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Freeze-substitution of dehydrated plant tissues: artefacts of aqueous fixation revisited

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Summary. This investigation assessed the extent of rehydration of dehydrated plant tissues during aqueous fixation in comparison with the fine structure revealed by freeze-substitution. Radicles from desiccation-tolerant pea (*Pisum sativum* L.), desiccation-sensitive jackfruit seeds (*Artocarpus heterophyllus* Lamk.), and leaves of the resurrection plant *Eragrostis nindensis* Ficalho & Hiern. were selected for their developmentally diverse characteristics. Following freeze-substitution, electron microscopy of dehydrated cells revealed variable wall infolding. Plasmalemmas had a trilaminar appearance and were continuous and closely appressed to cell walls, while the cytoplasm was compacted but ordered. Following aqueous fixation, separation of the plasmalemma and the cell wall, membrane vesiculation and distortion of cellular substructure were evident in all material studied. The sectional area enclosed by the cell wall in cortical cells of dehydrated pea and jackfruit radicles and mesophyll of *E. nindensis* increased after aqueous fixation by 55, 20, and 30%, respectively. Separation of the plasmalemma and the cell wall was attributed to the characteristics of aqueous fixatives, which limited the expansion of the plasmalemma and cellular contents but not that of the cell wall. It is proposed that severed plasmodesmatal connections, plasmalemma discontinuities, and membrane vesiculation that frequently accompany separation of walls and protoplasm are artefacts of aqueous fixation and should not be interpreted as evidence of desiccation damage or membrane recycling. Evidence suggests that, unlike aqueous fixation, freeze-substitution facilitates reliable preservation of tissues in the dehydrated state and is therefore essential for ultrastructural studies of desiccation.

Keywords: Aqueous fixation; Desiccation; Freeze-substitution; Rehydration artefact; Resurrection plant; Microscopy.

Abbreviations: LM light microscopy; TEM transmission electron microscopy; CF conventional (aqueous) fixation; FS freeze-substitution; ER endoplasmic reticulum.

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Introduction

Mature seeds, pollen and, in a few cases, vegetative tissues of a variety of plants can withstand exposure to extreme desiccation for different periods. In contrast, many others exhibit varying degrees of sensitivity to water loss and die following exposure to severe dehydration. Ultrastructural studies have contributed significantly towards increasing current understanding of the processes underlying the acquisition, or lack, of desiccation tolerance. However, many of those investigations have relied on the use of aqueous-based preparative protocols, which introduces uncertainty about the extent of rehydration that occurs during tissue processing. Rehydration of plant cells in the dry state can occur in a matter of seconds or minutes following immersion in aqueous media (Swift and Buttrose 1972, Buttrose 1973, Tiwari et al. 1990). Straightening of otherwise convoluted cell walls, discontinuity of the plasmalemma, and rounding of organelles showing diffuse membranes have been reported to occur during aqueous fixation (Swift and Buttrose 1972, Buttrose 1973, Webb and Arnott 1982, Vigil et al. 1984, Öpik 1985). Thus, use of aqueous-based fixatives is unlikely to give an accurate impression of cells and tissues as they occur in the dehydrated state.

Attempts to circumvent rehydration artefacts during processing of plant tissues have been made by truly anhydrous chemical processing methods using osmium vapour fixation (Perner 1965; Hallam 1976; Öpik 1980, 1985; Smith 1991) or aldehyde-based fixatives in anhydrous dimethyl sulfoxide or glycerol (Hallam 1976, Hallam and Gaff 1978, Smith 1991). In

other studies, rehydration was avoided initially by anhydrous primary fixation, but introduced later by exposure to aqueous media during subsequent processing (Thomson 1979; Altus and Hallam 1980; Fincher Chabot and Leopold 1982; Yatsu 1983; Vigil et al. 1984, 1985). Since the advent of cryotechniques (reviewed by Robards 1974, Robards and Sleytr 1985, Gilkey and Staehelin 1986, Echlin 1992), ultrarapid cooling of partially hydrated samples in combination with freeze-fracture (Swift and Buttrose 1972; Buttrose 1973; Fincher Chabot and Leopold 1982; Vigil et al. 1984, 1985; Platt et al. 1994) and freeze-drying (Lott 1974) have also been successfully used to prevent rehydration artefacts. Although these protocols yield a relatively more accurate representation of cells in the dry state than does conventional (aqueous) fixation, each technique has inherent limitations. Anhydrous chemical fixation using osmium vapour, which is undesirably prolonged (from 4 weeks [Hallam 1976] to 11 months [Öpik 1980]), can result in cytoplasmic shrinkage (Perner 1965), coalescence of oil bodies, reduced staining of organelles (Öpik 1980), limited perfusion and poor sectioning quality (Yatsu 1983, Smith 1991). Similarly, use of aldehydes in glycerol or dimethyl sulfoxide can result in poor definition of organelles and plasmalemma abnormalities (Thomson 1979, Smith 1991) as well as straightening of cell walls (Hallam 1976, Hallam and Luff 1980). While freeze-fracture is regarded as giving the most reliable ultrastructural preservation (e.g., Buttrose 1973, Swift and Buttrose 1972, Vigil et al. 1984), highly dehydrated tissues can cleave in unpredictable and irregular planes (Fincher Chabot and Leopold 1982, Vigil et al. 1985) and establishing the original location of cells in the sample can be hindered by extensive fragmentation of replicas during cleaning (Fincher Chabot and Leopold 1982, Platt et al. 1994).

Freeze-substitution (FS) and resin embedding for transmission electron microscopy (TEM) is an alternative low-temperature preparative technique that combines the advantages afforded by cryofixation with the convenience of room temperature sectioning (Steinbrecht and Müller 1987, Parthasarathy 1995). It involves the replacement of solidified water in the sample by an anhydrous solvent at temperatures precluding ice crystal growth. Following optimal cryofixation, FS can overcome most shortcomings of other anhydrous protocols, facilitating superior morphological preservation of hydrated plant tissues (Browning and Gunning 1977, Mersey and McCully 1978, McCully and Canny 1985, Lancelle et al. 1986, Kiss et al. 1990, Lancelle and Hepler 1992, Kellenberger 1991, Davidson and Newcomb 2000).

