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An Electron Paramagnetic Resonance Spin-Probe Study of Membrane-Permeability Changes with Seed Aging[1]

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We developed an electron paramagnetic resonance spin-probe technique to study changes in the barrier properties of plasma membranes in wheat (*Triticum aestivum* L.) seeds during aging under dry storage. The estimation of these barrier properties was based on the differential permeability of membranes for the stable free radical 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy and the broadening agent ferricyanide. The line-height ratio between the water and lipid components in the electron paramagnetic resonance spectra of 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (*R* value) allowed for the quantitative assessment of the plasma membrane permeability in small samples, enabling separate studies of the axis, scutellum, aleurone layer, and starchy endosperm tissue. High *R* values corresponded to low permeability and vice versa. Starchy endosperm cells had completely permeable plasma membranes even in mature, viable seeds. The loss of germinability with aging coincided with a considerably increased plasma membrane permeability of the embryo axis cells, but not of the scutellum and aleurone layer cells. The threshold *R* value for the individual axes associated with viability loss was established at 5 to 6, with the total ranging from 0 to more than 12. We suggest that the *R* value of an individual axis is the result of contributions from all individual cells, each of them characterized by a different permeability. The loss of viability, therefore, corresponds to the accumulation of cells having permeability above a critical level.

Seed viability loss is often attributed to the loss of plasmalemma integrity (Roberts, 1972; Maguire, 1977; Bewley and Black, 1994). Although it is not clear whether membrane damage is a primary reason or a secondary effect of seed deterioration (for discussion, see Priestley, 1986), it nevertheless can be considered as an early symptom of seed aging.

Several approaches are utilized to study failing plasmalemma integrity in aging seeds, and one that is often used is the measured conductivity of the soaking solution during imbibition (International Seed Testing Association [ISTA], 1985). Increased leakage of ions, amino acids, and sugars from aged seeds during imbibition is a definitive sign of loss of permeability of the plasma membranes (Bewley, 1986; Priestley, 1986). The rate and duration of this leakage and the type of solutes involved are indicative of the extent of the damage (Duke et al., 1983; McKersie and Senaratna, 1983). This type of damage assessment can be used only when the seed covers are permeable to ions, which is often not the case, even in aged seeds (Beresniewicz et al., 1995a, 1995b; Taylor et al., 1995). The increasing loss of barrier properties of the plasmalemma gradually leads to the inability of cells to maintain turgor (Parrish et al., 1982) and to respond osmotically (for refs., see Priestley, 1986). This eventually results in seed death.

At the ultrastructural level, aging-induced changes in membranes can be visualized from the earliest stages of imbibition (Hallam, 1973; Van Staden et al., 1975). As water uptake progresses, plasmalemma degradation becomes more pronounced. Furthermore, irregularity of the internal cellular structure is a clear indication of decompartmentalization, suggesting that the internal membranes have lost their integrity, too (Smith and Berjak, 1995). Some polar dyes, which penetrate cells only when the plasma membrane is disrupted, e.g. Evans blue (Duke and Kakefuda, 1981; Schoettle and Leopold, 1984), have been used to examine plasma membrane integrity. This method may give insight into the topographical distribution of nonviable cells in the sample. In spite of the valuable information that has been obtained by these approaches, each method has its disadvantages, which makes an interpretation difficult and uncertain in some cases (for review, see Priestley, 1986). The main problem with these methods is that it is not possible to quantitatively characterize membrane permeability in the different seed tissues.

EPR spectroscopy of spin probes is widely used in model membrane investigations (for review, see Marsh, 1981; Morse, 1985) and also in living systems. Plasma membrane permeability has been studied in fungi by applying water-soluble spin probes together with broadening agents (Miller and Barran, 1977; Miller, 1978). The dynamic properties of membranes have been studied with the help of hydrophobic spin probes (Miller and De La Roche, 1976; McKersie et al., 1978).

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Abbreviations: EPR, electron paramagnetic resonance; *R* value, ratio between the line heights of the water and lipid components of the spectrum; TEMPONE, 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy; TTC, tetrazolium chloride.

The EPR spin-probe technique can be useful in the study of seed plasma membrane integrity (Smirnov et al., 1992; Golovina and Tikhonov, 1994). We have shown that after more than 15 years of storage, the plasma membranes of wheat (*Triticum aestivum* L.) embryo cells completely lost their integrity. In addition, by applying apolar membrane spin probes, we have detected structural differences between membranes of viable and nonviable wheat embryos (Golovina and Tikhonov, 1994).

