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Acquisition of desiccation tolerance in developing wheat embryos correlates with appearance of a fluid phase in membranes

E. A. GOLOVINA[1,2] & F. A. HOEKSTRA[1]

[1]*The Graduate School Experimental Plant Sciences, Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands and* [2]*Russian Academy of Sciences, Timiryazev Institute of Plant Physiology, Botanicheskaya 35, Moscow, 127276, Russia*

ABSTRACT

Membrane behaviour in developing wheat (*Triticum aestivum* cv Priokskaya) embryos was studied in relation to the acquisition of desiccation tolerance, using spin probe techniques. Fresh embryos were able to develop into seedlings at day 15 after anthesis, but it took 18 d before fast-dried, isolated embryos could germinate. On the basis of membrane integrity measurements it was estimated that between 14 and 18 d after anthesis the proportion of embryonic cells surviving fast drying increased and the critical moisture content, to which embryonic cells could be dehydrated, decreased. Apparently, embryonic cells do not acquire the same level of desiccation tolerance simultaneously. Only when all cells had become desiccation tolerant was germination of air-dried embryos possible. Using 5-doxylstearic acid as the probe molecule, an approximately similar lipid–water interface ordering of membranes was observed in all hydrated embryos, irrespective of age. Dehydration had a dual effect on the lipid interface: further ordering of the major part of the interface and the appearance of additional, disturbed regions. The proportion of these regions correlated with the proportion of desiccation-tolerant cells. We propose that the membrane surface disturbance be caused by endogenous amphiphiles that partition from the cytoplasm into membranes during drying. The absence of such disturbed regions in dried, desiccation-sensitive embryos might reflect a lack of sufficient amphiphiles. The relevance of membrane surface disturbance for desiccation tolerance is discussed.

Key-words: *Triticum aestivum*; amphiphile partitioning; dehydration; electron paramagnetic resonance; fluidization; membrane integrity; mobility; ordering effect; spin probes.

Abbreviations: $2A_{max}$, hyperfine splitting between the outermost extremes; a_{iso}, isotropic hyperfine splitting constant; daa, days after anthesis; 5-DS, 5-doxylstearic acid; DSC, differential scanning calorimetry; DW, dry weight; EPR, electron paramagnetic resonance; FT-IR, Fourier transform infrared spectroscopy; MC, moisture content; PC, phosphatidylcholine; PDT, perdeuterated TEMPONE; RH, relative humidity; TEMPONE, 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy; T_m, gel-to-liquid crystalline transition temperature.

Correspondence: Elena A. Golovina. Fax: +31 317484740; e-mail: elena.golovina@wur.nl

INTRODUCTION

Desiccation tolerance, defined as the ability of organisms to resume their normal function after drying to the air-dry state and subsequent rehydration, is widespread in nature. Desiccation tolerance can be constitutive as in many seeds, or inducible as in whole plants, e.g. those from the genus *Craterostigma*. Different mechanisms of desiccation tolerance have been proposed (for reviews, see Bartels & Salamini 2001; Buitink, Hoekstra & Leprince 2002). As membranes are considered a primary target of desiccation injury, membrane stabilization is thought to be a key mechanism of desiccation tolerance (Crowe, Hoekstra & Crowe 1992; Crowe, Carpenter & Crowe 1998). Recently, we drew the attention to amphiphilic metabolites that might partition into membranes during dehydration and play a role in desiccation tolerance (Hoekstra *et al*. 1997; Golovina, Hoekstra & Hemminga 1998; Hoekstra & Golovina 2000, 2002; Hoekstra, Golovina & Buitink 2001; Golovina & Hoekstra 2002).

Partitioning of amphipathic small molecules into biological membranes has been extensively studied, particularly in relation to its impact on membrane structure and function. Initially, these studies were related to the mechanisms of anaesthesia (Franks & Lieb 1982). Later on, the research involved the membrane mechanisms of action of tranquilizers, alcohols, many other drugs, environmental pollutants, herbicides, insecticides, etc. Amphiphiles are molecules with two distinct domains, a hydrophilic one and a hydrophobic one. Partitioning of small amphiphiles into membranes may modify membrane structure, causing fluidization or immobilization depending on location in the bilayer, which is a crucial parameter in the control of many physiological processes (Aloia, Curtain & Gordon 1988).

Derangement of fluidity can even cause the development of pathological state (for references, see Lenaz & Castelli 1985).

Many cellular metabolites have amphiphilic properties. In fully hydrated cells, they are distributed over the lipid (membrane) and aqueous (cytoplasm) phases according to their partition coefficients. The amount of these compounds in membranes is expected to be low because of the high volume ratio of aqueous cytoplasm to membranes and/or the relatively low affinity of these compounds to the lipid phase. Membrane properties are thus only moderately influenced. Cellular metabolite partitioning into membranes has been studied mainly in relation to the uptake and passive transport across membranes. However, under stress conditions that cause water loss, such as drought, freezing, or osmotic stress the scenario might dramatically change (Hoekstra *et al*. 2001). An increased amphiphile concentration in the cytoplasm as a result of aqueous volume reduction would lead to an increased partitioning into membranes, with considerable consequences for membrane physical properties and cell physiology.

Using the amphiphilic spin probe 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPONE), we have shown that its partitioning into the lipid phase of plant cells during dehydration correlates with an increase in membrane permeability (Golovina *et al*. 1998) and fluidity (Hoekstra & Golovina 2000, 2002; Golovina & Hoekstra 2002). We have proposed that this partitioning with drying might have both adverse and beneficial effects (Hoekstra *et al*. 2001). The adverse effects could be associated with increased membrane permeability, whereas the beneficial effects relate to an automatic insertion of amphiphilic antioxidants into membranes with drying.

Progressive partitioning of amphiphiles from the aqueous cytoplasm into membranes during drying is a thermodynamically inevitable process and is expected to occur in all organisms, irrespective of possible desiccation tolerance. We assume that desiccation-tolerant organisms can cope with the adverse effects of membrane perturbance on the one hand, and benefit from the partitioning of possibly newly formed antioxidants into their membranes, on the other hand.

The aim of the present work was to compare the changes in membrane dynamics during dehydration of wheat embryos at the desiccation-sensitive and desiccation-tolerant stages of development. Dehydration is expected to cause immobilization of the membrane interface, whereas partitioning of endogenous small amphiphiles into membranes might cause fluidization. Using the electron paramagnetic resonance (EPR) spin label technique, we showed that dehydration has a dual effect on membranes. Dehydration indeed had a significant ordering effect on the major part of the lipid–water interface. In addition, a part of the interface exhibited fluid behaviour, which we attribute to the disturbing effects of partitioned endogenous amphiphiles in this membrane region. The appearance of this fluid phase was found to correlate with the acquisition of desiccation tolerance.

