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Magnetic resonance imaging of persimmon fruit (*Diospyros kaki*) during storage at low temperature and under modified atmosphere

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Abstract

Effects of time and storage atmosphere on relaxation properties in persimmon fruit (*Diospyros kaki* cv Fuyu) were investigated by nuclear magnetic resonance (NMR) imaging during the five weeks following commercial harvest. There were two treatments ($n = 4$): one in which fruit were hermetically sealed in individual polyethylene bags (modified atmosphere or MA treatment), and another sealed in individual perforated bags (control). Fruit were stored at 7°C for 4 weeks (conditions conducive to development of chilling-injury), before being removed to 20°C for 3 days to simulate poststorage shelf conditions. Every week, and at the end of the shelf-life period, a series of ^{1}H NMR images of median transverse and longitudinal planes were acquired from each fruit for calculation of spin–lattice (T_1) and spin–spin (T_2) relaxation times.

Relaxation times associated with the flesh, vasculature and locules in transverse sections, and flesh from basal, median and distal regions of longitudinal sections, were significantly shorter in MA-treated fruit, although no T_2 treatment difference was noted with vasculature. MA-treated fruit were further distinguished from controls in that all tissues exhibited a sharp increase in T_1 (but not T_2) relaxation between the second and third weeks in cold storage. Within a treatment, T_1 times in flesh and locules were similar (ca. 1120 ms), and shortest in vascular tissue (1036 ms). T_2 was a more sensitive indicator of tissue type (mean values of 71, 101 and 116 ms in the vasculature, flesh and locules of MA-treated fruit, respectively), than T_1. However the relative ranking of flesh and locule tissues was time-dependent. There was no gradient in relaxation times between basal, median and distal regions of the fruit.

Incipient stages of chilling-injury development were unable to be identified either by visual inspection of time-course images, or variation in relaxation properties. Our observations do indicate, however, that relaxation properties in fruit were strongly influenced by MA conditions. Increased concentrations of soluble metabolites arising from reduced respiration in MA-treated

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fruit is a possible mechanism consistent with these results, but not removal of a paramagnetic species like O_2, where T_1 values would be predicted to increase.

Keywords: Chilling-injury; *Diospyros kaki*; Fruit; Nuclear magnetic resonance imaging; Persimmon; Postharvest storage

1. Introduction

Like other subtropical fruits, persimmons (*Diospyros kaki* L.) are affected by chilling-injury when subjected to low or nonfreezing temperatures to extend their postharvest life (Lyons, 1973; Wang, 1990a). External symptoms commensurate with injury development are frequently absent. This has lead to interest in the use of nondestructive, noninvasive technologies like nuclear magnetic resonance (NMR) imaging and near-infrared spectroscopy, that allow repeated measurements of the same sample, to monitor the development and extent of internal injury.

In a previous study using a clinical scanner (Clark and Forbes, 1994), it was established that changes in image contrast indicative of fruit degradation were consistent with visual symptoms describing advanced stages of chilling-injury in this crop (MacRae, 1987). Beyond providing descriptive morphological information about tissues, proton NMR imaging is capable of generating spatially-resolved, quantitative data about specific tissue characteristics such as their spin–lattice (T_1) and spin–spin (T_2) relaxation times (Callaghan et al., 1994; Kuchenbrod et al., 1995). These are the unique parameters determined in a NMR experiment which relate to enthalpic (T_1) and entropic processes (T_2) whereby a stimulated protonaceous system returns to its unexcited groundstate. Both parameters are affected by the concentration and composition (viscosity) of aqueous solutions (Bovey et al., 1988). In addition, measurement of T_2 by different experimental approaches provides alternative information about susceptibility effects, and proton mobility and exchange processes in plant tissues (Duce et al., 1992; Hills and Duce, 1990; McCarthy et al., 1995). Comparison of temporal changes in relaxation measurements from time-course studies thus allows inferences to be drawn concerning how the water status of fruit responds to particular treatments. This investigation reports on changes in the relaxation properties of component tissues in persimmon recorded during a modified atmosphere (MA) experiment designed to mimic gaseous conditions prevailing when fruit are exported, and under temperature conditions expected to lead to development of chilling-injury within the storage period.

