Phyton (Austria) Special issue: "Free Radicals"	**Vol. 37**	Fasc. 3	(81)-(94)	1. 7. 1997

Use of Magnetic Resonance Techniques in the Study of Disease and Senescence Processes in Plants

By

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Key words: EPR spectroscopy, NMR microimaging, free radicals, paramagnetic metal ions, raspberry, strawberry, grape, apple, potato.

Summary

GOODMAN B.A. & GLIDEWELL S.M. 1997. Use of magnetic resonance techniques in the study of disease and senescence processes in plants.- Phyton (Horn, Austria) 37 (3): (81) - (94).

Some applications of NMR microimaging and EPR spectroscopy to the study of disease and senescence processes in plant organs are presented, with emphasis on the non-invasive nature of the techniques. A particular strength of the use of NMR microimaging in research on live specimens is its ability to perform repeated measurements on the same specimen. Images can be generated in either two or three spatial dimensions, depending on the objectives and timescale of any particular experiment. Contrast is determined by a combination of mobile proton concentration and its physical and chemical properties, but at the present time only a very small amount of molecular information is generated. EPR spectroscopy produces information specifically on free radicals and paramagnetic metal-containing species on a whole sample basis. Biological sample volumes that can be used with most conventional spectrometers are extremely limited, however, due to a combination of the small physical dimensions of microwave cavities and the fact that water strongly absorbs microwaves; the latter problem can be overcome to a large extent by freezing the specimen, but this is then at the expense of repeated measurements to obtain dynamic information. Both techniques largely avoid the production of artifacts from sample preparation, which limits the validity of results obtained with destructive analytical techniques, where damage-induced responses may represent an appreciable and unavoidable complication.

Introduction

Magnetic resonance techniques are dependent upon the magnetic properties of sub-atomic particles, nuclei for nuclear magnetic resonance (NMR)

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and electrons for electron paramagnetic or spin resonance (EPR or ESR). In both cases, the degeneracy of spin energy states is removed by large external magnetic fields and transitions are induced between these energy levels by the absorption of electromagnetic radiation of the appropriate frequencies. In NMR these are at radiofrequencies, whereas with EPR they are at microwave energies. For technical reasons, NMR spectra are usually obtained as a function of rf frequencies in a static magnetic field, whereas most EPR spectra are obtained as a function of magnetic field with a constant microwave frequency, although there is now a new generation of pulsed EPR spectrometers that use variable frequencies to study spectra that are spread over relatively small ranges.

Molecular information is obtained with both of these techniques because the positions of spectral peaks and patterns of associated structure are deternined by the chemical environments of the atoms being investigated. With NMR, information is obtained separately for each type of nucleus, e.g. ^{1}H, ^{13}C, ^{31}P, etc., and NMR spectroscopy is now one of the most important techniques for molecular characterisation in chemistry and biochemistry. EPR, in contrast, is more limited, depending on the presence of unpaired electrons, but it is an extremely sensitive technique for the characterisation of free radicals and paramagnetic metal ions and complexes.

In addition to their spectroscopic applications, it is also possible to use magnetic resonance techniques to generate images of the distribution and properties of selected spectral components by varying the magnetic field and radiation frequency in a controlled manner. This approach is well developed for NMR and (nuclear) magnetic resonance imaging (MRI) is regularly used for clinical diagnosis in major hospitals throughout the world. Its applications are based on the fact that experimental conditions can be chosen so that the intensity of the NMR spectrum of water differs for healthy and diseased tissues, and appropriate plots of the spatial distribution of spectral intensity show the location and size of any abnormalities in maps of soft tissue distribution (see e.g. WEHRLI & al. 1988). NMR microimaging systems have also been developed over the past decade and are being used increasingly to study a wide range of non-medical biological problems (e.g. CALLAGHAN 1991). EPR imaging, in contrast, is still in its infancy and there are currently few facilities outwith the instrument development laboratories. Both techniques are essentially non-invasive with the potential to make repeated measurements over extended periods of time on the same specimens, thus yielding dynamic information, although with EPR at the commonly-used X-band frequencies, there are practical limitations on the water contents of samples that can be studied, because of microwave absorption. This latter problem has meant that EPR imagers are being developed to operate at much lower frequencies (i.e. with much less sensitivity) than most spectrometers in order to avoid microwave absorption by water.