MATERIALS AND METHODS

Plant material

Plants of spring wheat (*Triticum aestivum* L. cv Priokskaya) were grown in pots under greenhouse conditions (16 h light/8 h dark and an average day temperature of 22 °C). Ears were tagged at the beginning of anthesis. Embryos were isolated from the kernels during development. Fast dehydration of the embryos was performed on the lab bench [relative humidity (RH) approximately 30%], half water loss being reached within 20–40 min of drying, depending on developmental age.

Germination of fresh and air-dried, isolated embryos ($n = 50$) was carried out in Petri dishes on 0.7% agar in tap water. Air-dried embryos were prehydrated in water vapour-saturated air for 1 h and then transferred to agar medium for germination. Water contents were analysed by weighing the samples before and after heating at 96 °C for 24 h.

EPR spin probe studies

Membrane integrity was measured with an EPR spin probe technique using perdeuterated TEMPONE (PDT, obtained from Professor Igor Grigoriev, Novosibirsk, Russia). Before labelling, the air-dried embryos were prehydrated in humid air for 1 h and then placed on wet filter paper for 3 h for further rehydration. Embryos that were partly dehydrated were immediately placed on wet filter paper (3 h), while fresh embryos were directly used for labelling. The hydrated embryos were incubated for 15–20 min in a 1 mM solution of PDT containing 120 mM of the broadening agent $K_3Fe(CN)_6$. The sample (usually one embryo) was then loaded into a capillary (2-mm diameter) together with a small amount of the solution. EPR spectra were recorded at room temperature with an X-band EPR spectrometer (Model 300E; Bruker, Rheinstetten, Germany). The PDT spectra were recorded at 2 mW microwave power and 0.25 G modulation amplitude. The scan range was 100 G.

The principles of the method have been described elsewhere (Golovina & Tikhonov 1994; Golovina, Tikhonov & Hoekstra 1997). Briefly, PDT spectra can be broadened to apparent invisibility by paramagnetic $Fe(CN)_6$ ions via spin–spin interactions (Eaton & Eaton 1978). Incubating a sample in this broadened solution of PDT allows compartmentation of the spin probe molecules in the cells with intact membranes to be detected. This is based on the fact that PDT can easily penetrate membranes, whereas $Fe(CN)_6$ ions cannot. If all cells contain intact membranes, ferricyanide broadens only the EPR signal from the extracellular solution to apparent invisibility. The EPR signal thus originates from PDT molecules located inside cells. However, when membranes are damaged, ferricyanide also broadens the EPR signals from PDT located inside cells. Compartmentation of label within the cells with undamaged membranes can be quantified from spectral line height ratios. Retention of membrane integrity as a criterion of cellular desiccation tolerance can thus be estimated from

PDT spectra after a dehydration/rehydration cycle. Owing to the high sensitivity of the method, even a few intact cells in one embryo of approximately 0.2 mg dry matter can be detected (Golovina, Hoekstra & van Aelst 2000, 2001).

Fluidity of the membrane interface was studied using the membrane spin label 5-doxylstearic acid (5-DS, from Sigma, St Louis, MO, USA). Fresh, isolated embryos were placed in a 2 mM solution (final solution containing 2% ethanol) of 5-DS. The solution of 5-DS was freshly prepared from a 100 mM stock solution in ethanol. Embryos were labelled for a period of 30 min, then washed in water and carefully blotted with filter paper. Subsequently, the embryos ($n = 3$–10) were placed into a capillary (2 mm diameter), immediately, or after air-drying inside a closed Petri dish. The 5-DS spectra (scan range 100 G) were recorded at 5 mW microwave power and 3 G modulation amplitude.

In the case of labelling egg-phosphatidylcholine (egg PC) dispersions, 1 mol% of 5-DS was added to the egg PC (Fluka, Buchs, Switzerland) in $CHCl_3$. The $CHCl_3$ was evaporated in a stream of nitrogen and, after the sample was held under vacuum for at least 2 h, 1 mL of water was added. Hydration was facilitated by five freeze–thaw cycles and vortexing. The egg PC dispersion was spread over a glass slide and allowed to dry overnight in a stream of dry air (3% RH). The dried material was scraped from the slide in a dry-air box (3% RH), transferred to 2 mm capillaries and flame-sealed.

Multi-component 5-DS spectra were decomposed by spectral titration, namely subtraction after adjusting for position and amplitude of peaks (Berliner 1976). Details of the decomposition procedure and the spectral parameters are described in the Results section.

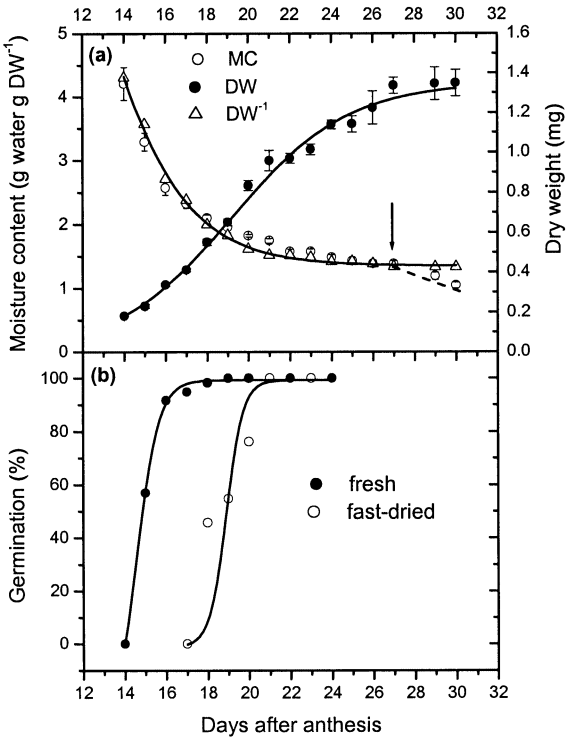


Figure 1. Timetable of wheat embryo development. (a) Changes in MC ($n = 7$; data ±SE) and DW ($n = 6$; data ±SE); open triangles represent reciprocal DW values plotted on a scale adjusted to the scale of MC (scale for DW^{-1}, values not shown). The arrow indicates the onset of maturation drying. (b) Germination of isolated embryos ($n = 50$), either fresh or after fast drying.

RESULTS

Acquisition of embryo desiccation tolerance during development

From 12 to 13 d after anthesis (daa) onward, it was possible to excise embryos from developing wheat grains, which allowed us to determine individual moisture content (MC) and dry weight (DW). Figure 1a shows that the embryo DW gradually increased with development up to 27 daa, which is characteristic for the maturation phase. The accumulation of DW was accompanied by a decrease in MC. This decrease is the result of dry matter accumulation rather than water loss, for the following reason. If MC is plotted as the amount of water per dry weight [MC = (W) DW^{-1}], then MC = constant DW^{-1} if the amount of water remains unchanged. Indeed, after adjustment of scale, the experimental values of MC and DW^{-1} plotted against daa practically coincided, which indicates that there is no net water loss. For developmental ages older than 27 daa, these values diverged (Fig. 1a), which indicates that the stage of maturation drying has begun at 27 daa.

