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Cold-induced imbibition damage of lettuce embryos: a study using cryo-scanning electron microscopy

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Abstract

The impact of rehydration on a multicellular organism was studied in lettuce (*Lactuca sativa* L.) embryos, using cryo-scanning electron microscopy (cryo-SEM). Naked embryos were sensitive to imbibitional stress, whereas embryos with an intact, thick-walled endosperm were not. Imbibitional injury to naked embryos was mainly confined to the outer 2–3 cell layers of the axis. These cells failed to swell and appeared poorly hydrated. Deeper layers were not affected even after extended periods of cold rehydration. The proportion of damaged cells (6–7% of total) roughly corresponded with the additional K^+ that gradually leached from the embryos. Damaged embryos were able to survive the loss of their surface layers and form adventitious roots. The swelling of inner tissues caused the dead surface layers to rupture into patches. Plasma membranes in dried embryos showed normal bilayer structure with a homogeneous distribution of intra-membrane particles (IMPs), also after non-injurious rehydration. Imbibitionally damaged plasma membranes showed many irregularities, such as globular insertions, that probably resulted from malfusions in the ruptured membrane, but the IMPs were still randomly distributed.

Keywords: cryo-planing, desiccation tolerance, imbibitional injury, K^+-leakage, *Lactuca sativa*, seed ultrastructure

Introduction

Organisms that are endowed with the ability to survive periods in the absence of water are called anhydrobiotes (Crowe *et al*., 1992). They can be found among fungi, algae, mosses and ferns, seeds, pollen, and even some whole angiosperm plants, as well as some invertebrates (Alpert, 2000). Desiccation tolerance permits the survival during extended episodes of low water availability at considerably restricted metabolic activity. In addition, desiccation tolerance facilitates the safe dispersal of propagules, such as spores (Hoekstra, 2002) and seeds (Leprince *et al*., 1993). Survival in the dry state requires that the organism copes successfully with three successive events: loss of water, some period in the dry state and rehydration. Desiccation tolerance requires the structural, chemical and physical stabilization of an organism, which enables it to endure these events. Dehydration often occurs over hours or days, during which there may be adaptive metabolism and gene expression (Ingram and Bartels, 1996), whereas rehydration is usually much faster. Algae and other sessile organisms in tidal zones exemplify this. When the seawater recedes, the shore dries gradually, but at high tide the organisms are rehydrated quickly. Also, moss clumps lose water slowly and are quickly rehydrated by rainfall. Slow desiccation occurs during seed maturation, whereas rehydration in the soil can be fast during rainfall or flooding. This paper investigates damage that arises during the transition from the dry to the hydrated state, which is known as imbibition(al) injury or imbibition(al) damage.

The hydrophobic effect, existing in aqueous surroundings, determines the conformation of membranes and proteins (reviewed in Hoekstra *et al*., 2001). Upon drying, the hydrophobic effect ceases, but the dried cells, nevertheless, maintain their cellular organization. Membranes and cell contents then form a highly viscous or glassy matrix, in which water as the driving force for structural order is replaced by other compounds, such as non-reducing di- and oligosaccharides and specific proteins, e.g. late embryogenic abundant (LEA) and heat-shock proteins (Hoekstra *et al*., 2001). On rehydration, this

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glassy matrix is fluidized, and hydrophobic interactions again prevail. Desiccation tolerance includes the resumption of normal metabolism after rehydration; failure to do so in otherwise anhydrobiotic organisms is often related to the loss of membrane integrity. In a physiological sense, imbibitional injury may be defined as an unsuccessful transformation from the chemically and structurally intact, metabolically inactive, dry state to the metabolically active hydrated state.

The best-studied symptom of imbibitional injury is the persistent leakage of cellular solutes, which points to reduced integrity of the plasma membrane. Several conditions may induce membrane injury during imbibition, such as low temperature and low initial moisture content (MC) of the organism. Imbibitional injury has been attributed to a membrane phase change from the gel phase to the liquid crystalline phase during water uptake (Crowe *et al.*, 1989; Hoekstra *et al.*, 1992a). Defects at the boundary between gel phase domains and the liquid crystalline phase are held responsible for the increased permeability. In addition, when membranes are in the dry state, their rigidity is much higher than when they are in the hydrated state (reviewed in Hoekstra and Golovina, 1999). The initially dry and rigid plasma membranes are exposed to considerable forces upon fast rehydration. As a result, plasma membranes may rupture and become permanently leaky (Hoekstra *et al.*, 1999). Low temperature and/or a low MC promote gel phase formation and increase rigidity, thereby increasing the likelihood of imbibitional injury (Crowe *et al.*, 1989). Membrane composition and vicinal solutes are additional factors involved in the phase behaviour of plasma membranes (Hoekstra *et al.*, 1992b, 2001; Hoekstra and Golovina, 1999; Bryant *et al.*, 2001). If a dried organism is preheated or prehumidified in moist air, imbibitional damage can be largely prevented (Hoekstra and Van der Wal, 1988), which has been attributed to the effects of these treatments on membrane phase behaviour (Crowe *et al.*, 1989).