This paper presents some of our recent studies on natural and disease-induced senescence processes in plants using NMR microimaging and EPR spectroscopy. Examples have been selected primarily to illustrate the scope and

limitations of the techniques at the present time. In addition, we consider briefly the likely developments of these techniques for advancing such studies in the near future.

Materials and Methods

Samples: The various plant products used in this work were either produced at the Scottish Crop Research Institute or purchased from local supermarkets. Full histories of specimens are provided in relevant cited references. However, neither the NMR imaging or EPR spectroscopic measurements required any specific sample pretreatments.

NMR Imaging: NMR images were generated using either Bruker AM300/WB or AMX300/SWB FT NMR spectrometers (7.1 T magnetic field, 300 MHz ^{1}H frequency) fitted with microimaging probes and using Bruker standard spin echo and gradient echo routines (e.g. Fig. 1).

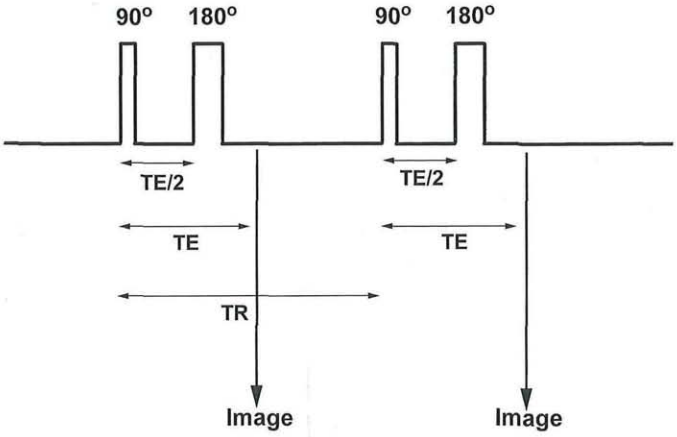

Fig. 1. Basic pulse sequence diagram used in NMR imaging showing the time to the echo (TE) and repetition time (TR). The 90° and 180° represent the lengths of the shaped rf pulses.

NMR images are generated primarily by water, although protons in other fluid-phase molecules also contribute to the overall spectral intensity and, when present in appreciable contents, such low molecular weight molecules may be imaged independently by chemical-shift-selective imaging sequences. Image contrast is also generated by variations in relaxation properties in addition to total mobile ^{1}H contents. The so-called spin lattice, T_1, and spin-spin, T_2, relaxation processes are influenced by a variety of factors such as cell size and solute concentrations, but they are particularly sensitive to the presence of paramagnetic ions. T_1- or T_2- weighted images are generated by using short pulse sequence repetition times (TR) or long times between the rf pulse and the echo (TE), respectively, whereas total mobile ^{1}H images require long TR and short TE. Times for image generation are generally a few minutes for 2-dimensional and a few hours for 3-dimensional data sets (depending on resolution required); with the former it is usual to sum together the data from several accumulations to produce improved signal-to-noise ratios. The various experimental parameters are presented in the legends for each figure or original references where cited. With the exception of the work with potatoes, which was performed in a super-wide-bore probe (maximum sample diameter 65mm) of "birdcage" design, all measurements were made in standard wide-bore probes with 25mm coils.

EPR Spectroscopy: EPR measurements were performed on a Bruker ESP300E computer controlled spectrometer operating at X-band frequencies with the relevant instrumental parameters shown in the various figure captions.

Results and Discussion

The principal objective of this work was to evaluate the potential contributions of magnetic resonance techniques to the study of natural senescence processes in fruit and to understanding the physiological and biochemical processes involved in the development of physical damage as a result of biotic or abiotic stresses in a number of plant organs. Emphasis in this paper is placed on evaluating the use of NMR microimaging techniques, whose applications to these areas of biology have received little attention to date. The roles of EPR spectroscopy in helping with understanding the origins of features in NMR images is described briefly along with its use to study oxidative (free radical) processes. Attention is also drawn to some practical limitations in its applications.

