EPR Studies on γ-Irradiated Barley Seeds: Identification of Trapped Electrons

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Electron paramagnetic resonance (EPR) studies were conducted on barley seeds exposed to normal (H_2O) and deuterated (D_2O) moisture, irradiated with 750 Gy at 77 K. Reported here, for the first time, are the trapped electrons formed on γ-irradiation of seeds at 77 K. Electrons are stabilized/solvated with an increase in the moisture content (H_2O/D_2O) of seeds. The recombination of the trapped electron with radical cation gave intense thermoluminescence emission at 110 K. With the increase in temperature and the destruction of singlet, unmasking of an underlying heterogeneous population of free radicals was observed. These free radicals emanate mainly from the endosperm (~95% by wt of the seed), whereas irradiated embryos show a broad multiplet of comparatively low amplitude. Radiolysis of carbohydrate, proteins (~95% of endosperm), and lipids could possibly be responsible for the heterogeneous population of free radicals. Peroxyl radicals were also observed on annealing.

Keywords: *Radiation effects; electron paramagnetic resonance; thermoluminescence; barley seeds; trapped electrons; organic peroxyl radical; endosperm; embryo*

INTRODUCTION

Radiation damage in seeds and other agricultural products is of intense current interest, both from a fundamental radiobiological point of view and also with respect to radiation preservation of seeds (*1*−*5*). In this context, a first logical step is the identification of radiation-induced free radicals as "transients"/"reaction intermediates" and their thermal stability leading to final stable species. Electron paramagnetic resonance (EPR) spectroscopy is an ideal technique for elucidation of the radiation-induced paramagnetic defect centers. Some of these free radical species could be the precursors of the radiation-induced damage in the biological systems. It may be noted that the investigations reported herein are primarily directed toward understanding the reactive species amenable to EPR investigations. In this context the diamagnetic species created during the radiolytic processes were considered to be ineffective for radiobiological changes. In this paper, we present the result of our investigations on the EPR of γ-irradiated barley seeds, which have several essential features to investigate mechanistic aspects in basic radiobiology (*6*, *7*). Barley seeds are very suitable for the study of both the physicochemical and biochemical stages of development of the radiobiological damage (*8*). The seeds of low moisture content (<5%) represent relatively inert media within which physicochemical reactions could occur in the near absence of metabolism. Israni et al. (*9*) have reported EPR results of barley seeds γ-irradiated at room temperature. The EPR spectra obtained by these authors were broad and had many unresolved components, which made the unambiguous identification of radical species rather difficult. Furthermore, the species that are observable at room temperature are the final equilibrium products and do not necessarily give direct information about the primary radiation damage and subsequent radical reactions, finally becoming observable at room temperature. Ehrenberg et al. (*10*) investigated the trapped radicals at 77 K in endosperm and embryo. The EPR spectra were broad and unresolved. Considerable improvement is made in the investigations of these two aspects compared to the earlier studies by changing the experimental conditions.

Seeds were irradiated at 77 K, which facilitates the detection of primary free radicals produced at 77 K, which acts as precursor to the stable radicals observed at room temperature. The sequence of electronic rearrangement following irradiation may be blocked at low temperature. By raising the temperature of the substances, these events may happen gradually. During this rearrangement, some detectable radiative transitions occur among a sequence of electronic transistions.

Moisure content in the seeds was deuterated, which is expected to reduce the line broadening created by the dipolar interactions with ^1H of the water molecules. Furthermore, this would help in the identification of the unpaired electron interaction with the moisture content of the seeds.

The study of the EPR spectra above 77 K and also its correlation with sub-room temperature thermoluminescence (TL) were also done with a view to identifying the thermal "destruction"/"recombination" of these centers. Our EPR and TL studies on the γ-irradiated seeds

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indeed lead to the identification of trapped electrons and other paramagnetic species. The trapped electrons were stabilized by the seed moisture content. These centers highlight the role of moisture contents in the retention of reactive species (unpaired electrons). These studies are indeed a step forward in a better understanding of the free radicals formed on irradiation of the cereals. These could indeed be playing a role in the formation of the precursors of the postirradiation oxygen effect observed in seeds.

MATERIALS AND METHODS

Test System. Pure-line barley seeds (*Hordeum vulgare*) of a hull-less strain (IB 65) were used for these experiments. The seeds were desiccated over fused calcium chloride to reduce the moisture content to ~2.4% (*9*, *11*). TL experiments were performed on these seeds.