Permanent gross leakage, which may result from imbibitional stress, inevitably leads to cell death in unicellular organisms. In multicellular organisms, however, death of one or more of their cells or cell layers might be overcome. The aim of the present work is to better characterize the phenomenon of imbibitional injury in multicellular organisms, such as a seed. Cryo-scanning electron microscopy (cryo-SEM) was used to study cell contents and membrane surfaces in lettuce seeds in the dry state and after different imbibition treatments. Intact lettuce seeds are not normally sensitive to imbibitional stress (Ellis *et al.*, 1995), but isolated embryos that are devoid of an endosperm layer appear to be highly sensitive.

Materials and methods

Seed material

Lettuce seeds (*Lactuca sativa* L. cv. 'Little Gem', selection 'Ferro') were chosen for this study because of their contrasting reactions to different imbibition treatments, their simple anatomy, and the availability of large, homogeneous seed batches. Seeds of other species that are inherently sensitive to imbibitional stress often show a more heterogeneous response to the stress. Mature high-grade lettuce seeds (approximately 100% germination) were provided by Nunhems Zaden BV (Haelen, The Netherlands). The seed, which is an achene, has three distinguishable layers that surround the embryo: an outermost layer, or pericarp; a thin median layer or integument; and an inner layer, the endosperm (Nijsse *et al.*, 1998). Of these surrounding layers, only the endosperm is alive. The endosperm consists of two cell layers and tightly surrounds the embryo. The endosperm cells have thick walls, particularly to the outside. The length of the seeds used ranged between 4 and 4.5 mm and the width was approximately 1 mm.

Embryo isolation

Seeds were allowed to imbibe for 2–3 h at 20°C between two layers of wet filter paper. Then embryos were isolated from the endosperm and pericarp by gently pressing the seed at the cotyledon end. Alternatively, embryos with an intact endosperm were excised from the pericarp with a forceps. The embryos thus isolated were re-dried in air of approximately 30% relative humidity and reached an MC of 0.040 ± 0.001 g H_2O (g DW)$^{-1}$.

Imbibition and treatments

Imbibition treatments differed in the following aspects:

- embryos were naked or covered with endosperm;
- imbibition temperature was 0°C (ice water) or 20°C;
- embryos were prehumidified from the vapour phase or not.

Prehumidification was accomplished by placing dried embryos for 24 h in an open Petri dish that was placed on a grid in a closed, water-containing box at 22°C. To ensure standardized imbibition, tap water was added to a vial containing the dried or prehumidified embryos, and the vial was gently shaken to ensure fast and complete wetting. After 1 h of imbibition, the embryos of all treatments were incubated at 20°C on filter paper that was wetted by a wick in ample tap water. Two days after the onset of

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imbibition, all embryos were photographed and analysed for seedling abnormalities, and cotyledon and axis damage. All visual aberrations from normal seedling development were regarded as damage, whereas only extensive damage (causing malformations of the seedling) was considered as seedling abnormality. These macroscopic observations were made on at least 50 seeds per treatment.

Cryo-SEM

Embryos were placed in a drop of Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) on top of small rivets (4-mm long aluminium pins with a head) and immediately plunge-frozen in liquid propane. These rivets fitted into sample holders for fracturing, cryo-planing and SEM investigation of the specimens. The embryos were cryo-planed (reviewed by Nijsse and Van Aelst, 1999) to examine cell contents, or cryo-fractured to inspect membrane surfaces. For cryo-planing, the frozen samples were sectioned with a glass knife in a cryo-ultramicrotome (Reichert-Jung Ultracut E/FC4D, Vienna, Austria) to the desired plane through the material. The last sections were cut using a diamond knife at decreasing section thicknesses from 0.5 µm to 20 nm, and at decreasing sectioning speeds down to 0.2 mm s^{-1}. During planing, the sample temperature was –90°C and the knife temperature –100°C. The samples were stored in liquid nitrogen and later cryo-transferred to a cryo-SEM (JEOL 6300F Field Emission SEM, Tokyo, Japan) equipped with an Oxford 1500 HF cryo-system (Eynsham, UK). The planed samples were freeze-etched for 3 min at –89°C to enhance contrast and remove water-vapour contamination, sputter-coated with platinum, and subsequently analysed at –190°C with an accelerating voltage of 5 kV. To examine the hypocotyl surface of embryos, the same procedures were followed, but without cryo-planing. Cryo-fractures were made using a scalpel blade at approximately –150°C. After sputter-coating with platinum, the fractured samples were analysed in the JEOL cryo-SEM.