(1) Natural senescence in fruit: The raspberry fruit is a very convenient subject for NMR microimaging studies and has featured strongly in our research programme in recent years (GOODMAN & al. 1992a,b, WILLIAMSON & al. 1992a,b, 1994). Development of senescence involves distinct physical changes, which have been followed in vivo using a 2D spin echo imaging sequence; NMR images of fruit developing from the red ripe to the overripe states are shown in Fig. 2. A number of changes can be seen, the most distinct being the physical separation of the drupelets from the receptacle and an increase in the size of the gas spaces associated with drupelet junctions. This figure also shows evidence of physical damage to a drupelet, caused by a parasite which moved during the experiment (Fig. 2b,d). Fruit ripening is accompanied by a change in relaxation properties of intracellular water as a consequence of changing solute concentration. An example for strawberry is illustrated in Fig. 3, which shows differences in the T_1 relaxation profiles (expressed as the ratio of intensities for parenchymal and vascular tissues) for green and red ripe fruit.

(2) Biotic damage in fruit: Substantial differences in relaxation parameters are often observed between damaged and healthy tissue. It is possible, therefore, by appropriate choice of relaxation weightings, to design NMR imaging measurements to highlight either healthy or damaged tissues (it is also possible to generate images that do not make such discrimination, which can be useful in some instances to demonstrate that no major structural changes have occurred). Over recent years, we have successfully used NMR microimaging to study fruit infected by the fungal pathogen *Botrytis cinerea*, e.g. raspberry (GOODMAN & al. 1992b), blackcurrant (WILLIAMSON & al. 1992b), strawberry (GOODMAN & al. 1996), grape

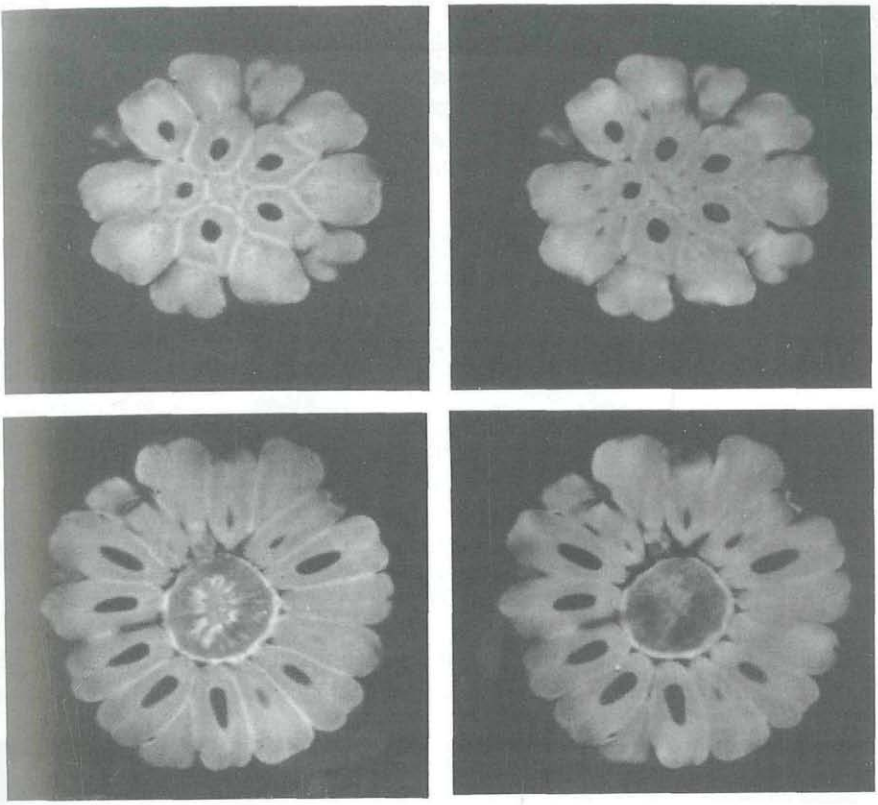

Fig. 2. 2-dimensional spin echo images of transverse slices at the distal (a,c) and proximal (b,d) ends of a single raspberry fruit (*Rubus idaeus* L. cv. Autumn Bliss) in the red ripe (a,b) and over ripe (c,d) states. Note the damage caused by an insect parasite in the top left hand corners of images b, d.Full experimental details are given in WILLIAMSON & al. 1992a, in which images a and b are also reported.