EPR investigation of membrane integrity using water-soluble spin probes has a considerable advantage over other methods that are in use. Due to the high sensitivity of the method it is possible to study, in small amounts of tissue, slight changes in membrane permeability of organisms under stress. In the case of seed aging, this technique permits the study of plasmalemma permeability in the different seed tissues at any time during germination. In the present paper we estimated, using an EPR spin-probe technique, the permeability of plasma membranes in different tissues of wheat grains of different harvest years to measure the progress of plasma membrane damage during aging under dry storage.

MATERIALS AND METHODS

Plant Material and Germination Test

All experiments were performed on wheat (*Triticum aestivum* L.) grains after 1 to 18 years of storage. We will refer to these grains as seeds, in spite of the fact that botanically they are considered fruits. Seeds of the cvs Albidum, Bezenchugskaya, Lutescens, Priokskaja, and Zarja were obtained from the Russian regional seed stations and seeds of cv Herzog were from the Department of Agronomy of the Wageningen Agricultural University (Wageningen, The Netherlands). The seeds were maintained in open storage at ambient temperature. RH of the surrounding air was not controlled.

Total percentages of germinated seeds were determined three times using 100 seeds that were placed on moistened filter paper in glass Petri dishes at room temperature over a period of 7 d. Seeds were considered germinated when protrusions reached more than one-half of the seeds size.

Staining by TTC was performed according to ISTA rules (ISTA, 1993).

EPR Measurements

The EPR spectra were obtained at room temperature with EPR spectrometers (X-band, model E-4, Varian, Sunnyvale, CA; and X-band, model 300E, Bruker Analytik, Rheinstetten, Germany). Microwave power was 12 mW, and the modulation amplitude was 1 G. The water-soluble nitroxide radical TEMPONE (Sigma) was used as a spin probe for testing the integrity of plasma membranes, according to Golovina and Tikhonov (1994).

Seeds were soaked in water for approximately 5 h at room temperature. This rehydration time before applying the spin probe is necessary to ensure that the data obtained can be linked with aging-associated plasma membrane permeability and not with a transiently increased permeability during cell rehydration (E.A. Golovina, F.A. Hoekstra, and M.A. Hemminga, unpublished data). Subsequently, the different tissues were dissected from the grain. Embryo axes can be easily excised because they are attached to the scutellum over a small region only. By contrast, the scutellum is tightly connected to the underlying layers of the endosperm and can be excised only from hydrated seeds. Aleurone layers were obtained by stepwise removal of the starchy endosperm. Starchy endosperm tissue was cut from the central part of the endosperm.

The excised, prehydrated embryos, embryo axes, scutella, aleurone layers, and small endosperm parts were immediately incubated for 15 min in a solution of 1 mM TEMPONE, completely broadened by 120 mM potassium ferricyanide. The sample was then loaded into a capillary (2 mm diameter), and a very small amount of solution was injected on the of the sample to prevent drying. Fifteen minutes of incubation in the TEMPONE solution appeared to be sufficient for the equilibrium distribution of the spin probe, because the amplitude reached its maximum within this period. The signal was stable for a period of at least 1 h.

Neutral Lipid and Phospholipid Extraction and Analysis

Approximately 20 mg of excised axes or scutella was homogenized with a mortar and pestle and a little sand in $CHCl_3$:methanol (2:1, v/v), and triheptadecanoin and diheptadecanoyl-phosphatidylcholine were used as the internal standards. Further lipid extraction and analyses were performed according to previously described methods (Hoekstra and van Roekel, 1988; Tetteroo et al., 1996).

RESULTS AND DISCUSSION

Use of TEMPONE to Estimate Cellular Membrane Integrity

A typical EPR spectrum from an aqueous 1 mM TEMPONE solution is a triplet with narrow, equidistant lines having an isotropic hyperfine splitting constant (the distance between peaks) of about 17 G (Fig. 1, top spectrum) (Griffith et al., 1974). TEMPONE also dissolves in apolar organic solvents because of its amphipathic character. Due to polarity effects, the distance between peaks in an EPR spectrum from TEMPONE in a hydrophobic environment decreases to 15 G (Fig. 1, middle spectrum [corn oil]). Also, the g value of the spectrum increases from 2.0056 to 2.0061, which causes an overall shift of the spectrum to the left (Griffith et al., 1974). In the case of the coexistence of a polar and an apolar phase in one sample, the superposition of the two EPR spectra was observed (Fig. 1, bottom spectrum). This two-component spectrum is resolved only in the high-field region, because of the combined effects of the changes in the g value and an isotropic, hyperfine-splitting constant on the peak positions of both components.