Fresh, isolated embryos acquired the morphological competence to germinate at 15 daa (Fig. 1b), which corresponds to the end of embryo length increase (Golovina *et al.* 2001). At this developmental age, isolated embryos showed some organized growth when seeded on agar medium. At later developmental age the accumulated storage reserves allowed for further axis growth.

To determine the developmental age, at which wheat embryos acquire desiccation tolerance, we fast-dried isolated embryos on the laboratory bench at the ambient air (approximately 30% RH). Dehydration of embryos within the grains is not an appropriate drying method here, because this takes several days, during which embryos continue their development. Desiccated, isolated embryos did not germinate until 18 daa (40% germination), and at 21 daa germination was 100% (Fig. 1b). The 60% non-germinated embryos harvested at 18 daa were probably younger than 18 d, because anthesis within the ear lasts at least 2 d, and ears were labelled when the first anthers appeared. Therefore, 18 daa can be considered as the developmental age at which embryo desiccation tolerance is acquired, which leaves the possibility that cellular desiccation tolerance is consecutively acquired.

Heterogeneity in the acquisition of cellular desiccation tolerance

To study the heterogeneity of cells within one embryo with respect to the acquisition of desiccation tolerance, we

applied a spin probe technique, using PDT (for structure image, see Fig. 2a) in combination with the broadening agent, potassium ferricyanide. Typically, PDT in solution gives an isotropic EPR spectrum containing three narrow lines (triplet; Fig. 2a). The distance between the lines, the isotropic hyperfine splitting constant a_{iso}, characterizes the polarity of the environment. The more polar the environment of the spin probe, the larger a_{iso}. As follows from Fig. 2a, an a_{iso} of 16.7 G characterizes the aqueous environment of PDT molecules.

Figure 2b shows EPR spectra of PDT in isolated embryos of the developmental ages 14–17 daa after a rapid drying/rehydration cycle. In contrast to the spectrum in Fig. 2a, the spectra in Fig. 2b contain two superimposed triplets with different distances between the lines. These two triplets are well resolved at the high-field (right-hand) side of the spectra. The triplet with the smaller distance between the lines of approximately 14.6 G characterizes a hydrophobic environment and originates from PDT molecules in lipid bodies (Golovina *et al*. 2001). The triplet with the larger distance between the lines (approximately 16.7 G) represents PDT in the aqueous cytoplasm of cells with intact membranes. This follows from the same values of a_{iso} in this triplet and the spectrum of PDT in water in Fig. 2a. Cells with disrupted membranes would not give such an aqueous signal (see Materials and methods).

The spectra in Fig. 2b were plotted in such a way that the amplitudes of the high-field (right-hand side) peak of the 'lipid' triplets were the same. In other words, all spectra were normalized to approximately the same sample size, at least with respect to the amount of lipid bodies present. This amount did not increase significantly over the period 14–17 daa, but did so at a later developmental age (data not shown). The legitimacy of using 'lipid' peaks for normalizing spectra is based on the fact that ferricyanide ions are unable to partition into lipid bodies, which renders the 'lipid' signal independent of membrane damage and only dependent on the total amount of lipid bodies in a sample (Golovina *et al*. 1997). The amplitude of the cytoplasmic component of the normalized spectra thus correlates with the amount of desiccation-tolerant cells that maintained plasma membrane integrity. From Fig. 2b it is clear that the proportion of the cytoplasmic component gradually increased with developmental age.

We quantified the proportion of surviving desiccated cells during embryo development as $W\ (W + L)^{-1}$ (see Fig. 2b) and plotted these values against embryo developmental age (Fig. 3a). It is evident that the proportion of desiccation-tolerant cells in an embryo gradually increased from 14 to 18 daa. Because embryos acquire desiccation tolerance at 18 daa, it can be concluded that germination of previously dried embryos is possible only when most of the embryonic cells survive desiccation.

The critical MC to which cells within the same embryo can be dehydrated decreased during development (Fig. 3b). Inspection of the proportion of surviving cells in 15 daa embryos during drying showed that some cells were sensitive to water loss just below 0.5 g H_2O g^{-1} DW, whereas

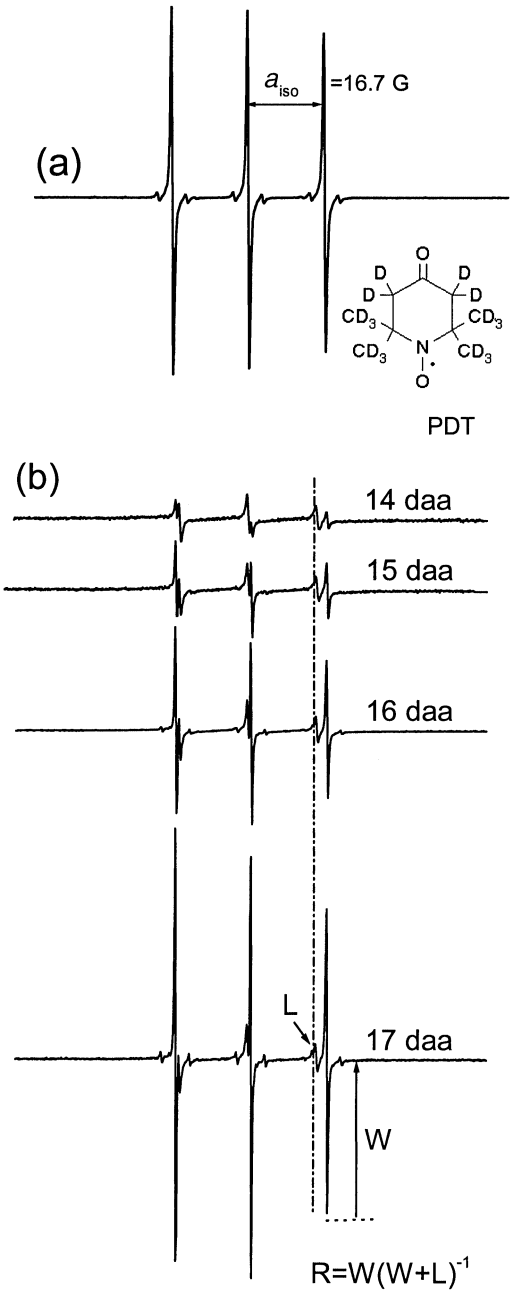


Figure 2. EPR spectra of PDT. (a) Spectrum (triplet) from a 1 mM PDT solution, consisting of three equidistant narrow lines with a_{iso} = 16.7 G; insert: structure image of PDT. (b) Spectra from single wheat embryos. The embryos were isolated from fresh kernels of different developmental ages and fast dried. Before incubation in a 1 mM PDT solution containing 120 mM potassium ferricyanide, the embryos were prehydrated in humid air for 1 h. Peaks linked to lipid bodies (L) and aqueous cytoplasm (W) are indicated. All the PDT spectra from wheat embryos are plotted in a way that the triplets originating from PDT in lipid bodies have equal amplitudes. The position of the high-field lines of the lipid triplets is indicated by a dashed line. The ratio $R = W(W + L)^{-1}$ can be used to estimate the proportion of viable cells.