(GLIDEWELL & al. 1997). Diseased and healthy tissues are readily distinguished, but the nature of these differences varies from one type of fruit to another. Thus, with *Botrytis* infection in raspberry, tissue discrimination is best observed using a gradient echo sequence that is sensitive to magnetic susceptibility variations within the specimen, because leakage of intracellular fluids into intercellular gas spaces produces a major change in overall magnetic susceptibility as a result of a decrease in the area of gaseous interfaces (GOODMAN & al. 1992b). With strawberry, diseased tissue is more readily identified in T_2-weighted images, because of a much longer T_2 in diseased than in healthy tissue (GOODMAN & al. 1996). By using 3D

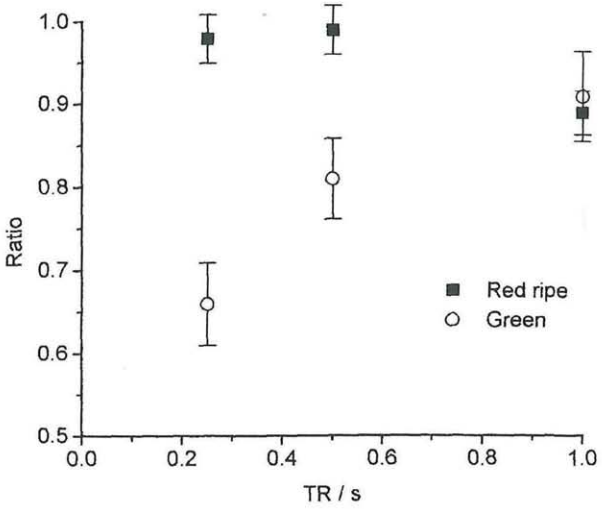

Fig. 3. Variations in the ratios of absolute signal intensities of parenchymal and vascular tissues in green and red ripe cultivated strawberry (*Fragaria* x *Ananassa* cv. Marmolada) as a function of TR (i.e. T_1 weighting) in spin echo images (adapted from GOODMAN & al. 1996).

surface rendering techniques, volumes of diseased tissue can be measured, thereby making it possible to study disease developmental processes (Fig. 4).

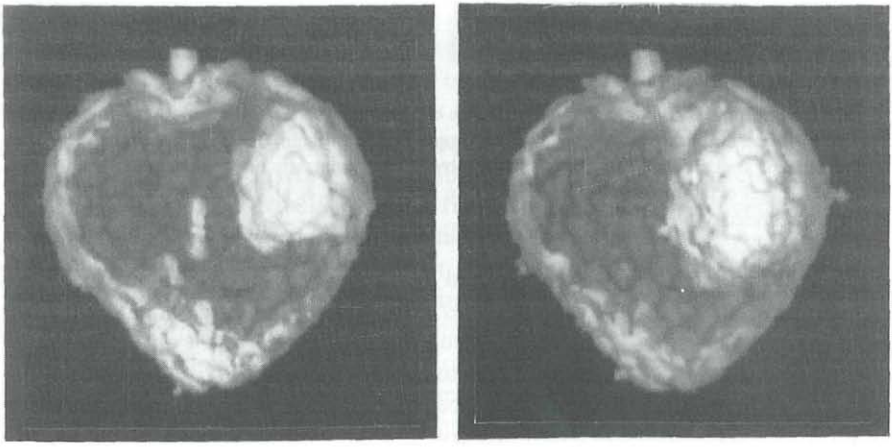

Fig. 4. Surface rendered 3D reconstructions of red ripe strawberry fruit illustrating the development of a lesion caused by *Botrytis cinerea* (a) 48 h and (b) 60 h after inoculation. Full parameter details are given in GOODMAN & al. 1996.

(3) Abiotic damage in fruit: An example of physical damage to fruit caused by a parasitic insect is illustrated in Fig. 2, which essentially shows a hole where

the tissue has been consumed. This figure also shows a feature which we believe corresponds to the insect, which moved during the course of the study (Fig. 2b,d). It is also possible to visualise chemical damage to fruit tissue, and we have recently used spin echo procedures to study the development of stalk end rot (putative SO_2 damage) in grape berries (GLIDEWELL & al. 1997). With appropriate T_2 weighting, clear distinction can be made between healthy and damaged tissue (Fig. 5).