2. Materials and methods

Fruit samples and destructive measurements

Trays of graded persimmon fruit (cv. Fuyu) of export quality standard (190–210 g; average soluble solids content of 14%) were selected from a commercial packhouse at Pukekohe, 50 km south of Auckland, N.Z., on 16 May 1994. The fruit, all of which originated from a local orchard, had been harvested 2 days earlier and stored at ambient temperatures during the interim. Individual fruit were subsequently weighed

and divided into two treatments — one in which single fruit were hermetically sealed in polyethylene bags (60 μm thickness; modified atmosphere or MA treatment), and another in which separate fruit were sealed in perforated bags (40 pin holes per bag — control) which allowed gas exchange, but drastically reduced water loss (aqueous protons being the source of the NMR signal). The polyethylene bags were effectively transparent in the imaging process and created a steady-state O_2 concentration of 2.4% within less than a week after fruit had been sealed: equilibrium CO_2 concentrations were not measured, but averaged 12 to 15% in identical bags containing single fruit (S.K. Forbes; unpublished data).

All fruit (260) were stored at 7°C for 4 to 6 weeks. Local industry protocols for long-term storage of persimmon in polybags require a storage temperature of 0°C — use of 7°C was employed to enhance the likelihood that chilling-injury would occur during storage and within the time frame available for the imaging experiment (S.K. Forbes, unpublished data). At harvest, and at weekly intervals thereafter, fruit were analyzed for firmness (SoftSense — a nondestructive measure of firmness; McGlone and Schaare, 1993), soluble solids (Atago Co. digital refractometer), soluble tannins, skin and flesh color, weight loss, and, in the case of the MA treatment, oxygen concentration (Model 3500 Oxygen Head Space Analyzer, Illinois Instruments, IL). Fruit color was determined using a Minolta Chromameter CR200 (Minolta, Japan) where chromaticity readings were based on the Hunter (L a b) scale (Voss, 1992). Tannin content was determined using an adaptation of the Folin-Ciocalteu technique (Singleton and Rossi, 1965). Samples of flesh from each fruit were disintegrated in methanol using a Polytron homogenizer. After settling, aliquots of the supernatant were analyzed for total phenolic content. The standard curve for spectrophotometric calculations was prepared using catechin, a major phenolic component in Fuyu fruit (Yonimori et al., 1983).

Imaging techniques

Details concerning the theory and application of NMR microscopy to biological systems can be found in Morris (1986) and Callaghan (1991). In our study, proton (^{1}H) NMR experiments were performed in a 2 Tesla superconducting magnet, part of a GE CSI Imager for Magnetic Resonance Imaging (Freemont, CA) located at Duke University, North Carolina.

Fruit required for NMR microscopy were treated similarly to those retained in New Zealand for destructive measurements. However, because of the length of time in transit, and availability of instrument time, the first NMR measurements on fruit were not recorded until one week after they had been sealed in their bags. In addition, imaged fruit were stored for only 4 weeks at 7°C (weeks 1 to 5 from harvest), after which time the bags were removed and the fruit were held at 20°C for 3 days before being imaged one final time.

Eight fruit were used in the imaging experiments — four from each treatment. Each week fruit were withdrawn from coolstorage in a predetermined sequence for imaging. Before being placed in the magnet, three small glass capillary tubes containing $CuSO_4$ internal standards were taped along the top, and along the side of the fruit, so that they would appear in the images of the longitudinal and transverse planes selected for analysis. The imaging planes, coincident with median slices, were drawn on the skin of

the fruit prior to bagging to assist in orienting the fruit within the magnet, and to ensure, as far as possible, that data were always collected from the same regions of the fruit. Individual fruit were positioned vertically (stem end up) in a 10 cm diameter 'birdcage' coil and aligned within the magnet bore using cross-sectional locator spin echo images (repartition time (T_R) = 100–300 ms, echo time (T_E) = 10 ms).

