Use of Cryogenically Prepared Samples in the Scanning Electron Microscopic Study of Dry-to-Wet Transitions

John N. A. Lott
McMaster University

Patrice Kerr
McMaster University

Utah State University
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USE OF CRYOGENICALLY PREPARED SAMPLES IN THE SCANNING ELECTRON MICROSCOPIC STUDY OF
DRY-TO-WET TRANSITIONS

John N.A. Lott* and Patrice Kerr

Department of Biology, McMaster University,
Hamilton, Ontario, Canada

Abstract

Cryogenic preparation has been used to study changes that occur as a dry specimen undergoes hydration. Conventional techniques such as critical point drying or anhydrous fixation are unsuitable for studying such changes. Seed tissue, a dry moss and various foods were chosen to show dry-to-wet transitions that take place after wetting of the dry sample begins. Cryogenically prepared specimens have great potential, not only in the study of changes which occur during imbibition of one sample, but also in the study of samples with different moisture content.

*Address for correspondence:
Department of Biology,
McMaster University, 1280 Main Street West,
Hamilton, Ontario, Canada, L8S 4K1.
                    Phone No: (416) 525-9140

Introduction

Most biological tissues consist mainly of water. However there are a number of important structures that have variable moisture contents depending on their environment. In some cases such as dry seeds, dry lichens and dry resurrection plants there may be very little free water present. A variety of important structural and physiological processes occur as such tissues lose water during desiccation or gain water during times of increased availability of water. In addition to the importance of studying living tissues at different moisture content there are many other important situations where samples undergo changes in water content. In some cases products are deliberately dried to very low water content as is the case with the preparation of many food products. Fibers, including paper, are subject to variable moisture content as they are processed. This manuscript describes the use of cryogenic preparation for the study of samples at different moisture content. We have illustrated only a few of the potential applications of this method.

Most specimen preparation procedures begin with fixation in aqueous solutions, a procedure suitable for hydrated samples or for dry samples where imbibition during fixation is of no concern. The slowness of chemical fixation and dehydration makes this procedure unsuitable for studies involving relatively rapid changes in hydration. There are also procedures which allow dry samples to be prepared anhydrously. However there is a lack of procedures that permit study of samples at different moisture levels or permit study of transitions between regions of greatly different moisture levels. One way of studying structural changes in such samples with different moisture levels is freeze-fracturing. While some interesting results have been obtained by workers such as Buttrose (1973) and Buttrose and Soeffky (1973), this technique has limitations including a restriction to small sized samples. The freeze-etching results of Buttrose (1973) and Buttrose and Soeffky (1973) thus deal mainly with changes in lipid vacuoles, membranes and other subcellular components. The freezing, fracturing and shadowing portions of the freeze-fracturing procedure are relatively straightforward. The

great difficulty is in digesting the dry plant tissue without damaging the thin platinum-carbon replica. The replica adheres very tightly to the tissue so that the inevitable swelling that occurs during cleaning with acid results in numerous rips in the replica. This paper will explore the use of cryogenic preparation for the study of samples involved in dry-to-wet or wet-to-dry transitions.

While it has been over ten years since the potential for studying frozen biological tissue in an SEM was demonstrated by Nei et al., (1973), the actual usage of the method by a number of investigators has only taken place recently. Commercially available equipment that can be attached to a scanning electron microscope now permits the study of fully frozen hydrated tissue samples as well as frozen samples from which the surface ice has been removed by etching (Robards and Crosby, 1979). The three main operational phases required for the study of cryogenically prepared samples in a SEM, as discussed by Beckett and Read (1986), are briefly described below. In the first phase a specimen is mounted on a metal holder and rapidly frozen, often by plunging it into subcooled nitrogen. Once the specimen is frozen all future steps are carried out under vacuum or in a dry gas atmosphere. The second phase of preparation allows the frozen sample to be manipulated in various ways so that it may be fractured or etched or both and then coated with metal or carbon. The third operational phase involves transfer of the frozen sample under vacuum to a cryo-stage in the SEM where the specimen can be studied. Samples can often be viewed in the SEM within fifteen minutes of the sample being frozen.

