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Use of NMR spectroscopy and magnetic resonance imaging for discriminating *Juglans nigra* L. seeds

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Summary

Black walnut (*Juglans nigra* L.) seeds are large and require stratification for germination. However, many seeds fail to germinate following stratification. Radiography can be used to select empty seeds, but cannot determine which full seeds will germinate. The objective of this study was to determine if any discrimination could be achieved through use of nuclear magnetic resonance (NMR) spectroscopy or magnetic resonance imaging (MRI) of seeds. Both NMR spectroscopy and MRI were as effective as radiography for detecting empty seeds. NMR spectroscopy before stratification showed that most full seeds gave proton spectral peaks for both water and lipids; some full seeds, however, showed no major lipid peak and consistently failed to germinate following stratification. NMR spectra of seeds following stratification were similar to those obtained before stratification. Results of MRI experiments mirrored those of spectroscopy experiments: seeds lacking large amounts of lipid produced images with very low intensity relative to those containing abundant lipid. Images of all embryos were more intense following stratification. Among seeds containing large amounts of lipid, germinable seeds were indistinguishable from non-germinable ones by either method.

Introduction

Forest tree seeds often fail to germinate. Climate, genetics, morphology, physiology and seed-handling techniques may account for reduced germination in any given seed lot. Some of these factors are characterized by visible signs which provide clues to the expected viability of the seed. For example, incomplete flowering due to non-optimum rain or temperature, animal feeding, and seed cracks may provide visible signs of seed abnormality. Conversely, physiological constraints such as dormancy (Vozzo and Young, 1975), embryo damage (Vozzo and Song, 1989), and incomplete embryo development (Vozzo, 1973) provide no visible clues to their presence, but may be demonstrated by various laboratory tests. Radiography is useful to determine if a seed is empty or full, internally whole or undeveloped, and with or without insect infestations. It also may be used to indicate gross water imbibition through an increase in radiopacity (Vozzo, 1988).

Black walnut seeds (*Juglans nigra* L.) require approximately 90 days stratification followed by 28 days at alternating 20–30°C to germinate-(Brinkman, 1974). This long period, their need for large germination containers, and their large size, make them rela-

tively expensive to germinate. Many stratified seeds fail to germinate, thereby increasing the relative cost of obtaining germinable seeds and ultimately seedling production. Methods are needed to reduce the proportion of non-germinable seeds prior to stratification, and. to select germinable seeds following stratification, but before planting. Empty seeds can imbibe sufficient water to appear full but these are easily distinguished by radiography before imbibition. However, a major disadvantage of conventional radiography is that the resulting image will not distinguish full, viable seeds from full, non-viable ones. Full seeds, both viable and non-viable, have 25 to 35% moisture content when freshly collected. This is sufficient water to confuse interpretation, due to the radiopacity of water.

Magnetic resonance imaging (MRI) provides an alternative technique to conventional radiography for the non-destructive study of seed embryos (Foucat, Chavagnat, Renou, 1993). This technology yields high resolution images of plant tissues (Connelly, Lohman, Loughman, Quiquampoix, Ratcliffe, 1987; Veres, Cofer, Johnson, 1991) and has the additional potential advantage of yielding important physiological information about those tissues. Changes in the degree of water binding in apple buds during vernalization, a process analogous to stratification, were demonstrated with MRI by Faust, Liu, Millard, Stutte (1991). Gussoni, Greco, Consonni, Molinari, Zannoni, Bianchi, Zetta (1993) and Halloin, Cooper, Potchen, Thompson (1993) used MRI to demonstrate the structure and distribution of lipids in seeds of olive (*Olea europaea* L.) and pecan [*Carya illinoensis* (Wangenh.) K. Koch], respectively.

Materials and methods

Juglans nigra L. seed is large (30 mm long) with clearly defined cotyledons, embryo axis, and seedcoat. Seeds were collected in Starkville, MS (east central MS in the southeastern USA). After husk removal, fresh seeds were briefly stored dry at 4°C until analyses. Dry seeds were radiographed at 30 kVp, 3 mA, 180 sec at 65 cm on Kodak Industrex Type M[1] film and developed manually. Each seed was individually identified for later MRI analyses, as well as for germination determination. Seeds containing embryos were imbibed for 24 h in water, subjected to MRI and NMR spectroscopy (see below), and pre-treated for germination tests by stratifying for 90 days at 4°C inside moistened, black plastic bags. Seeds then were reimaged with MRI and were germinated on moistened Kimpack[1] in germination boxes with alternating 20°C and 30°C regimes with 8 h of light during 30°C and 16 h during 30°C (Brinkman, 1974).

The magnetic resonance T1 and T2 relaxation constants, and spectra were measured and spin echo images acquired with a 4.7 Tesla, General Electric, Omega, chemical shift imaging system as described by Halloin et al. (1993). The spin echo image acquisition parameters for images were: sequence repetition time, 1 sec; echo time, 16 msec-

[1] The use of trade, firm, or corporation names in this paper is for the information and convenience of the reader. Such use does not constitute official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be available.

onds, slice thicknesses, 30 mm (to encompass the entire seed); numbers of acquisitions, 4; and total imaging time, 17 min, 4 sec. Data were acquired at a resonance frequency midway between the resonance frequencies of water and lipid.