To prepare samples for high-resolution microscopy of membrane surfaces, the hypocotyl region of the embryo was excised, rapidly placed between two aluminium cups in a droplet of hexadecene and fixed by high-pressure freezing (Moor and Riehle, 1968; Müller and Moor, 1984) without any chemical pre-treatment. An EM HPM high-pressure freezer, as described by Studer *et al.* (1995), was used (manufactured and distributed by Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland). The samples were fractured in a BAF 300 freeze-etching device (Bal-Tec, Liechtenstein) by detaching one of the aluminium holders, and immediately double-layer coated with 3 nm platinum at an angle of 45° and with 5–10 nm carbon at 90° (Walther *et al.*, 1995). These samples were analysed at –125°C and 10 kV with an in-lens field emission SEM Hitachi S-5200 (Hitachi, Tokyo, Japan), equipped with a Gatan cryo-stage 626 (Gatan Inc., Pleasanton, California, USA). Imaging was performed by collecting the backscattered electron signal (Walther and Hentschel, 1989). All cryo-SEM analyses were performed at least twice.

K^+-leakage measurements

Potassium leakage was measured on naked embryos imbibed at 0°C under damaging conditions (imbibition of initially dry embryos) and non-damaging conditions (imbibition after overnight prehumidification). Four replicates of ten embryos were rehydrated in 10.5 ml demineralized water at 0°C, and 1.5 ml aliquots were taken during the next 200 min. After 60 min the temperature was raised to 20°C as in the other experiments. The concentration of K^+ in these aliquots was determined by flame photometry (Jenway PFP7, Felsted, UK). K^+ leakage was calculated as nmol K^+ (mg embryo DW)$^{-1}$ and as percentage of the total amount of K^+ present in the sample. Total K^+ content (mg embryo DW)$^{-1}$ was measured after overnight incubation of embryos of the same batch in 0.02 N HCl at 20°C.

Volumetric calculations

An attempt was made to calculate the amount of damaged tissue as a percentage of the entire embryo. On the basis of measurements on dry embryos, the average embryo dimensions were estimated to be $3.5 \times 1.0 \times 0.5$ mm. The surface of the hypocotyl was estimated as one-third of the total surface, in which the adaxial surface of the cotyledons (lying appressed together) was not included. The embryonic volume was calculated assuming an ellipsoid shape, as follows: volume = $3.5 \times 1.0 \times 0.5 \times \pi/6 = 0.92$ mm^3. Damage to the two outer cell layers of the hypocotyl was a typical result of imbibitional stress (see below). These two layers had a total thickness of approximately 33 µm. The volume of the embryo without the volume of the two outer cell layers was calculated with the given ellipsoid volume formula: volume = $(3.5 - 0.066) \times (1.0 - 0.066) \times (0.5 - 0.066) \times \pi/6 = 0.73$ mm^3. Since it was assumed that the hypocotyl area comprised one-third of the total surface area, it was estimated that the outer two cell layers of the hypocotyl were $(0.92 - 0.73)/3 = 0.063$ mm^3, which is approximately 6–7% of the total volume.

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Results and discussion

Macroscopic observations during imbibition

In lettuce seeds, the presence of an intact endosperm kept the whole embryo free of imbibitional injury, even during imbibition at 0°C. Naked embryos suffered most from the 0°C imbibition (Fig. 1); almost all of them had a brown hypocotyl surface and reduced root hair development. When imbibed in water at 20°C, or prehydrated from the vapour phase (0 and 20°C), naked embryos generally developed normally. There was some incidental damage, mainly to the cotyledons, but this might be ascribed to the isolation procedure rather than to imbibitional stress (Figs 1 and 2).

Seedlings from the embryos imbibed at 0°C survived for at least 14 d. Apparently they were able to overcome the surface injury, but had retarded and less homogeneous growth. The average root length of 6-d-old seedlings was significantly less for the 0°C-imbibed, naked embryos than for all other treatments (Table 1). Figure 3 shows a 6-d-old seedling with heavy surface damage after imbibition of the naked embryo at 0°C. The hypocotyl surface has ruptured into patches, apparently as a result of swelling of the internal tissues. Two adventitious roots have grown out of the internal tissues to replace the damaged primary root.

Embryos that were surrounded by an endosperm and immersed at 0°C showed radicle protrusion within 1 d and developed into normal seedlings. The endosperm obviously slowed the first water entrance to an extent that the 0°C imbibition did not constitute a stress. That surrounding layers protect seeds against imbibitional injury has been found for other species (Larson, 1968; Powell and Matthews, 1978). Thickness and pigmentation of the seed coat in proso millet, for example, are positively related with resistance to imbibitional stress (Khan *et al*., 1996). In practice, sensitive seeds can be protected against imbibitional damage successfully by an artificial seed coat (Priestley and Leopold, 1986; Hwang and Sung, 1991). Duke and Kakefuda (1981) found that the cause of imbibitional injury in seemingly intact soybean seeds often can be traced back to cracks in the testa.