As discussed by Lott et al.(1985), there are a number of advantages to cryogenic preparation of samples for SEM studies. Evidence to date indicates that specimens are likely to retain more normal volume and shape than are samples that have been chemically dehydrated (Beckett et al., 1984; Read and Beckett, 1983). Another major advantage is that some samples, such as liquids and certain gels can be studied with low temperature procedures but cannot be studied with traditional fixation-dehydration-critical point drying routines. Using the cryogenic preparation procedure it is possible to determine if internal spaces in a tissue are filled with water, air or mucilaginous material (Beckett and Read,1986). Since freezing is much more rapid than chemical fixation, it is possible to study changes occurring over shorter time periods. Quick freezing prevents movement of soluble materials, a feature that has promoted interest in combining cryogenic specimen preparation with x-ray microanalysis (Echlin et al., 1980, 1982; Fuchs and Fuchs, 1980; Marshall, 1977, 1980). One advantage of cryogenic preparation that has received very little attention, is the potential for studying transitions that take place as a dry sample is hydrated or as a wet sample undergoes drying.

Materials and Methods

Seeds used in this study were squash (Cucurbita maxima cv. Warted Hubbard), and sunflower (Helianthus annuus L. cv. Russian Mammoth). Mature squash seeds with different moisture contents were obtained by using seeds recently removed from a moist fruit and after air drying. The moss, Polytrichum commune was collected near Parry Sound, Ontario. Cubes of freeze-dried carrot root and chicken boullion were purchased from a food store.

Samples were cryogenically prepared using an EMscope SP2000 cryo-unit (EMscope Laboratories Ltd. Ashford, England.). Those samples used to demonstrate the presence of a dry-to-wet transition were either immersed in water for one to ten seconds, as was the case with Polytrichum or were brought into contact with water on one surface only. Food samples were imbibed for several seconds prior to freezing whereas seed tissues were soaked for periods of up to one hour. Mounting of samples onto the EMscope metal specimen holder was achieved with an adhesive called Tissue-tek O.C.T. compound (Miles Scientific). The samples were then plunge frozen in liquid nitrogen sub-cooled to below its boiling point. This was prepared by placing liquid nitrogen under vacuum until it formed nitrogen slush.

The frozen samples seen in figures 1-4 were coated with chromium, to allow subsequent x-ray analysis. Chromium, in the form of pure metal chips, was evaporated onto the surface of the frozen specimen from a basket for 10-30 seconds at 10mA. The remaining samples were gold coated. All metal coating was done in the work chamber of the EMscope SP2000. Once coated, a sample was transferred under vacuum to the EMscope cryo-stage in an ISI model DS-130 scanning electron microscope. A variety of accelerating voltages ranging from 1 to 15 kV were used to image the samples.

Results

The structure of the cells in the embryo of a seed were found to be influenced by the moisture content. When mature squash seeds removed from the moist pericarp were frozen, fractured, metal coated and studied in a SEM the cell walls were smooth (Fig. 1). When other seeds removed from the same fruit were allowed to air dry the cotyledon cells lost volume and the cell walls became wrinkled (Fig. 2). The profound influence of moisture content upon seed structure can be seen by comparing cotyledon cells in Fig. 1 with those in Fig. 2. The shrinkage of embryo cells that occurs normally as seeds dry out is reversed when a dry seed is hydrated. Using cryogenically prepared tissue it was possible to show that the dry-to-wet transition can occur rapidly. The deeply wrinkled cell walls found in the dry state smooth out as the cells take up water. Cells undergoing this dry-to-wet transition show different degrees of wrinkling of the cell walls. The dry-to-wet transitions in the spongy mesophyll (Fig. 3) and
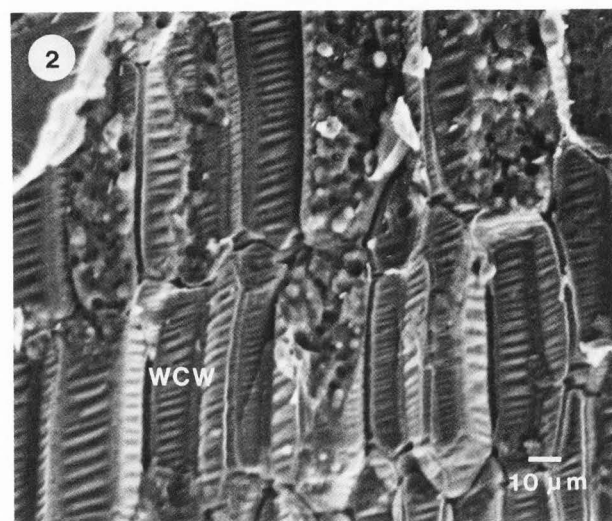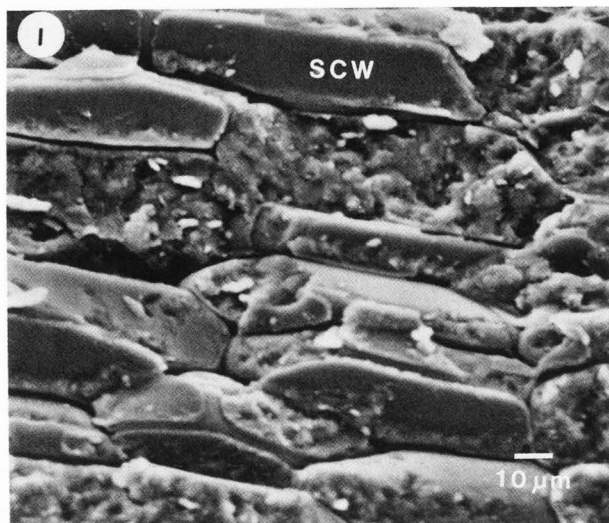
Fig. 1. Portion of a squash (Cucurbita maxima) seed just removed from a fruit. Cotyledon tissue was frozen, fractured, metal coated and viewed on a cryo-stage in a SEM. Cell walls were smooth (SCW) and the cells appeared turgid.

