IN VIVO DIGITAL PHYTO IMAGING (IDPI) IN *JUGLANS NIGRA* SEEDS

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A major disadvantage of conventional seed radiography is that the resulting image will not distinguish full-viable seeds from full-nonviable. Empty seeds will imbibe sufficient water to appear full, but these are easily distinguished by radiography before imbibition. Full seeds, both viable and nonviable, have 25 to 35% moisture content when freshly collected. This is sufficient water to confuse interpretation due to radiopacity of water density. While the full-viable seeds will germinate, the full-nonviable ones will not germinate due to physiological rather than morphological conditions, even while they maintain a moisture content sufficient to support germination.

Speculation points to distribution and availability of free water and fat as promising factors to determine if an otherwise healthy seed will germinate. Magnetic resonance imaging (MRI) will determine these distributions.

MRI is a nondestructive, noninvasive method suitable to seed research and ultimate germination of viable seeds. Although it is a widely used medical procedure, there are only limited applications to tree seed physiology.

Specific differences reported in this paper are primarily the distribution and abundance of mobile hydrogen (H^+) protons in bulk free water and in lipid molecules or parts of long chains. The difference in images was obtained by using fat and water suppression techniques utilising the small differences in resonant frequencies between H^+ in bulk water and lipids.

Introduction

Conventional radiography presents one means of determining nondestructive quality testing for tree seeds (Vozzo 1988). It represents, or images, the density of biological tissue which is compared with both absolute density at one end of the scale and no density at the other end. The 256 shades of gray visible in the density range represent the biological and physical qualities within the seed. For example, a healthy plant tissue fully hydrated will be very dense (bright gray) while a dead, dry tissue will be much less dense (dark gray to black). Radiography, or X-rays, provides interpretive value to understanding anatomy and morphology. A specialised X-ray technique, computerized tomography (CT), allows single-plane images of the seed at every 0.5 mm. This results in a flat-plane image showing all density structures as they would appear if hand-sliced at that level. All the serial sections can be combined by computer to reconstruct a 3-dimensional model. This procedure is also nondestructive.

However, other factors are equally significant to understand and discriminate seed viability which may not be relative to X-ray imaging. Seed physiological properties, such as a dormancy (Vozzo & Young 1975), embryo challenge (Vozzo & Song 1989), and incomplete embryo development (Vozzo 1973), provide no absolute visible images but are strongly related. Magnetic resonance imaging (MRI) is also nondestructive to tree seeds. It is an imaging technique to identify and localise protons-(the hydrogen nuclei) bound to bulk water and long-chain fatty acids. As both these metabolites are physiologically active during seed storage stratification, and germination, MRI has the value of imaging relative physiology. By comparing anatomy (X-ray images) and physiology (MRI), we can relate form and function as they interplay during prescribed seed treatments.

Pecan [*Carya illinoensis* (Wangenh.) K. Koch] seeds have been reported by Halloin *et al.* (1993) for their lipid distributions using MRI. Vozzo *et* al. (1996) relates the viability of black walnut seeds with water and lipid patterns determined by nuclear magnetic resonance (NMR) spectroscopy and MRI. Also, other agricultural seeds have been shown to image favorably using MRI (Foucat *et al.* 1993).

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 s 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 and pre-treated for germination tests by stratifying for 90 days at 4 °C inside moistened, black plastic bags. Seeds were then reimaged with MRI and were germinated on moistened Kimpack 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 s; echo time, 16 m seconds; slide thicknesses, 30 mm (to encompass the entire seed); numbers of acquisitions, 4; and total imaging time, 17 min, 4 s Data were acquired at a resonance frequency midway between the resonance frequencies of water and lipid.

Results

Seeds examined with conventional radiography were easily divided into two groups based upon density of the radiographs: those that were empty (no well-developed embryos), and those that contained well-developed embryos. Initial CT and MRI experiments showed that seeds that appeared empty on radiographs also appeared empty in CT and MRI images. Seeds that appeared empty consistently failed to germinate following stratification, whereas some, but not all, of those with embryo images germinated following stratification. All subsequent CT and MRI experiments were done on seeds that appeared in radiographs to contain well-developed embryos.

