C-3), 74.3 (C-4′), 76.7 (C-3′), 81.5 (C-5′), 92.3 (C-1), and 103.8 (C-2′). Additional lines from sucrose were also detected by the ^{1}H MAS NMR spectra in the region of 3.5–3.9 ppm (data not shown). Similarly, dissolved sucrose was found in the ^{13}C NMR spectra of germinating seeds of silver fir (Rutar *et al.*, 1988) and lodgepole pine (Sayer and Preston, 1996), and in germinated non-dormant seeds like soybean (Colnago and Seidl, 1983; Ishida *et al.*, 1987; Rutar, 1989). Sucrose probably accumulates during the development of the western white pine seed where it plays a role in the acquisition of desiccation tolerance (see later discussion); upon imbibition of the mature dry seed, the sucrose becomes mobile and is detected in the ^{13}C MAS NMR spectra (Fig. 1b). During post-germinative growth, the sucrose arises from reserve (oil) mobilization, and during this stage the depletion of oil reserves of the western white pine seed was clearly visible in the spectrum. In this regard, the most notable change (i.e. a decreased intensity of bands) occurred in the olefin–carbon region; this is particularly evident in Fig. 5, in which the decreased intensity of these signals correlates with increasing radicle length. Despite the decline of oil reserves during mobilization, the ratios of fatty acids (i.e. the fatty acid composition of the seed) did not change significantly.

New signals that did not correspond to oils or sucrose were also detected in the spectra of post-germinative seedlings (inset in Fig. 1c); the chemical shifts unequivocally identified two amino acids: arginine (R) (^{13}C NMR: δ 24.1 (C-γ), 27.9 (C-β), 40.8 (C-δ), 54.6 (C-α), 157.1 (C=N), 174.6 (broad, C=O)), and asparagine (N) (^{13}C NMR: δ 34.9 (C-β), 51.7 (C-α), 174.6 (broad, C=O)).

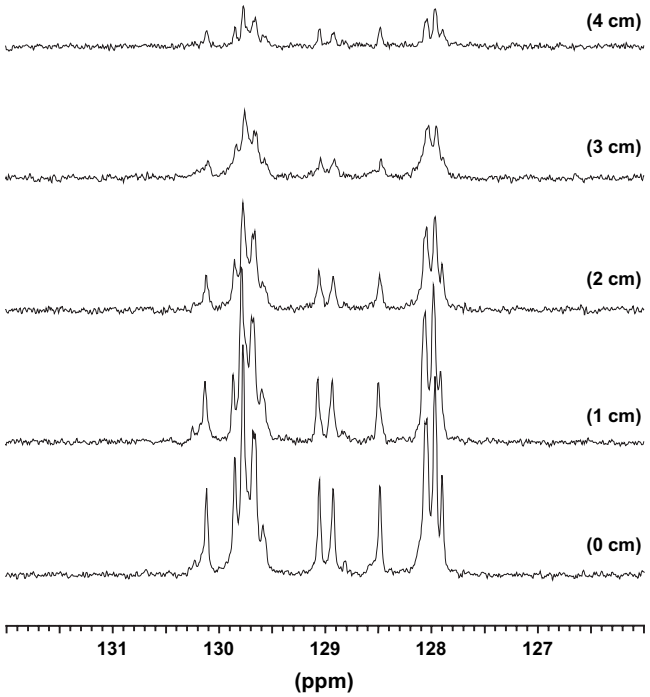


Fig. 5. The olefin-carbon region in the *in vivo* ^{13}C MAS NMR spectra of germinated western white pine seeds during early seedling growth demonstrating a gradual decline in the signals corresponding to triglycerides. Approximate length of the radicle is shown with each spectrum.

Arginine in particular was easily identified by a characteristic signal from the terminal N=C moiety at 157.1 ppm (Fig. 1c). Appearance of these two amino acids in the ^{13}C MAS NMR spectra is expected, based on studies of storage protein mobilization in conifer seeds (King and Gifford, 1997).

Amino acid signals in the ^{13}C NMR spectra of germinated seeds were also reported for soybeans (Rutar, 1989; Ishida *et al.*, 1990) and lodgepole pine seeds (Sayer and Preston, 1996). However, in these earlier studies the resolution and signal-to-noise ratios were not sufficient to allow for amino acid identification.