Fig. 2. Portion of a squash seed allowed to air dry following removal from the fruit (same fruit as in Fig. 1.). This cryogenically prepared specimen shows that in the air dry state the cotyledon cells had extensively wrinkled cell walls (WCW).
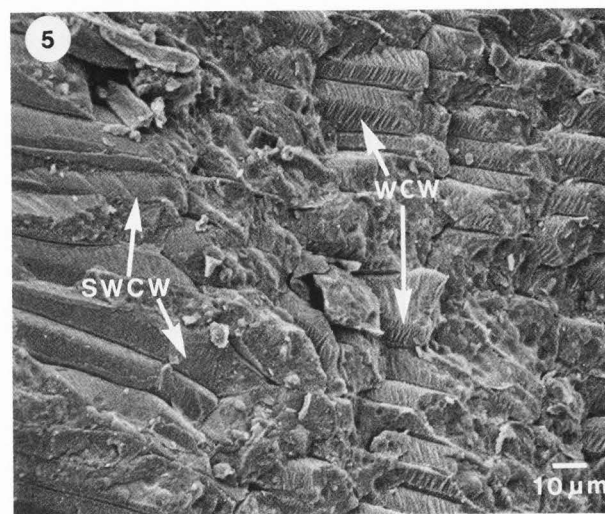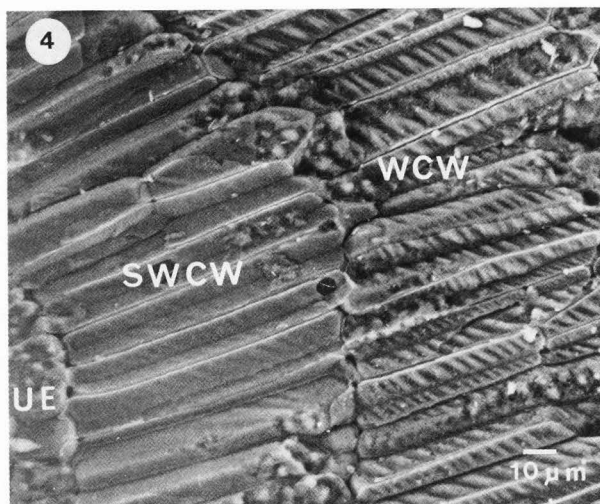
Fig. 3. Portion of an air dry squash seed (same fruit as used for Figs. 1 and 2) showing spongy mesophyll cells undergoing a dry-to-wet transition as the seed was imbibed. The dry portion had wrinkled cell walls (WCW) whereas the moist region had slightly wrinkled cell walls (SWCW).

Fig. 4. Similar to Fig. 3 except that the upper epidermis (UE) and the first layer of palisade mesophyll have been at least partially hydrated. The first palisade layer had only slightly wrinkled cell walls (SWCW) whereas the second palisade layer had the wrinkled cell walls (WCW) characteristic of air dry seed tissue.