The relationship between anatomy (CT) and physiology (MRI) shows definite interactions relating specimen density to proton distribution. Serial reconstructions from computer-generated 3-dimensional images illustrate localisation and densities of major seed structures: seedcoat, cotyledons, and embryos. False coloring assigned by spectral energy distribution ranges allow separation of shades of gray otherwise not obvious. Comparing empty, full nongerminated,with full, germinated seeds, there is distinction by the amount and localisation of false-color red. Wavelength energy of false-color red represents minimal viability to affect germination as densitometrically assigned levels of gray form CT radiographs. Empty seeds have minute or no red energies, while full nongerminated seeds show sparse and poorly localised or diffused red energies. The fully germinated seeds are readily distinguished by their relative abundance of false-color red (Figure 1). Each seed representative is reconstructed and projected in 3-dimensional presentations of their X, Y, and Z axes during an eight-minute video.

In order to define the densities represented by red spectral wavelengths, magnetic resonance images show mobile proton distributions of hydrogen nuclei (H^+). Comparing density patterns from CT with the mobile proton distribution of MRI, the false-color density red pattern is translated to bulk water and long-chain lipid localisation.

Initially, MRI was a composite of both water and lipid protons (Figure 2). Later, however, we were able to separate the two entities by altering the relaxation constants which separate water and lipid echo

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times (Figure 3). The resulting image verified that lipid and water protons were primarily responsible for image intensity.

Images acquired following stratification of seeds were more intense than those acquired before stratification. Intensification was due to increased water and fat during the stratification process (Figure 4). The increase is interesting as it reflects both the amount and the distribution internally. Water binding as a result of stratification was described by Faust *et al.* (1991).

Germination data showed that all embryos lacking sufficient amount and distribution of lipid (as indicated by images before stratification) failed to germinate.

Discussion

The relationship between proton abundance, proton decay constants, instrument parameters, and image intensity has been discussed in detail by Werhli *et al.* (1983), Halloin *et* al. (1994) and MacFall *et al.* (1994). The net result of this interaction is that the components with the longest T2 relaxation constants provide the greatest proportional 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 on the instrument used) resulted in lipid being responsible for most of the observed image intensity of the embryo. This is confirmed by the faint appearance of the embryo.

Images acquired following stratification of seeds were more intense than those acquired before stratification. This change was probably due to increased proton relaxation times, possibly parallelling the changes attributed to decreased water binding as a result of vernalisation described by Faust et *al.* (1991). The increased intensity was undoubtedly due to decreased water binding, as no NMR lipid peak was present in the spectrum of the embryo following stratification.

Previously, 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 (Vozzo *et al.* 1996).

Tissue densities are routinely imaged with conventional radiographic procedures and provide substantial interpretations regarding health and structure. As quality control techniques, they are limited to gross observations to distinguish full from empty, insect-infested, and mechanically-damaged seeds. CT provides single-plane-imaging which allows interpretations unimpaired by multiple strata of small but densely structured tissue. Hydration affects interpretation as water is a naturally-occurring radiopaque contrast agent. However, water is also integral to the germination process as well as highly significant regarding seed storage. MRI gives an advantage in interpreting water movement internally. Any biological entity capable of undergoing MRI imaging is nondestructively observed as water moves within it. The correlations described here using walnut seeds are valid for detecting and localising water and long chain lipids in other specimens as well.

Magnetic resonance imaging (MRI) provides an alternative technique to conventional radiography for the non-destructive study of seed embryos (Foucat *et al.* 1993). This technology yields high resolution images of plant tissues (Connelly *et* al. 1987, Veres *et al.* 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 vernalisation, a process analogous to stratification, were demonstrated with MRI by Faust *et al.* (199 1). Gussoni *et* al. (1993) and Halloin *et* al. (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.

In the walnut industry, for example, this technique can identify the amount and distribution of oil deposits (long-chain fatty acids) within different sources of seeds. In turn, this enables a selection for various grades/qualities of food-producing walnut meat designed at marketing and sales.

Quality control can be implemented by quantifying the state and status of internal water. Specifically, CT and/or MRI can provide beneficial procedures used to determine germination potential in walnut seeds. CT not only clearly provides density gradients related to seed viability, but also offers spatial fiduciaries to accurately (within 0.25 mm) measure internal structures. We imaged 48 walnuts at one time on standard 14x17" radiographic film. For MRI, we imaged nine walnuts at one exposure; however, this can be expanded to image more seeds by using an instrument commonly found in large hospitals where MRI bores typically have inside diameters up to 40 cm. Neither CT nor MRI are sample destructive, meaning all seeds may be used for their originally intended purpose after imaging. Both techniques are highly useful with practical applications in quality control.

References

Brinkman, K.A. 1974. *Juglans* L. Pp. 454-459 in Schopmeyer, C.S. (Coord.) *Seeds of Woody Plants.* USDA Forest Service, Agricultural Handbook 450, Washington, DC,

Connelly, A., Lohman, J.A.B., Loughman, B.C., Quiquampoix, H. & Ratcliffe, R.G. 1987. High resolution imaging of plant tissues by NMR. *Journal of Experimental Botany 38:* 17 13-l 723.