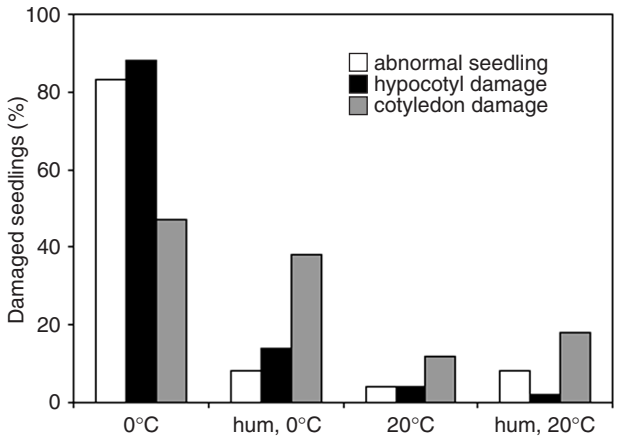

Figure 1. Visual assessment of the damage brought about by different imbibition treatments in 2-d-old lettuce seedlings ($n \geqslant 50$). Naked embryos were immersed in water at 0°C or 20°C for 1 h, followed by further incubation on wet filter paper at 20°C; prior to imbibition the embryos were either dry [MC = 0.040 ± 0.001 g H_2O (g DW)$^{-1}$] or prehumidified overnight (hum) in moist air [MC = 0.26 ± 0.01 g H_2O (g DW)$^{-1}$]. All visual aberrations from normal seedling development were regarded as damage, whereas only extensive damage leading to malformations of the seedling was considered as seedling abnormality. All the embryos with an intact endosperm (imbibition at 0°C) were free of injury (data not shown).

Cryo-SEM

The use of high-resolution cryo-SEM to analyse freeze-fractured surfaces has been developed by Walther and Müller (1997), and has several advantages over the freeze-fracture replica technique.

Table 1. Effect of different imbibition treatments on the average root length of lettuce seedlings (n = 50) after 6 d of incubation. Embryos were allowed to imbibe for 1 h at either 0°C or 20°C, followed by further incubation on wet filter paper at 20°C; prior to imbibition they were either dry [MC = 0.040 ± 0.001 g H_2O (g DW)$^{-1}$] or prehumidified overnight (hum) in moist air [MC = 0.26 ± 0.01 g H_2O (g DW)$^{-1}$]. If a seedling had more than one root, the lengths were summed. Root length values followed by a different letter are significantly different at $P < 0.001$ (Student's t-test). CV (coefficient of variation) = SD, expressed as a percentage of the average root length.

Imbibition treatment		Average root length (mm) at day 6	CV (SD%)
With intact endosperm	0°C	31.3a	26
Naked embryo	0°C	21.7b	49
Naked embryo	hum, 0°C	30.0a	27
Naked embryo	20°C	34.1a	25
Naked embryo	hum, 20°C	35.2a	36

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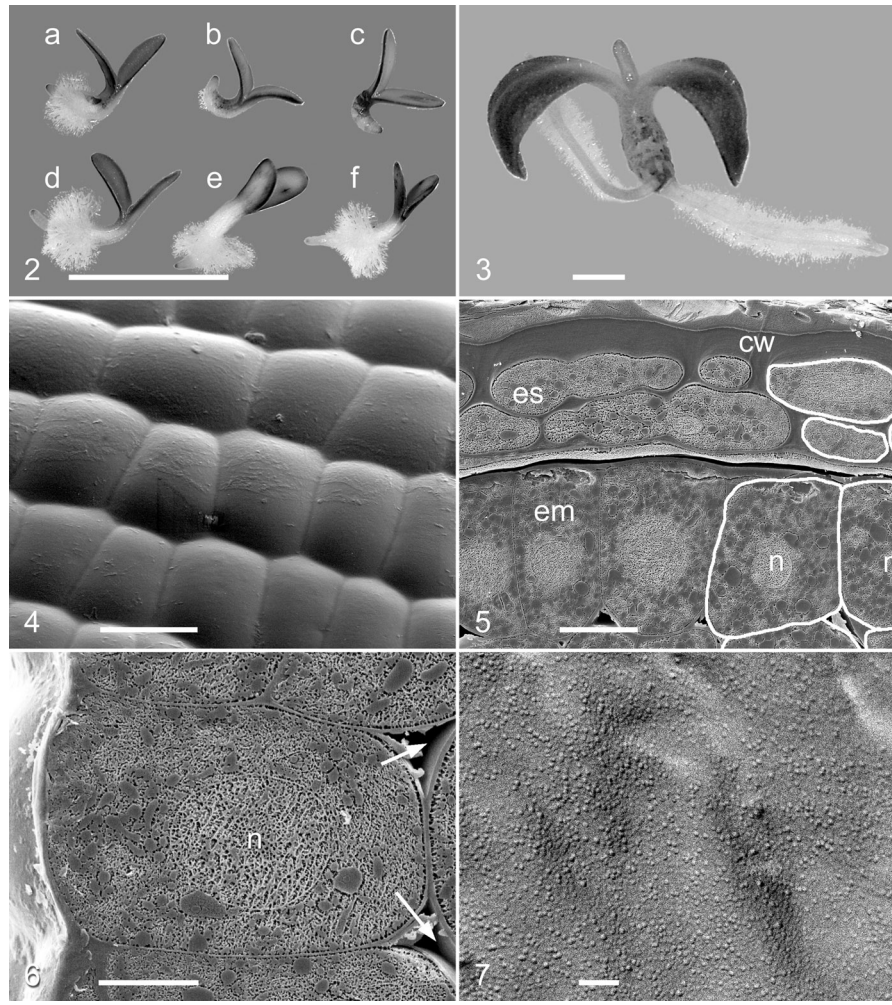

Figure 2. Lettuce seedlings 2 d after imbibition of dry naked embryos. (a–c) Immersed in water at 0°C during the first hour of incubation; (d–f) immersed in water at 20°C during the first hour of incubation. Further incubation was on wet filter paper at 20°C. (a) Normal seedling with minor hypocotyl damage; (b) and (c) abnormal seedlings with considerable hypocotyl damage; (d) normal seedling; (e) and (f) normal seedlings with minor cotyledon damage. Bar = 5 mm.