The relevance of FS to the study of desiccation was highlighted by studies on pollen (Tiwari et al. 1990) and the resurrection plant *Selaginella lepidophylla* (Hook & Grev.) (Platt et al. 1997, Thomson and Platt 1997). Improvements in the field of cryofixation (reviewed by Sitte 1996) and refinements to FS protocols (e.g., Steinbrecht and Müller 1987, Ding et al. 1991, Parthasarathy 1995) make it possible to reassess critically rehydration artefacts that occur during preparation of dehydrated tissues for microscopy. The present investigation evaluated the ultrastructural preservation of dehydrated plant specimens by cryofixation and FS and compared it with that attained following aqueous two-step glutaraldehyde-osmium tetroxide fixation. Embryonic or mature vegetative tissues from three species were chosen for their developmentally diverse characteristics at the cellular level and relevance to the study of desiccation. Seeds of desiccation-tolerant (orthodox) species undergo a genetically programmed phase of drying to equilibrium with ambient relative humidity, during which metabolic activity is reduced to quiescence (Bewley 1979, Kermode and Bewley 1985). Radicles from embryonic axes of air-dried pea (*Pisum sativum* L.) seeds were selected to illustrate the situation of cells of orthodox seeds following maturation drying. In contrast, desiccation-sensitive (recalcitrant) seeds do not undergo a similar drying phase and are shed at high water content as metabolically active propagules. Radicles excised from embryonic axes of *Artocarpus heterophyllus* Lamk. (jackfruit), dehydrated to a sublethal 20% of their water content at shedding, represent the condition of desiccation-sensitive cells during dehydration stress. Finally, inner leaves of the resurrection plant *Eragrostis nindensis* (Ficalho & Hiern.) tolerate extreme desiccation (Vander Willigen et al. 2001) and illustrate the third category. Unlike meristematic tissues in embryonic axes, the vegetative cells of the mature leaves are fully differentiated and adjust their metabolism to withstand water stress.

The present investigation assessed the extent of rehydration that occurs in dehydrated plant tissues during conventional aqueous fixation (CF) for microscopy and compared it to the preservation attained following FS. In addition to the morphological appearance at the light and electron microscopical levels, the areas of cells resulting from each processing

protocol were measured with image analysis software and compared.

Material and methods

Collection and preparation of plant material for fixation

Pea seeds (*Pisum sativum* L. cv. Greenfeast) were obtained commercially and maintained at the initial water content of 0.08 g of water per g of dry mass. The embryonic radicles from these seeds were excised and either immersed in aqueous fixative or further sliced into 1–2 mm thick transverse sections and immediately cryofixed as described below. In order to assess the structural changes that take place during imbibition, pea radicles were immersed in distilled water for 2 h prior to conventional fixation.

Hydrated samples were partially dried by different methods. Axes from jackfruit seeds were dehydrated from an initial water content of 3.5 to 0.7 g of water per g of dry mass. This was achieved over two days by placing the excised axes on a nylon mesh suspended inside a closed chamber containing a saturated salt solution of KH_2PO_4 that yielded a relative humidity of 96% at 25 °C (Winston and Bates 1960). After drying, radicles were excised and either immersed in aqueous fixative or sliced into 1–2 mm thick transverse sections and cryofixed immediately. The resurrection plant *E. nindensis* was grown under greenhouse conditions in Cape Town, South Africa. Watering was withheld while the plants air dried over a period of 14 days. Transverse sections (1–2 mm thick) were cut from an area 50 mm above the base of innermost leaves. The water content at the time of sampling ranged between 0.02 and 0.05 g of water per g of dry mass. These sections were fixed either conventionally or by cryofixation (described below). Segments from three replicates per treatment were randomly selected for microscopy and analysis of cell area.

Water contents were determined gravimetrically after drying to constant mass in an oven at 80 °C for 16 h.

Conventional processing protocol

Excised hypocotyls of axes of pea and jackfruit and inner-leaf segments of *E. nindensis* were immersed with agitation in a solution of 2.5% (v/v) glutaraldehyde in 0.1 M Sørensen phosphate buffer (pH 7.2) for 2 h at room temperature. Although longer primary fixation at 4 °C has been suggested to improve preservation of plant cell structure (Dong et al. 1994), the aqueous fixation protocol used in this study is typical of that commonly used in desiccation studies (e.g., Webster and Leopold 1977, Sack et al. 1988, Quartacci et al. 1997). After primary fixation, specimens were postfixed in 0.5% (w/v) osmium tetroxide for 1 h (with buffer rinses before and after), dehydrated in a graded acetone series, gradually infiltrated with low-viscosity epoxy resin (Spurr 1969) for 16 h, and finally polymerised at 70 °C for 10 h.

Cryofixation and substitution

Transverse sections of hypocotyls from embryonic axes and leaves were mounted edgeways on ultramicrotomy specimen holders (Leica, Vienna Austria) with a very small amount of low temperature adhesive (Tissue-Tek; Sakura Finetek, Torrance, Calif., U.S.A.). A Leica CPC cryofixation instrument was used to plunge samples rapidly into liquid propane at 83 K (–190 °C) immediately after mounting. Frozen specimens were placed in handling devices described by Parthasarathy (1995) and transferred while frozen to the freeze-substitution medium precooled to 193 K (–80 °C). A protocol modified from Ding et al. (1991) was used and consisted of specimen immersion in 0.1% (w/v) tannic acid in acetone at 193 K for 1 day followed by 3 days in a medium containing 2% (v/v) anhydrous glutaraldehyde in methanol (Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.), 2% (w/v) osmium tetroxide, and 2% (w/v) uranyl acetate in acetone at the same temperature (this medium contained a final concentration of 20% methanol introduced by the anhydrous glutaraldehyde solution). Subsequently, samples were warmed in a stepwise manner (–60 °C for 18 h, –40 °C for 12 h, –20 °C for 12 h, and 0 °C for 6 h) in a temperature-controlled substitution chamber (Reid and Associates, Durban, South Africa) placed inside a 193 K deep freezer. Samples were subsequently rinsed twice in acetone, infiltrated with increasing concentrations of low-viscosity epoxy resin (Spurr 1966) in acetone (1 : 3, 1 : 1, 3 : 1, 1 : 0) for 8–12 h at each stage and heat polymerised. Sections were cut on a Reichert Ultracut E (Leica) and contrasted for light microscopy (LM) and TEM according to standard techniques. Ultrathin sections showing copper/gold interference colours were collected on 600 mesh copper grids that provided increased support during staining and viewing.