Fig. 5. Portion of a cryogenically prepared sunflower (Helianthus annuus) cotyledon showing a dry-to-wet transition. The dry area had wrinkled cell walls (WCW) whereas the hydrated area had slightly wrinkled cell walls (SWCW).

palisade mesophyll (Fig. 4) of squash show similar changes as do cells from sunflower cotyledons (Fig. 5). In some cases cells adjacent to each other show marked differences as illustrated by the two layers of palisade mesophyll cells shown in Fig. 4.

Structural changes during dry-to-wet transitions are likely to occur in a variety of plants including lichens and resurrection plants. An example of this is illustrated here with the desiccation resistant moss Polytrichum. The upper side of Polytrichum leaves have rows of parallel flap-like lamellae that are one cell thick. When the leaf-like part of the gametophyte is air dried the cells are noticeably collapsed in appearance with the side walls frequently being concave (Fig. 6). As these cells hydrate (Fig. 7) they swell and become turgid when fully hydrated (Fig. 8). Figure 7 shows that only some cells became hydrated by the brief immersion in water given this sample.

Cryogenic preparation of samples permitted observations of dry-to-wet transitions in foods as diverse as a piece of freeze-dried carrot (Fig. 9) and a chicken boullion cube (Fig. 10). In both the carrot and the boullion it was possible to determine where the water had penetrated into air spaces inside the cube.

Discussion

Dry tissue can be prepared for scanning electron microscopy in several ways. In some cases air dry tissue may be broken, metal coated and examined. However for oil-rich seed tissue this procedure generally does not give satisfactory results since the oil smears on the specimen surface. Tissue may be frozen, fractured and freeze-dried prior to mounting and coating (Darley and Lott, 1973). Dry seed tissue fixed with osmium tetroxide vapour prior to fracturing and metal coating has proven to be a successful means of preparing oil-rich tissue for SEM (Webb and Arnott, 1980, 1982).

It is possible, with difficulty, to study the ultrastructure of dry tissue prepared for transmission electron microscopy in an anhydrous manner. Perner (1965) studied the cell structure of radicles from dry pea seeds that had been fixed in osmium tetroxide vapour and embedded. The ultrastructure of tissue from dry grains was studied by Öpik (1980, 1985) who used osmium vapour fixation or acrolein vapour fixation prior to embedding. Some dry seed tissue has also been studied by freeze-fracturing (Buttrose, 1971, 1973; Buttrose and Soeffky, 1973 ; Lott, 1974; Swift and Buttrose, 1973). Anhydrous fixation of tissue from dry resurrection plants has been investigated extensively by Hallam and co-workers (Hallam, 1976; Hallam and Gaff, 1978; Hallam and Luff, 1980a, 1980b; Moore et al., 1982). In addition to using osmium tetroxide vapour, Hallam (1976) used fixatives such as anhydrous glutaraldehyde in anhydrous glycerol followed by osmium tetroxide in glycerol or anhydrous glutaraldehyde in dimethylsulfoxide followed by osmium tetroxide in chloroform. In general all the anhydrous procedures used to study dry tissue have had

difficulty obtaining adequate infiltration of epoxy resin through cell walls (Yatsu, 1983).

Thus while it is possible to study dry tissue in an anhydrous state or tissue that has been thoroughly hydrated during conventional aqueous fixation, it is very difficult to prepare samples to show dry-to-wet transitions. As has been demonstrated here, it is possible to use cryogenically prepared specimens studied in a SEM for that purpose. Situations where there is a dry-to-wet transition within the same sample can be studied. We have used seed tissue, moss tissue and two different food samples to demonstrate this point but a wide variety of samples could be studied in this way. In some cases, such as the seed tissue and the moss tissue, there was swelling upon hydration so that there was a major change in structure. In other cases, such as the boullion cube, it was possible to see where the air spaces were partly filled with water even though rapid structural changes were less pronounced.