Figure 3. A lettuce seedling 6 d after the onset of imbibition (for 1 h) of a naked embryo in water at 0°C; further incubation was on wet filter paper at 20°C. Two adventitious roots have emerged, which have replaced the damaged primary root. The hypocotyl surface has broken up into patches. Bar = 1 mm.

Figure 4. Cryo-SEM image of the hypocotyl surface of an imbibed lettuce embryo without imbibitional injury. The epidermal cells have a turgid appearance. Treatment: naked embryo, overnight prehumidification followed by 1 h of immersion in water at 20°C. Bar = 10 μm.

Figure 5. A transversally cryo-planed hypocotyl of a lettuce embryo (em) with an intact endosperm (es) and no imbibitional injury. The endosperm has a very thick peripheral cell wall (cw). The cells of the embryo are turgid and their nuclei (n) are clearly visible. White lines mark the outline of individual cells. Treatment: 1 h immersion in water at 0°C followed by 4 h incubation on wet filter paper at 20°C. Bar = 10 μm.

Figure 6. Epidermal hypocotyl cell of a transversally cryo-planed naked lettuce embryo without imbibitional injury. Cells have large intercellular spaces (arrows). n, Nucleus. Treatment: 1 h immersion in water at 20°C followed by 4 h of incubation on wet filter paper at 20°C. Bar = 5 μm.

Figure 7. High-resolution cryo-fracture image of the plasma membrane of a rehydrated non-damaged cell situated five cell layers below the epidermis in a lettuce hypocotyl. The numerous dots are intra-membrane particles (IMPs). Treatment: naked embryo, imbibed for 1.5 h in water at 0°C. Bar = 100 nm.

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Using cryo-SEM, the replica does not need to be detached from the sample, which reduces uncontrolled loss of samples and improves the ability to localize the orientation of a specific area in the specimen. Moreover, it is difficult to acquire replicas from dry tissue because of the tension of the swelling material excised on the replica during the washing steps, which causes the replicas to rupture into many pieces (Platt *et al*., 1994). Use of the cryo-SEM method to examine dry and hydrated membrane surfaces holds promise in the study of desiccation-tolerance mechanisms.

Because the cryo-SEM images of dried embryos, and of embryos that were damaged upon rehydration, were somewhat difficult to interpret, images of intact, imbibed embryos are presented first. All images show the hypocotyl region of embryos. The epidermal cells at the hypocotyl surface of a prehumidified, 20°C-imbibed embryo have an ordered and turgid appearance (Fig. 4). Figure 5 shows a cross-section of the hypocotyl region of an embryo with intact endosperm. All cells look turgid, and organelles, including the nuclei, are clearly visible. Note the very thick outer cell wall of the endosperm. The cells of the embryo have thin walls and a rectangular shape in both the transverse and the longitudinal planes. They are ordered mainly in longitudinal files. Along the ribs of these files, intercellular spaces are often observed (Fig. 6). The plasma membranes of hydrated, non-damaged cells show a spaced, random distribution of intra-membrane particles (IMPs; Fig. 7).

Dried, naked embryos look shrunken and have a much less regular appearance than the hydrated embryos. The hypocotyl surface is irregularly folded (Fig. 8). Transverse (Fig. 9) and longitudinal fractures (Fig. 10) reveal that the cell walls are highly folded and that the intercellular spaces are as prominent as in the intact rehydrated embryos. These intercellular spaces can extend over at least 1 mm. At several places interconnections between these longitudinally arranged intercellular spaces were observed (image not shown). Wall curvature is mainly oriented in such a way that the cells are able to swell in the radial direction. Plasmodesmata are much more prominent in the transverse walls than in the longitudinal walls. Although heavily folded, the plasma membranes stay intact in the dry state, as depicted in Fig. 11. Membranes also remain structurally intact in dry cowpea seeds (Thomson and Platt-Aloia, 1982), and this is undoubtedly the case for all desiccation-tolerant organisms. No structural differences in the dry state were found after wetting and redesiccation of the seeds or embryos.