An aqueous solution of TEMPONE may be broadened by potassium ferricyanide via spin-spin interaction (Eaton and Eaton, 1978). The broadening leads to the apparent disappearance of the TEMPONE EPR spectrum. When viable cells are placed in such a broadening solution, the signal

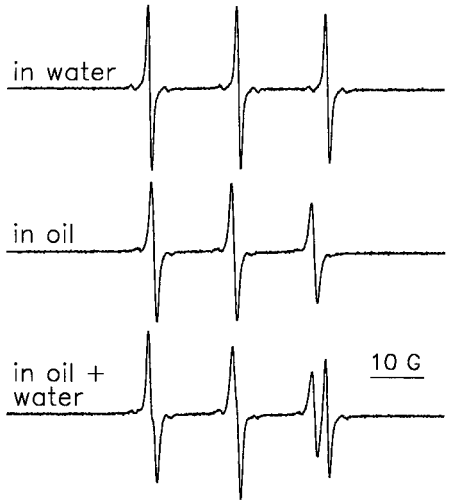

Figure 1. EPR spectra of 1 mM TEMPONE in water (top spectrum) and corn oil (middle spectrum). The spectrum at the bottom was obtained by a combination of capillaries containing either water or oil.

from TEMPONE will reappear because of the differential permeability of the plasmalemma for TEMPONE molecules and ferricyanide ions. TEMPONE molecules can pass through intact membranes within several minutes because of their amphipathic nature and relatively small size. In contrast, the charged ferricyanide ions cannot penetrate through an intact membrane. Thus, no broadening occurs inside the cells, and the triplet signal is exclusively derived from TEMPONE molecules inside of the intact cells. Because of the simultaneous presence in seed cells of polar (aqueous cytoplasm) and apolar (oil bodies and membranes) environments, superposition of the two types of spectra, as in Figure 1 (bottom spectrum) can be expected. Spectra of TEMPONE in viable wheat embryos (axis plus scutellum) show this to be the case (Fig. 2a). The two distinct peaks can be easily recognized in the high-field region of the spectrum. The left side of the lipid component, designated L, and the right side of the water component, designated W, were chosen for line-height measurement because interference of the two components with one another is least there.

The *R* value can be used to quantitatively characterize membrane permeability. In the case of reduced barrier properties of the plasma membranes, ferricyanide ions can diffuse into the cells and broaden the signal from TEMPONE localized in the aqueous, cytoplasmic environment. This will cause a partial decrease of the water component of the spectrum (Fig. 2b). The signal from the lipid environment cannot be broadened because of the inability of ferricyanide ions to dissolve in the hydrophobic phase. Thus, a decrease in *R* value describes an increase of membrane permeability.

Plasma Membrane Integrity in Different Tissues of Viable and Nonviable Wheat Seeds

Because the various seed tissues may contribute differently to the average membrane permeability of a seed and, therefore, may have a different impact on seed viability loss, we analyzed them separately. Due to the high sensitivity of the EPR spin-probe technique we were able to quantify the plasma membrane permeability of a single axis or less.

From 100% viable wheat seeds (1 year of storage) and from nonviable seeds (18 years of storage), the axes, scutella, aleurone layers, and starchy endosperm were excised. Figure 3A shows the EPR spectra of TEMPONE in the embryo axes. In the case of axes from nonviable wheat seeds, only the hydrophobic component of the EPR spectrum was observed, indicating that the broadening paramagnetic agent potassium ferricyanide penetrated into the cells and quenched the EPR signal from TEMPONE in the aqueous cytoplasm. A similar result, i.e. a considerable decrease of the hydrophilic component in the spectra, was obtained with aleurone-layer cells excised from the nonviable seeds (Fig. 3B). This sample also contained some seed-cover material, but the EPR water signal was exclusively derived from the viable aleurone-layer cells because seed covers consist of only dead cells. We conclude that in aged wheat seeds after 18 years of open storage, cellular membranes are completely disrupted in both the embryo axis and aleurone layer. The same was true for the scutellum (Fig. 3C). *R* values of scutellum cells from viable seeds were lower than those of embryo-axis and aleurone-layer cells, which may be due to the higher oil content in the scutellum cells (12.9 versus 9.3% in the axis). It also explains why *R* values of whole embryos, i.e. axis plus scutellum (Fig. 2), are intermediate to those of excised axes (Fig. 3A) and scutella (Fig. 3C).