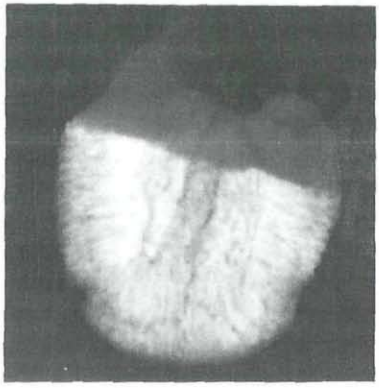

Fig. 5. T_2-weighted 2D spin echo NMR image of a Thompson seedless grape berry showing stalk end brown discolouration, probably due to overexposure to SO_2. TE = 70 ms, TR = 1000 ms, voxel dimensions = $(78\ \mu m)^3$.

When NMR microimaging is combined with 3D procedures, we have a technique that is capable of performing internal quality assessments in fruit (albeit uneconomically at the present time). Such procedures may take the form of surface rendering of the image of the damaged tissue, as shown with *Botrytis*-infected strawberry in Fig. 4, or maximum intensity projections, as used in Fig. 6a,b for an apple. In these T_2-weighted images, bruised tissue appears bright (as has been reported previously by MCCARTHY & al. 1995) and microbial lesions dark compared to the healthy tissue. Although maximum intensity projections provide a complete representation of all of the features in a specimen, individual features may be seen more clearly in 2D slices (e.g. Fig. 6c,d). The T_2-weighted image (Fig. 6c) shows the tissue damaged by the bruise spreading from the surface through to the core and the hole just below the surface near to the flower end of the fruit is clearly revealed in a T_1-weighted image (Fig. 6d)

(4) Biotic damage in potato tuber: Different pathogens may also manifest their presence in different ways in NMR images. For example, in studies of potato tubers infected with the fungal pathogens *Phytophthora infestans*, *Phoema foveata* and *Fusarium solani* var. *sulphureum,* SNIJDER & al. 1996 were able to discriminate, not only between healthy and diseased tissues, but also observed some differences between tissues infected with the different pathogens. Thus, tissue infected with *P. infestans* was readily distinguished from that infected

with either of the other two pathogens by its low intensity in T_2-weighted images. This decrease in intensity was much less than expected for the decrease in water content (from 85% to 69% on a fresh weight basis) and is consistent with increased levels of unpaired electrons in the infected tissue (see (6) below), with the result that much of the spectral intensity decays before the signal can be detected, even with short TE times. With such specimens, the diseased tissue is best visualised in 3D surface renderings of the healthy tissue component. An example of such an exercise is illustrated in Fig. 7, where two orientations of the image are shown for a potato in which the healthy ends were removed electronically. In an analogous manner to the *Botrytis*-infected strawberry fruit, it was possible to monitor the development of diseased tissue with time.

