X-Band Electron Paramagnetic Resonance (EPR) Spectroscopic Investigations of Free Radical and other Redox Processes in Whole Plant Tissues and *In Vivo* – a Review.

Bernard A. Goodman*

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources,
Guangxi University, China

Abstract

This paper reviews various types of application of electron paramagnetic resonance (EPR) spectroscopy to understanding live biological systems. These are then illustrated with examples from the study of plant free radical and redox processes in tissue samples and intact specimens *in vivo*. Experimental procedures are critically presented along with examples of applications that include the elucidation of normal biochemical processes, as well as the responses of plants to abiotic and biotic stresses. Procedures presented include various approaches for discriminating between different types of free radical, the real time observation of variations in the rates of total free radical generation in response to changes in a plant's environment, the identification of transition metal ion speciation, and the measurement of transport processes *in vivo*.

Keywords : EPR, plant, in vivo, free radical, redox

***E-mail:** bernard_a_goodman@yahoo.com

1. Introduction

Free radical reactions and redox processes are fundamental to life. However, their roles are complex, and still not fully understood. In this paper a brief summary is presented of contributions that electron paramagnetic resonance (EPR) spectroscopy has made towards developing our understanding of such processes in plants through work with tissue samples and intact specimens. This addresses normal metabolism and stress situations, and in the case of the latter considers responses to both biotic and abiotic challenges.

Redox signaling is a feature of all aerobic life, and is influenced by both normal metabolism and the environment. Foyer and Noctor (2005) have described a plant cell as a series of interconnecting compartments with different antioxidant buffering capacities determined by differences in synthesis, transport, and/or degradation, and as a consequence signaling is controlled independently at a set of discrete locations. Oxidants and antioxidants both provide information on general plant health, and antioxidants, especially glutathione and ascorbate, represent a dynamic metabolic interface between plant cell stress perception and physiological responses.

Reactive oxygen species (ROS), which are chemical species related to O_2, but more reactive than O_2 itself, form the foundations for many applications of EPR spectroscopy. ROS are crucial components of life, but also represent potentially toxic products generated during the process of living. Therefore, all forms of life have developed a vast array of defence processes to minimize tissue damage resulting from ROS generation. However, it is always a battle, and unlike most techniques which can only address the results of such conflict, EPR spectroscopy is able to give a glimpse of this battlefield whilst the fight is in progress.

ROS consist of various free radical species, such as the hydroxyl radical ($HO^{\bullet}$). the superoxide radical anion ($O_2^{\bullet -}$), its protonated form ($HOO^{\bullet}$), and peroxy radicals ($ROO^{\bullet}$), as well as non-radical species, which include hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), an unstable form of oxygen. As in all living systems, ROS play fundamental roles in plants, and are involved in normal metabolism as well as in responses to biotic and abiotic stress processes. They are constantly produced during photosynthesis and respiration, and also have important functions in signaling and regulation of a variety of cellular processes. The roles played by ROS in plant development through the regulation of cell growth have been reviewed by Gapper and Dolan (2006). The rationale behind various experimental approaches used for ROS measurement *in vivo* and in cell cultures has been critically reviewed by Halliwell and Whiteman (2004), and although the emphasis in that paper was on problems related to human diseases, much of the discussion is equally valid for other biological systems, including plants. Methodological problems specifically related to investigations of ROS in a plant context have been reviewed by Shulaev and Oliver (2006). The presence of ROS can be measured either directly, or their existence can be inferred indirectly as a result of the formation of oxidation products of lipids, proteins, or nucleic acids. However, *in vivo* measurement of ROS and elucidation of cellular responses to oxidative stress is complicated by their reactions often being localised in subcellular compartments. Also, although some ROS, such as hydrogen peroxide and superoxide, are relatively stable, they may be converted in the presence of low oxidation state transition metal ions, to more highly reactive species, such as hydroxyl radicals, Fe(IV)-oxo species, and $^1O_2$ (Freinbichler *et al*., 2011).

EPR is able to monitor in real time *in vivo* changes in concentrations of any paramagnetic species that produce EPR spectra at ambient temperatures. However, in practice concentrations of many free radicals are often below the limits of detection *in vivo*, and various "tricks" have been developed for their measurement. Methodology for measuring redox-related processes *in vivo* has been summarized by Khan and Swartz (2002), and Swartz *et al*. (2007), who described 3 complementary approaches: the

rate of reduction or reactions of nitroxides, spin trapping of free radicals, and measurements of thiols. Measuring nitroxide signal intensities is straightforward, but results are difficult to interpret because of contributions from several different mechanisms. *In vivo* spin trapping provides direct evidence for the occurrence of specific O-, C- and S-centered radicals and NO, but accurate quantification is not usually possible. Finally, the measurement of thiols *in vivo* is at an early stage of development, but is showing promise in mammalian systems. In addition, O_2 can be detected using O_2-sensitive paramagnetic materials, and some paramagnetic metal ions can be measured directly. However, the detection, quantification, and imaging of ROS in live biological specimens is challenging, and it is necessary to exercise caution in interpreting experimental results.

2. The EPR technique

2.1 Introduction to EPR spectroscopy

EPR spectroscopy is a versatile and sensitive technique for detecting and studying molecular systems possessing unpaired electronic spins; the technique is based on transitions between the spin states of unpaired electrons, whose energies are different in the presence of a magnetic field (Fig. 1a). These energies are typically in the microwave region of the electromagnetic spectrum, and an EPR spectrum is usually obtained by measuring the absorption of microwaves of a fixed energy whilst the magnetic field is varied. The g-value is a constant specific for the molecule under investigation, although for free radicals there is little variation from the value of 2.0023 for a free electron.

If the unpaired electron is able to interact with a nucleus with spin, $I \neq 0$, the energy levels are split into $2I + 1$ components, and the selection rules for the allowed transitions are $\Delta m_S = 1$, $\Delta m_I = 0$. The resulting structure in a spectrum is known as hyperfine structure (hfs), and is illustrated in Fig. 1b for the case of $I = ½$. The magnitude of the hfs is related to the unpaired electron

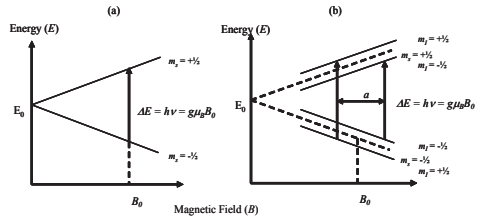

Figure 1. (a) Splitting of the energy levels of an electron in a magnetic field and the condition for electron paramagnetic resonance (EPR), (b) The origin of hyperfine structure from interaction of an unpaired electron with a nucleus of spin, $I = 1/2$

density at that nucleus, and it hence provides information on the delocalisation of the orbital containing the unpaired electron. Furthermore, hfs patterns when they are resolved can provide evidence for the identification of specific free radical species, though this is seldom the case with natural free radicals *in vivo*.

In addition to the characterisation of free radicals, EPR spectroscopy is also amenable to the study of other paramagnetic chemical species, especially those derived from transition metal ions containing an odd number of unpaired electrons, and the g-value and hfs patterns provide useful "fingerprints" for the presence of certain nuclei.

2.2 Instrumental requirements

Since EPR spectroscopy, like its better known relative NMR, is non-destructive and non-invasive, it can in principle be used for direct investigations of living organisms. However, because water strongly absorbs microwave radiation, there are severe limits placed on the water contents of samples that can be tolerated without the risk of heating and tissue damage. The absorption of microwaves by water is frequency dependent, and as a consequence spectrometers designed for use for *in vivo* biological studies normally operate at relatively low microwave frequencies (in the 3.5 GHz - 300 MHz range); such spectrometers have found valuable applications in investigations involving small mammals, especially in

the development of EPR imaging techniques (*e.g*. Krishna *et al*., 1998; Elas *et al*., 2003; Shen *et al*., 2009). Spectral sensitivity is, however, related to the transition energy, and with small samples there are considerable sensitivity gains to be made by working at the more common X-band frequencies (~9 GHz). Thus, in tissues with appropriate morphology (where only a small volume of water is present in the spectrometer resonance cavity), it is possible to perform high sensitivity *in vivo* EPR spectroscopy at X-band frequencies in addition to conventional measurements using frozen tissue samples (in which microwave absorption is generally not a problem).

2.3 Identification of specific radicals

Hfs patterns can be used as "fingerprints" for the presence of radicals with distinct EPR spectra. The most common is the ascorbate radical which has ^{1}H hyperfine splittings of $a(H_4)$ = 0.176 mT, $a(H_5)$ = 0.007 mT, $a(H_6)$ = 0.019 mT x 2, although in most biological samples it is necessary to use a modulation amplitude that is too high to resolve the small ^{1}H hf splitting. Another common radical in biological systems is ubisemiquinone. However, its EPR spectrum consists of a single peak with 1st derivative peak-to-peak linewidth of ~0.6 mT, which is thus quite similar to the spectra of melanoidins. Therefore there can be problems with the correct assignment of radicals in the EPR spectra of complex biological systems. The use of EPR for fingerprinting is much more common in *in vitro* measurements, *e.g*. in assessing the relative contributions of various green tea polyphenols to the free radical signal generated during the oxidation of aqueous extracts of green tea (Ferreira Severino *et al*., 2009, or in identifying the role of chlorogenic acids (caffeoyl quinic acids) in the oxidation of extracts of bitter tea (Pirker & Goodman, 2010). However, such discrimination is not usually possible *in vivo*, because the steady state concentrations of reactive free radicals are usually below the limit of detection by the technique.

2.4 Experimental procedures – spin trapping

Although in principle free radicals can be detected directly by EPR spectroscopy, the size of many specimens may be such that they contain too much water to study in an available spectrometer. Also, the physical properties of some species may render them undetectable at typical ambient temperatures. Some of these problems may be overcome by performing measurements at low temperatures, which also has the effect of allowing spectra to be acquired over extended periods of time, if necessary. The study of frozen samples can be effective if the original material contained relatively high equilibrium concentrations of the paramagnetic species, but it is extremely difficult to measure highly reactive radicals directly because of the lack of an adequate concentration at any particular time. In some situations the problem of their detection can be overcome by spin trapping, which is a procedure in which diamagnetic traps react with unstable radicals to produce (more) stable radicals that can then be detected. However, a major limitation in the use of the spin trapping technique *in vivo* is that it is necessary to infiltrate large quantities of spin trap molecules into cells, and thus to influence the biochemical processes that are being investigated. Furthermore, Khan *et al*. (2003) have reported considerable variations in the toxicity of different types of spin trap and in the stability of adducts in cell suspensions.

Spin traps are often nitrones, and form nitroxides by addition of a free radical to the carbon atom adjacent to the N-O group (Fig. 2); in principle, different radicals produce EPR spectra with different parameters (*e.g*. Buettner and Mason, 1990). However, caution has to be exercised in interpreting the results from spin trapping experiments, because different types of spin trap can (a) have different selectivities for different types of free radical, and (b) access different subcellular compartments.