The spectra of TEMPONE in dissected endosperm tissue were very similar in both viable and nonviable seeds, but differed considerably from the spectra in the axis, scutellum, and aleurone layer (Fig. 3D). They lack the narrow "polar" component, which means that the plasma membranes of starchy endosperm cells, even in viable wheat seeds, are permeable to ferricyanide ions (compare Fig. 3, A–C, with Fig. 3D). The occurrence of dead starchy en-

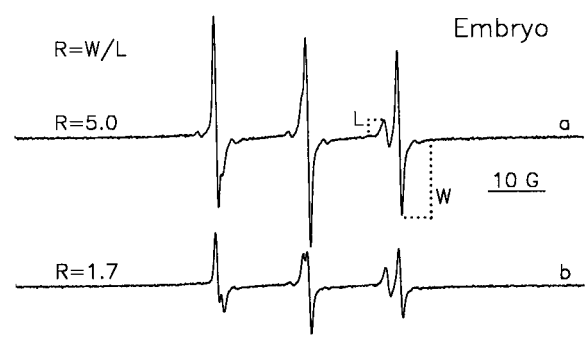

Figure 2. Representative EPR spectra of TEMPONE in embryos (axis plus scutellum) excised from viable wheat seeds (a) (1 year of storage; cv Herzog; 100% germination) and from nonviable wheat seeds (b) (9 years of storage; cv Zarja; 0% germination). *R* values were calculated as the ratio between the line heights of the water (right) and the lipid component (left) in the high-field region of the spectrum. The spectra were normalized according to the amplitudes of their lipid peaks, being a measure of sample size.

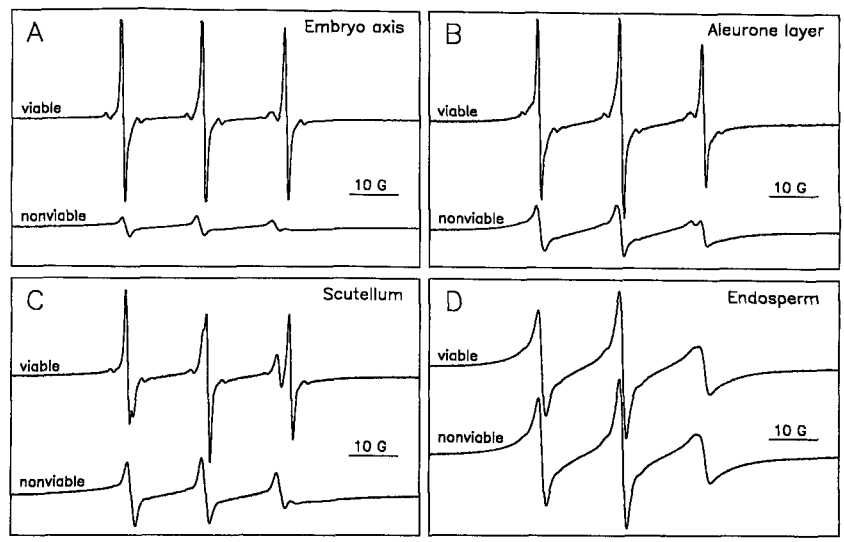

Figure 3. Representative EPR spectra of TEMPONE in the axis (A), aleurone layer (B), scutellum, (C) and starchy endosperm (D) excised from viable (top spectra) and nonviable (bottom spectra) wheat seeds after 1 year (100% germination; cv Herzog) and 18 years (0% germination; cv Albidum) of storage, respectively. The spectra were normalized according to the amplitudes of their lipid peaks.

dosperm cells in viable cereal caryopses has been described previously (Jacobsen, 1984; Bewley and Black, 1994), and is corroborated by the present EPR results.

Thus, the results of the EPR measurements indicate that in dry, viable wheat seeds only the embryo (embryo axis and scutellum) and aleurone layer contain cells with intact membranes, whereas starchy endosperm cells are completely permeable when the seed reaches the stage of maturity. Further experiments were conducted to follow the process of membrane degradation in embryo-axis, scutella, and aleurone-layer cells during natural aging and to establish the possible correlation between increased membrane permeability in these tissues and loss of germinability.