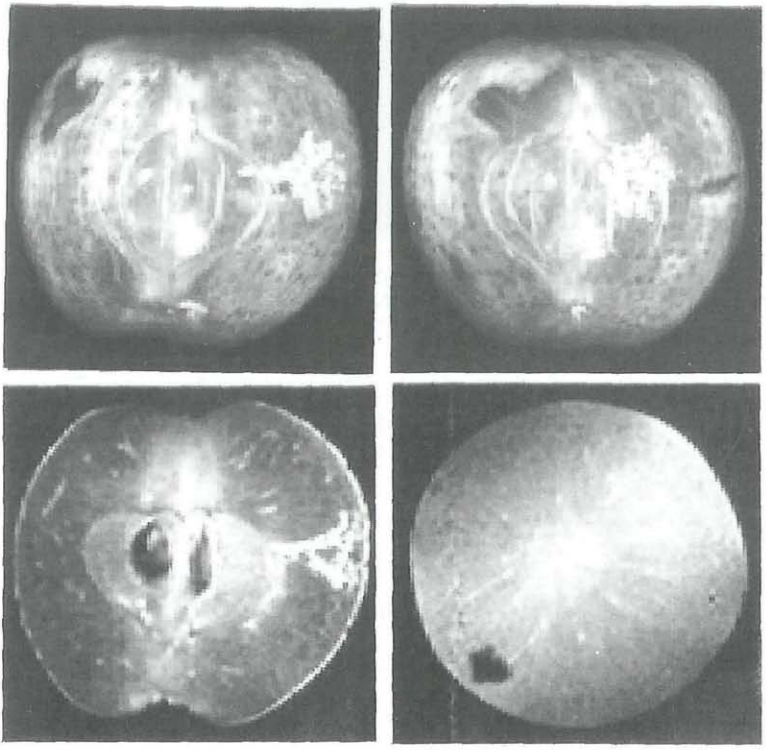

Fig. 6. Spin echo images of an apple. (a), (b) two orientations of a 3D maximum intensity projection, (c) 2D median longitudinal slice of a T_2-weighted image, each with TE = 30 ms and TR = 1750 ms, (d) 2D transverse slice near flower end of a T_1-weighted image with TE = 6.56 ms, TR = 100 ms. In all images, voxel dimensions are $(430\ \mu m)^3$. Note. The bright feature corresponding to bruised tissue is is best seen in the centre right of images a,c; the dark feature near to the surface of images a,b corresponds to a surface lesion; the dark feature near to the bottom left of image b is clearly revealed as a hole in image d.

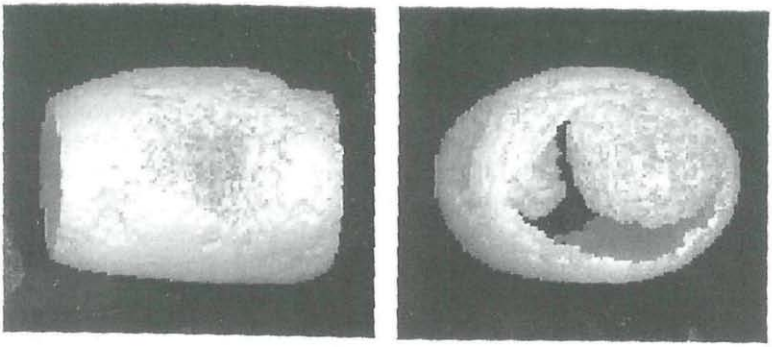

Fig. 7. Two orientations of a 3D surface rendering of the healthy tissue in a T_1-weighted gradient echo NMR image of a potato tuber infected with *Phytophthora infestans*, 33 days after inoculation. TE = 4.56 ms, TR = 100 ms, voxel dimensions = $(470\ \mu s)^3$.

(5) Free radicals in damaged tissue: Oxygen-derived free radicals have been implicated extensively in biological tissue damage caused by biotic and abiotic factors and there have been several studies in which attempts have been made to use EPR spectroscopy to measure free radical signals generated as a result of such processes (e.g. HENDRY & al. 1992, SEEL & al. 1991). Such applications are complicated by the ability of water to absorb microwaves, so that in any water-containing specimen the spectrometer sensitivity varies with the water content of the specimen. Few, if any, studies to date have incorporated internal standards to address this problem and as a consequence the quantitative conclusions of all such studies are invalid, although qualitatively the results are of value. The problem of microwave absorption is largely eliminated in frozen samples, but at the expense of disruption of the biological processes being studied (and hence losing much of the advantage of a non-invasive technique). Thus EPR spectroscopy needs to be used with great care for quantitative work on biological systems, but nevertheless may have considerable value when used properly.

The generation of unstable oxygen-derived free radicals is usually observed in biological systems by measuring the products of reaction with a chemical known as a spin trap. Spin traps have the ability to react selectively with unstable free radicals to produce new radicals with much greater stability. An example is the identification of the role of oxygen in free radical generation in the resistant response of potato tubers to the bacterial pathogen *Erwinia carotovora* (DEIGHTON & al. 1992). Occasionally, the generation of unstable oxygen-derived free radicals may be observed without any chemical intervention, as reported by GOODMAN & al. 1986 in the study of anoxia and post-anoxic recovery in wheat roots. In this latter work the quantitative aspects are valid because the same specimen was used throughout, with its hydration level maintained.

The generation of stable free radicals is more easily monitored (e.g. DEIGHTON & al. 1992, HENDRY & al. 1992, SEEL & al. 1991), but, with

biologically-viable tissue in general, care has to be taken to distinguish between stable free radical centres and unstable metabolically-generated radicals in steady state equilibrium or stabilised by quenching to low temperatures. In whole seeds in particular, there may be additional complications from the presence of large, but variable, quantities of stable free radicals in the testa, especially those with intense colour (HEPBURN & al. 1986). At the commonly used X-band frequencies, these radicals give EPR spectra that are essentially identical to those generated as a result of metabolic activity. Fig. 8 shows spectra for strawberry achenes at different stages of development and shows the intensity of the free radical signal decreasing with increasing maturity, presumably as a consequence of the slowing down of metabolic processes. It should be noted that this free radical signal (shown in expanded form in the inserts to each spectrum) was identical in each case with evidence for some anisotropy on the high field side of each resonance. Studying free radical generation as a result of damage to seeds is further complicated by the fact that the breaking of dormancy leads to an increase in EPR free radical activity similar to that generated by damage leading to viability loss (our unpublished results). In this respect, more measurements need to be made on high frequency equipment (e.g. ATHERTON & al. 1993), in order to assess fully the ability of EPR spectroscopy to distinguish between these different types of radical.