Highly unstable radicals such as ·OH react with the substrate at their site of formation, often extracting hydrogen atoms and producing secondary C-centred radicals that then react with the spin trap. In addition, side reactions may complicate the use of spin trapping to

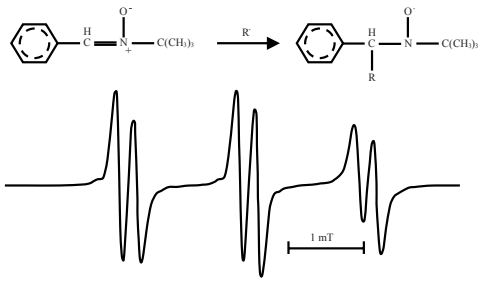

Figure 2. Formation of a spin trap adduct by reaction of a free radical R. with the spin trap N-t-butyl-α-phenylnitrone (PBN).

characterise free radical production in biological systems. For example, the redox reaction between nitroxides and ascorbic acid, leads to the production of the diamagnetic hydroxylamine and the ascorbate radical (Kocherginsky and Swartz, 1995) (Fig. 3). It is also possible for nitrones to undergo non-radical reactions to give false identification of the presence of free radicals (*e.g*. Ranguelova and Mason, 2011), and radical species can be generated by oxidation of the spin trap, the products of which may then be trapped by unreacted spin trap molecules (*e.g*. Mao *et al*., 1994; McCormick *et al*., 1995).

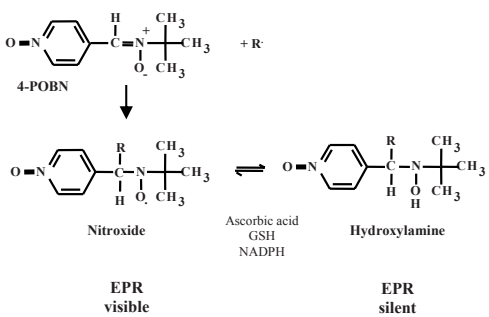

Figure 3. Reaction of nitroxides (adducts of nitrone spin traps) with reducing agents (antioxidants) to produce the diamagnetic hydroxylamines.

Discrimination between different types of free radical can be made on the basis of the EPR spectral parameters of their adducts with spin trap molecules (NIEHS Spin trap data base), and the performance of different spin-traps for qualitative analysis of radical-generating systems, has been reviewed by Bačić *et al*., (2008). These authors also discussed the ability of various spin traps to recognize previously unnoticed radicals, and reviewed the difficulties involved in detecting different O-centered radicals in chemical and biological systems. By using the spin-trap 5-diethoxyphosphoryl-5'-dimethyl-1-pyrroline-N-oxide (DEPMPO), they also demonstrated hydrogen atom (.H) generation in the Fenton reaction, and various biological systems. Nevertheless, despite the various problems associated with their use, spin trapping has aided considerably the development of our knowledge of the behavior of free radical ROS in biological systems. Furthermore, in recent years there have been major efforts to develop spin traps for specific purposes, such as improved radical selectivity, adduct stability, or for probing specific types of tissue. For example, Hideg *et al*. (1999) have synthesised adamantyl nitrone spin traps for detecting the locations of radical production within the thylakoid membranes, and Robertson and Hartley (2009) have described the use of N-arylpyridinium salts with a nitrone moiety for detecting generation of ROS in mitochondria.

2.5 Experimental procedures – use of spin probes and spin labels

Molecules known as spin labels and spin probes can provide information on physical and chemical properties of specific molecular sites in a biological system. These are stable free radicals or paramagnetic metal ions, which should produce minimal perturbation to the system and which have EPR spectra that can be distinguished from background signals. In principle any stable paramagnetic species could be used to probe mobility in biological fluids, as long as its rigid-limit spectrum is anisotropic. Aqueous and lipid phases can be probed selectively by changing functional groups on the probe, *e.g*. liposome-based pH sensitive nanoSPIN probes (nano-sized particles with incorporated nitroxides have recently been described by Woldman *et al*., (2009). By determining the extent to which the anisotropy of paramagnetic molecules is removed by molecular motion, information is produced

on their mobility, and hence the viscosity of the fluids in which they are located. Nitroxides are generally used for this work, and empirical relationships have been developed to relate the relative heights of the 2nd and 3rd peaks in Fig. 4 to their motional properties.

However, rotational correlation times can be calculated directly using modern spectral simulation programs, such as *Easyspin* (Stoll and Sweiger, 2006). Caution must be exercised in the use of nitroxide spin probes for *in vivo* studies, because of the reversible redox reaction with ascorbic acid produces the diamagnetic hydroxylamine (Fig. 3), although Bobko *et al*. (2007) have described nitroxides where the nitroxide-hydroxylamine equilibrium favours the nitroxide form.

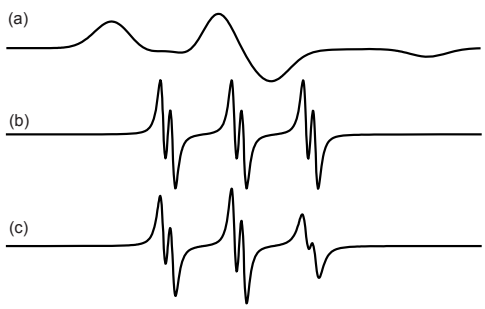

Figure 4. Typical nitroxide solution EPR spectra (a) frozen, (b) fluid, (rapid motion), and (c) fluid (restricted motion giving incomplete averaging of the anisotropy).

2.6 Measurement of redox-related parameters

EPR experimental methods for the measurement of tissue pO_2, redox, pH, and glutathione have been reviewed by Khan *et al*. (2010). Disulfide nitroxide biradicals (DNB) can be used for EPR detection of GSH *in vivo* if ^{15}N is substituted in the NO fragment to enhance the spectral resolution, and ^{1}H atoms are substituted by ^{2}H to reduce the linewidth (Roshchupkina *et al*., 2008). In addition, some new isotopically labeled isoindoline nitroxides and an azaphenalene nitroxide for use in EPR oximetry have been evaluated Khan *et al*. (2011). Trityl-nitroxide biradicals, which are both stable and have good EPR sensitivity, are able to provide simultaneous measurement of redox status and oxygenation (Liu *et al*., 2010), and trityl radical-conjugated disulfide biradicals have potential use as paramagnetic thiol probes (Liu *et al*.,. 2011). Such biradicals show broad EPR spectra at room temperature because of intramolecular spin–spin interactions, but reaction with thiol compounds such as glutathione or cysteine results in the formation of trityl monoradicals which have high spectral sensitivity to oxygen. The moderately slow reaction allows for *in vivo* measurement of GSH concentration without altering the redox environment in biological systems. Direct detection of $^{1}O_2$ in plants under stress conditions requires highly reactive and selective indicator reagents, localized at the presumed site of production, because of short life-times and diffusion distances over which it can travel. However, such determinations are especially important for identifying primary events of oxidative damage and exploring the potential role of ROS as signal molecules. As potential agents for such assays, Hideg *et al*. (2006a) have described double (fluorescent and spin) indicator reagents, in which partial fluorescence quenching of a dansyl moiety occurs as a result of nitroxide radical formation from a sterically hindered amine constituent.

2.7 Characterisation of coordination environments of transition metal ions

There is a large literature on the use of EPR to characterise transition metal species and standard texts on the subject should be consulted for more details (*e.g*. Abragam and Bleaney, 1970; Pilbrow, 1990; Mabbs and Collison, 1992). However, for a number of reasons, which will not be discussed here, only a relatively few transition metal species are amenable to *in vivo* characterisation. The principal exceptions are those that have a single unpaired electron and a non-zero magnetic moment, such as $^{51}V(IV)$ and $^{63,65}Cu(II)$, and EPR is a good fingerprint technique for these ions. It is also sensitive to the solvated Mn(II) ion, which is a highly symmetrical $3d^5$ species, but is much less sensitive to this ion when the symmetry is

distorted from cubic. A greater number of chemical species can be identified in solid (i.e. frozen) samples, and it may also be valuable to investigate frozen samples to obtain additional information on species that can be seen at ambient temperatures under true *in vivo* conditions. The chemical identification of any complex species is determined primarily by the g- and hyperfine (A-) values, and in addition, Cu(II) complexes with N-containing ligands often produce superhyperfine structure (shfs) from ^{14}N nuclei. Such structure can provide an unambiguous identification of the copper coordination environment (although the absence of such structure does not necessarily mean that there is no nitrogen coordinated to the copper).

2.8 Qualitative versus quantitative applications

Although in principle, the EPR spectral intensity is proportional to the concentration(s) of the paramagnetic materials responsible for the signal, there are a number of practical problems associated with the derivation of true quantitative information with complex biological systems. Because the amount of water in the specimen affects the intensity of the signal, great care has to be exercised in attempting to perform quantitative measurements *in vivo*. These should be made either with a dual sample cavity, or with a standard sample included in the spectrometer along with the experimental sample. It is also essential that the saturation characteristics of the radicals and standard samples are known, so that measurements can be made with optimum microwave powers. Nevertheless, even with care in experimental design, most EPR measurements are only of a semi-quantitative nature.

3. Plant Samples, Preparation, and Handling

Seeds are obvious materials for plant *in vivo* EPR measurements, but plants of the grass family, which include cereal crops, are also suitable for *in vivo* EPR measurements, and in principle it is possible to design experiments for measurements on root, stem or leaf tissues in intact specimens. However, because of the considerable sensitivity of normal biological processes to changes in various environmental parameters, it is essential that all plant *in vivo* work uses specimens grown under strictly-controlled conditions from seed lots of known age and origin. Ideally, seed germination and growth should be performed in a computer-controlled climate chamber, so that conditions can be chosen to produce if necessary controlled stress conditions during the early stages of growth of the plant. Although the ability to control these conditions is clearly essential for any investigations into "hardening", the tolerance of plants by pre-conditioning them to stress, any experiments that monitor stress responses are of limited value if the nature of the stress(es) experienced by the specimen is/are not fully known. There is extensive literature on the growing of plants under controlled soil-free conditions (*e.g.* Hoagland and Arnon, 1950), and it is essential in any investigation that all experimental conditions are fully documented. When plants are grown in soil, the soil should be one that has been fully characterized. However, for measurements on plant roots, soil should be avoided, because of problems of sample contamination, especially by the adherence of clay mineral particles to root surfaces. Clay minerals usually contain transition metals such as iron and manganese, which commonly exist as paramagnetic ions, and also free radical defect centres; these can, therefore, strongly interfere with measurements of paramagnetic species in plant tissues. It should also be borne in mind that although the acquisition of an EPR spectrum is relatively quick, it does involve the sample being held in a (fairly strong) magnetic field and bathed in microwaves, both of which could alter physiological processes. For this reason, it is probably best to avoid long term measurements, and the technique is most effectively used to investigate changes in the minutes-hours timescale (although of course as with all biological samples, several repetitions with different specimens are usually required). The major limitation in experimental design concerns the amount of water within the spectrometer resonator, otherwise the consequences of any conceivable stress

(biotic or abiotic) can be probed. Care has to be taken in the selection of the microwave power, not just to avoid signal saturation, but because tissue heating can occur as a result of absorption of microwaves by water in the specimen. Sample preparation and handling is less critical for investigations of seeds and excised tissues, although there are pitfalls that must be avoided. Seeds frequently contain microbial contaminants and should be cleaned and sterilized before being investigated. Also, it may sometimes be necessary to remove the testa from seeds in order to distinguish their EPR signals from those of the embryos or cotyledons, since they are often similar. This must be performed with care in order to avoid physical damage, which will initiate free radical reactions. Tissue excision, however, usually involves major physical damage, which can produce a rapid free radical response. Thus processes resulting from sample preparation must be able to be distinguished from those being investigated, if meaningful conclusions are to be obtained. Finally, there is the question of water content, and since microwaves are strongly absorbed by liquid water, any change in the water content of a sample during an experiment will affect the sensitivity of the spectrometer. Microwave absorption is much lower with frozen water (ice) (Fig. 5), and for this reason excised tissue measurements are often made with frozen samples.