Webb and Arnott (1982) studied anhydrously prepared seeds of different species and showed that the manner of cell wall collapse is characteristic of a given species. In some cases such as in the Cucurbitaceae, there was a regular folding pattern whereas other species such as Hibiscus had cell walls with random wrinkling. The way and extent to which the cell walls fold up determines not only shape changes during seed desiccation but also the direction in which expansion will take place upon imbibition. For example the long cylindrical palisade mesophyll cells of a squash cotyledon shrink to a greater extent in length than in cross section during seed drying, and elongate in the reverse plane during imbibition. Squash cotyledons, imbibed by placing the upper epidermis on a moist surface, were not subject to noticeable damage because of the direction of expansion. Soybean cotyledons, imbibed in a similar way swell sideways to such a degree that the cotyledon surface is ripped open. Clearly then, many factors including the species, the cell type, the moisture content and the presence of the testa will help determine the structure of cell walls in seeds.

Webb and Arnott discuss the importance of cell wall folding patterns as a means of permitting shrinkage while still retaining the vital plasmodesmata connections between cells. Obvious areas for future studies include documentation of the changes during seed desiccation and studies of the seed imbibition in the context of restraints placed on the embryo by a rigid seed coat. While we know that squash seeds removed immediately from a fresh fruit have smooth cell walls and that after air drying, the cell walls have become wrinkled, we have no knowledge of the events that occur during this process. Do all cells lose water and shrink at the same rate? Do the small wrinkles or the big ridges develop first? During imbibition, pressure developed by the embryo must assist rupture of the seed coats. Is this due to uniform unwrinkling throughout the cotyledon or do certain cells expand first?

While the dimensional changes seen in seed tissue during desiccation and imbibition are very
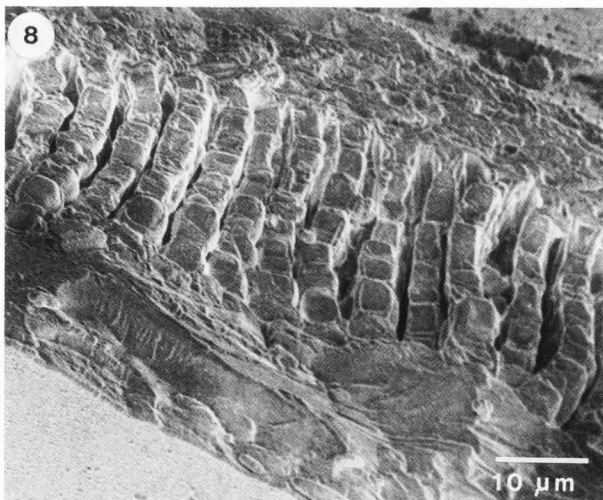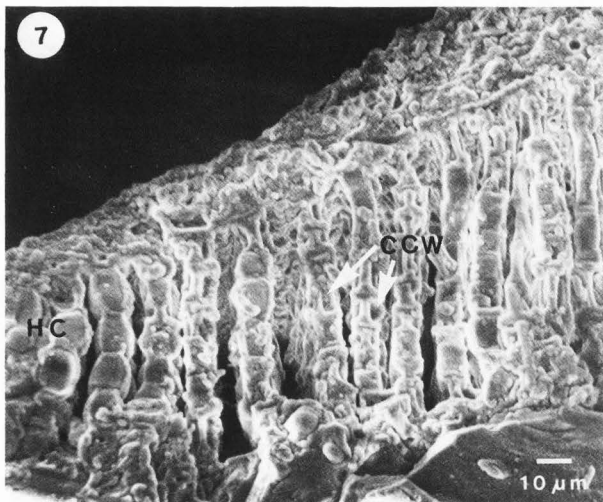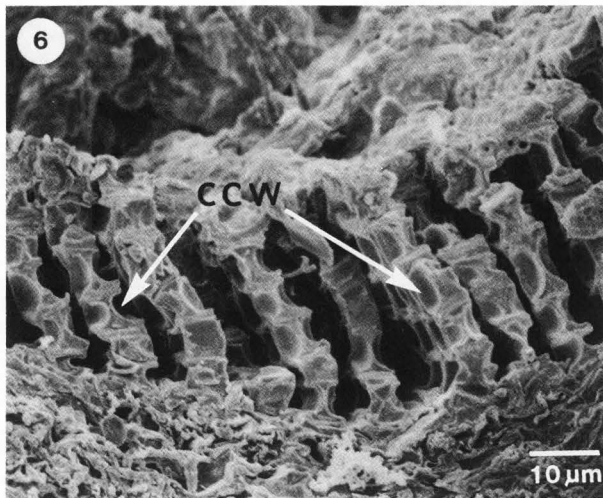
Fig. 6. Portion of a cryogenically prepared air dry leaf-like portion of the moss Polytrichum. Parallel lamellae of photosynthetic tissue on the upper surface of the leaf-like part of the gametophyte have concave cell walls (CCW).
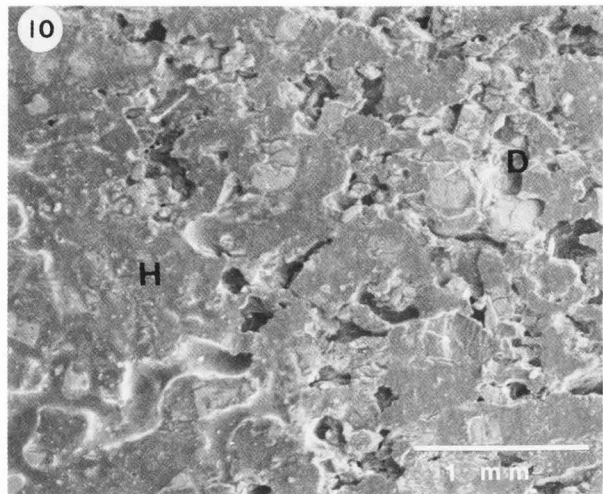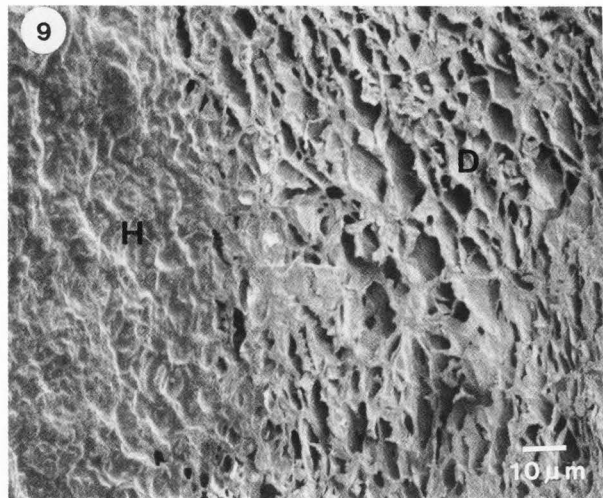