Moisture Content Determination. The moisture content of the seeds was determined by the change in the percentage weight of the seeds when they were dried at 373 K for 48 h.

For EPR studies, some of the seeds were cut into halves to yield a half containing only endosperm. From the other half the embryos were carefully removed and stored.

Deuteration of Moisture Content. The dry seeds of ~2.4% moisture, endosperm, and embryo were placed in desiccators saturated with D_2O vapor (99.4%) for 5 days. The moisture content of seeds and endosperm was found to be ~25% after 5 days of exposure to D_2O vapor. However, we did not determine the degree of deuteration that had occurred. Some of the deuterated seeds, endosperm, and embryo were lyophilized to reduce the moisture content to ~5 and ~7.8%.

Chemicals. All of the chemicals used in this study were of analytical grade and used without further purification. Deuterated water (D_2O, 99.4%) was obtained from the Heavy Water Division of the Bhabha Atomic Research Centre, Mumbai, India.

Irradiation. The deuterated and nondeuterated samples were transferred to liquid nitrogen for 10 min. Then these samples were irradiated with γ-rays from a ^{60}Co source (cumulative dose = 750 Gy) at 77 K. The seeds were irradiated with 375 and 750 Gy for TL and EPR spectra, respectively.

EPR Studies. The EPR spectra were recorded using a Bruker ESP-300 EPR spectrometer operating at X-band frequency. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as a field marker. The variable temperature assembly was employed to alter the temperature of the sample in the EPR cavity. The measurements at 77 K were done using a liquid nitrogen insert.

TL Studies. TL measurements were made for 77−320 K using a home-built instrument described earlier (*13*). The output from the IP 28 photomultiptier tube was amplified using a Keithley 610 °C electrometer amplifier and fed to a two-pen recorder. The output of the amplifier was also simultaneously fed through a voltage to frequency converter (Hewlett-Packard model 2211 BR Dymec) to a scalar for obtaining the integrated TL yields. An iron−constanan thermocouple attached to the sample stage was connected to the same recorder for monitoring and recording the rise in temperature. The accuracy of the peak temperature measurement is estimated to be ±2 K. The overall rate of heating over the entire temperature range was ~20 K min^{-1}.

RESULTS

EPR Studies on Un-irradiated Seeds. The EPR spectrum of un-irradiated barley seeds at 77 K shows six line spectra of very weak intensity characteristics of Mn^{2+} (Figure 1).

EPR Studies on Irradiated Seeds. Figure 2 shows the EPR spectra of the deuterated (a) and normal (nondeuterated; b) seeds after γ-irradiation at 77 K. Deuteration of moisture has a profound effect on the

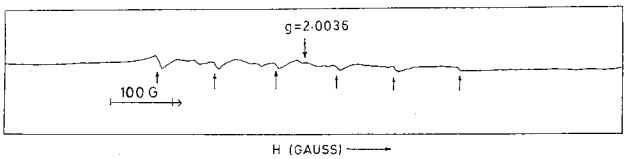

Figure 1. EPR spectra of the un-irradiated deuterated barley seeds (~25% moisture) recorded at 77 K. The sextet due to Mn^{2+} is marked with arrows. (Gain = 4×10^4.)

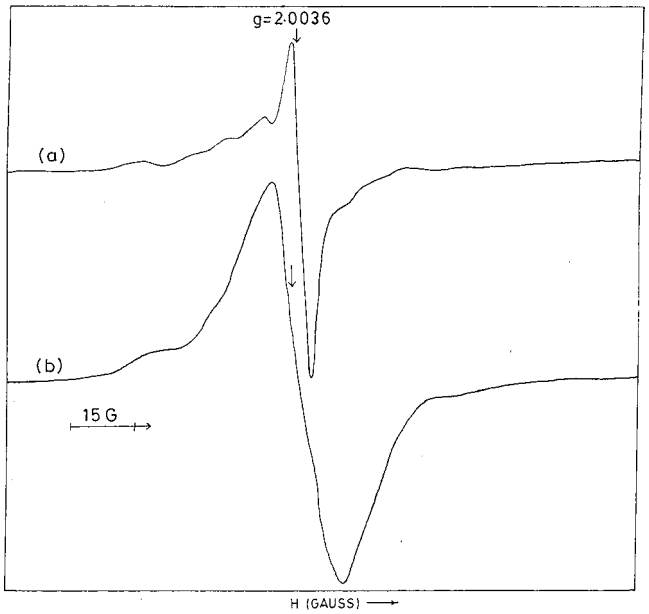

Figure 2. EPR spectra of γ-irradiated baley seeds (a) deuterated (~25% moisture) (gain = 4×10^4) and (b) normal nondeuterated (~2.4% moisture) (gain = 4×10^4). Note the large line width with ~2.4% of normal moisture.