Figure 12 shows a transverse fracture through a naked embryo that suffered imbibitional injury. The outer two or three cell layers are damaged and have heavily folded walls. Deeper-located cell layers have an intact, turgid appearance, which suggests that the rate of water entrance into the deeper cell layers was moderated. The outer, damaged cells appear to have a lower water content than the non-damaged cells in the interior of the embryo, as can be concluded from their dense appearance and poor ice crystallization pattern (Figs 13 and 14). One hour after rehydration, the embryos were fully turgid, and if the embryo was incubated in water at 0°C for longer than 1 h, still no more than two or three cell layers of the hypocotyl region were damaged (data not shown). The third cell layer in Fig. 13 shows multiple small vacuoles, which are not present in other intact cell layers. This abnormal vacuolization may depict an intermediate between the heavily damaged outer two layers and the intact deeper layers. The outside of the embryo has an overall turgid appearance, but the epidermal cells make longitudinally curved imprints (Fig. 15), which is in contrast to the bulging shape of the epidermal cells in the absence of imbibitional damage (Fig. 4). The plasma membranes of damaged cells have a very irregular surface, not only because of the folds that are present, but also because of indentations and the possible insertion of globules (Fig. 16). Some inclusions have a considerable size (0.3 µm) and might be malfused organelles. A high magnification (Fig. 17) of the damaged membrane shows undefined irregularities, but for the rest, the IMPs are randomly distributed. According to their size, the irregularities seen in this figure might also represent damaged plasmodesmata. These observations lead us to hypothesize that, upon adverse imbibition, the membrane often ruptures and that sometimes cellular contents (e.g. organelles, as suggested by Hoekstra *et al*., 1999) fuse into the membrane before it closes. If such inclusions have amphiphilic properties, they might contribute to the formation of stable holes that render cells permanently leaky, eventually leading to cell death. More work is needed to reveal the origin and structure of these putative leakage sites in the plasma membrane.

K^+ leakage

Dry embryos that were immersed in water at 0°C leaked more K^+ than overnight-prehumidified and further equally treated embryos (Fig. 18). The leakage pattern differed significantly between the two treatments, except at 10 min (*t*-test, $P < 0.05$). After a 200-min incubation, the still slowly increasing leakage difference between the two treatments was 4.7% of the total K^+ content of the embryo [total = 185 nmol K^+ (mg embryo DW)$^{-1}$]. This roughly corresponded with the visual observation that mainly the outer two cell layers of the axis were damaged (Figs 12 and 13), and with the calculated volume of

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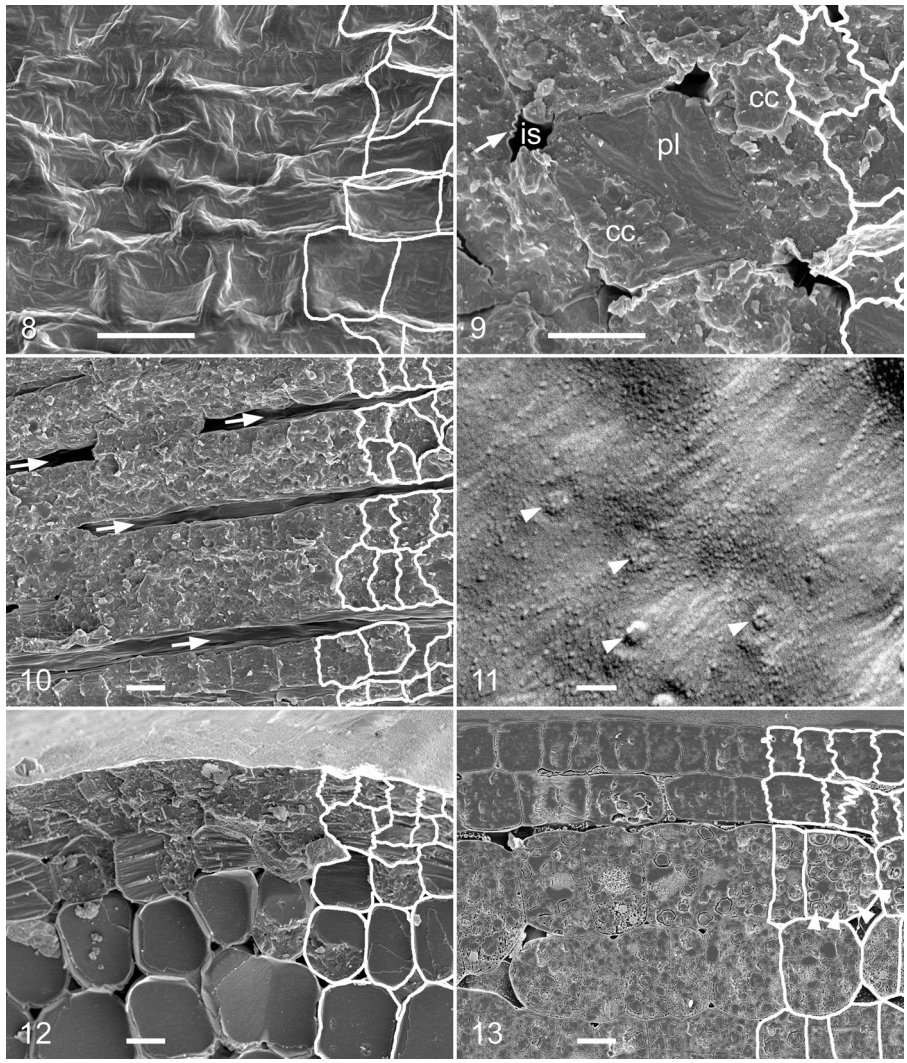

Figure 8. The hypocotyl surface of a naked, dried lettuce embryo. The surface has a shrunken appearance, and the epidermal cells are visible as depressions. White lines mark the outline of individual cells. Bar = 10 μm.