(6) Oxidation processes involving transition metal ions: EPR is a particularly valuable technique for investigating the roles of transition metals in oxidative processes in plants. Identification of different types of metal ion can be achieved from the nature of the characteristic hyperfine structure patterns that are generated through interactions of unpaired electrons with nuclei with non-zero spin. Information on the nature of the chemical environments of such metals may be obtained from the g-values and through the observation of hyperfine structure from ligand nuclei. Spectra are obtained readily from metal ions with an odd number of unpaired electrons, but ions with an even number of electrons often require special experimental conditions for their observation. Consequently, in redox systems, it is common for only one oxidation state of a metal to contribute to a spectrum (e.g. Fe(III), Mn(II), Cu(II)). Oxidation of iron has been observed in both abiotic (MONK & al. 1989) and biotic (SNIJDER & al. 1996) damage in potato tubers. SNIJDER & al. 1996 were further able to demonstrate that most of the increased paramagnetism which affected the NMR images of tubers infected with *P. infestans* was attributable to Fe(III) and that increases in free radical contents were relatively minor (Fig. 9).

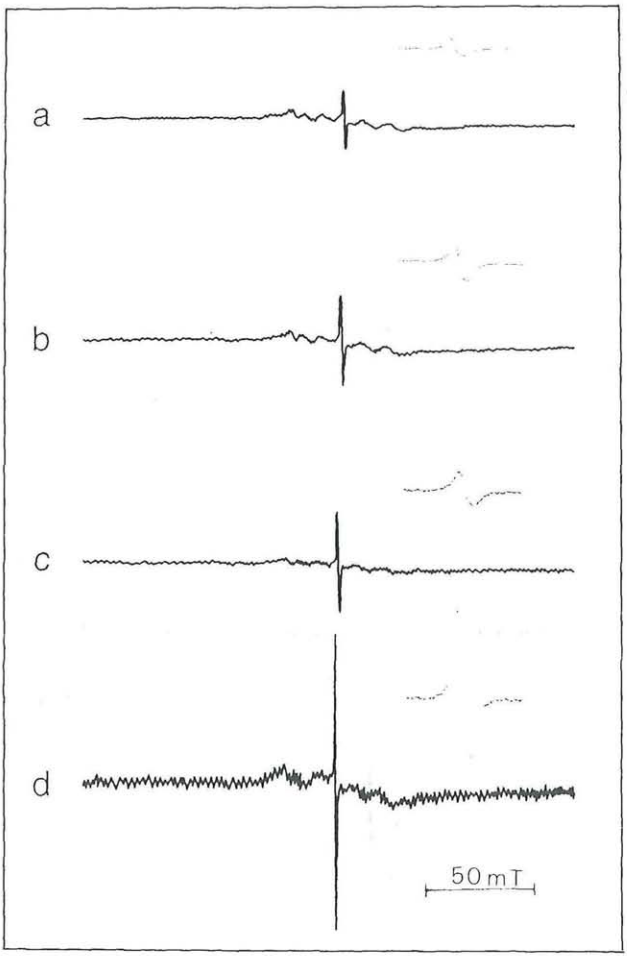

Fig. 8. X-band EPR spectra at ambient temperature of strawberry achenes (cv. Melody) scaled so that intensities are expressed relative to unit weight: (a) dark pigmented achenes from red-ripe fruit, (b) light coloured achenes from red-ripe fruit, (c) light coloured achenes from fully-developed green fruit, and (d) achenes from undeveloped fruit (ca. 10 mm). A modulation frequency of 100 kHz was used with 0.5 mT modulation amplitude and 10 mW microwave power. (adapted from GOODMAN & al. 1996).