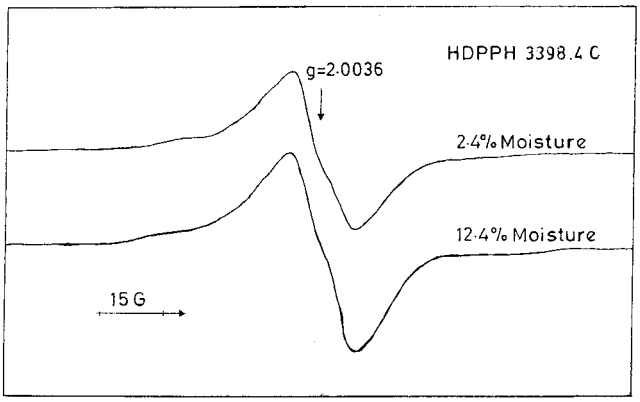

Figure 3. EPR spectra of γ-irradiated barley seeds with normal moisture (i) ~2.4% (gain = 4×10^4) and (ii) ~13% (gain = 2×10^4) irradiated and recorded at 77 K constant microwave power of 2 mW. When the normal moisture content was raised to ~13%, a 30% increase in the line width was observed.

EPR spectra. The EPR spectrum of the irradiated seeds with normal moisture (Figure 2b) was very broad, masking a number of spectral features that were better resolved in the case of deuterated seeds (Figure 2a). The EPR spectrum for the deuterated seeds shows a sharp central line with a line width of $\Delta H = 5.8 \pm 0.1$ G and $g = 2.0025$. The corresponding line width for ~13% normal moisture seeds is ~23 G (Figure 3). Due to the higher relative intensity of the sharp singlet, the spectral features of other centers were not clearly observable. The intensity of this central line increased

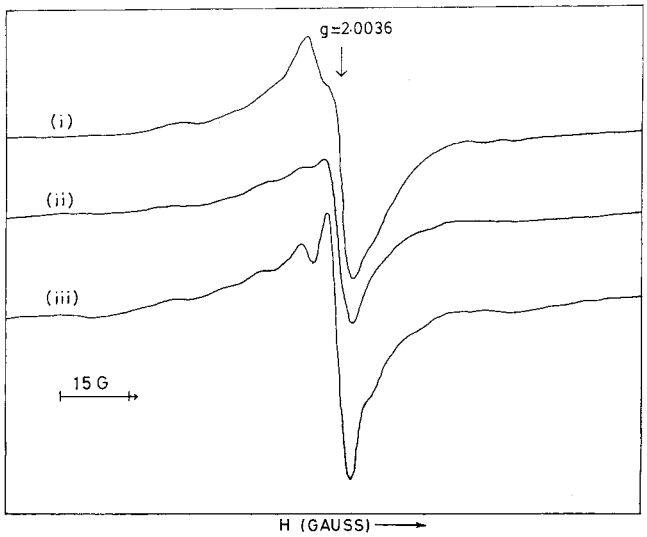

Figure 4. EPR spectra of γ-irradiated deuterated barley seeds of various moisture contents irradiated and recorded at 77 K constant microwave power of 2 mW: (i) 5.4% (gain = 4×10^4); (ii) 7.8% (gain = 2×10^4); (iii) 25% (gain = 4×10^4).