Fig. 7. Similar to Figure 6 except that the plant was briefly dipped in water prior to freezing. Some cells were hydrated (HC) but the dry regions still had cells with concave cell walls (CCW).

Fig. 8. Similar to Figure 6 except that the plant was allowed a longer time for hydration. Cells were swollen and had resumed the normal shape seen in the hydrated tissue.

Fig. 9. Portion of a cube of freeze-dried carrot root prepared cryogenically. A dry area (D) and an area that was hydrated (H), due to addition of water to one side of the dry tissue, are evident.

Fig. 10. Portion of a chicken bouillon cube that was prepared cryogenically, showing a dry area (D) and an area that was partially hydrated (H). The cavities in the hydrated area of the cube were at least partially filled with water prior to freezing.

pronounced, there is the potential for dramatic structural changes in other living systems. Dry Polytrichum cells, as shown here, rapidly changed from cells with concave side walls to turgid cells with convex side walls.

Cryogenic preparation of samples for SEM is also useful in situations where different specimens have been kept at different relative humidities. We have demonstrated the potential for such studies by studying mature squash seeds obtained under different conditions. Cotyledon cells in mature embryos of seeds removed from the moist pericarp of the fruit have smooth cell walls. Following air drying, cotyledon tissue from other seeds of the same fruit had wrinkled cell walls. From previous research by Buttrose (1973), Lott (1974) and Webb and Arnott (1982) it was expected that such a change would occur but proof has been difficult to obtain. Studies of samples held at various moisture levels may be useful not just for biological material but also for a range of food science and material science samples.

We believe that the cryogenic preparation of samples for the SEM opens up a new frontier in the study of specimens undergoing transitions from dry-to-wet or from wet-to-dry. The use of energy dispersive X-ray analysis in conjunction with this preparative method would make it possible to trace the advance of certain elements through a dry tissue as the tissue is imbibed.