At weekly imaging sessions, 28 single-slice, spin-echo images were acquired on each fruit using a Hahn spin-echo pulse sequence employing two-dimensional Fourier transform imaging according to a programmed script. Six images (T_E = 10 ms) were sequentially acquired through each transverse and longitudinal slice plane with T_R times ranging from 100 to 3200 ms for calculation of T_1 values. A further 8 images (T_R = 3200 ms) were acquired through each slice plane (T_E = 20 to 160 ms in 20 ms increments) using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence for calculation of T_2 values. The total imaging time for acquisition of a complete set of images was 3.5 h: fruit were unable to be maintained at 7°C during this period.

Images were reconstructed and displayed on a 256 × 256 pixel array using a Sun Workstation (Sun Microsystems, Mountain View, CA). Individual image slices were all 2 mm thick with an in-plane resolution of between 0.33 and 0.35 mm. Relaxation values were calculated after MacFall et al. (1987): spin–lattice relaxation being measured by the partial saturation method using a 3-parameter fit for flip angle, pseudo-density and T_1; and spin–spin relaxation by a 2-parameter fit for exponential decay determining pseudo-density and T_2.

Relaxation measurements at successive times were made on selected regions of interest (ROI) using the 'calculated' images arising from the fitting procedures of MacFall et al. (1987). Measurements in the transverse plane were made from small ROI selections of flesh, locule and vascular tissues, as well as a larger ROI selection containing both flesh and vascular tissue. Measurements in the longitudinal plane consisted of ROIs containing flesh and vascular tissue combined from basal, median and distal regions within the fruit. Relaxation measurements for a particular tissue were calculated as the mean of 6 ROI measurements, each in turn the average of relaxation data accumulated over 9 (flesh, locule and vascular tissue) or 316 pixels (flesh and vascular tissue combined). To account for between-image variability, measurements were normalized according to the T_1 and T_2 values calculated for the reference tubes in each image. This was done by dividing individual fruit measurements by the appropriate T_1 or T_2 value associated with the standards in that image, and scaling by 396.9 (T_1) or 91.4 (T_2), standard values associated with one fruit at the beginning of the study. Over all experiments, the coefficient of variation for the T_1 and T_2 standards was 6% (mean — 423 ms), and 11% (mean — 88.1 ms), respectively.

3. Results

Physicochemical measurements

No major treatment differences were observed during the 6-week trial period between fruit fresh weight, total soluble solids, soluble tannins, and fruit firmness. Fresh weight and soluble solids showed little variation with time, averaging 206 g per fruit and 16.5%, respectively: the perforated bags restricted moisture loss in the control treatment

to <0.2% of the weight at harvest. As expected, soluble tannins and fruit firmness both declined during storage (data not shown).

The most pronounced treatment differences were those related to change in color. By two weeks after treatments had been imposed, flesh and skin color in the control fruit had developed a markedly darker orange hue. Thereafter, both tissues in the control fruit consistently maintained color associated with a more advanced stage of ripening, i.e., darker orange/red colors in contrast to yellow/oranges indicative of less ripe fruit (data not shown).

Chilling-injury developed in nearly all the fruit imaged by NMR. Injury development was ascertained on a weekly basis by opening cohorts held in the same incubator as those being imaged, and by visual examination of interior of the imaged fruit at the end of the experiment. The only fruit not apparently injured in this study was a single fruit in the control treatment. Symptoms included change in the color of the skin and flesh from light to dark orange and formation of a transparent gel throughout the flesh. Texture remained relatively firm though, and there was no evidence of tissue breakdown.

NMR measurements

Figs. 1 and 2 typify reconstructed images used to obtain quantitative data about the nature of temporal changes in relaxation properties, and clearly illustrate the major morphological features associated with the fruit. In transverse image slices (Fig. 1) these included speckles of vascular tissue interspersed throughout the flesh (mesocarp), and the eight locules, predominantly seedless, radiating from the core. The locules were differentiated from the surrounding flesh by a thin gel-like layer of endocarp. Loosely-bound bundles of vascular tissue contained in the core cavity were not visualized in this experiment, implying — incorrectly — that the region is devoid of water, or tissue. Longitudinal images (Fig. 2) were dominated by the appearance of the vascular tissue extending from the calyx through the core, and out into the flesh at the basal end of the fruit. Traces of vascular tissue seen towards the distal end of the fruit tended to be differentiated from flesh as isolated strands, especially in T_1 images.