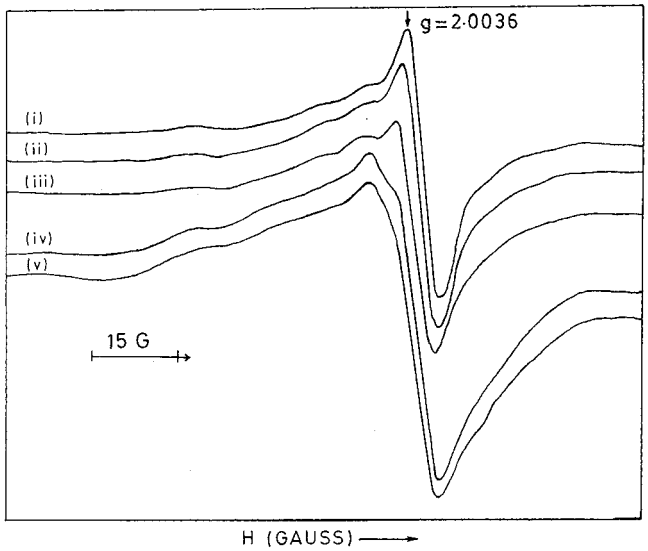

Figure 5. Microwave power dependence of the EPR spectra of γ-irradiated (dose = 750 Gy) deuterated barley seeds (7.8% moisture), irradiated at 77 K and spectra recorded at 77 K (gain = 2×10^4): (i) 0.2 mW; (ii) 0.63 mW; (iii) 2 mW; (iv) 6.3 mW; (v) 10 mW.

with the increase in initial seed moisture content (Figure 4). The sharp peak was found to exhibit power saturation around ~6.3 mW in seeds of high moisture, whereas in seeds of ~7.8% moisture, it was observed at a lower power, ~2 mW (Figure 5).

Effect of Change in Moisture Content. In seeds with normal moisture, it was observed that the line width increased with an increase in moisture content. At ~13% moisture content the line width was 30% more than that observed in seeds of minimal (~2.4%) normal moisture content (Figure 3). The spectra shown in Figure 2, therefore, are representative of contrasting line width in seeds with normal and deuterated moisture. These are not artifacts of changes in the moisture content per se (also see Figure 4).

The line widths of the progressively lyophilized seeds with initial ~25% D_2O moisture show an interesting behavior (Figure 4). At lowest moisture (~5.2% moisture, Figure 4) the line width increased to that of

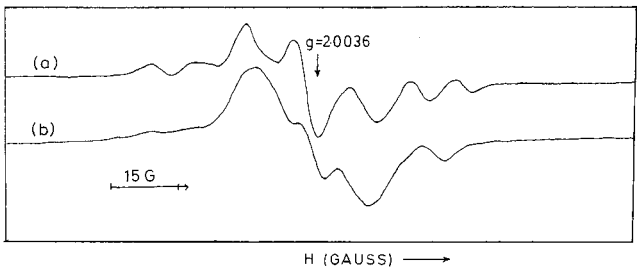

Figure 6. EPR spectra of barley seeds γ-irradiated (dose = 750 Gy, at 77 K) and later warmed to 173 K: (a) deuterated (~25% moisture) (gain = 4×10^4); (b) normal nondeuterated (~2.4% moisture) (gain = 4×10^4). Note a broad component present in (b) that is absent in (a). (b) is a superimposition of a broad line, and structure is seen in (a).

irradiated seeds of ~2.4% normal moisture (Figure 2b). This apparently suggests that the seeds are partially deuterated, and in the seeds of ~5.2% moisture it remains predominantly in normal ^1H.

Effect of Annealing of the Irradiated Seeds. When the irradiated seeds were warmed to 110 K and above, this sharp central signal was found to become thermally annealed (Figure 6). This signal did not reappear when the sample was cooled, clearly showing that the radical responsible for the central peak was destroyed at ~110 K.

Similar features, namely, the disappearance of intense single-line spectra and clearer appearance of other structures, were observed in normal seeds, also when subjected to the same heating cycle (Figure 6b). It may be noted that a broad component present in Figure 6b is not present in Figure 6a. Another interesting observation is that Figure 6b is a superposition of a broad line and also the structures that are seen in Figure 6a.

EPR Spectra of Seed's Endosperm and Embryo. The EPR spectra of irradiated pure endosperms and embryos (with deuterated moisture) were also recorded separately. The EPR spectrum of irradiated endosperm shows great similarity with that obtained in irradiated seeds (Figure 7a). However, EPR signals from the irradiated embryos consisted of relatively weak lines around $g = 2$ with broad shoulders (Figure 7b). These observations suggest that the EPR spectra of the irradiated seeds predominantly arise from the endosperm part of seeds.