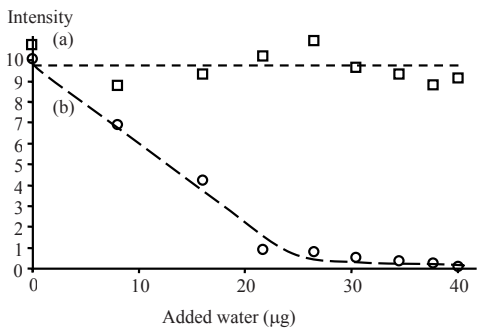

Figure 5. Variation in the intensity of the EPR signal for a standard DPPH sample with added H_2O (a) frozen, and (b) as liquid.

In many areas of biology it is common to work with freeze-dried specimens, because the freeze-drying process allows water to be removed from samples without the application of heat. However, this method of sample preparation is inappropriate for the study of free radical processes, because of reactions that are initiated between cellular components that are normally kept separate in live tissue. In work with strawberry fruits, Pirker *et al*. (2002) observed free radical concentrations (expressed relative to the dry weight) that were ~10 times higher in freeze dried fruit compared to frozen fruit.

4. Applications to physiological processes in seeds

4.1 Normal physiological processes

Many seeds have water contents that are sufficiently low for the direct recording of EPR spectra. As with many types of biological tissue, the EPR spectra from seeds consist of components that can be assigned to free radicals, along with the transition metal ions Mn(II) and Fe(III), the latter consisting of features corresponding to mononuclear complexes and polymeric species. However, the relative amounts of these paramagnetic components can vary greatly from one type of seed to another (Hepburn *et al*., 1986). Furthermore, especially in dark colored seeds, the testa frequently contain large quantities of free radicals in the form of melanoidins, and these thus complicate the interpretation of the results related to processes occurring in other parts of the seeds. Primarily for this reason, many studies have been performed on separated tissues, especially the embryonic axis.

In a review of the relationships between O_2, free radical processes and seed mortality Hendry (1993) presented evidence which showed that free radicals play a central if not causal role in promoting molecular damage from environmental stresses and induced ageing in mature plant tissue, although the evidence was less certain for ungerminated seeds. However, because free radical

reactions differ quantitatively and qualitatively in living and dead tissues attempts to correlate radical-mediated damage with viability in a population of seeds may be unreliable, particularly when measurements are made at uncertain intervals after death. Furthermore, Hendry speculated that O_2 plays a central role in seed mortality and may have significance in the evolution of seed persistence. In contrast, Schopfer *et al*. (2001) have shown that seed germination is accompanied by the active and developmentally controlled generation of various ROS (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase, which were considered to have a possible role in protecting emerging seedlings against pathogen attack.

The importance of pigments in the testa in protection against degenerative processes in seeds during artificial aging has been demonstrated by Zhang *et al*. (2006) for yellow and black-seeded rapeseed. After artificial aging, the yellow seeds showed faster deterioration with lower germination percentage, lower vigour index and lower reducing sugar contents, whereas markers of damage, such as electrical conductivity and malondialdehyde (MDA) content, were significantly higher, and the activities of antioxidant enzymes, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were significantly lower.

4.2 Desiccation damage to orthodox and recalcitrant seeds and embryos

EPR spectroscopy has contributed appreciably to the development of our understanding of the mechanisms of desiccation damage in orthodox and recalcitrant seeds. Orthodox seeds can survive drying and can remain viable in a dry form for extended periods of time, whereas recalcitrant seeds do not survive drying below a critical water content. Nevertheless, orthodox seeds are also susceptible to desiccation damage and Leprince *et al*. (1995) have shown that both O_2 and temperature influence desiccation-induced damage, which is associated with a free-radical process.

Desiccation tolerance in seeds and pollen is also affected by cytoplasmic viscosity which can be assessed with nitroxide spin probes, and Leprince and Hoekstra (1998) have reported the use of viscosity measurements for characterizing the relationship between desiccation and decreased metabolism. In addition, Buitink *et al*. (1998) observed a sharp increase in mobility of the spin probe 3-carboxy-proxyl in axes of pea seeds and cattail pollen during heating to a temperature which corresponded to melting of the glassy matrix. This temperature increased with decreasing water content of the samples, and molecular mobility was inversely correlated with storage stability. Buitink *et al*. (2000) have also shown that desiccation tolerance in seeds is related to the ability of amphiphilic spin probes to transfer from the cytoplasm into lipids during drying. They also found that incubation in polyethylene glycol (PEG) before drying could prevent the loss of desiccation tolerance in germinated cucumber radicles. Since partitioning of spin probes into the lipid components during dehydration occurred at higher water contents in desiccation-intolerant compared with tolerant radicles, it was suggested that cytoplasm microviscosity may control the partitioning of spin probes in dehydrating seeds. Dehydration also induces imbalanced metabolism before loss of membrane integrity in desiccation-sensitive germinated radicles, and Leprince *et al*. (2000) proposed that a balance between down-regulation of metabolism during drying and O_2 availability is associated with desiccation tolerance, and that products resulting from imbalanced metabolism may aggravate membrane damage induced by dehydration. The rate of drying is also an important factor in desiccation damage, and Walters *et al*. (2001) have reported a loss of viability, consistent with the induction of metabolic imbalances being induced in seeds that are tolerant of desiccation if dried rapidly. A similar effect has been reported recently by Ntuli *et al*. (2011) in work on cryopreservation of embryonic axes of *Quercus robur* L. These authors also reported that any given water content, rapid dehydration was associated with higher

activities of the free radical processing enzymes, superoxide dismutase, catalase and glutathione reductase and lower levels of hydroperoxide and membrane damage; it was also associated with lower malate dehydrogenase activity, and a reduced decline in phosphofructokinase activity and in levels of the oxidized form of nicotinamide dinucleotide.

4.3 Breaking of dormancy and seed germination

Although desiccation damage is associated with an increase in respiration (see previous paragraph) and an increase in free radical concentrations, a similar increase in the EPR free radical signal accompanies the breaking of dormancy (Fig. 6).

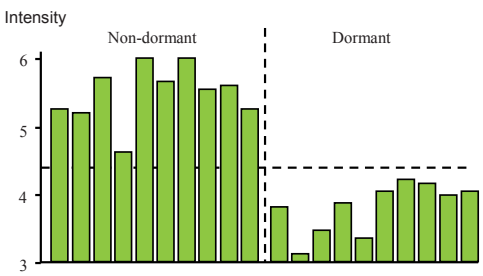

Figure 6. Free radical EPR signal intensity in non-dormant and dormant barley seeds.

By combining EPR measurements with NMR spectroscopy and imaging along with antioxidant assays Wojtyla *et al*. (2006) showed that such changes in free radical levels, along with antioxidant contents and enzymatic activities in embryo axes and cotyledons, are related to metabolic and developmental processes associated with preparation for germination, and do not correspond directly to the hydration state of the tissues. Further support for this conclusion has been produced by Leymarie *et al*. (2012), who found that imbibition of non-dormant seeds produced more ROS than dormant seeds, and that their catalase activity was lower. ROS production was found to be both temporally and spatially regulated: they were first localized within the cytoplasm upon imbibition of non-dormant seeds, then in the nucleus and finally in the cell wall, which suggests that ROS play different roles during germination.

During seed germination, hydroxyl radicals produced in the apoplast can facilitate cell wall loosening during cell elongation, and Kukavica *et al*. (2009) have shown that ·OH generation results from indigenous H_2O_2 and cell wall peroxidases. Furthermore, EPR measurements showed that production of semiquinone radicals accompanied ·OH generation. The formation ·OOH was observed when endogenous SOD was inactivated, and $O_2^{\bullet-}$ was converted to ·OH in an *in vitro* horseradish peroxidase (HRP)/H_2O_2 system to which exogenous SOD had been added. Taken together with the discovery of a cell wall-bound Mn-SOD isoform, these results suggest that hydroxycinnamic acids from the cell wall, act as reductants in the formation of H_2O_2 from O_2, and peroxidases and Mn-SOD couple to generate ·OH from this H_2O_2.

Although experimental evidence suggests that $O_2^{\bullet-}$ produced in the elongation zone of roots and leaves by plasma membrane NADPH oxidase activity is required for growth, Bustos *et al* (2008) showed that reductions in maize root-tip elongation by salt and osmotic stress do not correlate with apoplastic $O_2^{\bullet-}$ levels determined using nitro blue tetrazolium. As a consequence they hypothesised that under those conditions the role of apoplastic $O_2^{\bullet-}$ may be to participate in signalling processes, that convey information on the nature of the substrate that the growing root is exploring.

5. Applications to Metabolic Processes in whole plants

This was first demonstrated in the 1980's (Goodman *et al*., 1986) for the dependence of the total free radical signal intensity on the atmosphere around a wheat root (Fig. 7). These measurements showed that: (a) (most of) the EPR signal is O_2-dependent, and (b) the half life of the (major) radical is at most a few minutes. Monitoring O_2-derived free radical generation in mammalian tissues

usually requires the addition of spin probes or spin trap molecules in order to produce a species that can be detected and characterized by EPR spectroscopy, but plant tissue provides its own probe for such free radical generation. The measured g-value and linewidth for this free radical signal are very similar to those reported for ubisemiquinone (Salerno and Ohnishi, 1980; Suzuki and King, 1983), so it is probable that this or a similar radical is responsible for (or at least makes a major contribution to) the EPR spectrum.

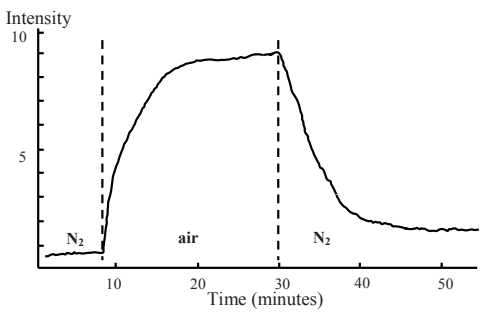

Figure 7. Intensity variation of the free radical signal in the wheat roots (Goodman *et al*. 1986).