Significant treatment differences ($P < 0.05$) were established between T_1 relaxation times for all tissues in both transverse and longitudinal image planes. In each case, average T_1 times were shorter in the MA treatment than the control. For example, 1114 versus 1264 ms for the large 316-pixel ROI measurements of flesh and vascular tissue in the transverse plane (Fig. 3a), and 988 versus 1175 ms for the same measurement in the longitudinal plane (Fig. 3b). Consequently, individual T_1 times in MA fruit were generally lower at various times throughout storage, although there were exceptions, most notably 3 weeks from harvest, after the NMR fruit had been in cold storage for two weeks. The sharp increase, followed by decline a week later, was evident in all tissues, in both image planes, and in all MA fruit but not in controls (see Figs. 4a,b).

Treatment differences ($P < 0.05$) associated with T_2 relaxation times were also noted. Again, T_2 times in the MA treatment were significantly lower than those in the control. For instance, 85.3 and 94.3 ms for the large ROI measurements of flesh and vascular tissue in transverse sections, and 88.4 and 99.6 ms in longitudinal sections (Fig. 3c,d). This was true of all measurements except those in vascular tissue where no treatment difference was observed. Over time, T_2 measurements were consistently

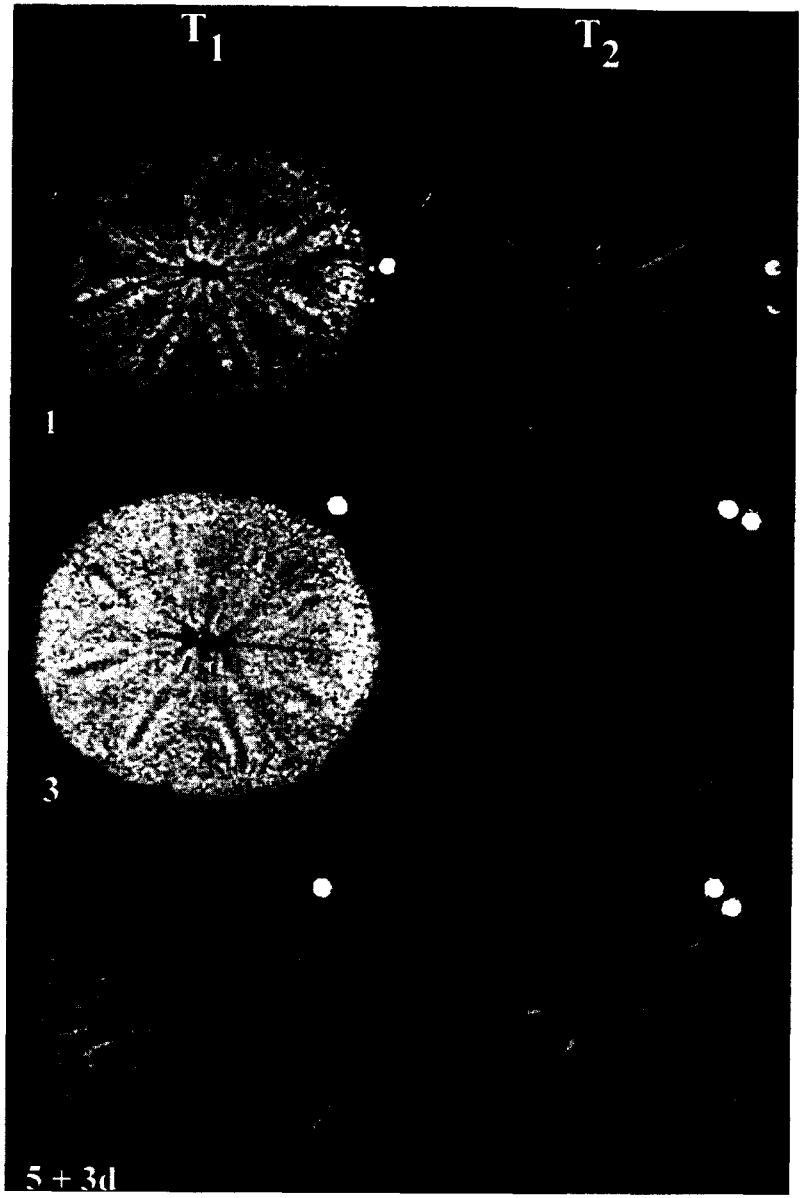

Fig. 1. Calculated spin–lattice (T_1) (left-hand column) and spin–spin (T_2) (right-hand column) relaxation images of a transverse equatorial slice through a fruit from the MA treatment. Numbered panels (1, 3, 5 + 3d) are of the same slice plane imaged successively during storage. Numbers indicate weeks from harvest whilst fruit were maintained at 7°C; 5 + 3d is the image obtained 3 days after fruit had been removed to 20°C to simulate shelf-life at the conclusion of 4 weeks cold storage. The resolution is 0.35 × 0.35 mm. Image intensity is based on a 256 point, continuous grey setting ranging from 0 (black) to 3000 ms (white) for T_1, and from 0 (black) to 200 ms (white) for T_2 images. Circular features adjacent to particular images are glass tubes (4 mm inner diameter) containing aqueous $CuSO_4$ standards which serve as scale markers. The polyethylene bag surrounding this fruit is transparent in the NMR process.