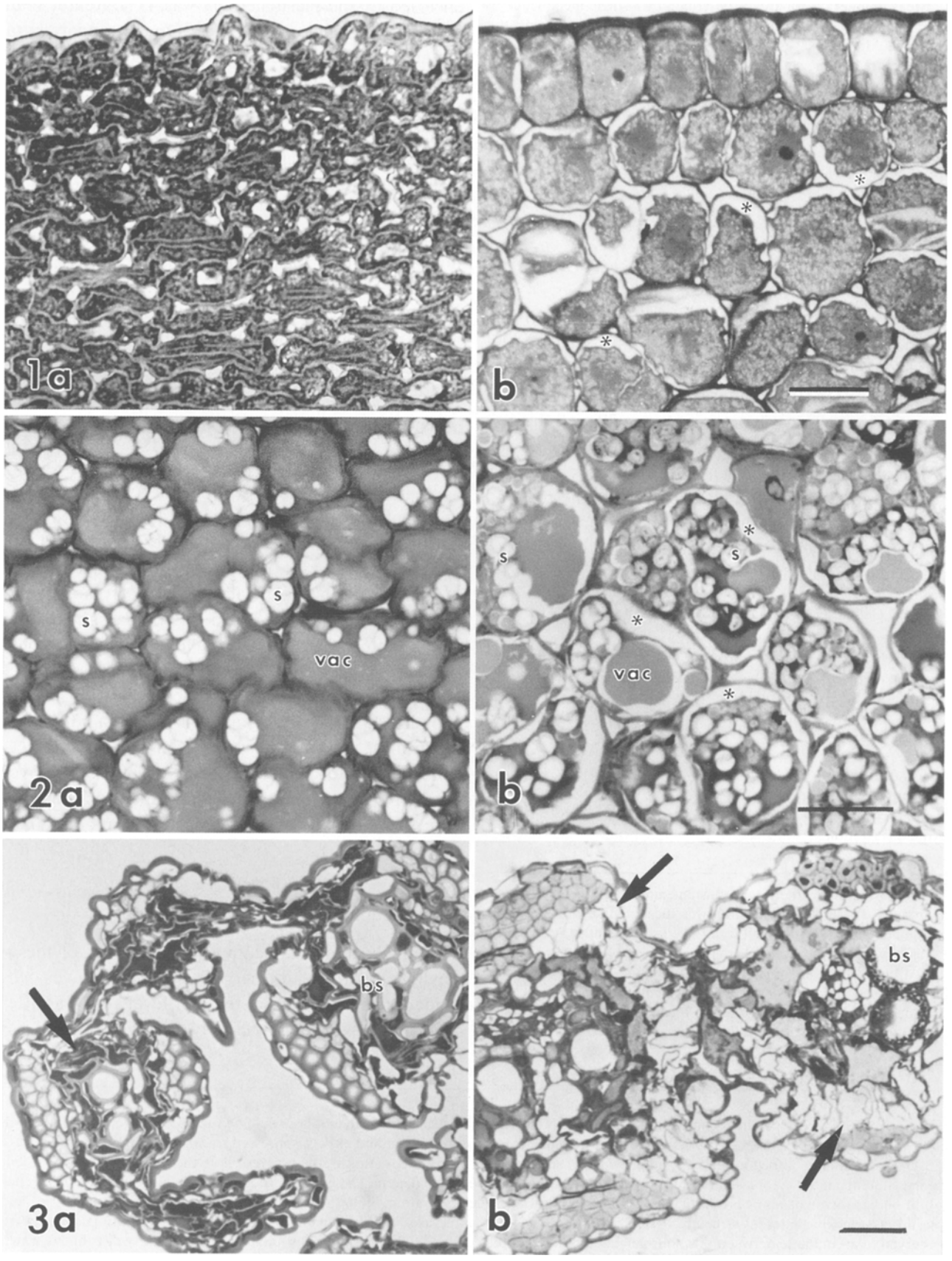
Microscopy, image recording and analysis

Toluidine blue-stained sections (1 μm thick) were viewed with a Nikon Biophot (Nikon, Tokyo, Japan) transmitted-light microscope. Ultrathin sections were viewed with a JEOL JEM 1010 TEM (JEOL, Tokyo, Japan) at 100 kV. Images from both instruments were recorded photographically and digitally. Measurements were performed on digitised images by the Zeiss KS100 image analysis software (Carl Zeiss Vision GmbH, Oberkochen, Federal Republic of Germany) after calibration to the appropriate magnification. In radicles of pea and jackfruit, cell areas were determined along four transects through the outermost 15–20 layers in transverse sections from each of three replicates. Leaves of *E. nindensis* prepared by either FS or CF were sectioned transversely, and the areas within the cell wall of mesophyll cells were also measured and compared.

Fig. 1a, b. LM of transverse sections of dry embryonic axes of pea. **a** FS. Radicle tissues had a dense, tightly packed appearance. **b** CF. An increase in cell area and separation of the plasmalemma from the wall (asterisks) was observed in cells after aqueous fixation. Bar for a and b: 40 μm

Fig. 2a, b. LM of transverse sections of dehydrated embryonic axes of recalcitrant jackfruit. **a** FS. Cells were tightly packed within these tissues and walls moderately folded. Vacuoles (*vac*) and starch deposits (*s*) were prominent. **b** CF. The outline of cells and vacuoles showed a rounded appearance not seen in FS axes. Asterisks indicate areas where the plasmalemma and cell wall became separated. Bar for a and b: 20 μm

Fig. 3a, b. LM of transverse sections of leaves of *E. nindensis*. **a** FS leaf showing marked cellular compaction. Note the dense appearance of mesophyll cells. **b** CF resulted in an expanded appearance of cells. *bs* Bundle sheath cells; arrows, mesophyll cells. Bar for a and b: 20 μm


1a
b
2a
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b

Measurement of cell area is described by a single mean value in specimens where the plasmalemma remained appressed against the cell wall. Alternatively, separate values are reported for either the area enclosed by the cell wall or that enclosed by the plasmalemma where separation between these structures had taken place. For greater clarity, results from LM and TEM observations are presented separately below.

Results

Light microscopy

Desiccation-tolerant tissues: pea

The protoplasm of cells from freeze-substituted pea hypocotyls appeared dense and tightly enclosed by moderately folded cell walls (Fig. 1 a). Measurements taken between the epidermis and the pericycle in transverse sections (comprising ca. 20 cell layers) showed that the average area enclosed within the cell wall was 203 μm^2 (Table 1). Following CF, cells had a rounder appearance and instances where the cell wall and the plasmalemma had separated could be seen in several layers below the epidermis (Fig. 1 b). The area enclosed by the plasmalemma was 41% greater than that in FS specimens (Table 1). One-way analysis of variance and multiple range tests showed that this difference was significant ($P < 0.05$). In cells where separation of the plasmalemma and the cell wall had occurred, the area enclosed by the cell wall increased by a further 14% (Table 1), which was identical to that from axes imbibed for 2 h prior to CF ($P > 0.05$; Table 1). Thus, equivalent rehydration of cell walls had occurred during the course of CF and during imbibition. In contrast, plasmalemma and cell walls maintained close contact if radicles were imbibed prior to CF (not shown). This indicated that both boundaries had expanded simultaneously and to the same extent during imbibition and that their close apposition was unaffected by CF.