Similar measurements can be made to determine plant root responses to any process that affects the respiration rate, and EPR spectroscopy is therefore a powerful technique for monitoring in real time changes in response to variations in environmental conditions. However caution must be exercised in the choice of spectral acquisition parameters. Signal intensities are often quite low, thus requiring the use of relatively high modulation amplitudes, which may then broaden narrow signals to such an extent that they may either be unobservable or misinterpreted. For example, Fig. 8 shows the effect of decreasing the modulation amplitude on the EPR spectrum from wheat roots; at modulation amplitudes 0.2 mT a weak signal from the ascorbate radical was revealed, although the majority of the intensity was still associated with the broad component that is assigned to ubisemiquinone.

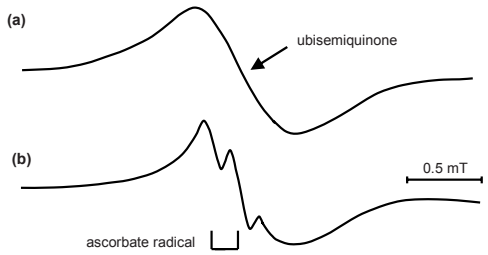

Figure 8. Effect of modulation amplitude on the 1st derivative EPR spectrum from the free radical component in the roots of wheat seedlings (a) 0.6 mT, and (b) 0.2 mT. Note the appearance of the signal from the ascorbate radical at the lower modulation amplitude.

Other works on the identification of the chemical nature of free radicals produced in plant roots have illustrated the potential importance of ROS in root physiological processes. The role of ROS in the control of root hair growth has been reviewed by Carol and Dolan (2006) who suggested that ROS are required for cell expansion during the morphogenesis of organs such as roots and leaves. ROS also control the activity of calcium channels required for polar growth. The production of ·OH radicals in the growing zone of maize roots (Liszkay *et al*., 2004) or cucumber roots (Renew *et al*., 2005) has been demonstrated using an assay based on the trapping by 4-POBN of the α-hydroxyethyl radical which is produced by hydroxyl radical attack on ethanol added to the plant. However, it is possible that the alcohol may perturb the metabolic processes sufficiently to influence ·OH production, although Mojović *et al*. (2004) demonstrated with DEPMPO the production of both ·OH and $O_2^{\cdot -}$ in isolated purified plasma membranes from maize roots. Procedures used for sample preparation may, however, contribute to radical generations, and it is very difficult to observe spin trap adduct formation in a truly *in vivo* situation.

6. Applications to of EPR spectroscopy to the study of abiotic stress processes

6.1 Background information on the role of antioxidants

Changes in the rates of generation of the superoxide radical anion represent an important response of biological systems to oxidative stress processes, which can result from a wide range of environmental factors including UV stress, pathogen invasion (hypersensitive reaction), herbicide action and oxygen shortage. Information relating to each of these can potentially be obtained from EPR spectroscopy. The complex relationships between antioxidant molecules, oxidative damage and oxygen deprivation stress has been reviewed by Blokhina *et al*. (2003), who described three physiologically different states of O_2 deprivation stress: transient hypoxia, anoxia and reoxygenation. Generation of ROS is characteristic for hypoxia and especially for reoxygenation, when it can cause damage to unsaturated lipids, proteins, carbohydrates and nucleic acids. Formation of ROS is inhibited by antioxidant enzymes and ROS are also scavenged by low molecular mass antioxidants, such as ascorbic acid, glutathione, tocopherols, and other phenolic compounds. Various factors affect the effectiveness of antioxidant protection under environmental stress conditions, including compartmentalization of ROS formation and antioxidant localization, synthesis and transport of antioxidants, the ability to induce the antioxidant defense and cooperation (and/or compensation) between different antioxidant systems.

Low molecular weight antioxidants, such as ascorbate, glutathione, and tocopherol, have crucial roles in cellular defence and as enzyme cofactors; cellular antioxidants also influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and death (De Pinto and De Gara, 2004; Potters *et al*., 2004; Tokunaga *et al*., 2005). Most importantly, antioxidants provide essential information on cellular redox status, and they influence gene expression associated with biotic and abiotic stress responses to maximize defense. Growing evidence suggests a model for redox homeostasis in which the reactive oxygen species (ROS)–antioxidant interaction acts as a metabolic interface for signals derived from metabolism and from the environment. This interface modulates the appropriate induction of acclimation processes or, alternatively, execution of cell death programs.

EPR spectroscopy combined with spin trapping using molecules, such as 5,5-dimethylpyrroline-N-oxide (DMPO) and related compounds is one of the various techniques available for measuring O_2-derived free radicals. Also, determination of methane sulfinic acid, which is formed by the oxidation of DMSO by hydroxyl radicals, can be used to monitor the production of hydroxyl radicals which have been shown by Babbs *et al*. (1989) to be formed in massive amounts in paraquat-treated duckweed or perennial ryegrass.

6.2 In vivo Response to physical damage

Physical damage can result in major free radical generation in plant tissues as was seen in experiments aimed at understanding the free radical processes that accompany the mastication of fresh plant material consumed as fresh fruit or salad vegetables (*e.g*. Goodman *et al*., 2002; Pirker *et al*., 2004). However, it is also possible to observe effects related to physical damage in a true *in vivo* situation, and Tada *et al*. (2003) have reported the observation of changes in redox states related to cellular damage in flower buds of the sweet cherry caused by exposure to low temperatures. EPR spectra of samples containing a carbamoyl-PROXYL spin probe were continuously recorded, whilst they experienced freezing and thawing conditions. *In vivo* EPR can also be used to detect directly free radical responses at one position in a plant stimulated by physical damage at another position (*e.g*. Fig. 9). In this example, the EPR spectra were recorded for the mid-region of the roots of a wheat seedling whilst the plant was cut just below the seed. This resulted in an almost immediate change in both the free radical signal intensity and its linewidth. However, if a corresponding sample was cut above the seed, there was little effect on the EPR signal.

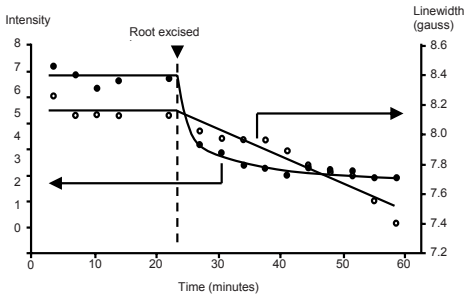

Figure 9. Variation with time of the free radical EPR spectral intensity in the mid-region of the roots of a wheat seedling before and after cutting the plant just below the seed.

Warwar *et al*. (2011) have recently described the EPR detection and imaging of $O_2^{\cdot -}$ in plant roots with appropriate morphology using a small imaging resonator and a stable spin probe that rapidly permeates the extracellular space in the plant and specifically reacts with superoxide with a high reaction rate constant. In those experiments, $O_2^{\cdot -}$ generation in a plant root was observed as a result of an apical leaf injury, a result which is in contrast to that described in the previous paragraph, where measurements were made without using spin traps. The mechanism for the production of $O_2^{\cdot -}$ is complex, and Schopfer *et al*. (2008) have reported that this involves a classical NAD(P)H:quinone-acceptor oxidoreductase that reduces menadione to menadionehydroquinone and subsequently undergoes autoxidation at pH ≥ 6.5. Autoxidation involves the production of the semiquinone as an intermediate, creating the conditions for one-electron reduction of O_2 and the subsequent generation of $O_2^{\cdot -}$ and H_2O_2 at the plasma membrane of plants.

6.3 Plant responses to toxic challenge

One of the earliest plant *in vivo* EPR measurements was an investigation of the responses of wheat seedlings to a short-term exposure to potentially toxic levels of the Cu^{2+} ion (Goodman and Linehan, 1979). The EPR spectra (Fig. 10) show signals from both Cu^{2+} and free radicals. The Cu^{2+} signal was originally anisotropic consistent with the Cu being immobilised by adsorption on cell walls, but there was the progressive generation of an isotropic signal indicating the formation of low molecular mass complexes with molecules produced by the root.

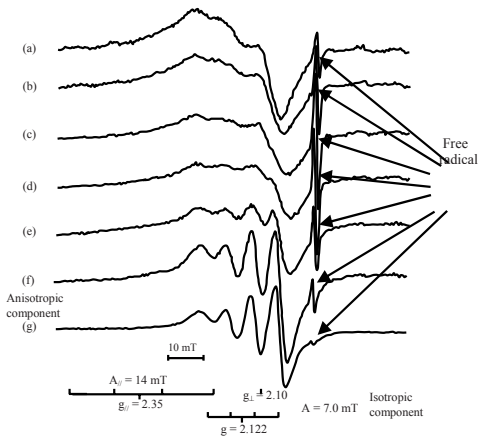

Figure 10. Variation with time of the EPR spectrum of wheat roots that had been exposed to a so lution containing 10 mM Cu(II) for 30 minutes and then washed with water. Spectra were recorded after (a) 0.5, (b) 1.5, (c) 2.5, (d) 4.0, (e) 6.0, (f) 11.5, and (g) 23.0 hours (adapted from Goodman and Linehan 1979).

These complexes were able to be removed by washing (Goodman *et al*., 1979) and were thus related to excretion processes. At the same time there were major changes in the free radical signal (Fig. 11), which increased progressively over a period of a few hours, reached a maximum and then decreased exponentially. Since this free radical signal is primarily related to respiratory activity, the experiment illustrates that there was an initial increase in respiration in response to the toxic challenge, but this then reached a maximum and then decreased as the plant died.

6.4 Investigations of long term environmental stresses on plant tissues

Such experiments need to be conducted using excised tissue samples, because the exposure to high magnetic fields and microwave radiation in an EPR spectrometer for an extended period of time would itself likely be stressful for a plant.

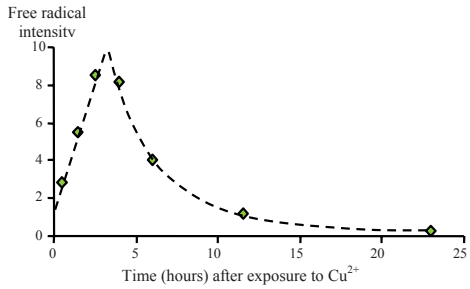

Figure 11. Variation with time of the free radical signal intensity in the spectra in Fig. 12.

The effects on EPR free radical signal intensities of drought stress under field conditions have been investigated for fruits of strawberry (Pirker *et al*., 2002) and tomato (Pirker *et al*., 2003). In both cases the effects were quite minor, whereas significantly higher free radical concentrations were observed in tomato fruit that had received moderate short term exposure to ozone (Pirker *et al*., 2003). However, there were no significant differences in free radical concentrations in tomato fruit after longer term ozone exposure. Taken together these results indicate that plants have considerable ability to adapt to long term stress conditions, although there may be appreciable short term responses.

Support for this conclusion has been observed in a study of drought stress in wheat (Goodman and Newton, 2005). These results showed that leaf free radical contents were similar for plants grown under conditions of drought and adequate water supply, but the release of drought resulted in a rapid short-term increase in free radical concentrations.