Results and discussion

Radiography

Seeds examined with radiography were easily divided into two groups based upon density of the radiographs: those that were empty (devoid of well developed embryos), and those that contained well developed embryos (Figure 1). Seeds that appeared empty consistently failed to germinate following stratification, whereas some, but not all, of those with embryos germinated following stratification. All subsequent MRI and NMR spectroscopy experiments were done on seeds that appeared in radiographs to contain well developed embryos.

NMR Spectroscopy

Spectroscopy revealed that most freshly collected, full seeds contained peaks for both water and lipid protons (Figure 2A); however, some showed no evidence of the lipid peak (Figure 2B). Images acquired at a resonance frequency midway between those of water and lipid protons produced strong images of embryos containing lipid (Figure 3A), but only weak images of those lacking a lipid peak (Figure 3B).

MRI

The relationship between proton abundance, proton decay constants, instrument parameters, and image intensity has been discussed in detail by Werhli, MacFall, Newton (1983), Halloin, Cooper, Potchen (1994), and MacFall, Spaine, Doudrick, Johnson (1994). The net result of this interaction, as related to the images in Figure 3, is that the components with the longest T2 relaxation constants (Table 1) provide the greatest pro-

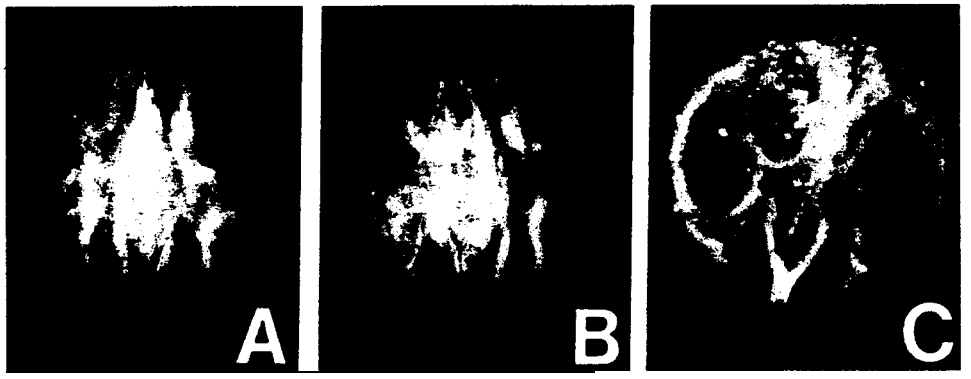

Figure 1. Radiographs of walnut seeds showing (A) a full seed that germinated. (B) a full seed that did not germinate, and (C)an empty seed.

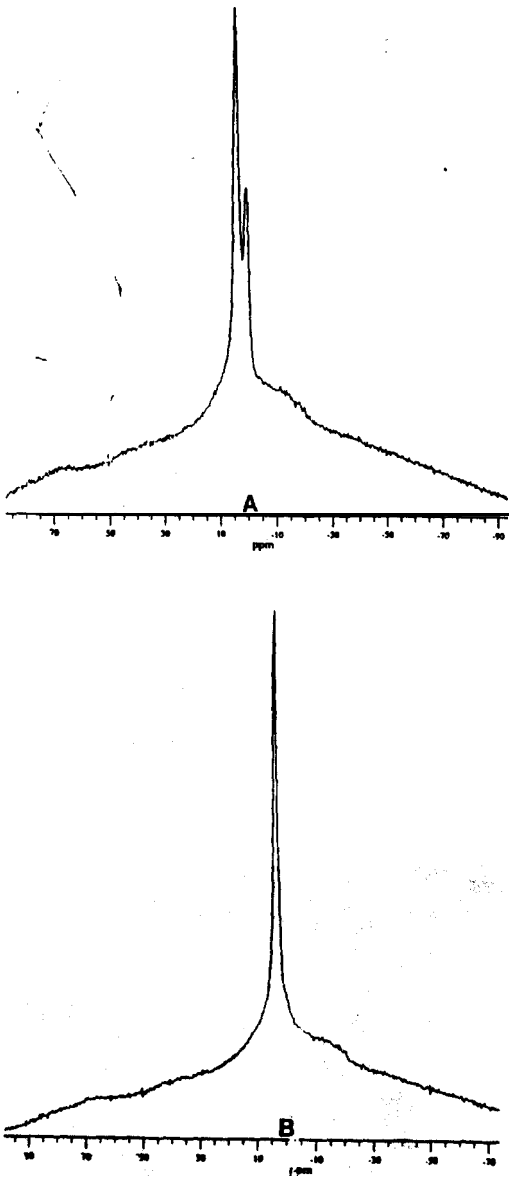

Figure 2. Nuclear magnetic resonance spectra of walnut seeds, prior to stratification of (A) a full seed that germinated, and (B) a full seed that failed to germinate.. Note the presence of peaks representing both water and lipid (left and right peaks, respectively) in the spectrum of the viable seed, and the absence of a lipid p&k in the spectrum of the nonviable seed. The vertical axis represents relative signal intensity with most intense peaks arbitrarily adjusted to full scale. Quantitative comparisons cannot be made.