Evaluation of Plasma Membrane Permeability Increase during Aging

For the quantitative characterization of the plasma membrane permeability, we used the ratio (R) between the amplitudes of the polar (water [W]) and apolar (lipid [L]) peaks of the EPR spectra ($R = W/L$) (Fig. 2). The lipid signal in EPR spectra of TEMPONE can be used as the internal standard for the amount of material that is to be analyzed only when the oil content remains stable with aging in the different tissues. Our analysis in the axes of the different cultivars of different ages indicated that on a dry weight basis the oil content was stable over the years (9.19% ± 0.26 [SD]) and the phospholipid content varied around 1.85% ± 0.17 (SD). Therefore, the use of the lipid component as the internal reference was legitimate.

To study the impact of changes in the barrier properties of the plasma membranes of the different seed tissues on viability, we analyzed the R values in the embryo axes, scutella, and aleurone layers for several seed lots of different storage times (Fig. 4). R values were determined on five embryo axes or scutella from the same grains. In the case of aleurone layers, parts from several grains were combined and measured. The 9-year-old sample, having 0% germination, had just lost viability, whereas the 18-year-old sample probably had lost viability much earlier. The further decrease of the R value to extremely low values in the 18-year-old sample can be considered as the result of postmortem deterioration of the plasma membranes.

A considerable decrease in the R value with storage time and loss of germination was observed for the embryo axes. The R values for the scutellum and aleurone layers decreased to a lesser extent. Similarly, stainability of the scutellum and the aleurone layer in the TTC test during storage was maintained longer than that of the axis (data not shown), suggesting that aleurone-layer and scutellum cells were better preserved. Therefore, their deterioration cannot be considered a primary reason for the loss of seed viability. These data are in agreement with other EPR data on plasma membrane deterioration in naturally aged seeds from dicotyledonous species, showing that cells in the embryo axes lose plasma membrane integrity faster than those in cotyledons (Golovina et al., 1997), and support the view that embryo-axis cells are more sensitive to aging than storage tissue (see ISTA, 1985; for review, see Priestley, 1986).

Figure 5 shows, in more detail, the relationship between the R values in the embryo axes and the percentages of

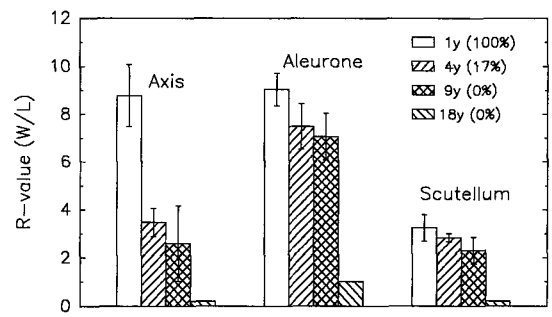

Figure 4. R values calculated from the EPR spectra of TEMPONE in the axes, aleurone layers, and scutella excised from wheat seeds of seed lots of different ages and different germination (1 y [year(s)], cv Herzog; 4 y, cv Zarja; 9 y, cv Zarja; and 18 y, cv Albidum). Data (±SD) are the average of three to five replicates of samples containing tissues from three to five grains each.

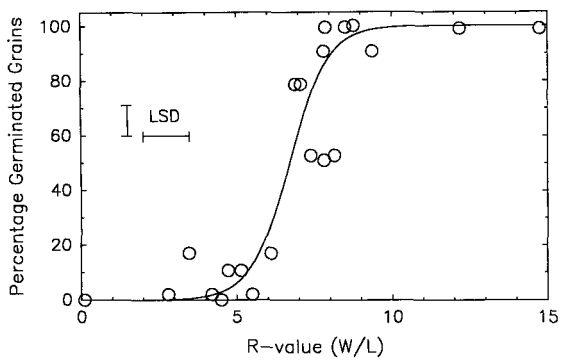

Figure 5. The relationship between the *R* values of embryo axes (5) and the percentages of germinated seeds from seed lots of different ages and all cultivars: cv Albidum, 18 years; cv Bezenchugskaya, 6 years; cv Herzog, 1 and 2 years; cv Lutescens, 6, 7, and 10 years; cv Priokskaja, 0.5 year; and cv Zarja, 3, 5, 7, 8, and 9 years. LSD ($P = 0.05$) values for *R* and percentages of germination are also indicated.

germinated seeds for the seed lots of different cultivars and storage periods. High-germination seed lots were characterized by *R* values above 8, up to values as high as 15. Below values of 5 the percentage of germinated seeds was low. Intermediate germination was characterized by a narrow range of *R* values between 8 and 5. The wide range in the *R* values above 8 in viable seeds could be a reflection of initial variation in vigor, whereas that below 5 may represent various degrees of postmortem decay (see also Fig. 4). The curve of Figure 5 also suggests that there is a critical *R* value in axes of around 5, below which no germination can occur.