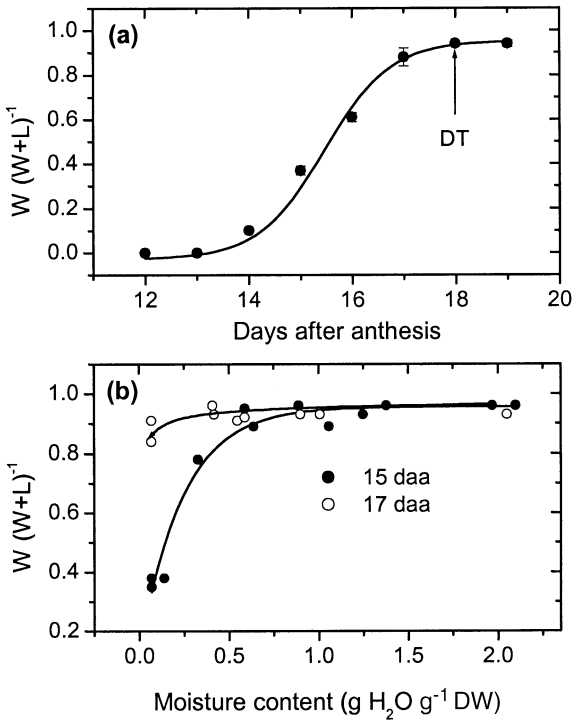


Figure 3. Plot of R-values [W(W + L)$^{-1}$] calculated from spectra of PDT in wheat embryos versus embryo developmental age or MC. The R-value relates to the proportion of desiccation-tolerant cells in the sample (R = 0 when all cells are leaky and R = 0.94 when all cells are alive). (a) During development (after desiccation to the air-dried state (approximately 0.07 g H_2O g^{-1} DW); n = 3–5, data ±SE; embryos were from different ears); (b) During fast drying of embryos (15 and 17 daa) from one ear. DT, desiccation tolerant.

others could withstand drying to lower MCs. The majority of cells in 17 daa embryos survived considerably lower MCs. Apparently, at the cellular level the acquisition of desiccation tolerance is a gradual process, occurring in different cells at different times.

Membrane interface ordering upon air-drying of desiccation-sensitive 13 daa embryos

The lipid–water interface is considered to be the most informative part of membranes in relation to membrane barrier function (Tieleman, Marrink & Berendsen 1997). Therefore, we selected the membrane probe, 5-DS, that provides information about mobility at the lipid–water interface of membranes. In this molecule the nitroxide doxyl group (stable radical) is attached to stearic acid at the fifth-C of the carbon chain (for structure image, see Fig. 4) in a way that the motion of the nitroxide group directly reflects the motion of the labelled part of stearic acid. The shape of EPR spectra of 5-DS depends on the motion and angular orientation of the nitroxide group with respect to the membrane surface (Marsh 1981). The parameters of mobility and order derived from this shape can thus be used to characterize the dynamic properties of membranes close to the lipid–water interface. There is a body of evidence that supports the hypothesis that 5-DS mainly resides in the plasma membrane (Kaplan, Canonico & Caspary 1973; Bales & Leon 1978).

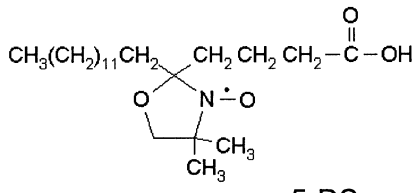
Since 12–13 daa is the earliest developmental age at which wheat embryos can reliably be excised, we used 13 daa embryos as the control for the desiccation-sensitive stage. Figure 4a shows 5-DS spectra from fresh (upper spectrum) and fast-dried (lower spectrum) 13 daa wheat embryos. The presence of the outer extremes in spectra is an indication of the ordered character of the interface, and the distance between them, $2A_{max}$, provides a measure of membrane structural order and motional freedom. An increase in this parameter corresponds to an increased ordering of spin label in the membrane environment. Dehydration causes $2A_{max}$ values to increase from 54.3 G in the hydrated specimen to 65.3 G in the desiccated one (Fig. 4a). This indicates that desiccation leads to further ordering of the interface. In hydrated membranes, the acyl chains have considerably higher freedom of angular motion than in dried membranes, as follows from the smaller $2A_{max}$ values in the former case. The spectra in Fig. 4a are comparable in shape and $2A_{max}$ values with those from air-dried egg PC dispersions obtained at 30 °C (relatively mobile) and –15 °C (relatively immobile) (Fig. 4b). Apparently, desiccation causes a similar ordering of the membrane interface in cells of desiccation-sensitive wheat embryos as does lowering the temperature in a dried model membrane system.

Other air-dried tissues of the ear, such as desiccation-sensitive pericarp tissue and flower parts gave 5-DS spectra that were similar in shape as those from the desiccated 13 daa embryos shown in Fig. 4a (spectra not shown).

Dual effect of desiccation on membrane interface mobility in desiccation-tolerant 21 daa embryos

Figure 4c shows 5-DS spectra from fresh and fast-dried 21 daa wheat embryos. The spectrum from fresh 21 daa embryos resembled that from 13 daa fresh embryos (Fig. 4a) in shape as well as in $2A_{max}$. This holds for the 5-DS spectra from fresh embryos of all developmental ages (Fig. 5 for $2A_{max}$, spectra not shown). It can therefore be concluded that the acquisition of desiccation tolerance does not influence lipid–water interface ordering in hydrated cells.