Another example of the application of EPR spectroscopy to the study of a long term stress condition is illustrated in Fig. 12, which shows the effects on the redox active Fe, Mn and free radical components of internal rust spot, a physiological disorder of potato tubers. This disorder results in the formation of lesions in the medullary tissue during the bulking phase of tuber growth. Compared to normal tissue, the EPR spectra of chlorotic tissue show increases in the Fe(III)(g=4.3) and free radical signals, whilst the Mn(II) signal is decreased. Each of these indicates the existence of oxidative processes, probably as a result of increased ROS production arising from a loss of cell membrane integrity.

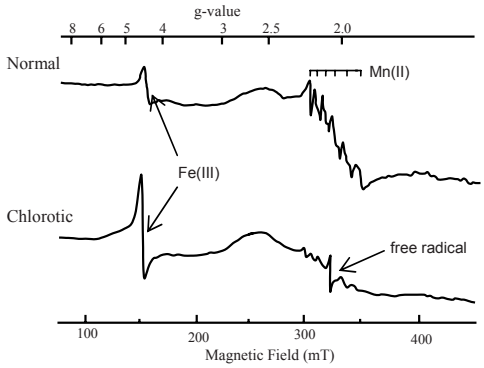

Figure 12. EPR spectra at 77K of tissue from a potato tuber with internal rust spot disorder normal tissue, and chlorotic tissue (adapted from Monk *et al*., 1989).

6.4 Measurements on Leaf Samples

There are relatively few direct EPR studies of free radical processes in leaf tissue, primarily because the leaf chemistry is dominated by photosynthesis, and many of the key components of the photosynthetic pathway do not produce EPR spectra at room temperature. However, Runcekles and Vaartnou (1997) have reported the generation of an ozone-derived signal in plant leaves which was assigned to the presence of an immobilized $O_2^{\cdot -}$ radical. EPR has also been used in investigations of stress effects induced by UV-B radiation, which has been shown to elicit multilevel oxidative stress (Hideg and Vass, 1996). Illumination of isolated thylakoid membranes induced the formation of ·OH and C-centered radicals, but not $^1O_2$, thus demonstrating that the primary site of UV-B induced electron transport impairment in photosystem II is different from that of photoinhibition by excess photosynthetically active radiation. Furthermore, membrane preparations from UV-B pre-illuminated leaves featured ·OH, C-centered and ROO· radicals in the thylakoid enriched pellet and increased production of ascorbate radicals

in the supernatant fraction. Thus UV-B irradiation elicits multilevel oxidative stress, and in addition to immediate free radical production, UV-B also initiates radical-yielding reactions detectable in leaves even minutes after ceasing irradiation. Šnyrychová *et al*. (2007) have also observed the production of •OH radicals and hydrogen peroxide which were derived from $O_2^{\cdot -}$ and were dependent on photosynthetic electron transport at an early stage of UV-B stress. However, after longer exposure of thylakoid membranes to UV-B, PSII centres were inactivated, but some •OH was still observed, even though no free hydrogen peroxide was detected, possibly as a result of direct cleavage of organic peroxides by UV-B. Hideg *et al*. (2006b) used EPR spectroscopy to investigate genotypic differences in the UV-B induced stress responses in barley cultivars. Stress tolerance was associated with a higher quantum yield of photosynthesis under photosynthetically active radiation, a smaller decrease in both electron transport rate and non-photochemical quenching under high PAR and less oxidized ascorbate.

EPR has also been used to investigate the effects of previous submergence and the addition of ascorbate on free radical generation and iron oxidation in leaves of rice grown in an iron toxic soil (Thongbai and Goodman, 2000). The survival rate of plants was increased appreciably by the addition of ascorbate one day before they were returned to aerobic conditions. However, there were also appreciable genotypic differences between tolerant and sensitive varieties. Compared to unsubmerged controls, submergence treatment of sensitive varieties resulted in larger increases in the Fe(III)(g=4.3) EPR signal and total Fe content of the leaves compared to a tolerant variety. Furthermore, free radical signal intensities were lowest in the submergence-tolerant plants, and only increased slightly on submergence, whereas higher free radical concentrations were found in submergence-sensitive varieties, and these increased appreciable on submergence.

Abiotic stress effects during the growing period can also influence the composition of plant-derived products, even after considerable processing, and Reichenauer and Goodman (2001) observed ozone-induced differences in the free radical contents of EPR spectra of freeze-dried samples from wheat plants. However, it must be borne in mind that the freeze drying process itself results in massive free radical generation (Pirker *et al*., 2002). Subsequently, Goodman and Reichenauer (2003) reported that ozone induced a fundamental change in the EPR free radical spectral characteristics and this change was accompanied by a large increase in intensity of the Fe(III) (g = 4.3) signal, both changes indicating stress-induced oxidative processes, that are visible even after the samples has been freeze-dried.

Finally, Reichenauer and Goodman (2003) have observed changes in the free radical contents of wheat flour during storage in air that reflected differences in exposure to ozone during the growing period of the plants.

7. Applications to Biotic stress processes

7.1 Introduction

There is a need for the scientific community to adopt standard procedures (both chemical and biological) to facilitate comparison between results from different groups, and the inconsistency in methodology adopted by different groups is a handicap to the development of the science. However, it is clear that the involvement of ROS in plant-pathogen interactions is a complex one, and that they are associated with the initiation and progression of many diseases.

ROS play a crucial signaling role in the establishment of plant immunity, and recognition of an avirulent pathogen stimulates an oxidative burst during which $O_2^{\cdot -}$ amd H_2O_2 are generated (Alvarez *et al*., 1998). These then activate the induction of defence genes and cell death in a restricted lesion as an initial action in the development of systemic acquired resistance to virulent pathogens. Alvarez *et al*. (1998) showed that inoculation of *Arabidopsis* leaves with *Pseudomonas syringae* induced a secondary oxidative bursts in discrete cells in

distant tissues and that both oxidative bursts are required for systemic immunity. Necrotrophic pathogens on the other hand utilize ROS production as an attack strategy in colonising plant tissues, and the interactions between H_2O_2 and other AOS produced by the fungus and the plant-based antioxidant systems in determining the outcome of the infection process have been reviewed by Lyon *et al*. (2007). Biochemical processes that appear to be of importance for lesion development involve perturbation of the free radical chemistry and transition metal redox processes (particularly those involving iron), the regulation of enzymes (of both plant and fungal origin), the production of toxic metabolites in the host, and host signalling and programmed cell death.

Most of the EPR investigations of biotic stress processes have used excised tissues, although it is possible in principle to perform truly *in vivo* measurements as described above for abiotic stress effects. Thus, the experiments described in this section all involved some form of tissue damage in order to make the measurements; nevertheless they were able to provide direct insight into *in vivo* processes induced by biotic stress. Examples are presented for tubers, leaves and fruit, and for fungal and bacterial pathogens.

7.2 Infection of potato tubers with fungal pathogens

Snijder *et al*., (1996) compared the effects of different fungal pathogens on the intensities of the free radical signals in different regions of the medullary tissue from potato tubers. The results from two replicate experiments are shown in Fig. 13.

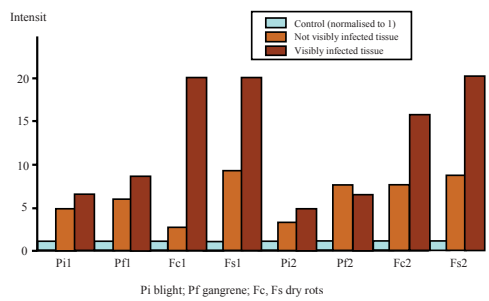

Figure 13. Intensity of the free radical EPR signal in potato tuber slices (Pentland Crown) infected with various fungal pathogens.

For each set of measurements, potato tubers were cut into 5 slices, which were either inoculated with one of four fungal pathogens, with the 5th slice being used as an uninoculated control. In both replicates, the free radical signal intensity in the dry rot lesions was ~20 times that of the control, whereas the increase as a result of blight or gangrene infection was in the range 5-10 times. Of particular note in these measurements, however, was the increase in free radical signal intensity in uninfected regions of infected tissue slices, a result which indicated a major increase in free radical generation at positions in the tuber remote from the infection.

7.3 Infection of potato tubers with Erwinia carotovora

Erwinia carotovora (*Ec*) is a bacterial pathogen that develops under anaerobic conditions and is responsible for major losses in stored potatoes. Although a free radical signal is associated with infected tissue, Deighton *et al*. (1992) showed it that resulted from a tissue response to the pathogen and not the pathogen itself (Fig. 14). By incubation of a spin trap along with the pathogen, Deighton *et al*. also showed that the radical generation occurred rapidly when infected tissue was exposed to O_2, but did not occur significantly when either O_2 or *Ec* were absent.

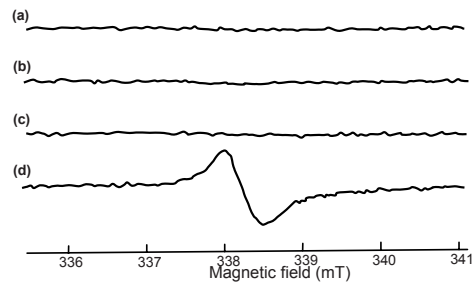

Figure 14. EPR spectra at 120 K of potato tuber tissue infected with the bacterial pathogen Erwinia carotovora: uninfected control,(a) no O_2, (b) in air, and infected samples (c) no O_2 and (d) in air.

7.4 Infection of leaves of French bean (Phaseolus vulgaris) *with* Botrytis cinerea

Botrytis cinerea is a necrotrophic fungal pathogen that causes soft rots that result in appreciable global crop losses. Soft rots in French bean leaves were shown by Muckenschnabel *et al*. (2001a) to be associated with various markers of oxidative damage, including increases in malondialdehyde and 4-hydroxy-2-nonenal and the EPR Fe(III)(g=4.3) signal, and decreases in ascorbic acid. There was also a change in the spectral characteristics of the free radical signal. In further measurements, Muckenschnabel *et al*. (2003) observed oxidative effects in uninfected tissue remote from the positions of the soft rot lesions (Fig. 15). In these measurements, the tissue samples were infiltrated with the spin trap 4-POBN, and the resulting solutions separated by centrifugation before recording their EPR spectra. In the absence of the spin trap, the EPR spectrum consisted of the ascorbate radical (from oxidized ascorbic acid) at both 24 and 56 hours post inoculation (hpi), whilst the signal from a radical adduct of 4-POBN was seen along with that of the ascorbate radical at 24 hpi and was the only signal seen at 56 hpi. There was also a progressive decline in ascorbic acid concentrations with time hpi, but no changes in other markers of oxidation (such as lipid peroxidation products or the Fe(III)(g=4.3) EPR signal) were detected.