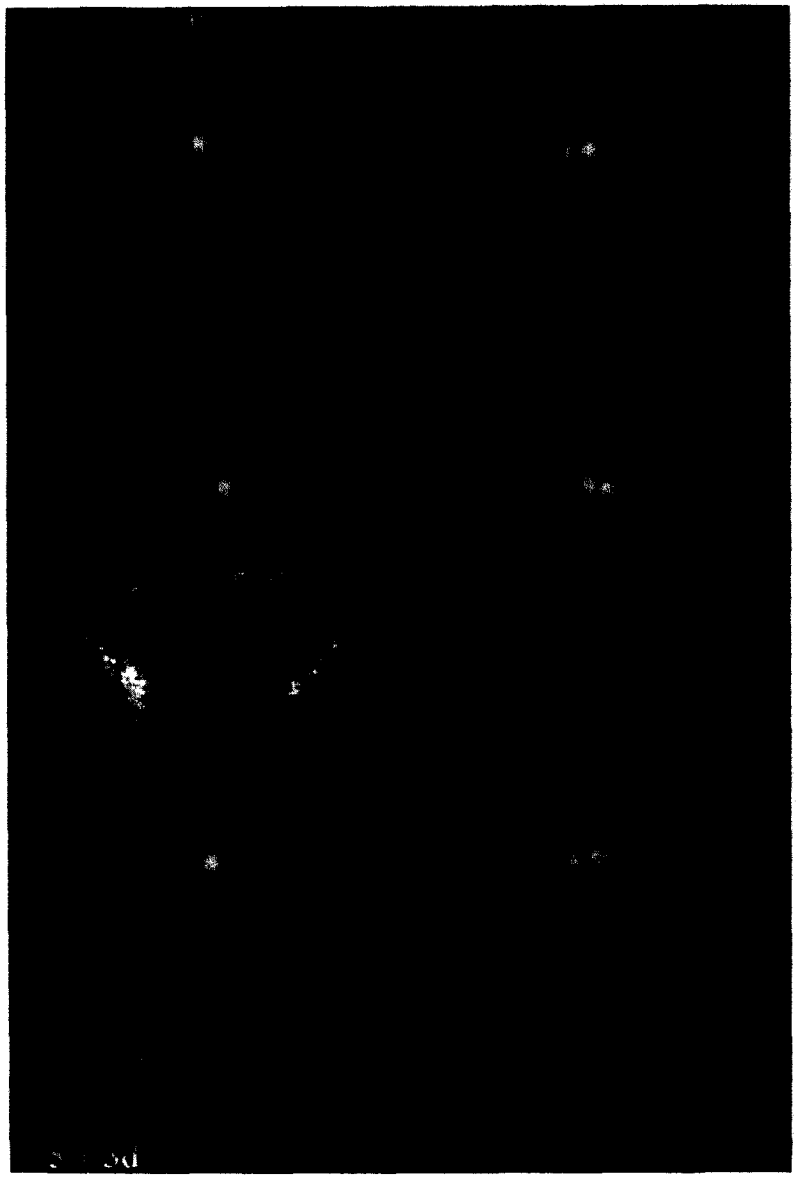

Fig. 2. Calculated spin–lattice (T_1) (left-hand column) and spin–spin (T_2) (right-hand column) relaxation images of a longitudinal equatorial slice through the same MA-treated fruit displayed in Fig. 1. Numerals, resolution, intensity scale and scale markers are the same as Fig. 1.

lower in MA fruit (Fig 3c,d), although statistically, treatment means were almost always within experimental error at separate times during storage. Unlike T_1, no sharp rise in T_2 values was observed 3 weeks after harvest. In fact mean T_2 values showed little tendency to vary at all during the course of the study.

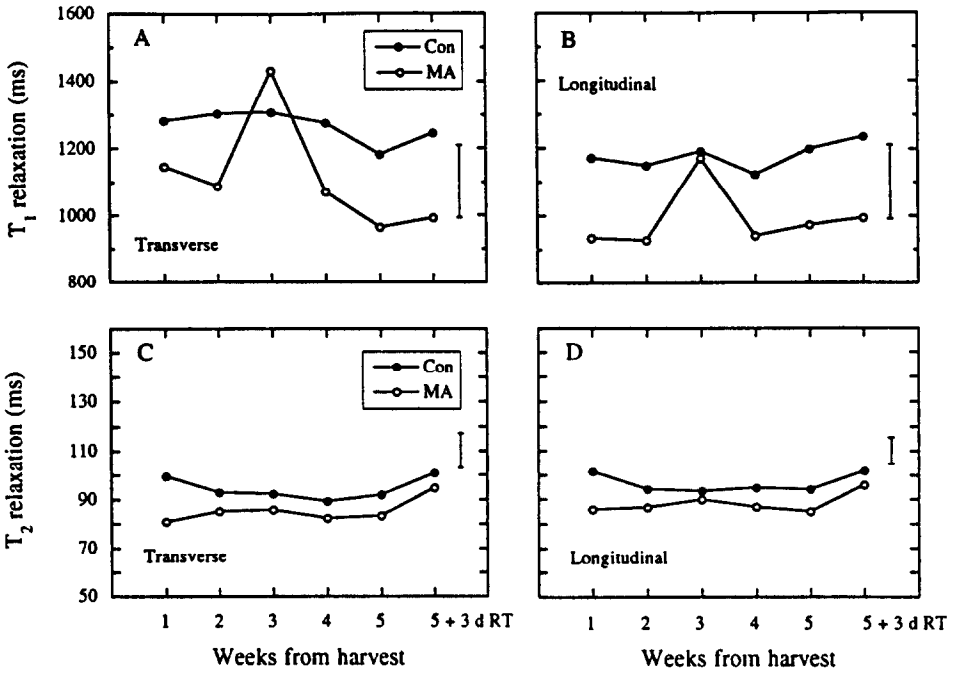

Fig. 3. Temporal variation in spin–lattice relaxation (T_1) times in flesh tissue in A) transverse, and B) longitudinal equatorial image slices of fruit from control and MA treatments stored at 7°C. Corresponding spin–spin relaxation (T_2) measurements for the same slice planes are presented in panels C) and D) respectively. Error bars (based on a pooled estimate of variance) represent a 95% confidence interval for an LSD for between-treatment comparisons at any individual imaging date. Note, the same ROIs were utilised when calculating T_1 and T_2 data: every datum point is the mean of six, 316-pixel measurements obtained from calculated images like those in Figs. 1–2.