Figure 9. Transversally cryo-fractured hypocotyl cortex cells of a dried lettuce embryo. White lines mark the outline of individual cells. cc, Cellular contents; is, intercellular space; pl, face-fractured plasma membrane; arrow, cross-fractured cell wall bordering the intercellular space. Bar = 10 μm.

Figure 10. Longitudinally cryo-fractured hypocotyl cortex cells of a dried lettuce embryo. Intercellular spaces (arrows) extend over long distances along many cells. White lines mark the outline of individual cells. Bar = 10 μm.

Figure 11. High-resolution cryo-fracture image of the plasma membrane along a transverse wall of a cortex cell in the hypocotyl of a dried lettuce embryo. Line patterns, mainly oriented from the lower left to the upper right, are imprints of the cell wall fibrils, which are typically visible in the dry state. Arrowheads, plasmodesmata. Bar = 100 nm.

Figure 12. Transverse cryo-fracture through the hypocotyl of a naked lettuce embryo suffering from imbibitional injury. The outer two or three cell layers (top) have heavily folded walls, while the deeper-lying cells have a normal, turgid appearance. White lines mark the outline of individual cells. Treatment: immersion for 1 h in water at 0°C. Bar = 10 μm.

Figure 13. A damaged, longitudinally cryo-planed lettuce hypocotyl. The outer two layers (top) have a dense appearance and lack the swelling and turgescence of the deeper layers. The third layer has cells with multiple small vacuoles (arrowheads) that are not found in deeper cell layers. White lines mark the outline of individual cells. Treatment: naked embryo, 1.5 h immersion in water at 0°C, followed by 4.5 h of incubation on wet filter paper at 20°C. Bar = 10 μm.

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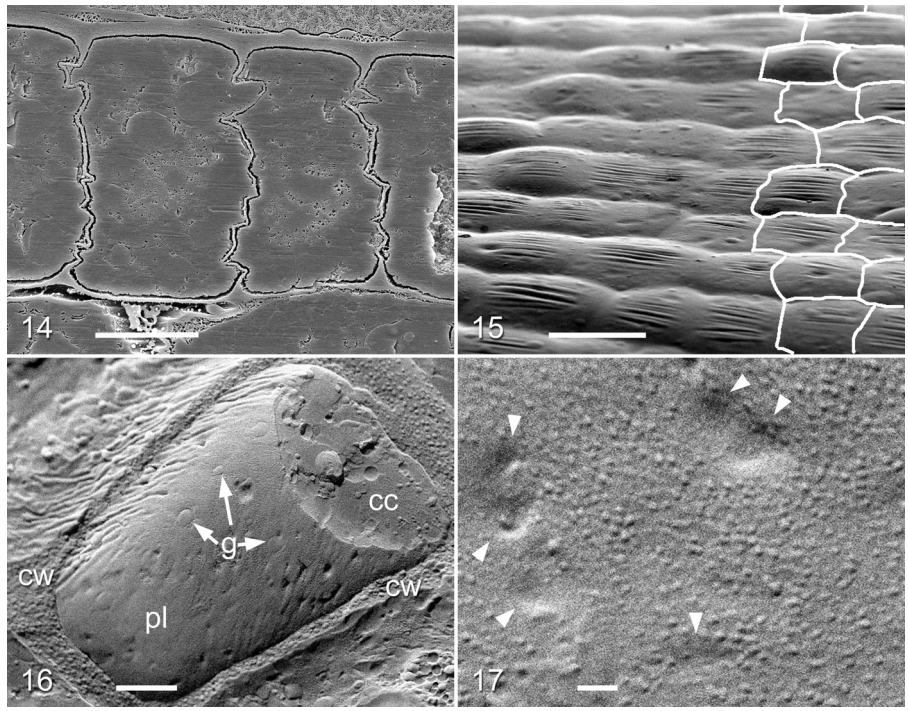

Figure 14. Detail of the epidermal cells shown in Fig. 13. Cell contents show a poor ice crystallization, which points to a low water content. Organelles are difficult to discern and seem to be disorganized. The transverse cell walls are highly folded. Bar = 5 μm.

Figure 15. The hypocotyl surface of an imbibed naked lettuce embryo suffering from imbibitional injury. The damaged epidermal cells cause longitudinally curved imprints. White lines mark the outline of individual cells. Treatment: immersion for 1 h in water at 0°C. Bar = 10 μm.

Figure 16. Cryo-fractured epidermal hypocotyl cell of a naked lettuce embryo suffering from imbibitional injury. The plasma membrane has a folded and irregular appearance, with globular insertions (g). pl, Plasma membrane; cc, cellular contents; cw, cell wall. Treatment: immersion for 1.5 h in water at 0°C. Bar = 1 μm.