Table 1. Cell area in transverse sections of pea radicles after fixation

Fixation method	Cell area boundary[a]	Cell area	
		mean ± SD[b] (μm^2)	% increase[c]
Freeze-substitution	PL = CW	203 ± 47 a	
Aqueous fixation, dry	PL	350 ± 92 b	41
	CW	463 ± 106 c	55
Aqueous fixation, imbibed	PL = CW	492 ± 131 c	55

[a] PL = CW, contact between plasmalemma and cell wall was maitained; PL and CW, cell area was measured separately within plasmalemma and within cell wall since loss of contact occurred
[b] Values with the same letters are not significantly different as established by one-way analysis of variance (least significant difference, $P < 0.05$) and multiple-range test (least significant difference)
[c] Values represent percentage of the mean area occupied by corresponding freeze-substituted cells

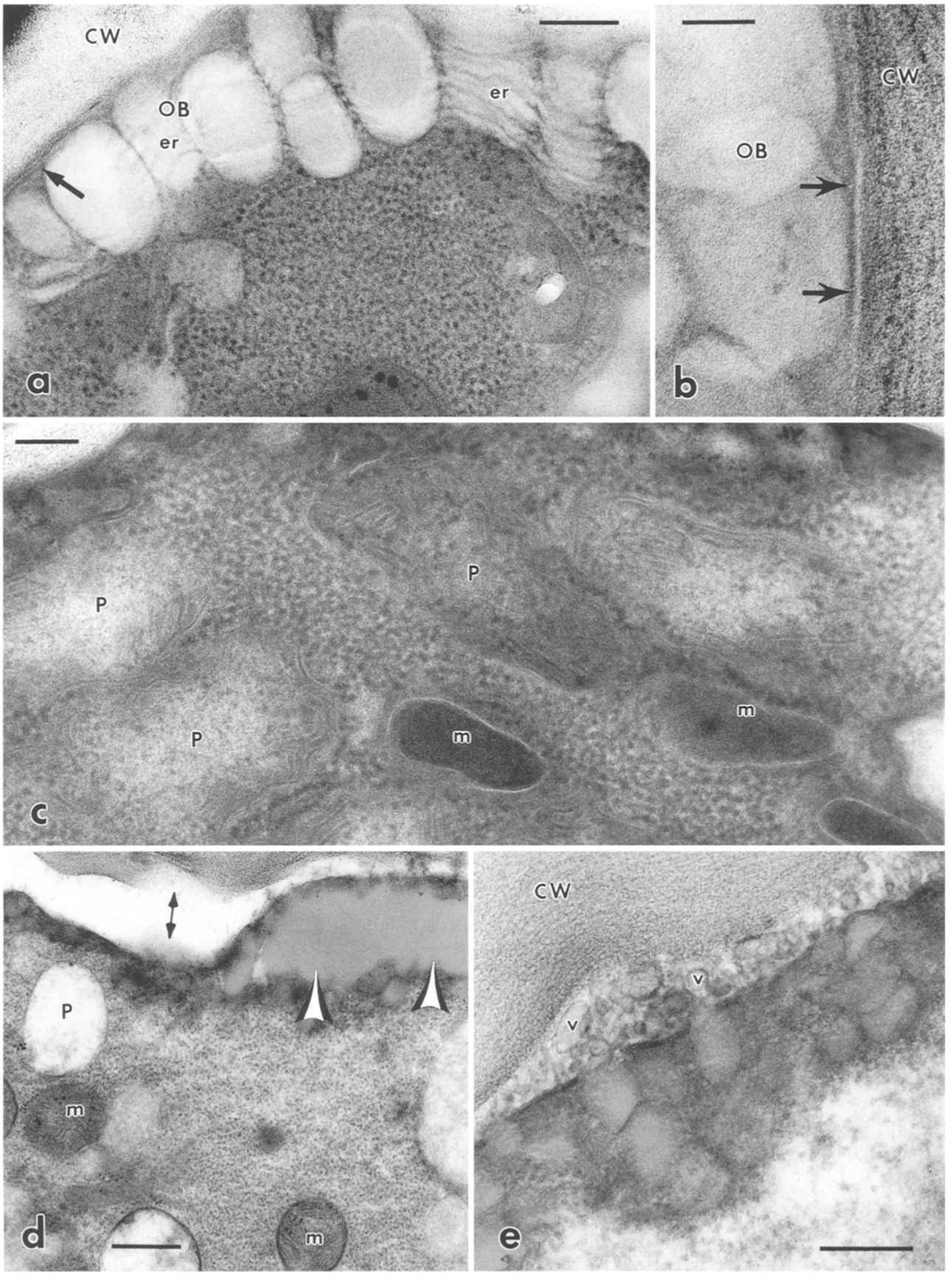
Desiccation-sensitive axes: jackfruit

Cells of cortical tissues dehydrated to 0.7 g of water per g of dry mass and processed by FS appeared tightly packed and bound by moderately folded cell walls. The cytoplasm of these cells had a dense, compacted appearance, and vacuoles were prominent and irregularly shaped (Fig. 2 a). CF of axes at similar water content resulted in relatively rounded contours of cells and vacuoles and in the appearance of spaces between the plasmalemma and cell walls of corresponding cortical cells (Fig. 2 b). The area within the cell wall of the outermost 15 layers in transverse sections of CF axes showed an increase of ca. 20% over those from corresponding FS specimens. Student's t-test showed this increase to be significant ($P < 0.05$; data not shown).

Resurrection plants: *E. nindensis*

The highly folded appearance of the walls of mesophyll cells from air-dried *E. nindensis* leaves was evident after FS even at the LM level (Fig. 3 a). Mesophyll cells stained intensely relative to their CF

Fig. 4 a–e. TEM of dry pea hypocotyls. **a** FS. Oil bodies (*OB*) lined the clearly trilaminate plasmalemma (arrow) and were closely associated with cisternae of ER (*er*). Bar: 0.2 µm. **b** FS. Plasmalemmas appeared intact and closely appressed to cell walls (*CW*). Bar: 0.1 µm. **c** FS. Densely staining mitochondria (*m*) and plastids (*P*) showing internal differentiation were observed. Note the even distribution of ribosomal subunits in the cytoplasm. Bar: 0.1 µm. **d** CF. Separation of the plasmalemma and cell wall (double-headed arrow) and coalescence of oil bodies (arrowheads) were frequently observed following aqueous fixation. Note the swollen appearance of mitochondria (*m*) and plastids (*P*) lacking internal structure. Bar: 0.5 µm. **e** CF. Numerous vesicles (*v*) presumably originating from the plasmalemma were observed to accumulate between this bounding membrane and the cell wall (*CW*). Ribosomal subunits were irregularly distributed, which gave the cytoplasm a patchy appearance. Bar: 0.25 µm


CW
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b
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m
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counterparts (Fig. 3b). CF of dehydrated leaves resulted in notable expansion of cell walls in these cells (Fig. 3b) and reduced the curled appearance characteristic of *E. nindensis* leaves in the dry state (Vander Willigen et al. 2001). Accurate measurement of cell areas within mesophyll cells was difficult due to the highly convoluted character of cell walls, especially after FS. The average area within the cell wall of FS mesophyll cells was $53 \pm 20\ \mu m^2$, and increased significantly ($P < 0.001$; Student's t-test) by approximately 30% to $76 \pm 20\ \mu m^2$ after CF.