TL Studies. The TL studies performed on the irradiated seeds (normal moisture, 2.4%) show a sharp glow peak at 110 K (Figure 8). The energy associated with the detrapping of the unpaired electrons at 110 K was ~0.23 eV (*13*). The glow peak was accompanied by the disappearance of the violet color from the inner core of the endosperm. Due to the experimental limitations other more dependable methods to evaluate the activation energy could not be employed.

DISCUSSION

EPR Spectra of Un-irradiated Seeds. Our results showed that the un-irradiated seeds had weak EPR spectral lines with $g = 2.0027$ and $A = 96$ G (Figure 1). This corresponds to divalent manganese (*14*). From the presence of nuclear forbidden transitions, it is noted that the site symmetry at Mn^{2+} is lower than axial symmetry.

EPR Spectra of Irradiated Seeds. The EPR spectral features of the γ-irradiated normal seeds (nondeuterated) presented in Figure 2b, and also reported

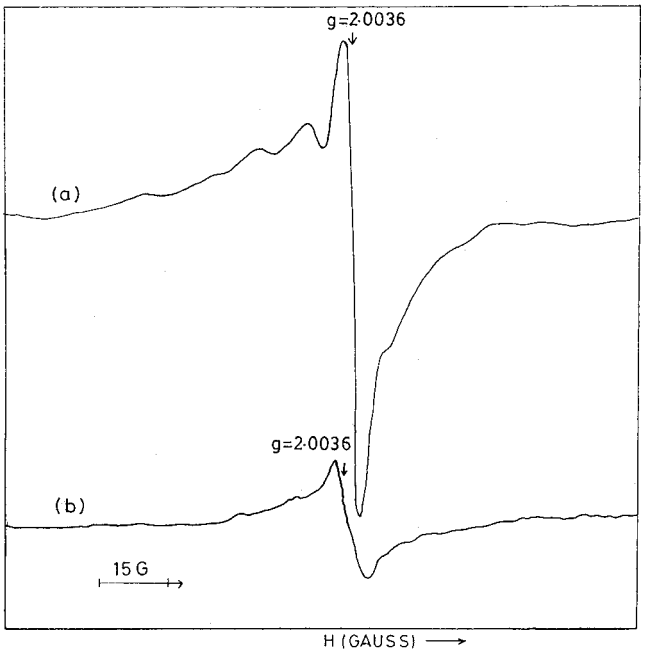

Figure 7. EPR spectra of γ-irradiated deuterated (~25% moisture) (a) endosperm (gain = 4×10^4) and (b) embryos (gain = 4×10^4).

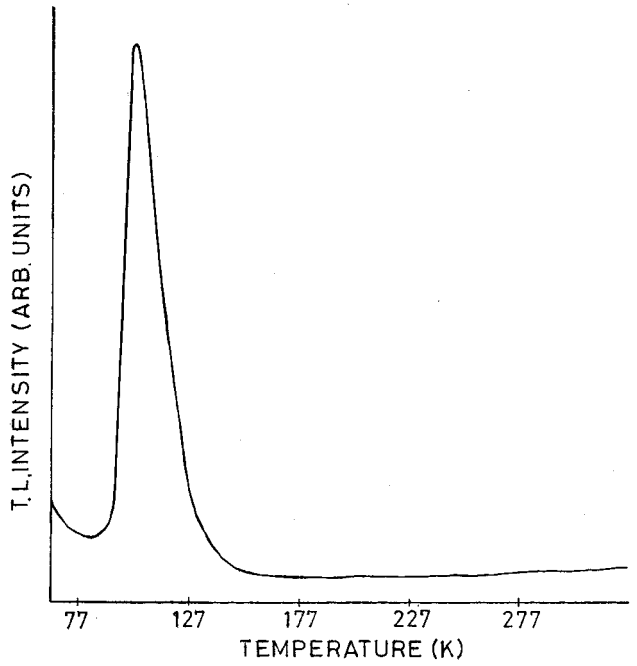

Figure 8. Thermoluminescence spectra of the irradiated seeds of ~2.4% nondeuterated moisture.

earlier (*9*, *10*), are broad multiplet resonances. The other spectral features are masked by the broad resonances, thereby making the identification of the other spectral features rather impossible. A more resolved spectrum was obtained on the deuteration of the moisture (Figure 2a). The noteworthy EPR spectral features of irradiated seeds include a singlet with $g = 2.0025$ observed on irradiation at 77 K. It was found to be thermally stable at and below 110 K. The sharp singlet is quenched irreversibly when the seed temperature is increased to ~110 K.