Figure 17. High-resolution cryo-fracture image of the plasma membrane of a damaged epidermal cell of a lettuce hypocotyl. The intra-membrane particles (IMPs) show the usual distribution, except for local irregularities (arrowheads). Treatment: as in Fig. 16. Bar = 100 nm.

these two layers being approximately 6–7% of the total. Although the outer cells had damaged plasma membranes (Figs 16 and 17), the cell walls seem to have slowed the release of K^+ from the cells.

Occurrence of imbibitional injury in multicellular organisms

Cell walls of lettuce embryos (this paper) and other seeds (Webb and Arnott, 1982) are highly curved in the dry state, and their rehydration requires coordinated cellular imbibition and cell-wall unfolding. If one cell rehydrates faster than its neighbours, this imposes a forced stretching of still highly viscous membranes, walls and cytoplasm, resulting in substantial tension. To avoid cell-to-cell friction, imbibition of the embryo should occur sufficiently slowly to accommodate a homogeneous swelling. A thick-walled surrounding layer, such as the endosperm in lettuce seeds, can act as a barrier for too rapid a water influx. Furthermore, a network of intercellular spaces permits water vapour to diffuse rapidly over large areas, thus allowing a homogeneous and mild hydration from the vapour phase. These intercellular spaces may also permit cells to swell at slightly different rates without friction.

Imbibitional injury occurs primarily at the outside of a multicellular organism, because the peripheral cell layers prevent too rapid a water influx into the inner layers (Simon and Raja Harun, 1972; Powell and Matthews, 1978; this paper). To understand the differing results in the literature on imbibitional injury, one has to consider membrane rupture *versus* cell wall/tissue rupture as physically different phenomena. Membrane injury has been studied

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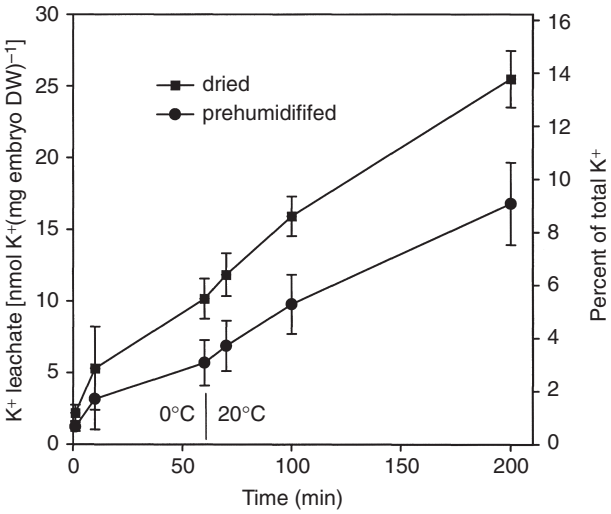

Figure 18. Effect of initial moisture content (MC) on K^+ leakage from naked lettuce embryos. The embryos used were either dry [MC = 0.040 ± 0.001 g H_2O (g DW)$^{-1}$] or prehumidified overnight [MC = 0.26 ± 0.01 g H_2O (g DW)$^{-1}$]; they were immersed for 1 h in water at 0°C, after which the temperature was raised to 20°C. Each point is the average of three or four replicates of 10 embryos ± SD.

extensively in unicellular organisms, and is mainly dependent on imbibition temperature and the fluidity and composition of the membranes (e.g. Hoekstra *et al.*, 1999). The damage noted in the present experiments on lettuce embryos is of the membrane type, since it was dependent on temperature, and, in the first hour of incubation, rupture of cell walls never occurred.

Alternatively, cell-wall injury and tissue rupture are the result of non-homogeneous swelling of tissues, leading to tension cracks within the cell walls. This second type of imbibitional damage occurred in the experiments of Duke and Kakefuda (1981), on soybean and bean cotyledons, and of Spaeth (1987), on bean and pea cotyledons. Both papers report on the immediate leakage of particulate matter, which is evidence for ruptures in the cell walls. Stelar lesions, which occur during rapid hydration of embryos having low moisture contents, have been found in maize kernels (Cohn and Obendorf, 1978) and isolated soybean embryos (Ashworth and Obendorf, 1980). In maize the stelar lesions were observed in cold-imbibed kernels, but not in warm-imbibed ones. However, in soybean, both cold- and warm-imbibed embryos showed lesions. Tissue rupture as caused by imbibition is clearly increased at low initial moisture content, but temperature dependency appears to vary with different samples.

The functional loss of (parts of) the epidermis and successive cell layers causes the deeper cell layers to be exposed to several secondary stresses, such as drought and microbial attack. The leakage of nutrients from the damaged cells into the surroundings promotes microbial growth. In imbibitionally damaged lettuce embryos, the swelling inner tissues led to a strain that caused the outer, dead cell layers to break up into patches. Under the given experimental conditions, the embryos appeared to be able to activate deeper cell layers to replace the lost functions of the epidermis, which led to delayed and less homogeneous growth. Whether or not an organism can recover from imbibitional injury depends on the extent of the damage, on whether the lost functions can be replaced, on the vigour of the organism and on environmental conditions.

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