Because of variation in molecular environment, individual tissues might be expected to be distinguishable on the basis of their relaxation properties. T_1 relaxation times proved to be an insensitive discriminator of tissue type compared with T_2. However, no consistent differences were established that were applicable to all tissues in either treatment at all imaging dates. Broad trends were evident. In the MA treatment, for example, there was no difference between mean relaxation measurements in flesh (1130 ms) and locule tissues (1102 ms) (Fig. 4a). However, T_1 times were lower ($P < 0.05$) in vascular tissue (1036 ms), especially at the beginning and end of the study (Fig 4a). There were no gradients in T_1 measurements taken from selected areas of combined flesh and vasculature in basal, median and distal regions of longitudinal sections (Fig. 4b). By contrast, T_2 relaxation measurements clearly distinguished individual tissue types, relative values in flesh, locule and vascular tissue being 101.4, 115.9 and 70.6 ms, respectively (Fig. 4c). Changes with time suggested that this ordering was appropriate for the first half of the study, but thereafter T_2 times in locule tissue declined to values similar to those in flesh (Fig. 4c). In longitudinal sections (Fig. 4d), T_2 values showed little temporal variation but were consistently higher in the middle of the fruit (96.6 ms) than at either extremity (88.2 ms).

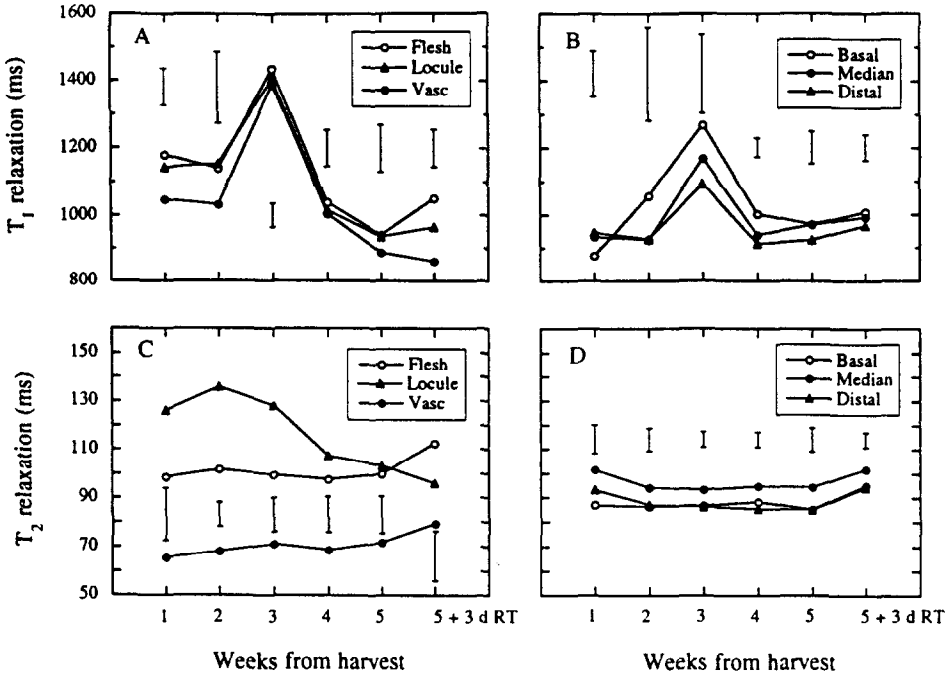

Fig. 4. Temporal variation in spin–lattice relaxation (T_1) times in flesh, vascular and locule tissues located in A) the transverse slice plane, and B) flesh in basal, median and distal regions of the longitudinal plane of fruit stored at 7°C. Corresponding spin–spin relaxation (T_2) measurements for the same slice planes are presented in panels C) and D), respectively. Error bars represent a 95% confidence interval for an LSD for between-tissue comparisons at their respective imaging dates. All measurements are from fruit in the MA treatment. Measurements in the transverse plane are based on analysis of a series of 9-pixel ROI areas; those in the longitudinal section from 316-pixel ROIs.

There were no obvious correlations between either relaxation parameter and our routine physicochemical measurements.