In mature seeds of conifers, storage proteins accumulate in protein storage vacuoles (1–20 μm) as a proteinaceous matrix and a water-insoluble protein aggregate (the crystalloid) (Bewley and Black, 1994; Kermode, 2003). Due to their solid nature, ^{13}C NMR signals from protein storage vacuoles can only be detected when a cross-polarization (CP/MAS) technique is applied (Haw and Maciel, 1983). As discussed above, experimental conditions in this study only detect signals from highly mobile species in the liquid state and the appearance of signals corresponding to free amino acids during early seedling growth can clearly be attributed to protein reserve breakdown. Generally, only two amino acids (arginine and asparagine), were detected by *in vivo* ^{13}C NMR; however, minor amounts of valine and alanine were also detected in some cases (characteristic

signals in the methyl region at 16.5–17.2 ppm possibly correspond to valine (C-γ) and alanine (C-β) amino acids). Destructive methods for the quantification of amino acids in germinated loblolly pine seeds detected significant amounts of glutamine, in addition to arginine and asparagine (King and Gifford, 1997). It is very likely that certain amino acids (possibly including glutamine) are undetectable by ^{13}C NMR because they are not in a 'free liquid state'; in addition, the stage of post-germinative growth may determine which free amino acids predominate. Additional ^{13}C MAS NMR experiments will address this question.

The present study demonstrates the advantages of *in vivo* ^{13}C MAS NMR spectroscopy to study aspects of reserve mobilization. Because of its non-destructive nature, the potential exists for directly relating the process of reserve mobilization to seedling vigour. This technique may well uncover additional basic information on how protein reserves are mobilized in seeds, a subject which is still poorly understood.

^{13}C NMR for detection of stress protectants, sucrose and oleoresin, in dry and imbibed conifer seeds

Another important process during seed development that has an impact on seedling vigour is the accumulation of various macromolecules (including proteins and sugars) that act as protectants, allowing seeds to survive desiccation and other stresses. Sucrose and other sugars (e.g. raffinose and stachyose) that accumulate during seed development (Konradova *et al.*, 2002) may help to preserve membranes and other cellular constituents during maturation drying (Amuti and Pollard, 1977; Leopold and Vertucci, 1986; Carpenter *et al.*, 1987; Williams and Leopold, 1989; Leprince *et al.*, 1990; Koster, 1991; Blackman *et al.*, 1992; Close, 1996; Wolkers *et al.*, 1999; reviewed in Bewley and Black, 1994; Kermode and Finch-Savage, 2002; Kermode, 2005*b*).

The first appearance of sucrose in the ^{13}C NMR spectra occurred following imbibition of the western white pine seed, during which time there were no net changes in the oil content or composition (Fig. 1b). Sucrose of the imbibed western white pine seed is obviously in the dissolved 'liquid' state, different from its state in the mature dry seed in which it is undetectable on the ^{13}C NMR spectra. Western white pine seeds are deeply dormant at maturity and operational dormancy-breakage requires not only a lengthy (e.g. 12 d) water soak (imbibition), but a subsequent and lengthy moist-chilling treatment, typically of 98–119 d (Feurtado *et al.*, 2003). In natural stands, 'moist chilling' may be interrupted by periods of dehydration. Thus, one would expect a preservation of stress protectants during this lengthy period in order for the seed to survive following shedding or dispersal when it can be subjected to cycles of desiccation–rehydration and other environmental stresses. Indeed, the ^{13}C NMR spectra of imbibed western white pine seeds showed no detectable changes in the oil and sucrose sub-spectra during 3 months of moist-chilling. The availability of dissolved sucrose in the seeds during moist chilling is also probably important to sustain minimal metabolic activity vital for survival. Despite no detectable changes in the oil subspectra during imbibition/moist chilling, the possibility cannot be ruled out that some of the sucrose derives from very limited oil reserve breakdown (Mullen and Gifford, 1997).

Many conifers produce oleoresin, a complex mixture of mono-, sesqui- and diterpenoids, as a primary defence against certain insect pests and fungal pathogens (Phillips and Croteau, 1999). Synthesis occurs in a constitutive and inducible manner and the oleoresin generally accumulates at the wound site to kill invaders and both flush and seal the injury (Trapp and Croteau, 2001). Although toxic to certain insects (e.g. bark beetles) and their vectored fungal pathogens, oleoresin also plays a central role in the chemical ecology of these boring insects, from host selection to pheromone signalling and tritrophic level interactions (reviewed in Trapp and Croteau, 2001). In some conifers, oleoresin is produced not only in wood, bark, or needles, but also in seeds. In seeds of the true firs, western hemlock and western redcedar, the oleoresin accumulates in specialized resin vesicles which form early in seed development within the middle or outer layer of the seed coat; generally the resin vesicles are more abundant on the lower surface of the seed (formerly in contact with the ovuliferous scale) (Kolotelo, 1997). The role of resin vesicles of seeds is unknown. They have been implicated in various processes including pathogen defence, preventing germination in the fall, protecting the embryo and seed from excessive drying and 'coat-imposed' dormancy (Kolotelo, 1997). When resin vesicles are damaged during seed processing, the result is generally a decline in seed germination.