Similar reports of a sharp central peak are mentioned in the literature of Smith and Pieroni (*15*) and also of VanLith et al. (*16*, *17*). This singlet is observed more predominantly when the deuterated endosperm is irradiated and recorded at 77 K. This was found to be less prominent in the irradiated spectra of deuterated embryo. The sharp singlet was quenched irreversibly when the seed temperature was increased to 110 K. Chachaty and Hayon (*18*) reported a sharp line at 77 K, which was bleached at 110 K with the disappearance of purple color. Smith and Pieroni (*15*) also made similar observations of trapped electrons. The diminution of the singlet showed correspondence with the disappearance of blue color. These results suggest that the narrow EPR singlet is due to the trapped electrons.

Effect of Initial Seed Moisture Content. The increase in the moisture content of the seeds with normal moisture led to an increase in the line width. An increase in the moisture content from 2.4 to ~13% led to an increase in line width by ~30% (Figure 3).

The deuteration of moisture shows the sharpening of the singlet (line width $\Delta H = 5.8 \pm 0.1$ G) with an increase in the moisture content of the seeds (deuterated moisture) (Figure 4). The sharpening of the singlet in the EPR spectrum (Figure 4) of the electron suggests that the electrons are increasingly solvated with an increase in moisture. The interaction of the electron with the dipolar field due to the unpaired electron with moisture (D_2O/H_2O) implies that the trapped electron ($e^-_{trapped}$) in barley seeds is solvated and these are in rigid coordination at 77 K with moisture (H_2O/D_2O). The increase in the moisture of the seeds increases the moisture content of the seed components—starch (~80%), proteins (~13%), lipids (~3%), sugars, and DNA. The moisture-dependent changes in the free radical yield could primarily be attributed to the hydration of the macromolecules in the seeds.

The major seed fraction is starch, constituted of amylose (~22%) and amylopectin (~78%). The amylopectin is associated with lipids to form a starch−lipid complex (*19*). In barley seeds these lipids are predominantly (98%) monoacyl lysophospholipid with a small amount of free fatty acid (2%) and are present inside the starch granules of the endosperm. The lipid−starch complex could be a good electron trap wherein, on annealing, these electrons then decay by reacting with the holes in the starch. The protein matrix surrounds the starch−lipid complex. The seed proteins include hordeins (seed storage proteins), albumins, globulins, and glutelins (*20*). It may be mentioned here that in low moisture content the water in the proteins is highly confined and the water motion is strongly retarded (*21*), although the overall water motion is insensitive to details of protein packing in high hydration.

However, it is known that the number and the combination rate of the radicals produced by radiation have an important role in regulating the damage to the biological materials. The initial seed moisture content plays an important role in the post-irradiation oxic damage in barley seeds (*4*, *22*, *23*). A reduction in the post-irradiation oxic damage in the irradiated seeds was observed with an increase in seed moisture content. Ours is the first in vivo investigation for the qualitative differences in the free radical population of the irradiated seeds of various moisture contents. With an increase in moisture the central peak sharpens, which decays irreversibly when the seed temperature is increased to 110 K. It is likely that the electronic radiative transitions and recombination of free radicals that

restore the original molecular structure in the seeds are closely linked.

TL Studies. Thermal annealing is accompanied by a sharp thermoluminescence peak at 110 K (Figure 8). Thermal recombination occurs as a result of diffusion of electrons to the positive charge. The period of comparatively rapid recombination is followed by the slow recombination of radicals. The different rate of recombination could be explained on the basis of uneven distribution of charged particles in the irradiated solids, the presence of several types of traps differing in depth, and the formation of a relatively stable electron hole pair due to irradiation.

The activation energy associated with a trapped electron is ~0.23 eV in barley seeds. According to Urbach's empirical formula the trap depth (E_{trap}) that an electron may arbitrarily leave is $T/500$. At 110 K E_{trap} = 0.23 eV, but the actual depth could be considerably greater. Thus, freeing of an electron apparently occurs as a result of destruction of trap due to perturbation by a favorable orientation of dipoles. This probably begins at a temperature lower than those for the translational motion of molecules. The thermal recombination of electron captured by a radical occurs at the same temperature range as the recombination of radicals themselves. This indicates the decisive role played by the molecular diffusion. At a temperature of 110 K the diffusion coefficient should be effectively zero. It is quite possible that both physical (conformational defects) and chemical defects (e.g., broken bonds) may be present in the irradiated seeds and may both trap electrons. The potential well model of Meunier and Quinke (*24*) suggests the residence time of $\sim 10^{-12}$ s, for the trapped electron of energy ~0.1−0.3 eV.