4. Discussion

The primary purpose of our study was to establish if NMR imaging was sensitive enough to allow visual detection of incipient stages of chilling-injury, and whether there would be differences in the quantitative measurements between relaxation properties of affected and unaffected tissues. In a previous investigation (Clark and Forbes, 1994), fruit kept at 0°C for 6 weeks showed no sign of injury development during cold storage, but degraded rapidly during the week following at ambient temperatures. Under these conditions injury development at the earliest stages occurred too quickly to be observed by the imaging schedule so that only images of gross tissue breakdown corresponding to advanced injury (MacRae, 1987) were recorded. Here, seven of eight fruit developed moderate symptoms of chilling-injury by the end of the experiment, including all those in sealed bags where it was expected that MA storage would delay, but not necessarily

prevent, injury development (Ben-Arie and Zutkhi, 1992; MacRae, 1987). Subsequent visual inspection of the time-course images failed to reveal unequivocal differences between affected fruit and the sole unaffected control sample. Furthermore, there was too much between-fruit variability in the individual relaxation property measurements to suggest that the unaffected fruit had characteristics markedly different from those of its affected counterparts. Without a greater number of undamaged fruit to facilitate comparisons, neither the qualitative nor quantitative approach successfully pinpointed commencement of injury development. What was more unexpected, however, was the way in which modified atmosphere profoundly altered the relaxation characteristics of the fruit, and from very early (less than a week) after treatments had been imposed.

Quantitative measurements of relaxation properties in fruit tissues have not been routinely reported. Values for T_1 and T_2 presented here are similar to those documented in other biological tissues (Morris, 1986; Veres et al., 1991). However, because of the dependence of relaxation on magnetic field strength (Bovey et al., 1988), and the necessity to normalize data to account for experimental variability, the more important aspect of our measurements are the relative differences between treatments, and interpretation of trends, rather than the absolute magnitude of the values themselves. In terms of the relative differences between tissues, the markedly lower relaxation times associated with vascular tissue are consistent with the presence of elevated carbohydrate. The ability to differentiate vascular tissue by its characteristic relaxation properties has been the basis for novel 3-dimensional studies which provide anatomical details about transport pathways in fruit and vegetables (MacFall and Johnson, 1994).

Several mechanisms are consistent with a reduction in relaxation times in the MA fruit. Paramagnetic species alter the magnetic properties of adjacent protons, effectively reducing their relaxation times (Morris, 1986). While changes in paramagnetic species may affect relaxation times in specialized organs (e.g., the nodules in soybean; MacFall et al. (1992)), we do not suspect that to be the case here. The main metallic paramagnetic species in persimmon are Fe and Mn (Clark and Smith, 1990), but there is no evidence that the distribution or ionic form of these elements changes in response to MA. Oxygen is also a paramagnetic species. Under the conditions of this experiment, relaxation times would be expected to increase as molecular O_2 was gradually reduced in the MA treatment. This was not observed.

A more likely scenario concerns changes in solution composition which in turn affect relaxation processes. An increase in protein or carbohydrate concentration, for example, promotes faster relaxation and hence leads to shorter relaxation times (Cho et al., 1993; MacFall and Johnson, 1994). Reduced losses of organic acids, simple sugars and metabolites such as amino acids and ascorbic acid have been reported in controlled atmosphere (CA) or MA trials as opposed to conventional storage conditions (Wang, 1990b). This would need to be confirmed by monitoring an extensive range of compounds in both treatments to determine the nature of any accumulated products at the lower respiration rate in MA fruit.

The cause of the dramatic change observed in MA fruit 3 weeks after harvest is unknown. It is probably not related to development of chilling-injury, since a similar response was not elicited in control fruit which were also affected. This emphasizes the inadequacy of our present knowledge concerning the nature of physiological

processes that occur during MA storage, and ripening/senescence generally. Further NMR experiments involving fruit from more sophisticated CA/MA studies employing a rigorously defined series of O_2 and CO_2 atmospheres and analysis of a range of metabolites, should help to begin unravelling the nature of the processes we have observed here.

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