Because the oleoresin of resin vesicles is a low-viscous liquid, it can be detected by high-resolution ^{13}C MAS NMR spectra *in vivo* (Fig. 6). The most abundant terpenes detected in seeds of Pacific silver fir, western hemlock, and western redcedar were the monoterpene olefins ($C_{10}H_{20}$), including limonene, myrcene, α- and β-pinenes, and thujone (Table 4). Thujone, a monoterpene was found in considerable amounts exclusively in seeds of western redcedar; as reported for *Thuja* spp., in oil extracted from western redcedar needles, thujone accounts for 65–85 wt.% of all terpenes (Rudloff *et al.*, 1988). Two monoterpenes, α-pinene and β-pinene, appeared to be present in about equal proportions in seeds of western hemlock (Table 4); these same monoterpenes predominated in emissions from western hemlock trees (Pressley *et al.*, 2004).

The most complex mixture of monoterpenes was found in seeds of Pacific silver fir in which limonene, myrcene, α-pinene, and β-pinene were detected (Table 4). However, these compounds occurred in varying amounts in individual seeds of the same seed lot (Fig. 6), such that generally

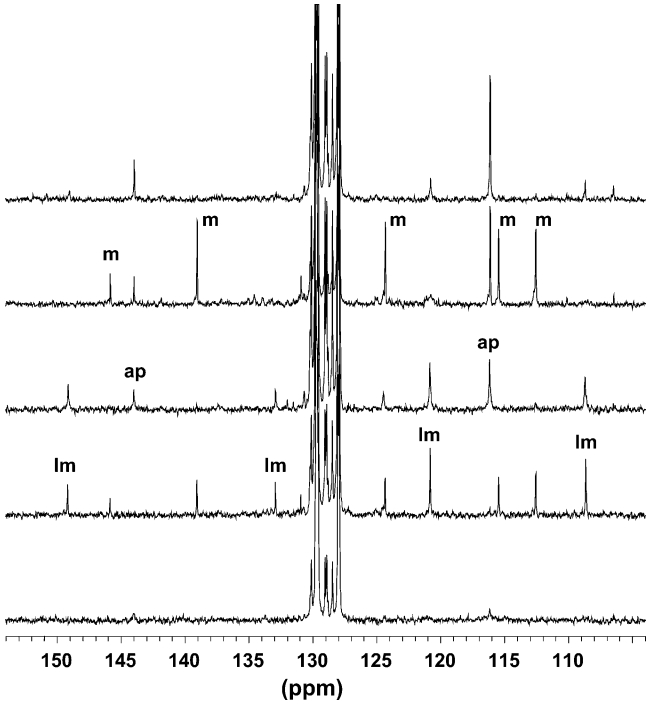
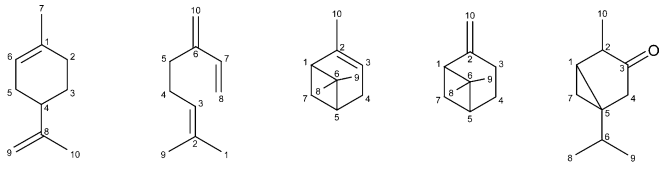


Fig. 6. ^{13}C MAS NMR spectra demonstrating the variability in monoterpene composition of single seeds of Pacific silver fir from the same seedlot. (lm) limonene; (m) myrcene; (ap) α-pinene. Chemical shift values for individual terpenes are summarized in Table 4. A group of intense signals in the 131–127 pm region is from triglycerides (oils).

Table 4. *Spectral assignment (δ ^{13}C, ppm) of major monoterpenes detected in ^{13}C NMR MAS spectra of dry seeds of Pacific silver fir[1], western redcedar[2] and western hemlock[3]*