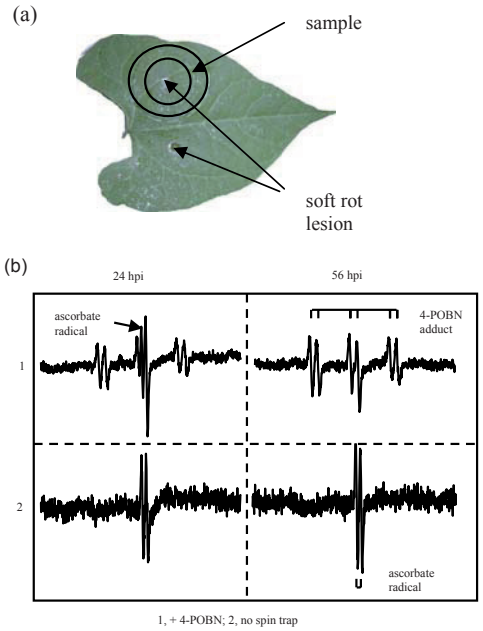

Figure 15. (a) Phaseolus vulgaris leaf infected with Botrytis cinerea showing (a) the disc of sample used for EPR investigation, and (b) EPR spectra after infiltration of the tissue disc with (1) an aqueous solution of the spin trap 4-POBN or (2) water after 24 or 56 hours.

7.5 Infection of fruit of pepper (Capsicum annuum) *with* Botrytis cinerea

Results similar to those described in the previous paragraph for leaves of French bean have been reported for tissue of green pepper fruit infected with *Botrytis cinerea* (Deighton *et al*., 1999; Muckenschnabel *et al*., 2001b). As illustrated in Fig. 16, the changes seen in tissue remote from the site of infection are strongly influenced by whether or not it is connected to the lesion by a major vascular bundle, even in the absence of any detectable fungal spores. These results show that although the concentrations of the ascorbate radical decreased with decreasing distance from the lesion, it was only in tissue connected to the lesion by a major vascular bundle that a 4-POBN radical adduct signal could be observed. Therefore, these results suggest that reduction of ascorbic acid concentrations and the generation of free radicals are not directly coupled.

7.6 Infection of leaves of Arabidopsis thaliana *with* Botrytis cinerea

The EPR and ascorbic acid results from tissue of leaves of the brassica *Arabidopsis thaliana* infected with *Botrytis cinerea* (Muckenschnabel *et al*., 2002) were similar to those observed with leaves of French bean (see above), but there were no corresponding increases in the aldehydic products of lipid peroxidation; the MDA contents of leaf tissue from infected plants were actually appreciably lower than in healthy controls. This result thus indicates the need for the use of caution in extrapolating results from one plant species to another, and is particularly

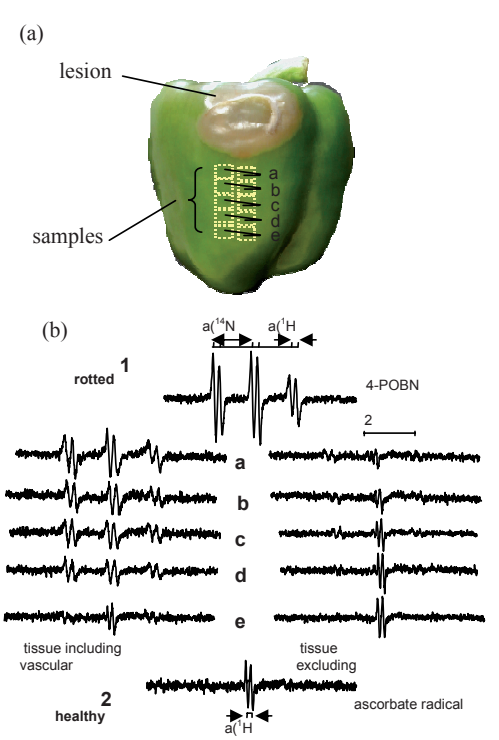

Figure 16. (a) Pepper fruit infected with Botrytis cinerea showing sampling positions, and (b) EPR spectra of the various samples after infiltration with 4-POBN

significant because of the extensive use of Arabidopsis as a model plant.

7.7 Plant-virus interactions

Fodor *et al*.. (2001) have described the detection *in vivo* of a tobacco mosaic virus (TMV)-induced local and systemic oxidative burst by EPR spectroscopy, and showed that TMV-induced oxidative stress in a necrotic host plant is signalled by an elevated level of monodehydroascorbate radicals. In addition, systemic acquired resistance induced in remote leaves of Xanthi-nc tobacco is also associated with stimulated ascorbate radical EPR signals indicative of a micro-oxidative burst.

8. Transport processes

Little work has been published in which EPR has been used to investigate transport processes, although the role of hydroxyl radicals in inducing fluxes of K^+ and Ca^{2+} has recently been investigated by Zepeda-Jazo *et al*. (2011) for roots of pea (*Pisum sativum*). However, it is a straightforward experiment to measure the time taken for a paramagnetic chemical species to move from the position of uptake to the region of the plant inside the spectrometer cavity, and the use of an imaging facility could provide detailed information on transport mechanisms for paramagnetic species. Furthermore, it is possible to monitor independently the movement of two separate non-interacting paramagnetic ions, provided they have distinctly different EPR spectra, or the dynamics of their movement into different compartments in the tissue (provided this involves changes in their spectral properties) as was described for seeds in Section 4.2. By incorporating nitroxide groups into xenobiotic molecules it should be possible to monitor their transport and degradation within root tissues, since nitroxides often show relatively low reactivities towards O_2-derived free radicals (Kocherginsky and Swartz, 1995). However, the reaction of nitroxides with antioxidant molecules to produce hydroxylamines as described in Fig. 3 is always a potential complication. It should also be borne in mind that a number of methods for assessing antioxidant contents of food products are based on their reaction with stable free radicals. Also, the introduction of a paramagnetic spin probe into a biological system could initiate chemical changes that might not otherwise occur, but by using probes with different levels of reactivity, it might also be possible to probe simultaneously free radical reactions in different cell compartments using *in vivo* EPR procedures.

9. Speciation of paramagnetic transition metal ions and complexes *in vivo*

This is one of the most straightforward *in vivo* application of EPR spectroscopy, since it follows conventional chemical applications, and was the approach used in the early applications of the technique. For example, Goodman and Linehan (1979) were able to follow changes in Cu(II) speciation in wheat roots following a short exposure

to the metal ion (Figs. 12, 13). McPhail *et al*. (1982) showed that a similar exposure to vanadium(V) resulted initially in reduction to V(IV), which was the form transported within the plant; in that work it was also possible to observe subsequent changes in the coordination environment of the V(IV). A similar result was obtained by Morrell *et al*. (1986) who also discussed the occurrence of vanadium in the biosphere, and the possible roles this element may play in the metabolism of living organisms, especially higher plants.

In these papers, the time resolution of the results was of the order of a several minutes, *i.e*. the time taken to record a spectrum with acceptable signal:noise ratios. The complete EPR spectrum of many transition metal complexes covers a wide energy range, and it is usually not feasible to acquire satisfactory data in less than a few minutes. However, selected regions of a spectrum can be acquired quicker, and as has been shown in Fig. 8, it is possible to monitor the changes in intensity at a selected position in a spectrum in real time (though the danger is that this type of measurement does not detect changes in metal speciation and thus should never be performed without additional measurements to ensure that the spectral parameters remain unchanged during the period of the measurement).

10. General Discussion and Conclusions

A particular strength of the EPR technique is its ability to characterise the chemical nature of paramagnetic molecules that are present in a sample in only trace quantities. However, this is offset by the fact that it provides no information on diamagnetic components, which represent the majority of chemical species. Thus EPR is very selective as to the types of biochemical process to which it can be applied, and experiments need to be planned with care in order to ensure that meaningful results are obtained. Nevertheless, with appropriate experimental design and execution, EPR spectroscopy can make important contributions to understanding the dynamics of plant behavior, and the *in vivo* technique is particularly valuable for measuring responses to short-term changes in the environment of a plant.

The biggest restriction to the routine use of EPR for investigating biological tissues is the problem of absorption of microwaves by water, and at the commonly-used X-band frequencies specimens can only be investigated if they contain < ~30 μL, although the exact volume varies with the type of microwave resonator used. Larger samples can be tolerated in lower frequency spectrometers, but there is a corresponding decrease in sensitivity because of the smaller differences between the populations of the ground and excited spin states. In addition, since spectrometer sensitivity is strongly affected by the amount of water in the resonator, quantitative information can only be obtained if a sensitivity standard is measured along with the sample being studied.

It is often difficult to obtain detailed information on the majority of transition element ions and free radicals in living systems. Many paramagnetic species give broad, weak signals, which make it quite hard to interpret their EPR data, or possess physical properties which preclude their observation in solution at ambient temperatures. For free radicals related to O_2 metabolism, even though spin traps can be used to observe the adducts, there are still problems, and it is a fundamental requirement that the spin trap is stable in the absence of free radicals and that its free radical adducts are sufficiently unreactive to allow their detection in real samples. Nevertheless, EPR has provided interesting and useful information on aspects of the dynamics of plant redox behaviour.

In vivo EPR spectroscopy is especially suitable for the observation of relatively short term responses to changes in a plant's environment. This is because (a) an EPR spectrum can be acquired in a timescale of seconds to minutes, and (b) being bathed in microwaves in the presence of a strong magnetic field for a prolonged period of time might produce a plant stress response that could mask its response to the stress process being investi-

gated. Early work used the technique to monitor spectral changes (and hence reactions) that followed short-term exposures to heavy metal ions, and by combining *in vivo* experiments with similar measurements using dead tissue, it was possible to differentiate between simple sorption and metabolic processes (*e.g*. Goodman *et al*., 1979). Furthermore, it is straightforward to investigate various types of *in vivo* process provided that they involve chemical species with unpaired electrons that produce EPR spectra at ambient temperatures, with the *proviso* that there are low water levels in the part of the sample tube that is in the spectrometer cavity.

Many plant tissues are too large for *in vivo* study by EPR, and the only feasible approach to investigate processes occurring in them is by using excised tissue. The major complication in such experiments is the free radical response to damage resulting from the excision process, especially since this could be considerably greater than the processes one might wish to investigate. Nevertheless, as can be seen from examples presented in this review, there are many situations where measurements on excised tissue can provide valuable information on stress-induced processes in plant tissues.

Overall EPR is able to monitor variations in respiration rates through the behaviour of free radical components, and to probe changes in redox status through free radical and Fe components; in appropriate systems the chemistry of some other metal ions can also be investigated. Paramagnetic probes can be used to investigate transport processes, and also changes in viscosity of cellular fluids when subjected to stress. Thus there is considerable potential of the EPR technique to further our knowledge of plant biochemical processes.

Acknowledgements

Much of my original research on plant *in vivo* and whole tissue processes was funded by the Scottish Office Agriculture Environment and Fisheries Department (SOAEFD); Dennis Linehan and Donald McPhail from the (then) Macaulay Institute for Soil Research and Ingo Muckenschnabel and Nigel Deighton from the (then) Scottish Crop Research Institute are gratefully acknowledged for their contributions to the initial development of this work. I also thank the Austrian Institute of Technology for the use of the EPR facilities for some of the more recent measurements and to Chris Wood for the results shown in Fig. 5 and Jiang Lei (Sophia) for the results in Fig. 9.

References

Abragam, A., & Bleaney, B. (1970). *Electron Paramagnetic Resonance of Transition Ion*s. Dover Publications Inc., New York.