Electron microscopy

Desiccation-tolerant tissues: pea

The cytoplasm of FS cortical cells of pea was densely packed with dissociated ribosomal subunits (Fig. 4a). The plasmalemma of these cells was closely appressed to the walls and showed a continuous, trilaminar appearance in favourable cross sections (Fig. 4a, b). Numerous discrete oil bodies lined the plasmalemma intracellularly, and these were closely associated with tubular structures presumed to be cisternae of endoplasmic reticulum (ER) (Fig. 4a). Similar cisternae were observed in close proximity to protein vacuoles, oil bodies, and other structures within these cells (not shown). Irregularly shaped mitochondria and plastids were observed (Fig. 4c). Mitochondrial matrices were extremely electron dense, which obscured internal detail. On the other hand, membranes within plastids could be resolved clearly in negative contrast against the dense background (Fig. 4c).

CF introduced marked ultrastructural distortion of dehydrated cells. Separation of plasmalemma and cell walls was commonly observed (Fig. 4d), and this was often accompanied by other ultrastructural abnormalities at the cell boundary. Oil bodies previously observed lining the inside of the plasmalemma (Fig. 4a) often fused into large pools that lacked visible boundaries (Fig. 4d, and compare with Fig. 4a). Loss of plasmalemma integrity and extensive vesiculation of this bounding membrane was also observed, with many resultant vesicles situated in spaces between the plasmalemma and the cell wall (Fig. 4e). Oil-body-associated cisternae of ER could not be discerned in CF cells (Fig. 4e). CF also seemed to result in sectioned plastids having rounded profiles (Fig. 4d) and in uneven distribution of ribosomal subunits, which gave the cytoplasm of these cells a patchy appearance (Fig. 4e).

Desiccation-sensitive axes: jackfruit

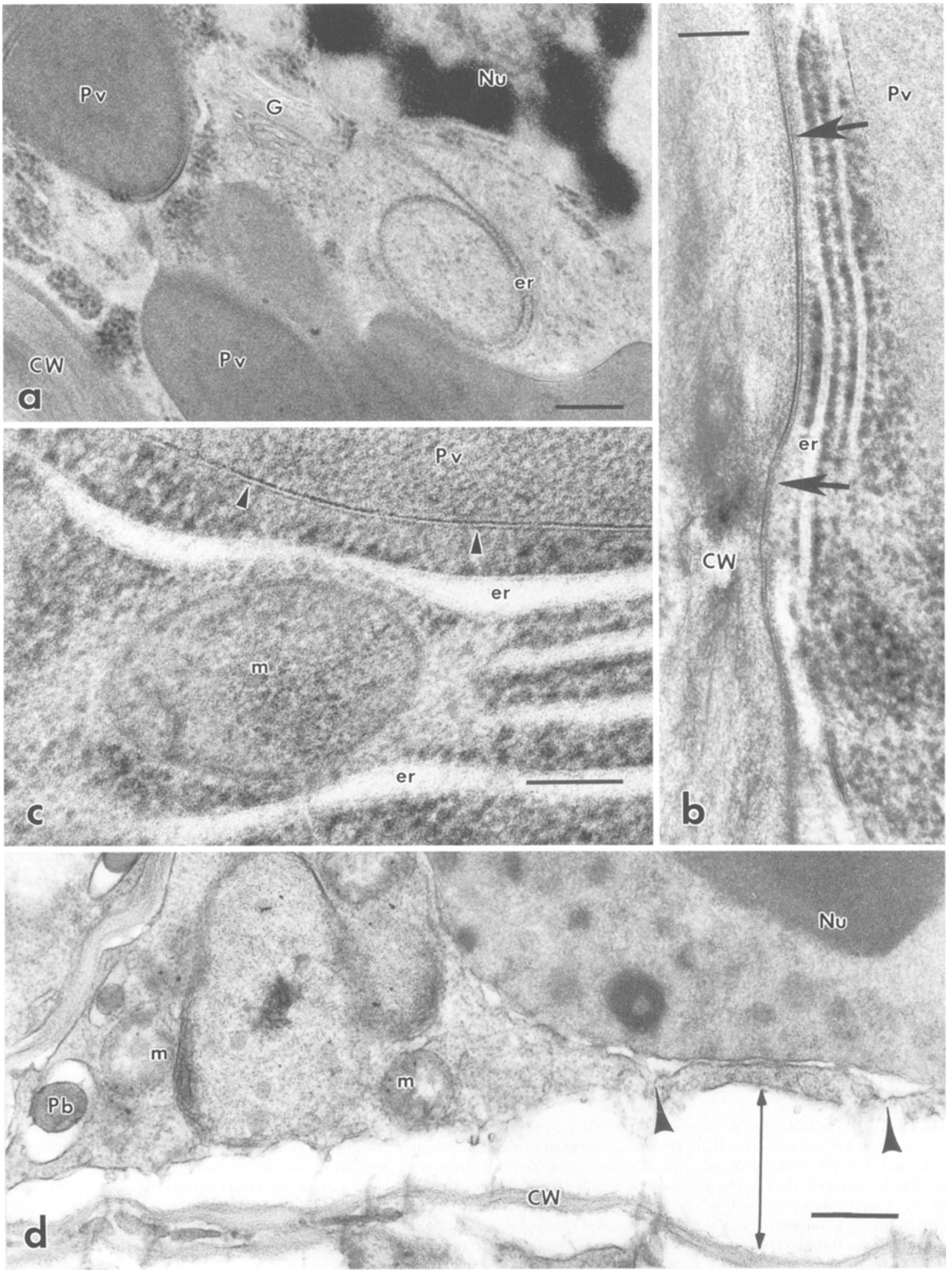
FS of dehydrated cells revealed reduced cell volumes, condensed nuclear chromatin as well as compaction of organelles and other cellular compartments (Fig. 5a), compared with the hydrated condition (not shown). Cell wall folding was common in these cells, but, significantly, separation of the plasmalemma and the wall was not observed (Fig. 5a, b). Furthermore, plasmalemma and tonoplast integrity was invariably maintained in partially hydrated cells prepared by FS (Fig. 5b, c). Ribosomal subunits were evenly distributed, Golgi bodies appeared well developed (Fig. 5a) and close associations of ER profiles with nuclear envelope, plasmalemma, and mitochondria (Fig. 5a–c) were observed.