Site	Limonene[1]	Myrcene[1]	α-Pinene[1,3]	β-Pinene[1,3]	Thujone[2]
1	132.9	26.8	47.0	51.7	25.1
2	120.8	124.3	144.0	150.8	47.0
3	30.6	130.9	116.1	23.6	219.1
4	41.1	25.6	31.2	23.5	39.2
5	27.9	31.4	40.8	40.5	29.2
6	30.9	145.8	37.8	40.5	32.9
7	23.4	139.0	31.4	27.9	18.6
8	149.2	115.4	26.3	26.0	19.6
9	108.6	17.5	20.8	21.7	19.9
10	20.6	112.5	22.9	106.4	17.9



only one or two monoterpenes predominated. Previously, ^{13}C NMR detected only limonene in silver fir seeds (Rutar *et al.*, 1988). This is probably the result of the large variability that exists between individual seeds (Fig. 6) and from one seed lot to another. Notably, some of the seeds contained little or no detectable monoterpenes, which may be the result of damage during seed processing in which resin vesicles can break or collapse. The variability in monoterpenes of individual Pacific silver fir seeds may originate from a pre-existing variability in monoterpene production in individual parent trees, which is a known phenomenon in *Abies* spp. (Zavarin *et al.*, 1973). It is unclear whether the amount and composition of oleoresin influences seed survival and post-germinative performance. The capability of ^{13}C NMR to determine the dominant monoterpenes of individual conifer seeds in a non-destructive manner may aid in seed selection for breeding programmes.

Conclusions

The successful establishment of the new plant, both temporally and spatially, as well as the vigour of the young seedling is largely determined by processes that occurred earlier, during seed maturation. In particular, the accumulation of reserves ultimately allows the seedling to survive before it commences photosynthesis and autotrophic growth; likewise the synthesis of protective compounds allows the seed to withstand some degree of water loss and to survive for long periods under adverse environmental conditions. Most seeds also accumulate toxic compounds during development, many of which confer some resistance to pathogens, insects, and herbivores. A systematic ^{13}C NMR profiling of several primary and secondary metabolites detectable *in vivo* has been performed in whole intact seeds of eight conifer species as a means of selecting suitable 'markers' for seedling vigour. *In vivo* ^{13}C NMR provided reliable quantitative information on the total oil content of conifer seeds as well as allowing for accurate determinations of the fatty acid composition of the major storage lipids (triglycerides). Furthermore, the data generated by NMR correlated very well with that generated by the more traditional analyses (GLC and lipid quantification of seed extracts by biochemical methods). *In vivo* ^{13}C NMR was also successful in detecting and identifying some of the products of reserve mobilization during early post-germinative seedling growth, including the free amino acids, arginine and asparagine, released by cleavage of storage protein reserves. Conifer seedling vigour (especially emergence) is critically dependent on the quantity of seed reserves accumulated during seed maturation as well as on the rate of reserve breakdown following germination; thus the sensitive and non-destructive detection of oil reserves and products of storage protein breakdown (in mature dry seeds and germinated seeds, respectively) are key to the development of markers for seed quality.

Finally, NMR was also useful for detecting the presence of protective compounds in conifer seeds, including a

putative desiccation protectant (sucrose) and complex terpenoid mixtures (oleoresin) effective in warding off bark beetle pests and their vectored fungal pathogens. With respect to the latter, *in vivo* ^{13}C NMR provided the terpene composition within an individual seed and was further capable of discerning the variability in monoterpenes between individual Pacific silver fir seeds, even within the same seed lot, a factor which may, in part, determine differences in survival/performance.

The non-destructive nature of NMR spectroscopy allows studies on the same seed throughout the course of imbibition, moist-chilling, and germination/post-germinative growth, thus providing less ambiguous metabolic information compared with that generated from destructive biochemical analyses. One potential application of using NMR as a screening tool is the optimization of environmental conditions in tree seed orchards for achieving maximum production of high quality seeds (i.e. those that yield the highest accumulation of reserves and stress protectants in conifer seeds). ^{1}H and ^{13}C NMR also hold promise as tools for the rapid screening of conifer seeds to identify/predict those seed lots with compromised viability due to developmental problems or as a result of deterioration during prolonged seed storage.

Supplementary material

Detailed results of the total lipid and fatty acid methyl ester (FAME) analysis in seeds of four *Pinus* species, a correlation between GLC and NMR data, and some ^{13}C MAS NMR spectra recorded for dry western white pine seeds are provided as supplementary data associated with this article and are available at JXB online.

Acknowledgements

We are grateful to Dave Kolotelo and Dean Christianson (of the BC Ministry of Forests Tree Seed Center, Surrey, Canada) and to Gary Delaney (of the Louisiana Forest Seed Company, Lecompte, LA, USA) for their help in obtaining seeds, and, in addition, to DK for valuable comments. Thanks are also extended to Brock Chatson (Plant Biotechnology Institute, Saskatoon, Canada) for his assistance with some of the NMR experiments. This research is supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic grant awarded to ARK, SRA and others.

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