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A., & Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, 92, 773-784.

Babbs, C.F., Pham, J.A., & Coolbaugh, R.C. (1989). Lethal hydroxyl radical production in paraquat-teated plants. *Plant Physiology, 90*, 1267-1270.

Bačić, G., Spasojević, I., Šećerov, B., & Mojović, M. (2008). Spin-trapping of oxygen free radicals in chemical and biological systems: New traps, radicals and possibilities. *Spectrochimica Acta Part A, 69*, 1354-1366

Bobko, A.A., Kirlyuk, I.A., Grigor'ev, I.A., Zweier, J.L., & Khrantsov, V.V. (2007). Reversible reduction of nitroxides to hydroxylamines: the roles of ascorbate and glutathione. *Free Radical Biology and Medicine, 42*, 404-412.

Buettner, G.R., & Mason, R. P. (1990). Spin-trapping methods for detecting superoxide and hydroxyl free radicals in vitro and in vivo. In: *Oxygen Radicals in Biological Systems, Part B, Oxygen Radicals and Antioxidants (Methods in Enzymology, Vol. 186)*, L. Packer and A.N. Glazer, Eds., Academic Press, Inc., San Diego, pp. 127-133.

Buitink, J., Claessens, M.M.A.E., Hemminga, M.A., & Hoekstra, F.A. (1998). Influence of water content and temperature on molecular mobility and intracellular glasses in seeds and pollen. *Plant Physiology, 118*, 531-541.

Buitink, J., Leprince, O., & Hoekstra, F.A. (2000). Dehydration-induced redistribution of amphiphilic molecules between cytoplasm and lipids is associated with desiccation tolerance in seeds. *Plant Physiology, 124*, 1413-1425.

Carol, R.J, & Dolan, L. (2006). The role of reactive oxygen species in cell growth: lessons from root hairs. *Journal of Experimental Botany, 57*, 1829-1834.

Deighton, N., Johnston, D.J., Glidewell, S.M., Lyon, G.D. & Goodman, B.A. (1992). The involvement of oxygen in the resistant response of the potato tuber to *Erwinia carotovora. Protoplasma, 171*, 167-169.

Deighton, N., Muckenschnabel, I., Goodman, B.A., & Williamson, B. (1999). Lipid peroxidation and the oxidative burst associated with infection of *Capsicum annuum* by *Botrytis cinerea. The Plant Journal, 20*, 485-492.

De Pinto, M.C., & De Gara, L. (2004). Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. *Journal of Experimental Botany, 55*, 2559-2569.

Elas, M., Williams, B.B., Parasca, A., Mailer, C., Pelizzari, C.A., Lewis, M.A., River, J.N., Karczmar, G.S., Barth, E.D., & Halpern, H.J. (2003). Quantitative tumor oxymetric images from 4D electron paramagnetic resonance imaging (EPRI): methodology and comparison with blood oxygen level-dependent (BOLD) MRI. *Magnetic Resonance in Medicine, 49*, 682-691.

Ferreira Severino, J., Goodman, B.A., Kay, C.W.M., Stolze, K., Tunega, D., Reichenauer, T.G., & Pirker, K.F. (2009). Free radicals generated during oxidation of green tea polyphenols: electron paramagnetic resonance spectroscopy combined with density functional theory calculations. *Free Radical Biology and Medicine, 46*, 1076-1088.

Fodor, J., Hideg, E., Kecskés, A., & Kiraly, Z. (2001). *In vivo* detection of tobacco mosaic virus-induced local and systemic oxidative burst by electron paramagnetic resonance spectroscopy. *Plant and Cell Physiology, 42*, 775-779.

Foyer, C.H., & Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell, 17*, 1866-1875.

Freinbichler, W., Colivicchi, M.A., Stefanini, C., Bianchi, L., Ballini, C., Misini, B., Weinberger, P., Linert, W., Varešlija, D., Tipton, K.F., & Della Corte, L. (2011). Highly reactive oxygen species: detection, formation, and possible functions. *Cellular and Molecular Life Sciences, 68*, 2067-2079.

Gapper, C., & Dolan, L. (2006). Control of plant development by reactive oxygen species. *Plant Physiology, 141*, 341-345.

Goodman, B.A., Glidewell, S.M., Arbuckle, C.M., Bernardin, S., Cook, T.R., & Hillman, J.R. (2002). An EPR study of free radical generation during maceration of uncooked vegetables. *Journal of the Science of Food and Agriculture, 82*, 1208-1215.

Goodman, B.A., & Linehan, D.J. (1979). An electron paramagnetic resonance study of the uptake of Mn(II) and Cu(II) by wheat roots. In: *The Soil Root Interface*, (Eds. J.L.Harley and R.Scott Russell), Academic Press, 67-82.

Goodman, B.A., Linehan, D.J., & Powell, H.K.J. (1979). An electron paramagnetic resonance study of the "uptake" of copper (II) by wheat roots. *Proceedings of the 2nd New Zealand Seminar on Trace Elements and Health* (Pub. Dept. Commun. Health, Univ. Auckland, N.Z.), 63-70.

Goodman, B.A., McPhail, D.B. & Linehan, D.J. (1986). Oxygen - induced free radical in wheat roots. *Free Radical Research Communications, 2*, 173-178.

Goodman, B.A., & Newton, A.C. (2005). Effects of drought stress and its sudden relief on free radical processes in barley. *Journal of the Science of Food and Agriculture, 85*, 47-53.

Goodman, B.A., & Reichenauer, T.G. (2003). Formation of paramagnetic products in leaves of wheat (*Triticum aestivum*) as a result of ozone-induced stress. *Journal of the Science of Food and Agriculture, 83*, 1248-1255

Halliwell, B., & Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *British Journal of Pharmacology, 142*, 231-255.

Hendry, G.A.F. (1993). Oxygen, free radical processes and seed longevity. *Seed Science Research, 3*, 141-153.

Hepburn, H.A., Goodman, B.A., McPhail, D.B., Matthews, S., & Powell, A.A. (1986). An evaluation of EPR measurements of the organic free radical content of individual seeds in the non-destructive testing of seed viability. *Journal of Experimental Botany, 37*, 1675-1684.

Hideg, É., Kálai, T., Kós, P.B., Asada, K., & Hideg, K. (2006a). Singlet oxygen in plants - its significance and possible detection with double (fluorescent and spin) indicator reagents. *Photochemistry and Photobiology, 82*, 1211-1218.

Hideg, É., Rosenqvist, E., Váradi, G., Bornman, J. & Vincze, É. (2006b). A comparison of UV-B induced stress responses in three barley cultivars. *Functional Plant Biology, 33*, 77-90.

Hideg, É., Takátsy, A., Sár, C.P., Vass, I., & Hideg, K. (1999). Utilizing new adamantyl spin traps in studying UV-B-induced oxidative damage of photosystem II. *Journal of Photochemistry and Photobiology, B: Biology, 48*, 174-179.

Hideg, É., & Vass, I. (1996). UV-B induced free radical production in plant leaves and isolated thylakoid membranes. *Plant Science, 115*, 25l-260.

Hoagland, D.R., & Arnon, D.I. (1950). *The Water-Culture Method for Growing Plants without Soil*. Circular 347, California Experimental Station, University of California, Berkeley.

Khan, N., Blinco, J.P., Bottle, S.E., Hosokawa, K., Swartz, H.M., & Micallef, A.S. (2011). The evaluation of new and isotopically labeled isoindoline nitroxides and an azaphenalene nitroxide for EPR oximetry. *Journal of Magnetic Resonance, 211*, 170-177.

Khan, N., Khramtsov, V.V., & Swartz, H.M. (2010). Methods for tissue pO_2, redox, pH, and glutathione measurements by EPR spectroscopy. In: *Methods in Redox Signaling* (Ed. D. Das), Mary Ann Liebert, Inc., 81-89.

Khan, N., & Swartz, H. (2002). Measurements *in vivo* of parameters pertinent to ROS/RNS using EPR spectroscopy. *Molecular and Cellular Biochemistry, 234/235*, 341-357.

Khan, N., Wilmot, C.M., Rosen, G.M., Demidenko, E., Sun, J., Joseph, J., O'Hara, J., Kalyanaraman, B. & Swartz, H.M. (2003). Spin traps: in vitro toxicity and stability of radical adducts. *Free Radical Biology & Medicine, 34*, 1473-1481.

Kocherginsky, N., & Swartz, H.M. (1995). Chapter 3 Chemical reactivity of nitroxides. In *Nitroxide Spin Labels, Reactions in Biology and Chemistry*, Eds. N. Kocherginsky and H.M. Swartz, CRC Press, Boca Raton, Florida, pp. 27-66.

Krishna, M.C., Kuppusamy, P., Afeworki, M., Zweier, J.L., Cook, J.A., Subramanian, S., & Mitchell, J.B. (1998). Development of functional electron paramagnetic resonance imaging. *Breast Disease, 10*, 209-220.

Kukavica, B., Mojović, M., Vuččinić, Ž., Maksimović, V., Takahama, U., & Veljović Jovanović, S. (2009). Generation of hydroxyl radical in isolated pea root cell wall, and the role of cell wall-bound peroxidase, Mn-SOD and phenolics in their production. *Plant and Cell Physiology, 50*, 304-317.

Leprince, O., & Hoekstra, F.A. (1998). The responses of cytochrome redox state and energy metabolism to dehydration support a role for cytoplasmic viscosity in desiccation tolerance. *Plant Physiology, 118*, 1253-1264.

Leprince, O., Vertucci, C.W., Hendry, G.A.F., & Atherton, N.M. (1995). The expression of desiccation-induced damage in orthodox seeds is a function of oxygen and temperature. *Physiologia Plantarum, 94*, 233-240.

Leprince, O., Harren, F.J.M., Buitink, J., Alberda, M., & Hoekstra, F.A. (2000). Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration of germinating radicles. *Plant Physiology, 122*, 597-608

Leymarie, J., Vitkauskaité, G., Hoang, H.H., Gendreau, E., Chazoule, V., Meimoun, P., Corbineau, F., El-Maarouf-Bouteau, H., & Bailly, C. (2012). Role of reactive oxygen species in the regulation of Arabidopsis seed dormancy. *Plant and Cell Physiology, 53*, 96-106.

Liszkay, A., van der Zalm, E., & Schopfer, P. (2004). Production of reactive oxygen intermediates ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$) by maize roots and their role in wall loosening and elongation growth. *Plant Physiology, 136*, 3114-3123.

Liu, Y., Song, Y., Rockenbauer, A., Sun, J., Hemann, C., Villamena, F.A., & Zweier, J.L. (2011). Synthesis of trityl radical-conjugated disulfide biradicals for measurement of thiol concentration. *Journal of Organic Chemistry, 76*, 3853-3860.

Liu, Y., Villamena, F.A., Rockenbauer, A., & Zweier, J.L. (2010). Trityl-nitroxide biradicals as unique molecular probes for the simultaneous measurement of redox status and oxygenation. *Chemical Communications, 46*, 628-630.