CF led to the separation of plasmalemma and cell wall in cells of partially hydrated jackfruit axes (Fig. 5d). This separation was almost invariably accompanied by severing of plasmodesmatal connections between cells, tearing of the plasmalemma, and structural abnormalities that occasionally included irregularity between the membranes of the nuclear envelope (Fig. 5d). Membrane-bound organelles appeared distended after CF, and mitochondria also showed electron-translucent areas that were not observed in FS preparations (Fig. 5d).

Resurrection plants: *E. nindensis*

Mesophyll cells from FS leaves of *E. nindensis* showed extensive wall folding, but no separation of

Fig. 5a–d. TEM of cortical cells of jackfruit. **a** FS. Nuclear chromatin (*Nu*) appeared condensed, and cellular compartments were brought into close proximity by the drying treatment. In spite of this, the integrity of Golgi stacks (*G*), protein vacuoles (*Pv*), and plasmalemma was maintained. Bar: 0.2 μm. **b** FS. Plasmalemmas (arrows) were intact and closely appressed to cell walls (*CW*) in partially hydrated cells. Cisternae of ER (*er*) were frequently observed in close proximity to the plasmalemma. Bar: 0.1 μm. **c** FS. Protein vacuoles (*Pv*) were delineated by well-defined bounding membranes (arrowheads). ER cisternae (*er*) were also found in close association with protein vacuoles and oval mitochondria (*m*). Bar: 0.1 μm. **d** CF. Separation of the plasmalemma from walls was observed following aqueous fixation (double-headed arrow). Tearing and vesiculation of the plasmalemma and severing of plasmodesmatal connections were observed. Underlying structures were also affected by CF, as evidenced by the localised distension of the nuclear envelope (arrowheads), protein bodies (*Pb*), and mitochondria (*m*), which also showed abnormal clearings within their matrices. Bar: 0.5 μm


Pv
G
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plasmalemma and cell wall was observed (Fig. 6a). In many cells a large central vacuole reduced the cytoplasm of mesophyll cells to a narrow band along the periphery (Fig. 6a). Vacuoles were irregularly shaped, and in bundle sheath cells these often interdigitated with adjacent plastids containing very large starch grains (Fig. 6b). Dehydration induced severe compaction of the cytoplasm and organelles in cells of *E. nindensis*, which also gave membranes a negatively stained appearance (Fig. 6c, d). In spite of this compaction, structural integrity was apparently maintained, as illustrated by the favourable preservation of mitochondria (Fig. 6c) and the orderly distribution of thylakoid membranes in sections of chloroplasts (Fig. 6d).

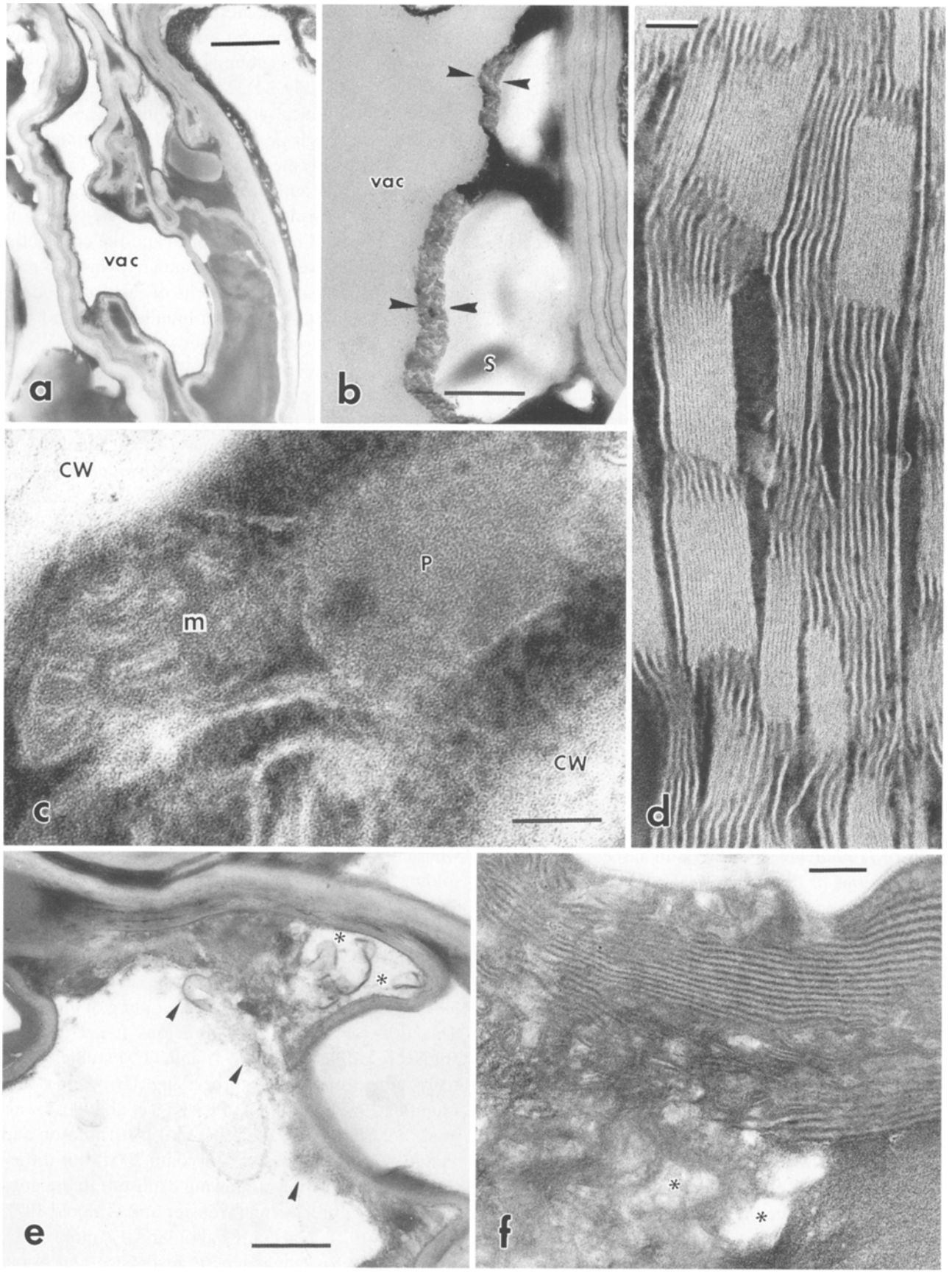
The orderly ultrastructural preservation observed after FS was absent from CF leaves. Tonoplasts frequently appeared ruptured, leading to mixing of vacuolar and cytoplasmic contents (Fig. 6e). Irregularities of the plasmalemma (Fig. 6e), disruption of chloroplast structure, and localised cytoplasmic clearing were also commonly observed (Fig. 6f).