Faust, M. Liu, D., Millard, M.M. & Stutte, G.W. 1991. Bound versus free water in dormant apple buds – a theory for endodormancy. *HortScience 26: 887-890.*

Foucat, L., Chavagnat, A. & Renou, J.P. 1993. Nuclear magnetic resonance microimaging and X-radiography as possible techniques to study seed germination. *Scientia Horticulture 55: 323-33* 1.

Gussoni, M., Greco, F., Consonni, R., Molinari, H., Zannoni, G., Bianchi, G. & Zetta, L. 1993. Application of NMR microscopy to the histochemistry study of olives *(Olea europaea* L.). *Magnetic Resonance Imaging* 11: 259-268.

Halloin, J.M., Cooper, T.G. & Potchen, E.J. 1994. Magnetic resonance imaging, a technology for noninvasive plant analysis. Pp. l-9 in Vozzo, J.A. (Comp.) *Research and Applications of Chemical Sciences in Forestry.* USDA Forest Service, New Orleans, LA, General Technical Report SO-104.

Halloin, J.M., Cooper, T.G., Potchen, E.J. & Thompson, T.E. 1993. Proton magnetic resonance imaging of lipid in pecan embryos. *Journal of the American Oil Chemists Society 70:* 1259-1262.

MacFall, J.S., Spaine, P., Doudrick, R. & Johnson, G.A. 1994. Alterations in growth and water-transport processes in fusiform rust galls of pine determined by magnetic resonance microscopy. *Phytopathology 84: 288-293.*

Veres, J.S., Cofer, G.P. & Johnson, G.A. 1991. Distinguishing plant tissues with magnetic resonance microscopy. *American Journal of Botany 78:* 1704- 17 11.

Vozzo, J.A. *1973. Germination Analyses of Excised Embryo Cylinders and Whole Acorns of Water Oak.* USDA Forest Service, New Orleans, LA, Research Note SO- 153. 3 pp.

Vozzo, J.A. 1988. Seed radiography. *Materials Evaluation* 46(11): 1450-1455.

Vozzo, J.A., Halloin, J.M., Cooper, T.G. & Potchen, E.J. 1996. Use of NMR spectroscopy and magnetic resonance imaging for discriminating *Juglans nigra* L. seeds. *Seed Science and Technology 24: 457-463.*

Vozzo, J.A. & Song, M.J. 1989. High-voltage electron microscopy of cell wails in *Pinus taeda* seeds. Pp. 78-80 in Turnbull, J. W. *(Ed.) Tropical Seed Research, Australian Centerfor International Agricultural Research.* Proceedings No. 28, Canberra, Australia.

Vozzo, J.A. & Young, R.W. 1975. Carbohydrate, lipid and protein distribution in dormant, stratified and germinated *Quercus Nigra* embryos. *Botanical Gazette 136: 306-3* 11.

Werhli, F.W., MacFall, J.R. &Newton, T.H. 1983. Parameters determining the appearance of NMR images. Pp. 81-117 *in* Newton, T. H. & Potts, D. *G.* (Eds.) *Modern Neuroradiology, Volume 2. Advanced Imaging Techniques.* San Anselmo, CA: Clavadel Press.