Lyon, G.D., Goodman, B.A., & Williamson, B. (2007). *Botrytis cinerea* perturbs redox processes as an attack strategy in plants. In: Botrytis: *Biology, Pathology and Control* (Eds. Y. Elad, B. Williamson, P. Tudzynski and N. Delen), Springer, Dordrecht, 119-141,

Mabbs, F.E., & Collison, D. (1992). *Electron Paramagnetic Resonance of d Transition Metal Compounds*. Elsevier, Amsterdam.

McCormick, M.L., Buettner, G.R., & Britigan, B.E. (1995). The spin trap alpha-(4-pyridyl-1-oxide)-N-tert-butylnitrone stimulates peroxidase-mediated oxidation of deferoxamine. Implications for pharmacological use of spin-trapping agents. *Journal of Biological Chemistry, 270*, 29265-29269.

McPhail, D.B., Linehan, D.J., & Goodman, B.A. (1982). An electron paramagnetic resonance (EPR) study of the uptake of vanadyl by wheat plants. *New Phytologist, 91*, 615-620.

Mao, G.D., Thomas, P.D., & Poznansky, M.J. (1994). Oxidation of spin trap 5,5-dimethyl-1-pyrroline-1-oxide in an electron paramagnetic resonance study of the reaction of methemoglobin with hydrogen peroxide. *Free Radical Biology and Medicine, 16*, 493-500.

Mojović, M., Vuletić, M., Bačić, G.G., & Vučinić, Ž. (2004). Oxygen radicals produced by plant plasma membranes: an EPR spin-trap study. *Journal of Experimental Botany, 55*, 2523-2531.

Monk, L.S., McPhail, D.B., Goodman, B.A., & Davies, H.V. (1989). An electron spin resonance investigation of internal rust spot : a physiological disorder of the potato tuber. *Free Radical Research Communications, 5*, 345-350.

Morrell, B.G., Lepp, N.W., & Phipps, D.A. (1986). Vanadium uptake by higher plants: Some recent developments. *Environmental Geochemistry and Health, 8*, 14-18,

Muckenschnabel, I., Williamson, B., Goodman, B.A., Lyon, G.D., Stewart, D., & Deighton, N. (2001a). Markers for oxidative stress associated with soft rots in French beans (*Phaseolus vulgaris*) infected by *Botrytis cinerea. Planta, 212*, 376-381.

Muckenschnabel, I., Goodman, B.A., Deighton, N., Lyon, G.D., & Williamson, B. (2001b). *Botrytis cinerea* induces the formation of free radicals in fruits of *Capsicum annuum* at positions remote from the site of infection. *Protoplasma, 218*, 112-116.

Muckenschnabel, I., Goodman, B.A., Williamson, B., Lyon, G.D., & Deighton, N. (2002). Infection of leaves of *Arabidopsis thaliana* by Botrytis cinerea: changes in ascorbic acid, free radicals and lipid peroxidation products. *Journal of Experimental Botany, 53*, 207-214.

Muckenschnabel, I., Schulze Gronover, C., Deighton, N., Goodman, B.A., Lyon, G.D., Stewart, D., & Williamson, B. (2003). Long range oxidative effects in leaves of bean (*Phaseolus vulgaris*) infected by *Botrytis cinerea. Journal of Agriculture and Food Chemistry, 83*, 507-514.

Ntuli, T.M., Finch-Savage, W.E., Berjak, P., & Pammenter, N.W. (2011). Increased drying rate lowers the critical water content for survival in embryonic axes of english oak (*Quercus robur* L.) seeds. *Journal of Integrative Plant Biology, 53*, 270-280.

Pilbrow, J.R. (1990). *Transition Ion Electron Paramagnetic Resonance*. Oxford University Press.

Pirker, K.F., Goodman, B.A., Pascual, E.C., Kiefer, S., Soja, G., & Reichenauer, T.G. (2002). Free radicals in the fruit of three strawberry cultivars exposed to drought stress in the field. *Plant Physiology and Biochemistry, 40*, 709-717.

Pirker, K.F., Reichenauer, T.G., Pascual, E.C., Kiefer, S., Soja, G., & Goodman, B.A. (2003). Steady-state levels of free radicals in tomato fruit exposed to drought and ozone stress in a field experiment. *Plant Physiology and Biochemistry, 41*, 921-927.

Pirker, K.F., & Goodman, B.A. (2010). Caffeoylquinic acid derived free radicals identified during antioxidant reactions of Bitter tea *(Ilex latifolia and Ilex kudincha). Food and Function, 1*, 262-268.

Pirker, K. F., Reichenauer, T.G., Goodman, B.A., & Stolze, K. (2004). Identification of oxidation processes during simulated eating of uncooked foods using EPR spectroscopy. *Analytica Chimica Acta, 520*, 69-77.

Potters, G., Horemans, N., Bellone, S., Caubergs, R.J., Trost, P., Guisez, Y., & Asard, H. (2004). Dehydroascorbate Influences the plant cell cycle through a glutathione-independent reduction mechanism. *Plant Physiology, 134*, 1479-1487.

Ranguelova, K., & Mason, R.P. (2011). The fidelity of spin trapping with DMPO in biological systems. *Magnetic Resonance in Chemistry, 49*, 152-158.

Reichenauer, T.G., & Goodman, B.A. (2001). Stable free radicals in ozone-damaged wheat leaves. *Free Radical Research, 35*, 93-101.

Reichenauer, T.G., & Goodman, B.A. (2003). Free radicals in wheat flour change during storage in air and are influenced by the presence of ozone during the growing season. *Free Radical Research, 37*, 523-528.

Renew, S., Heyno, E., Schopfer, P., & Liszkay, A. (2005). Sensitive detection and localization of hydroxyl radical production in cucumber roots and *Arabidopsis* seedlings by spin trapping electron paramagnetic resonance spectroscopy. *The Plant Journal, 44*, 342-347.

Robertson, L., & Hartley, R.C. (2009). Synthesis of N-arylpyridinium salts bearing a nitrone spin trap as potential mitochondria-targeted antioxidants. *Tetrahedron, 65*, 5284-5292.

Roshchupkina, G.I., Bobko, A.A., Bratasz, A., Reznikov, V.A., Kuppusamy, P., & Khramtsov, VV. (2008). *In vivo* EPR measurement of glutathione in tumor-bearing mice using improved disulfide biradical probe. *Free Radical Biology and Medicine, 45*, 312-320.

Runeckles, V.C., & Vaartnou, M. (1997). EPR evidence for superoxide anion formation in leaves during exposure to low levels of ozone. *Plant, Cell and Environment, 20*, 306-314.

Salerno, J.C., & Ohnishi, T. (1980). Studies on the stabilized ubisemiquinone species in the succinate-cytochrome *c* reductase segment of the intact mitochondrial membrane system. *Biochemical Journal. 192*, 769-781.

Schopfer, P., Plachy, C., & Frahry, G. (2001). Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiology, 125*, 1591-1602

Schopfer, P., Heyno, E., Drepper, F., & Krieger-Liszkay, A. (2008). Naphthoquinone-dependent generation of superoxide radicals by quinone reductase isolated from the plasma membrane of soybean. *Plant Physiology, 147*, 864-878.

Shen, J., Sood, R., Weaver, J., Timmins, G.S., Schnell, A., Miyake, M., Kao, J.P.Y., Rosen, G.M., & Liu, K.J. (2009). Direct visualization of mouse brain oxygen distribution by electron paramagnetic resonance imaging: application to focal cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism, 29*, 1695-1703.

Shulaev, V., & Oliver, D.J. (2006). Metabolic and proteomic markers for oxidative stress. new tools for reactive oxygen species research. *Plant Physiology, 141*, 367-372.

Šnyrychová, I., Kós, P.B., & Hideg, É. (2007). Hydroxyl radicals are not the protagonists of UV-B-induced damage in isolated thylakoid membranes. *Functional Plant Biology, 34*, 1112-1121.

Snijder, A.J., Wastie, R.L., Glidewell, S.M., & Goodman, B.A. (1996). Free radicals and other paramagnetic ions in interactions between fungal pathogens and potato tubers. *Biochemical Society Transactions, 24*, 442-446.

Stoll, S., & Sweiger, A. (2006). EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *Journal of Magnetic Resonance, 178*, 42-55.

Suzuki, H., & King, T.E. (1983). Evidence of an ubisemiquinone radical(s) from the NADH-ubiquinone reductase of the mitochrondrial respiratory chain. *Journal of Biological Chemistry, 258*, 352-358.

Swartz, H.M., Khan, N., & Khramtsov, V.V. (2007). Use of electron paramagnetic resonance spectroscopy to evaluate the redox state *in vivo Antioxidants and Redox Signaling, 9*, 1757-1772.

Tada, M., Shiraishi, T., Takashina, T., Ohya, H., & Ogata, T. (2003). Direct observations of the redox states of frozen cherry buds by a unique *in vivo* ESR. *Biochemical and Biophysical Research Communications, 310*, 72-77.

Thongbai, P., & Goodman, B.A. (2000) Free radical generation and post-anoxic injury in rice grown in an iron-toxic soil. *Journal of Plant Nutrition, 23*, 1887-1900.

Tokunaga, T., Miyahara, K., Tabata, K., & Esaka, M. (2005). Generation and properties of ascorbic acid-overproducing transgenic tobacco cells expressing sense RNA for L-galactono-1,4-lactone dehydrogenase. *Planta, 220*, 854-863.

Walters, C., Pammenter, N.W., Berjak, P., & Crane, J. (2001). Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research, 11*, 135-148.

Warwar, N., Mor, A., Fluhr, R., Pandian, R.P., Kuppusamy, P., & Blank, A. (2011). Detection and imaging of superoxide in roots by an electron spin resonance spin-probe method. *Biophysical Journal, 101*, 1529-1538.

Woldman, Y.Y., Semenov, S.V., Bobko, A.A., Kirilyuk, I.A., Polienko, J.F., Voinov, M.A., Bagryanskaya, E.G., & Khramtsov, V.V. (2009). Design of liposome-based pH sensitive nanoSPIN probes: nano-sized particles with incorporated nitroxides. *Analyst, 134*, 904-910.

Wojtyla, Ł., Garnczarska, M., Zalewski, T., Bednarski, W., Ratajczak, L., & Jurga, S. (2006). A comparative study of water distribution, free radical production and activation of antioxidative metabolism in germinating pea seeds. *Journal of Plant Physiology, 163*, 1207-1220.

Zepeda-Jazo, I., Velarde-Buendía, A.M., Enríquez-Figueroa, R., Bose, J., Shabala, S., Muñiz-Murguía, J., & Pottosin, I.I. (2011). Polyamines interact with hydroxyl radicals in activating Ca^{2+} and K^{+} transport across the root epidermal plasma membranes. *Plant Physiology, 157*, 2167-2180.

Zhang, X.K., Yang, G.T., Chen, L., Yin, J.M., Tang, Z.L., & Li, J.N. (2006). Physiological differences between yellow-seeded and black-seeded rapeseed (*Brassica napus* L.) with different testa characteristics during artificial ageing. *Seed Science and Technology, 34*, 373-381.