Discussion

The occurrence of rehydration artefacts during fixation of dehydrated cells in aqueous media has been recognised since the initial study of Perner (1965). In spite of this, aqueous fixation has continued to be used in desiccation studies, possibly due to expediency in contrast to the relatively more demanding anhydrous protocols. The uncertainty regarding the magnitude of rehydration that occurs during aqueous fixation prompted the present study. The assessment of rehydration artefacts by freeze-substitution was facilitated by the increasing number of works outlining this procedure (e.g., Robards and Sleytr 1985, Gilkey and Staehelin 1986, Lancelle et al. 1986, Kellenberger 1991, Echlin 1992, Parthasarathy 1995). Results from the present investigation showed that similar kinds of artefacts were found in dehydrated tissues of widely different origins following aqueous fixation, viz., separation of the cell wall and the plasmalemma, membrane irregularities, and distortions of cellular substructure. These are considered below.

Separation of plasmalemma and cell walls was not observed in cells that had been freeze substituted (Figs. 1a, 2a, and 5b) or imbibed prior to CF (not shown) but was evident in dehydrated samples after CF (Figs. 1b, 2b, 4d, and 5d). This indicates that the close apposition between plasmalemma and cell walls observed in FS tissues had been maintained during imbibition and was not altered during subsequent CF. Swift and Buttrose (1972) proposed that during aqueous fixation of dehydrated tissues, a hydration front precedes that of the cross-linking chemical, resulting in ultrastructural changes that resemble early stages of imbibition. Hallam (1976) reported cell walls to "spring away" from the cytoplasm during fixation, and later Öpik (1980) added to this view by suggesting that cross-linking chemicals restrict the expansion of protoplasts during this process. While there is no direct evidence to support the existence of such hydration fronts, differences between the composition of cell walls and the protoplasm are likely to result in different responses to aqueous fixation. Glutaraldehyde and osmium tetroxide are efficient cross-linking agents of proteins and lipids, respectively (Weakley 1981, Bullock 1984), and therefore affect predominantly the plasmalemma and cellular contents without stabilising cell wall components. The folded conformation of walls in dehydrated cells (as observed in FS preparations) appears to relax during CF (compare Figs. 1a, 2a, and 3a with Figs. 1b, 2b, and 3b). This observation is supported by the study of Lott (1974), which showed that cell wall conformational changes were determined by the water status of the tissue and not influenced by glutaraldehyde fixation itself. It is possible that while the plasmalemma and cellular contents are immobilised early during fixation, expansion of the cell wall in the presence of water leads to loss of contact (Figs. 1b, 2b, 4d, and 5d) and possibly to the

Fig. 6a–f. TEM of transverse sections of dehydrated leaves of *E. nindensis*. **a** FS. Mesophyll cells showed extensive infolding of cell walls and large central vacuoles (*vac*) that limited the cytoplasm to a narrow peripheral band. Bar: 1 μm. **b** FS. Vacuoles (*vac*) had irregular outlines, and in bundle sheath cells vacuoles often interdigitated with neighbouring plastids (arrowheads) containing prominent starch grains (*s*). Bar: 0.5 μm. **c** FS. The cytoplasm of mesophyll cells appeared compacted and electron dense. The extent of this compaction is illustrated by the close proximity of a well-preserved mitochondrion (*m*) and plastids (*P*) between cell walls (*CW*). Bar: 0.1 μm. **d** FS facilitated the orderly preservation of structure, such as thylakoids of chloroplasts. Bar: 0.1 μm. **e** CF. Tonoplast dissolution (arrowheads) and plasmalemma discontinuities (asterisks) became apparent after aqueous fixation. Bar: 0.5 μm. **f** CF. Distortion of chloroplast structure and clearings in the cytoplasm (asterisks) were also frequently observed. Bar: 0.25 μm


vac
a
vac
S
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P
m
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c
e
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plasmalemma abnormalities observed (Figs. 4e and 5d). Therefore, the description of the phenomenon commonly referred to as "withdrawal of the plasmalemma from the cell wall" as a sign of desiccation stress (or injury) could be inaccurate and should be revised. Instead, this separation is likely to reflect the artefactual expansion of the cell wall away from the plasmalemma during aqueous fixation.

Distortion of structures during CF of dehydrated material was not limited to the cell wall, but occurred intracellularly as well. A significant ($P < 0.05$) increase in the area enclosed by the plasmalemma was observed in cells from pea radicles (Table 1), and the rehydrated appearance of cells from jackfruit axes and leaves of *E. nindensis* was also evident even at the LM level (Figs. 2b and 3b). Ultrastructural evidence from this and other investigations shows that plastids and mitochondria acquire a relatively rounded appearance after CF (Figs. 4d and 5d) (Buttrose 1973, Swift and Buttrose 1972, Vigil et al. 1984, Platt et al. 1997). The close association between cisternae of ER and discrete oil bodies visible in FS cortical cells of pea (Fig. 4a) was not observed after CF (Fig. 4d, e), as reported by others (Öpik 1985, Smith 1991). Extensive fusion of oil bodies occurred (Fig. 4d), and this was also reported after osmium vapour fixation (Öpik 1980). Coalescence of oil bodies is thus not particular to aqueous fixation, but may indicate sensitivity of oleosins to changes in pH (Huang 1992) during fixation. Electron-translucent areas in the ground cytoplasm of pea (Fig. 4e) and in mitochondria of jackfruit (Fig. 5d) were not observed in FS cells and may also be ascribed to artefacts of CF. Loss of vacuolar integrity in mesophyll cells of *E. nindensis* (Fig. 6e) is in agreement with the observations of Platt et al. (1997) on dehydrated cells of the resurrection plant *S. lepidophylla* after aqueous fixation. Collectively, these findings highlight the relatively slow nature of conventional fixation (Mersey and McCully 1978) and changes that can occur during this process (McCully and Canny 1985; reviewed by Gilkey and Staehelin 1986).