Figures

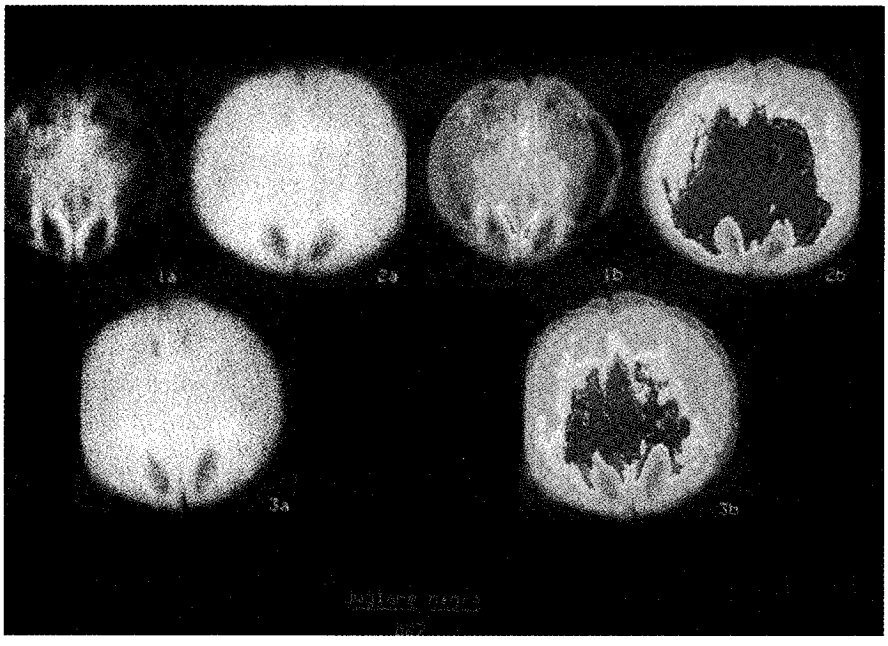

Figure 1. Conventional radiographs and false-colored CT of *Juglans nigra* seeds. la and lb; empty 2a and 2b, full, viable; 3a and 3b full but nonviable.

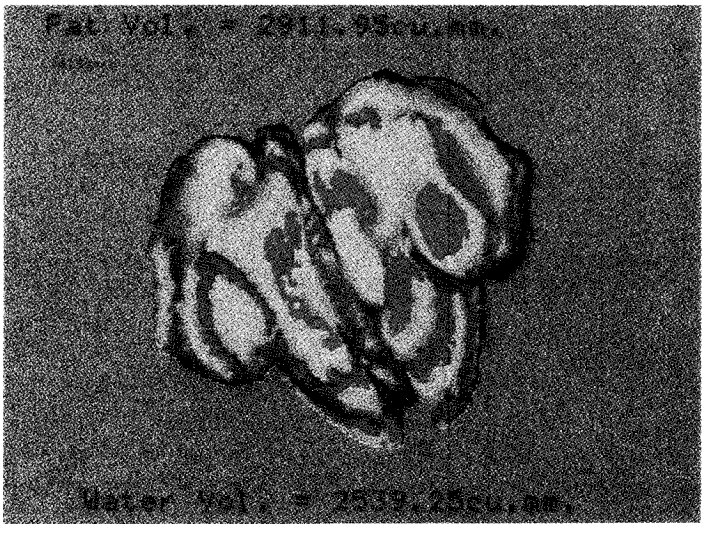

Figure 2. Composite MRI of *Juglans nigra* seed with false-colored fat = yellow.

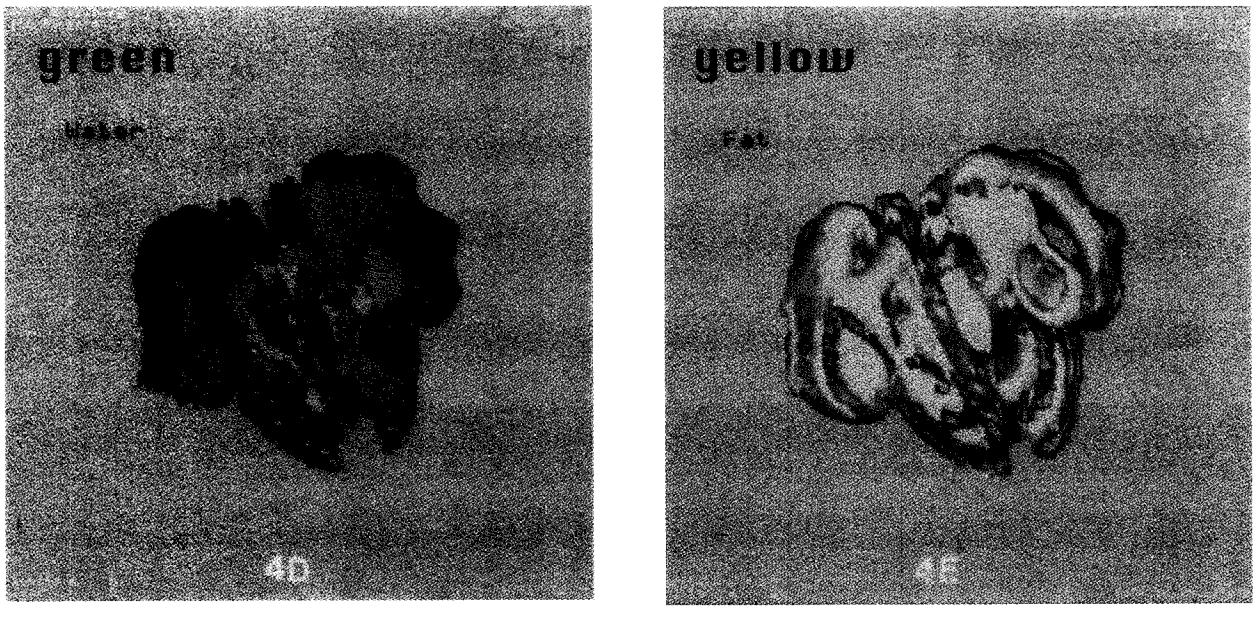

Figure 3. Same seed as Figure 2 but only water component (green) and fat component (yellow).