The 5-DS spectrum from air-dried, desiccation-tolerant, 21 daa embryos differed from that from the desiccation-sensitive, 13 daa embryos in that an additional peak appeared in the spectrum from the 21 daa embryos (cf. Fig. 4a & c). This peak is located at the low-field (left-hand) side of the spectrum adjacent to the outer-most peak (arrow in Fig. 4c) and indicates that the nitroxide moiety of some 5-DS molecules has a relatively high degree of motional freedom. The two peaks at the low-field side of the spectrum from desiccated 21 daa embryos (Fig. 4c) thus represent two spectral components originating from



5-DS

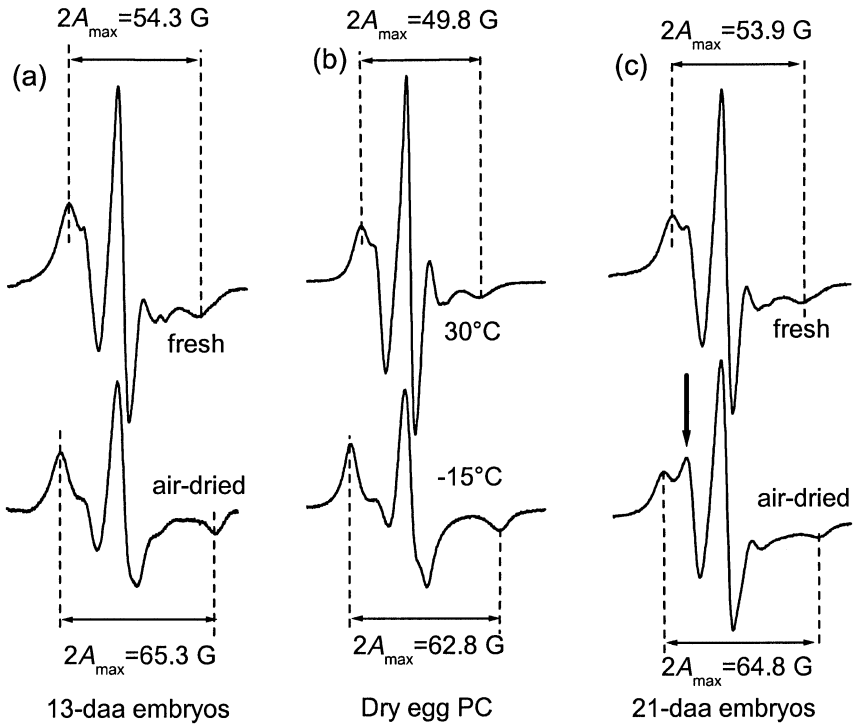


Figure 4. Representative EPR spectra of 5-DS. (a) In fresh and air-dried 13 daa wheat embryos (desiccation sensitive); (b) in air-dried egg PC dispersions at 30 °C and –15 °C; and (c) in fresh and air-dried 21 daa wheat embryos (desiccation tolerant). Dashed lines indicate the position of the outer extremes of anisotropic spectra, with the distance $2A_{max}$ as a measure of the local membrane order and mobility. The arrow in the spectrum of the desiccated embryo in (c) indicates the position of the low-field peak of a second ('fluid') spectral component. Insert: structure image of 5-DS.

the nitroxide moiety in two different membrane environments – an ordered and a disordered one that we will further refer to as the fluid component. The motional parameters of the ordered environment could be derived from $2A_{max}$, because the outer-most extremes of the ordered spectrum did not overlap with the fluid one. Upon desiccation of the 21 daa embryos, $2A_{max}$ increased, in a similar way as for the 13 daa embryos, from 53.9 G for the spectrum from the fresh, hydrated embryos to 64.8 G for the spectrum from the desiccated embryos. Desiccation therefore causes further immobilization of the ordered phase at the membrane interface. An approximately similar immobilization of the ordered membrane phase with drying was observed for all wheat embryos irrespective of their age as follows from the similar $2A_{max}$ values (Fig. 5).

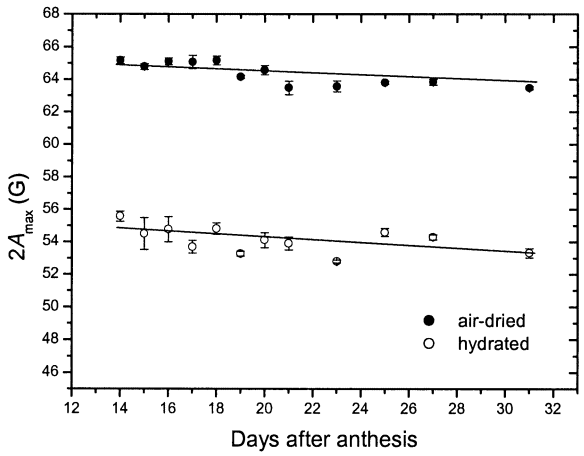


Figure 5. Effect of wheat embryo developmental age on $2A_{max}$ in 5-DS spectra from hydrated, fresh embryos and from air-dried embryos. Data ±SE (n = 5–23).

While the 5-DS spectra in air-dried, desiccation-sensitive, 13 daa embryos show a single ordered component (Fig. 4a), those in desiccation-tolerant 21 daa embryos display both ordered and fluid components (Fig. 4c). To characterize the motional freedom of the environment corresponding to the fluid component, the spectrum from desiccated 21 daa embryos was decomposed. To accomplish this, we subtracted the spectrum obtained from desiccated 13 daa embryos from that obtained from the desiccated 21 daa embryos after adjustment for peak position and amplitude of the outer-most left peak (Fig. 6a). The difference spectrum (Fig. 6b) did not contain outer-most extremes, but still

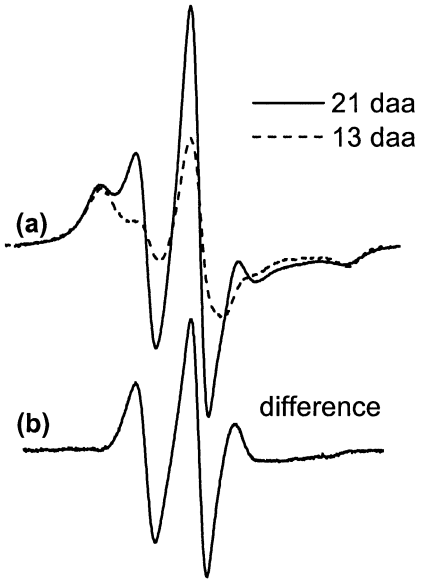


Figure 6. Decomposition of 5-DS spectra obtained from desiccated wheat embryos. (a) 5-DS spectrum from 13 daa embryos (dashed line) and 21 daa embryos (solid line). Adjustment for amplitude and position of the low-field (left-hand side) outer-most peaks of the ordered components in both spectra (normalizing) allows the shape of the fluid component to be revealed after subtraction. (b) The difference spectrum obtained after subtraction of the 13 daa spectrum from the 21 daa spectrum.

displayed a small degree of anisotropy, as follows from the different amplitudes of the low-field (left-hand side) and central peaks. Such a spectral shape previously has been described for membranes and referred to as corresponding to pseudoisotropic motion (Teixeira *et al*. 2001; and references therein).