Distribution of *R* Values of Individual Embryo Axes in Seed Lots of Different Germinability

Seeds do not age and die simultaneously. In every seed lot a distribution of the *R* values for the individual axes can be expected. Average *R* values, as shown in Figures 4 and 5, can give information only about the approximate position of the median of the *R* value distribution in individual axes. We expect that viable axes will have higher *R* values than nonviable axes, with a certain distribution around the average. In an attempt to confirm this, the frequency distribution of *R* values (integers) of a number of individual axes (more than 50) in seed lots of different germination capacity was studied (Fig. 6). The histograms correspond to the probability of finding an axis with a certain *R* value in every seed lot. For very old seeds (18 years of storage, cv Albidum) all *R* values were between 1 and 0. This means that there were very few cells with intact membranes in these embryo axes.

The seed lot of 100% germination (1 year of storage, cv Herzog) had a frequency distribution of the *R* value between 5 and 12, whereas the seed lot with 0% germination (cv Zarja, 9 years of storage) had a frequency distribution between 0 and 6. A slight overlapping of *R* values around $R = 5$ for these two seed lots was observed. Therefore, all embryo axes that have an *R* value above 6 may be considered as viable, whereas axes with an *R* value below 5 have a high probability of being nonviable. This further supports the concept that the threshold for viability may be at approximately $R = 5$ to 6.

If our assumption about a threshold for *R* values is correct, then in a seed lot with intermediate germination representing a mixture of viable and nonviable seeds the portion of *R* values above the threshold (above 6) should correspond to the germination percentage. Indeed, the distribution of the *R* values in the seed lot with 17% germination (cv Zarja, 4 years of storage) was from 1 to 10, with 16% of embryo axes characterized by *R* values > 6. It is worth noting that the percentage of embryo axes with *R* values between 5 and 6 (18%) approximately corresponded to the relative percentage of seeds that had signs of the beginning of germination but could not develop into normal seedlings (15%).

How Can the *R* Value Be Interpreted?

If it were possible to study the *R* values of individual cells in an embryo axis, then a decreased *R* value would

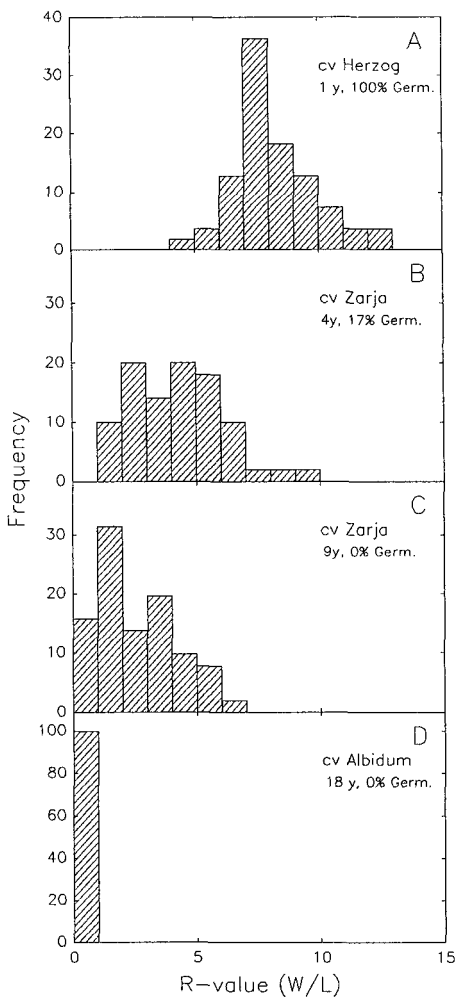

Figure 6. Distribution of the *R* values calculated from the EPR spectra of TEMPONE in individual axes within seed lots of different ages: A, 1 year (cv Herzog); B, 4 years (cv Zarja); C, 9 years (cv Zarja); and D, 18 years of storage (cv Albidum).