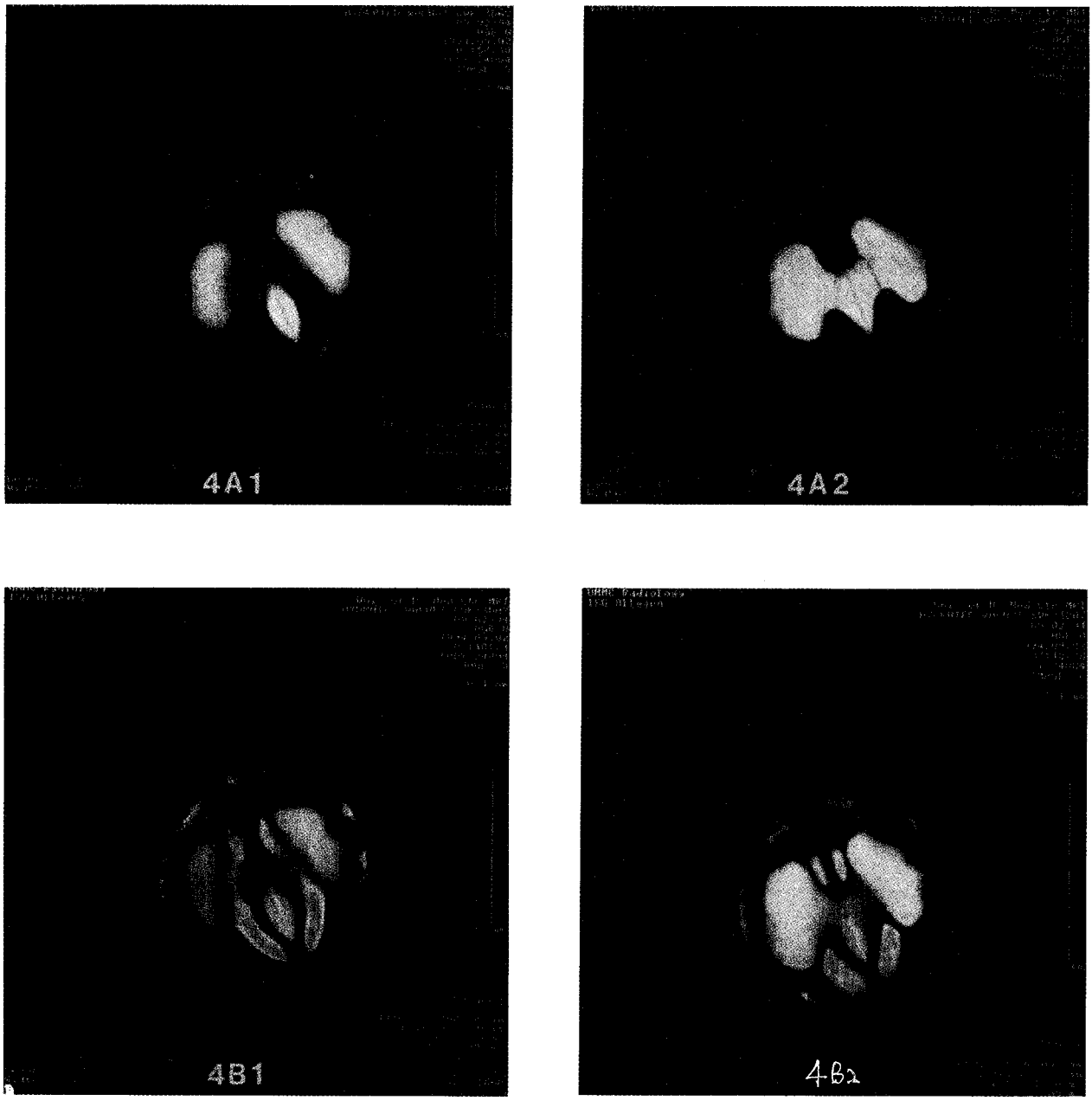

Figure *4.* MRI of *Juglans nigra* seeds after stratification. Al after one week and A2 after three weeks to show fat distribution. B | and B2 as A l and A2 but to show water distribution.