Proportion of the fluid component in 5-DS spectra from desiccated embryos of different developmental ages

The fluid component was observed not only in the 5-DS spectrum from desiccated 21 daa embryos, but also in those from desiccated embryos of developmental ages greater than 13 daa (Fig. 7). Spectra were plotted in such a way that the amplitudes of the outer-most, low-field peaks of the ordered component were approximately equal. Then, the amplitudes of the low-field peaks of the fluid component become indicative of the proportion of nitroxide moieties in the disordered environment, increasing with embryo developmental age. The proportion of the fluid component was obtained by spectral subtraction (as in Fig. 6) and subsequent double integration of the two individual components. Subtraction was performed using the 5-DS spectrum from desiccated 13 daa embryos (bottom spectrum in Fig. 4a) as the ordered component. The proportion of the fluid component is indicative of the proportion of 5-DS molecules with their nitroxide moiety in the disordered environment. This proportion was calculated as a percentage of the total and was found to rise from zero at 13 daa just prior to the acquisition of desiccation tolerance to approximately 23% at 18 daa when embryos had become desiccation tolerant (Fig. 8).

Changes in membrane fluidity in the course of dehydration of embryos at the desiccation-sensitive and -tolerant stages

The increase in proportion of the fluid component in 5-DS spectra with developmental age was clearly observed only in desiccated wheat embryos and not in fresh embryos (cf. Figs 4a & 7). This indicates that the rise in proportion of the fluid component must take place during dehydration. Figure 9 shows the changes in the 5-DS spectra from 21 daa embryos with drying. All spectra were plotted in such a way, that the amplitude of the low-field peak of the ordered component was the same. With drying the low-field peak of the ordered component downshifted and the amplitude of the fluid component began to rise long before drying was

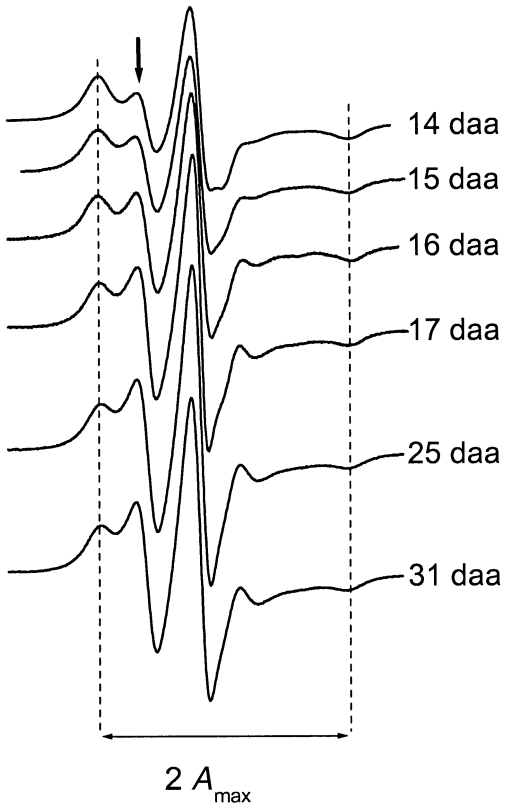


Figure 7. EPR spectra of 5-DS in dried wheat embryos of different developmental age. Spectra represent the superposition of ordered and fluid components. The occurrence of the ordered component is evident from the presence of outer extremes (indicated by the dashed lines with the distance between them $2A_{max}$ = 63–65 G). The occurrence of the fluid component is evident from the presence of a peak (indicated by arrow) adjacent to the outer-most peak at the low-field (left-hand) side of the spectra. Spectra are plotted in a way that the amplitudes of the outer-most, low-field peaks are equal.

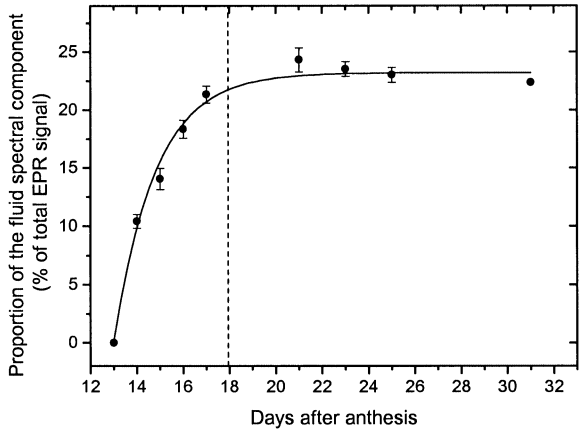


Figure 8. Changes in proportion of the fluid spectral component in 5-DS spectra from desiccated wheat embryos during embryo development. The proportion was calculated from the number of 5-DS molecules in fluid phase as a percentage of that responsible for the total EPR signal. Data points (± SE) for each developmental age were averaged from five to 23 samples. A dashed line separates the desiccation-sensitive state from the -tolerant state.

completed. The downshift is indicative of a decrease in mobility of the ordered phase. A plot of $2A_{max}$ against MC for embryos of different developmental age showed that the downshift was similar for all embryos, irrespective of desiccation tolerance (Fig. 10). An MC of 1 g H_2O g^{-1} DW appears crucial in that below this MC the immobilization of the ordered phase at the lipid–water interface began. However, the fluid component appeared in the spectra already below 1.62 g H_2O g^{-1} DW, which is ahead of the dehydration-induced immobilization of the ordered component. With further drying the proportion of the fluid component gradually increased (Fig. 9). At MC values ≥ 1.62 g H_2O g^{-1} DW the 5-DS spectra were similar (spectra not shown).

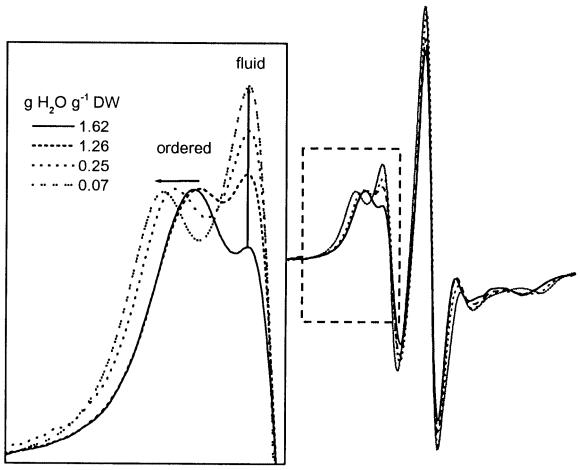


Figure 9. Changes in EPR spectral shape (5-DS) in 21 daa wheat embryos during dehydration. Insert: magnified low-field part of the spectra.

DISCUSSION

In this paper we showed the differential behaviour of membranes during dehydration at the desiccation-sensitive and desiccation-tolerant stages of wheat embryo development. Several aspects are now discussed.