In contrast, cryofixation can immobilise cellular process within milliseconds (Staehelin and Chapman 1987, Craig and Staehelin 1988; reviewed by Gilkey and Staehelin 1986), and subsequent processing of cells under anhydrous conditions precludes rehydration artefacts. The plasmalemma of FS cells was invariably appressed to cell walls and appeared intact in all samples examined (Figs. 4b and 5b). The cytoplasm of dehydrated cells appeared compacted and very electron dense, especially in mesophyll cells of *E. nindensis* (Fig. 6c). The cytoplasm was studded with evenly distributed ribosomal subunits in cells of pea (Fig. 4a, c) and jackfruit (Fig. 5c). Mitochondria and plastids were mostly elliptical and well defined (Figs. 4c, 5c, and 6d), although internal detail was sometimes obscured by highly electron-dense matrices. Cisternae of the ER were prominent and clearly resolved in cells of pea and jackfruit (Figs. 4a and 5b, c). Nuclei in jackfruit cells had regular outlines and the chromatin appeared condensed. Tonoplast integrity was also preserved in freeze-substituted cells of jackfruit and *E. nindensis* (Figs. 5c and 6b). In bundle sheath cells of *E. nindensis* clearly demarcated interdigitation was maintained between the vacuoles and surrounding starch-containing plastids. The tightly packed distribution of organelles in mesophyll cells of *E. nindensis* (Fig. 6c) agrees with the findings of Platt et al. (1997) and Thomson and Platt (1997) on *S. lepidophylla*. The highly ordered structure of the chloroplasts attests to the excellent preservation attainable by FS (Fig. 6d). The superior ultrastructural preservation of plant structures attained by cryotechniques is well documented (Staehelin and Chapman 1987, Craig and Staehelin 1988, Kiss et al. 1990, Davidson and Newcomb 2000; for a review, see Gilkey and Staehelin 1986), which enhances the reliability of observations made on tissues prepared by such methods.

Evidence suggests that cellular volume was reduced during dehydration by cell wall folding, irrespectively of whether cells were desiccation tolerant or desiccation sensitive. However, this does not exclude the possibility of plasmolysis *sensu stricto* occurring in species where the rigidity of the cell wall does not allow folding in response to dehydration (Webb and Arnott 1982) or to extra-protoplasmic freezing (Murai and Yoshida 1998). Electron microscopy revealed that the plasmalemma maintains its trilaminar structure, continuity, and apposition against cell walls even at the very low water content of 0.08 g of water per g of dry mass. This is in agreement with previous freeze-fracture studies (e.g., Platt et al. 1994) and TEM studies using truly anhydrous tissue processing (Browning and Gunning 1977; Öpik 1980, 1985; Kiss et al. 1990; Tiwari et al. 1990; Smith 1991; Platt et al. 1997; Thomson and Platt 1997; Davidson and Newcomb 2000) but different from observations following exposure to aqueous media during processing (Webster and Leopold 1977, Thomson 1979, Fincher Chabot and Leopold 1982, Yatsu 1983). Nuclear magnetic resonance and X-ray

studies of orthodox seeds have also shown that membrane integrity is maintained even in the dry state and that membranes retain a lamellar configuration (Seewaldt et al. 1981). Plasmalemma discontinuities and vesiculation were presently observed only following aqueous fixation from the dehydrated state, and particularly in areas where separation of this membrane and the cell wall had occurred (Fig. 3b). These findings provide a caveat that interpreting plasmalemma irregularities at the electron microscope level as a sign of desiccation damage, or as a mechanism to conserve membranes during dehydration, demands careful consideration of the processing conditions (see Platt et al. 1997, and compare, e.g., Webster and Leopold 1977, Fincher Chabot and Leopold 1982, and Sargent et al. 1981 with Staehelin and Chapman 1987, Tiwari et al. 1990, Thomson and Platt 1997).

Results from this investigation are particularly relevant to desiccation studies relating ultrastructure and survival. It is generally agreed that maintenance of plasmalemma and tonoplast integrity is critical for cell survival. Therefore, it seems counterintuitive to attempt reconciling recovery of plants from sublethal desiccation with ultrastructural evidence obtained by aqueous fixation, which almost invariably shows artefacts similar to those described above. It must be stressed that those artefacts are often cited as indicating the nature of dehydration damage in a diversity of plant material by many authors using TEM (e.g., Webster and Leopold 1977, Fincher Chabot and Leopold 1982, Sargent et al. 1981, Sack et al. 1988, Berjak et al. 1989, Schneider et al. 1993, Quartacci et al. 1997; reviewed by Walters et al. 2001). Therefore, conventional preparative methods for microscopy do not allow unequivocal distinction between damage caused by rehydration per se and ultrastructural changes induced during aqueous fixation.

The confounding effects of aqueous fixation on dried and rehydrating vegetative tissues of the lower-order resurrection plant *S. lepidophylla* were previously addressed by Platt et al. (1997). Those authors endorsed the superiority of FS and concluded that ultrastructural damage to dehydrated vegetative cells occurred upon rapid rehydration, either during imbibition of detached leaves in water or during aqueous fixation. Results from the present investigation not only support their findings, but also extend those observations to vegetative cells of an angiosperm resurrection plant (*E. nindensis*) and to developmentally diverse tissues such as embryonic axes of both desiccation-tolerant pea and desiccation-sensitive jackfruit seeds. Additionally, measurement of cell areas provides convincing evidence of the distortion of cell structure introduced during aqueous fixation and of the unreliability of ultrastructural information obtained from tissues processed in this way.

In conclusion, FS is ideally suited for ultrastructural investigations of dehydrated plant tissues. The low water content in desiccated samples reduces, or even precludes, the biggest obstacle to any cryotechnique, viz., the distortion of ultrastructure due to intracellular crystallisation during cooling. Similarly, recrystallisation during freeze-substitution at or above −80 °C is also unlikely due to the high solute concentration within dehydrated cells. A drawback associated with FS is the likely extraction of lipids in organic solvents (Lancelle et al. 1985, Lancelle and Hepler 1992, Thomson and Platt 1997). Additionally, obtaining adequate thin sections can be challenging due to inadequate resin infiltration into cells. However, this problem is common to most anhydrous protocols for plant tissues using resin embedding (e.g., Öpik 1980, Smith 1991, Thomson and Platt 1997). Ironically, this obstacle may arise from the superior preservation of the plasmalemma attained by such methods. Nevertheless, while FS is technically more demanding than conventional aqueous fixation, the resultant improvement in the preservation of structure in the dry state (e.g., Tiwari et al. 1990, Platt et al. 1997, Thomson and Platt 1997) more than rewards the effort invested.

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