portional contribution to image intensity. Selection of the resonance frequency for image data acquisition between the frequencies of water and lipid resulted in both components contributing to image intensity. However, differences between the T2 relaxation constants of water and lipid, together with the selected echo time, the shortest possible

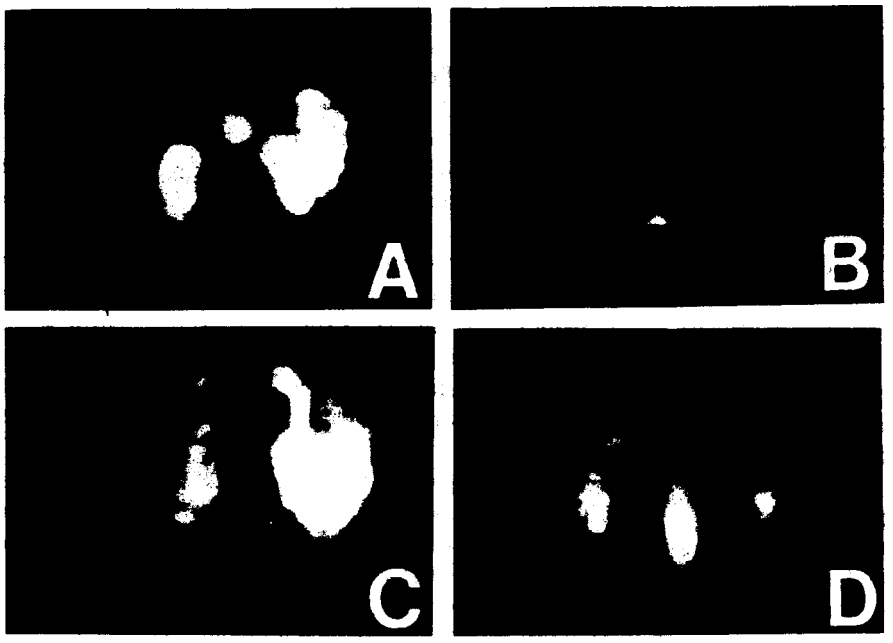

Figure 3. Magnetic resonance images of freshly collected (A and B) and stratified (C and D) walnut seeds showing the appearance of the embryos in a germinable (A and C) and a nongerminable (B and D) seed. Images A and B are of the same seeds as C and D, respectively.

on the instrument used, resulted in lipid being responsible for most of the observed image intensity of the embryo in Figure 3A. This is confirmed by the faint appearance of the embryo in Figure 3B.

Table 1. Proton spin-lattice (T1) and spin-spin (T2) relaxation constants in milliseconds of water and lipid in imbibed walnut embryos before (fresh) and after stratification for 90 days at 4°C. The seeds all exhibited both water and lipid proton peaks in the NMR spectra. Germinability determinations were based upon germination tests following the imaging and spectroscopy experiments.

Group	Relaxation constants			
	Water		Lipid	
	T1	T2	T1	T2
	(milliseconds)			
Fresh				
Germinable	58	2	129	9
Nongerminable	91	2	172	9
Stratified				
Germinable	268	5	316	22
Nongerminable	317	8	329	34

Images acquired following stratification of seeds (Figures 3C and D) were more intense. than those acquired before stratification. This change was probably due to increased proton relaxation times (Table 1), possibly parallelling the changes attributed to decreased water binding as a result of vernalization described by Faust *et al.* (1991). The increased intensity of Figure 3D, relative to that of Figure 3B was undoubtedly due to decreased water binding, as no lipid peak was present in the spectrum of the embryo following stratification (not shown).

Germination data showed that all embryos lacking a significant lipid peak in their NMR spectra (28 of 54 seeds), and therefore yielding weak images before stratification, failed to germinate. Also, many of the embryos exhibiting 'normal' NMR spectra and yielding intense images failed to germinate (11 of 54 seeds). We were unable to determine characteristics of images, either before or after stratification, that would allow differentiation between germinable and non-germinable embryos when lipid peaks were present in NMR spectra.

NMR spectroscopy an&or MRI can provide useful methods for discriminating samples of walnut seeds. Spectroscopy can be done with more readily available instrumentation. Any seed lacking au obvious lipid peak is either empty or contains an abnormal and non-germinable embryo. The major disadvantage of NMR spectroscopy is that evaluation must be done on individual seeds. MRI provides the advantageof allowing simultaneous evaluation of many seeds. Our MRI experiments were done on groups of nine walnut seeds. However, larger instruments used for human imaging commonly use imaging coils with inside diameters as large as 40 cm. Use of such a coil would allow simultaneous imaging of 100 or more seeds. While not providing spectral data, these instruments would allow discrimination between seeds with high intensity and those with low intensity images, and therefore could indicate a differentiation in germination potential. Because both NMR spectroscopy and MRI provide information that in part duplicates information derived from conventional radiography, use of radiography is not required where either of these methods is used.

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