Immobilization of the ordered phase at the membrane interface with embryo dehydration

Irrespective of embryo developmental age, drying caused a decrease in freedom of angular motion of the major part of the acyl chains close to the membrane lipid–water interface. This follows from the rise in $2A_{max}$ of the ordered component in 5-DS spectra with embryo dehydration from 52–54 to 63–65 G (Fig. 5). This rise commenced just below the relatively high MC of 1 g H_2O g^{-1} DW (Fig. 10). Immobilization of the membrane interface could logically be expected in concert with the loss of the water shell below 0.3 g H_2O g^{-1} DW (Hoekstra *et al*. 2001), or at best below 0.6 g H_2O g^{-1} DW when the cytoplasmic viscosity greatly increases (Leprince & Hoekstra 1998). In the presence of bulk water such an immobilization might result from the interaction with some ions (Turchiello *et al*. 2000), the concentration of which increases with water loss. In addition, the immobilization might result from the established interaction of certain cytoplasmic biopolymers with the membrane surface. A number of such molecules have been identified, ranging from polysaccharides (Vereyken *et al*. 2001) to proteins (Tsvetkova *et al*. 2002; Koag *et al*. 2003), which have been attributed a protective role under conditions of drought (Vijn & Smeekens 1999; Buitink *et al*. 2002). In this respect it is interesting to note that desiccation-sensitive, 15 daa wheat embryos contained cells that largely remained alive during drying to 0.5 g H_2O g^{-1} DW; that is, these cells were at least drought tolerant. Apart from immobilization of the membrane interface, the increase in $2A_{max}$ could result from an upward movement of 5-DS molecules to the more rigid environment because of carboxylic group ionization at elevated pH (Barrat & Laggner 1974;

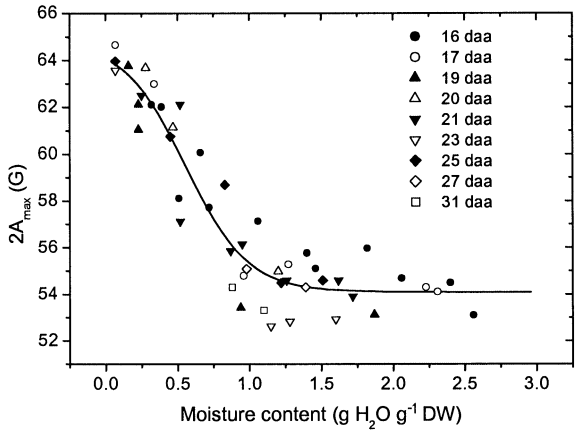


Figure 10. Effect of drying on $2A_{max}$ of the ordered component in 5-DS spectra from wheat embryos of different ages (16–31 daa).

Sanson *et al*. 1976). High electrolyte concentration has no effect on the position of 5-DS in bilayers (Bartucci, Gulfo & Sportelli 1990).

Immobilization of the membrane interface with drying does not oppose the established opinion that membrane protection against desiccation includes a decrease in T_m (Crowe *et al*. 1992, 1996). Methods to determine T_m, such as DSC or FT-IR, mainly detect the average melting of acyl chains at considerably deeper levels in the bilayer than the level of sensing by 5-DS. Membranes are characterized by a gradient of mobility along the acyl chains, which decreases from the core to the surface, so that melting occurs at a much higher temperature at the membrane interface than in the core. Interactions of the headgroups of phospholipids with sugars during drying further decrease the mobility of the bilayer interface (Lee, Waugh & Griffin 1986; Tsvetkova *et al*. 1998). However, these interactions do not necessarily have to increase T_m as determined by DSC or FT-IR. On the contrary, such interactions may decrease T_m via an increase in the space between headgroups, which has been convincingly shown by Crowe *et al*. (1992, 1996).

Partial disordering of the membrane interface during dehydration of desiccation-tolerant embryos

Immobilization of the major part of the membrane lipid–water interface during embryo dehydration is one side of the story. Another important finding is the increase in proportion of a fluid component in 5-DS spectra during dehydration and its correlation with the acquisition of desiccation tolerance. The shape of the difference spectrum (Fig. 6b), which corresponds to so-called pseudoisotropic motion, is an indication of loosely packed regions in membranes. These regions might result from direct fluidization of the lipid–water interface. However, the fluid component could also be the result of a downshift of 5-DS molecules in the membrane so that the nitroxide moiety will experience another, more disordered environment. Indeed, the shape of the spectrum in Fig. 6b is nearly the same as that observed for the methyl ester derivative of 5-DS (5-MeSL) in the bilayer (see Bianconi, do Amaral & Schreier 1988; for an example). The neutral and less polar character of the 5-MeSL headgroup leads to a loss of strong anchoring to the interface as compared to 5-DS and therefore explains the deeper position of 5-MeSL in the bilayer (Sanson *et al*. 1976).

Acidification of the local environment can also downshift the position of anchoring of 5-DS molecules in the headgroup area and thus bring the nitroxide moiety to a less ordered environment (Barratt & Laggner 1974). However, the degree of disordering derived from the difference spectrum in Fig. 6b was much greater than could be expected from a pH decrease.

The presence of a fluid component in 5-DS spectra therefore does not necessarily mean that this fluid component originates from direct fluidization of the membrane interface. In our opinion, it is more probable that some changes at the interface other than those caused by pH cause the loss of strong anchoring of 5-DS in the headgroup region. This leads to vertical movement of the spin label downward into regions where the degree of motional freedom is higher. However, even if the fluid component in 5-DS spectra results from deeper anchoring of 5-DS molecules in the bilayer, this is nevertheless indicative of some disturbance at the membrane interface, which allows such a downshift to take place.

Air-drying of desiccation-tolerant, 21 daa embryos therefore has a dual effect on the membrane interface. Apart from the expected further ordering observed for the major part of the lipid interface, it also caused local disordering of some part of the membrane interface. The disordered areas appear at the onset of the acquisition of cellular desiccation tolerance and reach maximum when all embryonic cells survive desiccation at 18 daa (Fig. 8). Intercalation of small amphiphilic molecules between membrane lipids can create such a disorder in lipid packing. The hydrophobic moiety of these molecules would allow some penetration into the bilayer, whereas the hydrophilic moiety would anchor them at the membrane interface. Indeed, it has been shown that arbutin (a glycosylated hydroquinone) can partition with its hydrophobic moiety between the lipids, whereas the glucose moiety prevents deeper penetration into the bilayer (Oliver *et al*. 1998; Hincha, Oliver & Crowe 1999). This partitioning causes spacing between the polar lipids and, as a consequence, T_m is decreased and leakage increased. Thus, when the cellular volume decreases, partitioning of small amphiphiles from the cytoplasm into the region of lipids close to the membrane interface might be responsible for the partial disorder of the membrane interface rather than a fall in pH or increase in ion concentration.