Acknowledgements

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Discussion with Reviewers

A. Beckett: The authors state that cryogenically prepared tissue shows that the dry to wet transition can occur rapidly. No times are given for hydration treatment for any of the samples. Were these measured? and if so, what does "rapidly" actually mean? - for example for a squash seed.

Authors: The time required for a dry sample to become hydrated varies greatly depending upon the type of specimen and the place of sampling within that specimen. For instance, a one centimetre cube of sucrose immersed in water is hydrated to the centre in about 10–15 seconds whereas an intact dry seed may have to be immersed in water for several hours before all cells are hydrated. The speed with which a water front moves through a sample will depend on a variety of factors including the amount of open space and the chemical composition of the specimen. In our samples the dry-to-wet transitions frequently occurred within 30 seconds in a given region of the sample but the seed tissue was much slower to respond.

A. Beckett: Were water droplets found on the cell walls in intercellular spaces within tissues which were freeze-fractured following hydration by immersion in water? How is it envisaged that the "hydration front" (e.g. Figs. 4 and 7) moves through the tissue? Is it symplastically, apoplastically or both, and is there a continuous film of intercellular water moving through the tissue?

Authors: In biological tissue that has begun hydration, intercellular spaces often appear to be open, as can be seen in Figure 4 between the upper epidermis and the uppermost palisade layer. Perhaps a film of water moves along the wall surface but it does not seem to fill up the air spaces, nor do beads of water appear to form. We suspect that water may move through the cell walls and from there enter the adjacent cytoplasm. We base this upon some trials in which seed tissue was imbibed from one direction with 10% (wt/vol) cobalt chloride. The salt penetration was followed both visually, since cobalt chloride is a burgundy colour and with EDX analysis. Since water may move in a different way than a concentrated salt solution the test is not foolproof. With EDX analysis we did find salt in the area with smooth or slightly wrinkled cell walls and no salt in the area clear of the transition zone where major cell wall wrinkling occurred. This provides further support for our contention that the areas with distinctly wrinkled cell walls are dry and the areas with smooth cell walls are at least partially hydrated. The salt detected in the transitional area was, if anything, concentrated in the cell walls and not in the adjacent cytoplasm. This probably means that the solution moves through the walls first. In food samples such as the boullion cube our results suggest that water forms a layer along the edge of air spaces and in some cases fills the spaces completely.

A. Beckett: Does the rate of hydration vary with cell type within a tissue or does it depend upon the water moving from one point to another through the tissue as seems to be the case for Fig. 4?

Authors: We expect that both the cell type and the rate at which water can move from one point to another play a role in determining the rate of hydration. While we did not study hydration through hypodermal or endodermal cells, which would be expected to restrict apoplastic movement of water, we would expect such cell types to influence the rate of hydration. Similarly, the presence of an intact cuticle on epidermal surfaces would greatly retard the uptake of water. It also seems likely that the content of the cells may have an influence on the rate at which water is taken up. For example cells in some seeds contain numerous lipid vacuoles located next to the plasmalemma. Water entering such cells would have to move through the small regions of cytoplasm between the lipid vacuoles,

a process that must be slower than that occurring in cells without such lipid storage vacuoles.

Reviewer IV: What specific information may be gained in the study of dry-to-wet transitions?
Authors: Since we are particularily interested in seeds we have included in the revised text a discussion of several problems in seed structure and function that could be tackled with this technique. In addition we believe this procedure will be of use to a number of other disciplines with which we are less familiar. For example there is great interest in food science in the development of products suited for rapid food preparation such as by microwave cooking. Some of these products will be partially or completely dehydrated and must be capable of rapid rehydration if they are to be successful. Cryogenic specimen preparation is therefore likely to be a technique useful to the food scientist. Additional areas where this type of preparation may be applied includes the study of soils, materials such as cements and commercial fibers.

Reviewer IV: Some authors have noted the production of intercellular artifacts on bulk plant samples which were frozen in a liquid nitrogen slush. Were any samples fractured to reveal internal cellular structures and if so, were any artifacts observed?
Authors: When highly hydrated leaf cells are frozen and fractured open there is considerable evidence that ice crystal damage resulted from the freezing process. The seed tissue which we have studied most extensively is unusual in that it has a very high organic content due to the presence of storage reserves and likely due to this does not form ice crystals noticeable at the level of resolution used with this procedure. We have noticed that certain samples will crack during the freezing process, indicating that in some cases the freezing process produces tension inside the sample.