The EPR spectra and the TL studies highlight the similarity in the spectral pattern of the endosperm and the seeds. At 77 K the sharp singlet corresponding to a trapped electron is observed in the endosperm part of the irradiated seeds.

The TL studies on irradiated plant powdered seeds of *Pisum*, *Brassica*, and *Raphanus* species showed two peaks at 125 and 175 K (*25*). The biological structure was destroyed in the lyophilized powder so these peaks could be attributed to the components of the seeds. The glow curves of the lipidic components consist of a glow peak at ~125 K and could be due to the fatty acid. In barley seeds, the lipid constitutes 2−3% by seed weight of the total seeds (*26*). The endosperm of the hull-less barley seed contains 77.1% of this fraction and ~20% is found in the embryo (*27*). These lipids are associated with starch and form a starch−lipid complex (*19*). In barley seeds these lipids are predominantly monoacyl lysophospholipid with a small amount of free fatty acid and are present inside the starch granules of the endosperm. We suggest that, in the barley seed system also, the lipid−starch complex might be acting as an electron trap. More of these electrons are better trapped with an increase in moisture. On annealing, these electrons trapped in lipids could decay harmlessly by reacting with the holes in the starch. Studies in the literature highlight the high reactivity of lipid (free radicals) with molecular oxygen at low temperature (110−140 K) (*28*). These authors suggested that the trapped oxygen becomes mobilized at this temperature and migrates to a carbon-centered radical that remains immobile because of high viscosity.

Thermal annealing on irradiated seeds resulted in a decay of the sharp singlet in the EPR spectra of the seeds and unmasked the underlying heterogeneous population of free radicals. Irradiation of deuterated seeds showed better resolution of spectra (Figure 6). The spectra showed α and β coupling of protons. More detailed investigations are in preparation and are not discussed herein. Because the temperature of irradiation and also the recording of EPR spectra are low, the free radicals trapped would indeed be dependent on the composition of barley seeds. Barley seed composition on a weight percentage basis could be given as follows: carbohydrate, ~80% seed weight; proteins, ~13% seed weight; ~3% lipids. The free radical entrapped could possibly be from the major seed fraction, that is, carbohydrate in the seeds at low temperature.

We did not separate the various constituents of the seeds and did not perform separate studies on them for two reasons. The powdering of the seeds led to a total loss in the anisotropy signal, and hence the purpose of the in vivo studies was not met. Second, enough information is available in the literature on the composition of seeds and also on the radiochemistry of the constituents.

In vitro EPR investigations on the irradiated seed storage proteins zein (similar in composition to hordeins), polysaccharides, and a molecular mixture of proteins and polysaccharides are available in the literature (*29*−*32*). The residual signal could emanate from the peptide backbone in proteins (*33*). The reactivity of these radicals is indeed known to be very high (*34*). We have also observed the formation of peroxyl radical (Figure 6). The peroxyl radical concentration varies with temperature (*35*).

SUMMARY

γ-Irradiation of barley seeds of ~5.4, ~7.8, and ~25% moisture at 77 K resulted in the formation of qualitatively similar types of free radicals. Irradiation of these seeds at 77 K resulted in the generation of trapped electrons. These electrons are solvated by the increase in the seed moisture content. Identification of this species in vivo in seeds is reported herein for the first time. It was made possible by the deuteration of moisture. The higher yield of electrons in seeds of high moisture suggests that these are more stable in the seeds of high moisture than in seeds of low moisture. This sharp intense peak is thermally annealed at 110 K, accompanied by the emission of violet light. These electrons are trapped mainly in the starch−lipid complexes in the endosperm part of the seeds. In addition to trapped electrons ($e^-_{trapped}$) we have observed other radical components. Deuteration of moisture improved the resolution of EPR spectra obtained on the thermal annealing of the seeds. The free radical is localized on the carbohydrate/protein fraction of the seeds. An organic peroxyl radical is also observed and reported herein. Our results also reveal that there is qualitatively no difference in the EPR spectra of γ-irradiated dry (~2.4% moisture) and wet seeds (~25% moisture).

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