Partitioning of endogenous amphiphiles into membranes during dehydration – previous studies

Although partitioning of amphiphiles into membranes has been extensively studied, this phenomenon has never been considered in relation to dehydrating systems until our first publications (Hoekstra *et al*. 1997; Golovina *et al*. 1998). Using the spin probe TEMPONE as a model amphiphilic small molecule we have shown its partitioning into the lipid phase during drying of *Typha latifolia* pollen. Although TEMPONE is a xenobiotic compound, its partitioning into the lipid phase implies that endogenous cellular amphiphiles might partition into the lipid phase, including cellular membranes, in a similar manner. Furthermore, an increase in membrane permeability with dehydration has been observed, which we have attributed to the partitioning of endogenous amphiphiles into membranes. This interpretation was based on the linear correlation between the amounts of TEMPONE that had partitioned into the lipid phase of pollen samples at different MCs and the membrane permeability upon rehydration at the respective MCs (Golovina *et al*. 1998; Hoekstra & Golovina 2002).

Later on, we obtained additional indication that endogenous amphiphiles partition into membranes during dehydration. Using 5-DS for the inspection of the membrane lipid–water interface, a mobile (fluid) phase has been noticed during dehydration in a number of plant organisms (Golovina & Hoekstra 2002).

Correlation between appearance of a fluid phase in the interface region and acquisition of desiccation tolerance

We showed that dehydration causes the appearance of a disturbed phase at the membrane interface in wheat embryos and that the appearance of this phase coincides with the acquisition of desiccation tolerance. The combination of data from Figs 3a and 8 reveals the relationship between the proportion of the fluid component in 5-DS spectra and the proportion of desiccation-tolerant embryonic cells (Fig. 11). The maximum proportion of fluid component of approximately 23% was attained when all embryonic cells had acquired desiccation tolerance (Fig. 8), which means that each desiccation-tolerant cell has approximately 23% of disturbed areas at the membrane interface. There is a linear correlation between the proportion of the fluid component and the proportion of viable cells above 10%. The lack of linearity below 10% viable cells can be explained by the gradual accumulation of fluidizing amphiphiles before any cell acquires desiccation tolerance.

Indeed, the acquisition of cellular desiccation tolerance during embryo development was not homogeneous. Not only was the proportion of desiccation-tolerant cells different at the different stages of development (Fig. 3a), but, in addition the desiccation-sensitive cells had different critical MCs to which they could be dehydrated without loss of viability (Fig. 3b). This finding is in agreement with previously published data on the gradual decrease of the critical MC during development of soybean seeds (Sun & Leopold 1993). The relationship in Fig. 11 allows us to assume that at the cellular level the increase in the proportion of fluidizing amphiphiles is a gradual process, and that those cells that reach the 23% level of interfacial loosening can withstand air-drying. An intermediate level of fluidizing amphiphiles can then be referred to as the drought-tolerant stage.

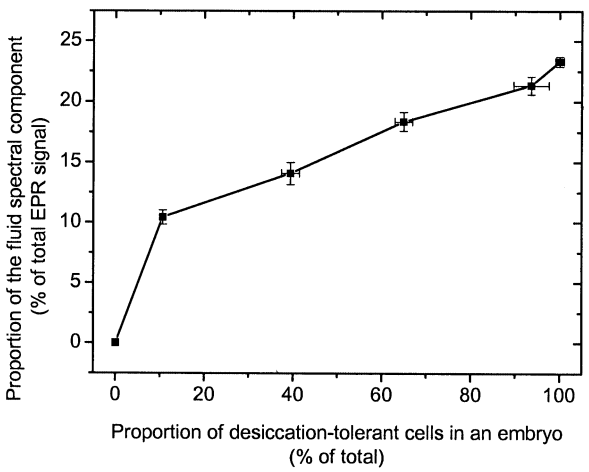


Figure 11. Correlation between the proportion of the fluid component in 5-DS spectra from desiccated wheat embryos (data from Fig. 8) and the proportion of desiccation-tolerant cells in such embryos during development (calculated from Fig. 3a, with R = 0.94 for 100% of viable cells). All the data concerning the desiccation-tolerant embryos in Fig. 8 (21 daa and older) were averaged and plotted as one point.

However, correlation does not mean causal relationship. We therefore forward three propositions that put in perspective the relevance of membrane fluidization for desiccation tolerance.

1 There is no causal relationship. The increase in the proportion of fluid phase in membranes of desiccated embryos (Fig. 8) just coincides with the beginning of embryo DW accumulation (Fig. 1a). This accumulation might involve low molecular weight metabolites that later will be converted into storage biopolymers, but without a specific protective function. Some of these metabolites might have amphiphilic properties, for example some aromatic amino acids (Popova, Heyer & Hincha 2002), and inevitably partition into membranes during premature drying. This partitioning therefore might not represent a desiccation-tolerance mechanism. Nevertheless, even if partitioning does not relate to protective mechanisms, the embryonic cells are able to cope with the disturbance of the lipids at the membrane interface, which in it self can be considered an indication of the presence of a protective mechanism.
2 Partitioning of amphiphiles does have a causal relationship with the acquisition of desiccation tolerance. It might be that during the transition to desiccation tolerance, specific amphiphiles with a special protective role such as lipid-soluble antioxidants are synthesized and automatically inserted into membranes during dehydration. Desiccation-intolerant embryos lack these antioxidants, so that their membranes are not well protected against oxidative damage.
3 Amphiphiles that appear in cells during the acquisition of desiccation tolerance are non-specific and do not have antioxidative properties. The main benefit of their partitioning might be a partial disturbance of the membrane interface, which provides elasticity to membranes, thus alleviating mechanical stress during dehydration/rehydration-induced folding/unfolding of membranes (Hoekstra, Golovina & Nijsse 2003).

We do not know what cytoplasmic amphiphiles might relate to desiccation tolerance. The general requirements of such compounds are that they are relatively small, having sufficient hydrophilicity to reside in the cytoplasm in the hydrated condition, and have antioxidant properties. Arbutin might be one such compound. This product of secondary metabolism occurs at high concentrations in certain resurrection plants (Suau *et al*. 1991; Bianchi *et al*. 1993) and has been shown to be an antioxidant (Ioku, Terao &

Nakatani 1992). It can intercalate into membranes and stay near the lipid interface, thus increasing spacing between the headgroups (Oliver *et al*. 1996, 1998; Hincha *et al*. 1999). Arbutin decreases T_m of dry vesicles (Oliver *et al*. 1996, 1998) and prevents the formation of non-bilayer structures (Hincha *et al*. 1999; Oliver *et al*. 2001). Previously, we considered in this respect the possible role of quercetin and rutin (Hoekstra & Golovina 2000). However, these compounds cause leakage and might therefore be harmful if cells would not have other protective mechanisms.

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