indicate a partial penetration of ferricyanide ions into the cell's interior. The question is, by how much ferricyanide is this? Experimental data on oil suspensions (E.A. Golovina, unpublished data) indicate that 1 mM ferricyanide causes a 50% decrease in the *R* value. Because the outside concentration used in the present tests was 120 mM, a 50% reduction of the water component would mean that only 1 ferricyanide ion out of every 120 was able to penetrate into the cell. Likewise, it was found that 10 mM ferricyanide ions reduce the *R* value to 1, and 100 mM ferricyanide ions reduce it to 0 (complete disappearance of the water signal). However, because single-cell analysis is impossible we attempted to interpret the averaging effect of the whole-axis measurement in terms of events in individual cells.

If we assume that all viable cells in an axis are characterized by a high *R* value and all nonviable cells are characterized by $R = 0$, then the *R* value obtained for an individual axis would be determined by the relative proportion of dead cells. Indeed, the spectrum of the combination of one embryo axis with $R = 0$ and one with $R = 10$ gave an *R* value of 5, representing 50% dead cells. If we interpret the distribution of the *R* values of the 100% viable seed lot (Fig. 6A, $R = 6$–12) this way, it would mean that in the embryo axes of $R = 6$, up to 50% of the cells are fully permeable in comparison with those of $R = 12$. Because such a high proportion of dead cells in 100% viable axes is not likely and also was not observed with TTC staining, we assume that individual cells within one axis have a distribution of the *R* values, which is indicative of a range of different plasma membrane permeabilities. Further support for this assumption comes from our experiments with tetrazolium staining of embryo axes from seed lots of different viability. Nonstained embryo axes had average *R* values around 0, slightly colored axes (rose) had values around 1, and well-stained axes (purple) had values of 4 and higher.

Microscopic analysis of the degree of TTC staining in individual cells of axes from seed lots of intermediate viability revealed that the rose color may stem from intermediate staining and not from a few cells with intense staining in the midst of many unstained cells.

We suppose that aging begins with the slight increase in the permeability of some cells, probably caused by an accumulation of free fatty acids in membranes (Senaratna et al., 1988). This would not cause seed death but could reduce seed vigor. Indeed, the 100% germinating seed lot (cv Herzog; $R = 6$–12) germinated faster than the 17% viable grains of cv Zarja (4 years of storage; *R* = presumably 6–9) (Fig. 6). The further accumulation of free fatty acids in membranes with time of storage could increase membrane permeability in some cells to an extent that is incompatible with viability. Thus, the number of nonviable cells will increase with time, as will the probability of damage to crucial meristematic cells. This is in agreement with the mathematical model proposed by Roberts (1972) that a certain number of randomly damaged, key cells determines the loss of seed viability. The presence of viable cells within nonviable wheat embryos, as demonstrated by Innocenti et al. (1983), concurs with our observation that the average *R* values in seed tissues of the seed lot of 0% germination (Fig. 4, 9 years of age) can be relatively high.

CONCLUSIONS

The present study has demonstrated the application of an EPR spin-probe technique in the investigation of membrane involvement in seed aging. Membrane permeability in the embryo axis, scutellum, aleurone layer, and endosperm was quantitatively estimated by the *R* value.

We confirmed, in contrast to embryo axes, scutella, and aleurone layers, that starchy endosperm in mature wheat caryopses does not contain cells with intact membranes. Plasma membrane permeability of embryo axes increases more rapidly with aging than that of the scutella and aleurone layers, indicating that embryo axes are more sensitive to aging. Aging in axes begins with a slight increase in permeability (decrease of *R* from approximately 15 to 8), without much effect on the germination percentage. The loss of viability is connected with a decrease of *R* from 8 to 5, whereas further decreases of *R* below 5 characterize nonviable embryos at different stages of debilitation. It has to be stressed that an *R* value of 5 does not characterize full membrane disruption, because only a few percent of the ferricyanide ions that were present in the solution penetrated in this case. At this critical *R* value, tetrazolium can still stain the axis. Axes with *R* values below 1, lacking tetrazolium staining, may be considered to have fully disrupted membranes. Such axes were found only in very old seed lots.

Due to its high sensitivity, the EPR spin-probe technique has potential applications in stress physiology, where barrier properties of membranes are believed to be changed, e.g. osmotic, temperature, and freezing stress. It allows small changes in membrane permeability to be localized and quantitatively estimated.

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