Conclusions and Forward Look

NMR images are able to reproduce accurately the main histological features of a wide range of fruit and vegetables, although the values of optimum experimental parameters are dictated by the nature of the tissues of interest. By

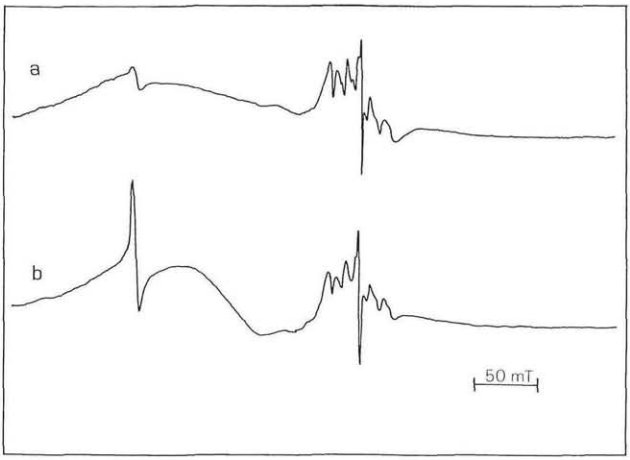

Fig. 9. X-band EPR spectra at 77 K of tissue from the medula of potato (*Solanum tuberosum* L. cv. Pentland Crown) from (a) control slice and (b) slice from the same tuber infected with *P. infestans*. A modulation frequency of 100 kHz was used with 0.6 mT modulation amplitude and 20 mW microwave power. Note. The only major difference between these spectra is the intensity of the g = 4.3 Fe(III) peak at ~160 mT.

appropriate choice of imaging sequence and parameters, damaged tissue (caused as a result of biotic or abiotic processes) can be distinguished from healthy tissue, as can changes associated with aging and senescence; the nature of tissue damage can also be determined where this is the consequence of different types of biochemical process. In the case of damage to potato tubers caused by important fungal pathogens, EPR spectroscopy has been able to characterise changes in paramagnetic components, which may be responsible at least in part for the observed contrast between healthy and diseased tissues, but most of its applications have been made with relatively small specimens, many of which are not ideal subjects for NMR imaging.

At the present time, NMR microimaging is performed in different laboratories on instrumentation operating over a wide range of ^{1}H frequencies (20 to 500 MHz), with consequent considerable variations in cost, sensitivity and resolution. In general, these all increase with increasing experimental frequency, but there are also accompanying complications from greater sensitivity of image quality to local variations in magnetic susceptibility. Identification of the optimum instrumentation for investigating different types of biological problem has still not been achieved.

In typical instruments, 2D and 3D representations can be obtained for the fluid-containing components of biological structures with dimensions in the range from a few millimetres to a few or several centimetres, with voxel (volume element) dimensions in the range from a few tens to several hundred micrometres.

Scope for increasing resolution is limited and only in relatively rare cases, and then only with small samples and high field instruments can individual cells be imaged (e.g. BOWTELL & al. 1990). For most practical purposes, therefore, discrimination in measurements will be confined to the different tissue types in a specimen.

Pulse sequences have been developed that allow computation of velocity of flow and diffusion (CALLAGHAN 1991, CALLAGHAN & al. 1994) and such procedures have applications in studies of transport processes in intact plants. Some chemical information is available from chemical shift selective methods in addition to the use of relaxation weightings; in this respect the development of imaging techniques based on 2D spectroscopic procedures holds particular promise for establishing the location and diffusion properties of major plant metabolites, such as glucose, sucrose, amino acids etc. (METZLER & al. 1994). When these procedures are combined with full environmental control in NMR probes, then we will have the facilities for a completely new approach to the study of plant responses to stress processes.

In the short-term EPR developments are likely to be less dramatic than those involving NMR imaging and will probably be concerned largely with the exploitation of the increased range of experimental frequencies that is now available. This is likely to lead to two separate types of development; an increase in the scope for in vivo studies with increasing availability of low frequency instrumentation, and a larger number of measurements with high frequency equipment to provide greater spectral discrimination.

Acknowledgements

This work was carried out with funding provided by the Scottish Office Agriculture, Environment and Fisheries Department. Mylnefield Research Services is gratefully acknowledged for the provision of the Bruker AMX300/SWB imaging NMR spectrometer. We are especially indebted to our many colleagues, but particularly, Dr. J.A. CHUDEK of Dundee University and Drs. N. DEIGHTON and B. WILLIAMSON of the Scottish Crop Research Institute, who have contributed substantially to various aspects of this work.

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