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Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive oilseeds

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Abstract. In order to clarify further the physiological role of oleosins in seed development, we characterized the oil-body proteins of several oilseeds exhibiting a range of desiccation sensitivities from the recalcitrant (*Theobroma cacao* L., *Quercus rubra* L.), intermediate (*Coffea arabica* L., *Azadirachta indica* A. Juss.) and orthodox categories (*Sterculia setigera* Del., *Brassica napus* L.). The estimated ratio of putative oleosins to lipid in oil bodies of *Q. rubra* was less than 5% of the equivalent values for rapeseed oil bodies. No oleosin was detected in *T. cacao* oil bodies. In *A. indica* cotyledons, oil bodies contained very low amounts of putative oleosins. Oil bodies both from *C. arabica* and *S. setigera* exhibited a similar ratio of putative oleosins to lipid as found in rapeseed. In *C. arabica* seeds, the central domain of an oleosin was partially sequenced. Using a low temperature field-emission scanning electron microscope, the structural stability of oil bodies was investigated in seeds after drying, storage in cold conditions and rehydration. Despite the absence or relative dearth of oleosins in desiccation-sensitive, recalcitrant oilseeds, oil bodies remained relatively stable after slow or fast drying. In *A. indica* seeds exposed to a lethal cold storage treatment, no significant change in oil-body sizes was observed. In contrast, during imbibition of artificially dried seeds containing low amounts of putative oleosins, the oil bodies fused to form large droplets, resulting in the loss of cellular integrity. No damage to the oil bodies occurred in imbibed seeds of *Q. rubra*, *C. arabica* and *S. setigera*. Thus the rehydration phase appears to be detrimental to the stability of oil bodies when these are present in large amounts and are lacking oleosins. We therefore suggest that one of the functions of oleosins in oilseed development may be to stabilize oil bodies during seed imbibition prior to germination.

Key words: Desiccation sensitivity – Imbibition – Oil body – Oleosin – Recalcitrant seed – Seed storage

Introduction

All seeds store lipids in the form of small spherical intracellular organelles, termed "oil bodies", which generally range in size between 0.5 and 2.0 μm in diameter. Oil bodies are composed of a matrix of triacylglycerols, surrounded by a phospholipid monolayer, and a set of interfacial proteins termed "oleosins" (Huang 1992, 1996; Murphy 1993). Oleosins are small alkaline proteins of 15–26 kDa, which represent 0.5–4% (w/w) of the oil body. Oleosins are characterised by a highly conserved hydrophobic domain of about 70 non-polar residues, which is flanked by more-polar N-terminal and C-terminal amphipathic domains. The central hydrophobic domain is embedded into the aprotic oil-body interior whereas the terminal domains are probably located on the surface of the oil body. The ratio of oleosin to storage oil is sufficient for the oleosins to form a continuous mono-molecular coating around the entire surface of the oil bodies (Huang 1992; Tzen and Huang 1992). It is believed that oleosins are a major factor in maintaining the integrity of oil bodies as discrete organelles in aqueous solution and that the ratio oleosin:oil will determine the oil-body size (Tzen and Huang 1992; Cummins et al. 1993; Ting et al. 1996). It has been proposed that one of the physiological roles of oleosins in oilseeds is to prevent the coalescence of oil bodies as they become evermore closely appressed to one another during maturation drying (Huang 1992; Cummins et al. 1993). Hence, the presence of oleosins enables oil bodies to be maintained as relatively small and

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Abbreviations: LTFESEM = low-temperature field emission scanning electron microscope; RH = relative humidity; WC = water content

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discrete organelles with a relatively large surface area:volume ratio. This would then facilitate the rapid mobilisation of the oil bodies following germination, since the activity of lipases is directly proportional to the interfacial surface of their substrate, i.e. the oil bodies. Mesocarp tissues of olive and avocado are lacking in oleosins and accumulate large oil droplets of over 20 μm (Ross et al. 1993). However, these tissues neither undergo desiccation, nor store lipid as a long-term energy reserve. In contrast to the mesocarp tissues, the seeds of such species contain both small oil bodies and plentiful oleosins and undergo germinative mobilisation of their storage oil. Thus oleosins appear to be required only in tissues which store oil for subsequent mobilisation.

It is believed that oleosins are ubiquitous in oil-storing seeds (Huang 1996). Oleosin genes and/or proteins have been found and characterised in all oilseed species that have been analyzed to date (Ross and Murphy 1992; Ross et al. 1993; Tzen et al. 1993). The species analyzed so far produce seeds that undergo drying during maturation and can be stored safely for long periods in dry, cold conditions. Such behaviour has been referred to as orthodox (Roberts and Ellis 1989). However, there are many examples of other oilseed species, including important crops such as coffee, cocoa and neem, which do not conform to the orthodox behaviour (Roberts and Ellis 1989; Ellis et al. 1991). On the one hand, seeds (e.g. cocoa) may not undergo extensive desiccation during the later stages of development. They are usually killed at high water contents (WCs) and low temperatures, and are termed "recalcitrant". On the other hand, seeds of certain species (e.g. coffee and neem) do undergo desiccation to WCs roughly similar to those survived by orthodox seeds but are sensitive to storage temperatures below 0 °C. They belong to the "intermediate" behaviour (Ellis et al. 1991). There are currently no standard protocols designed for the long-term storage of recalcitrant and intermediate seeds, except for a few successful instances with cryopreservation (Pritchard et al. 1995). Preliminary observations have indicated that oil-body proteins may be lacking from a few recalcitrant oilseeds (Murphy et al. 1995). With the increasing concern of maintaining the genetic diversity of plants, factors involved in stabilizing cell constituents during drying and long-term storage must be assessed. This study aims to characterize the oil-body proteins in a range of oilseeds exhibiting different desiccation sensitivities and storage behaviours with a view to establish the physiological role of oleosins. The structural stability of oil bodies both during drying and storage in unfavourable conditions and during rehydration was also assessed. The main problem posed by examining the effects of drying and rehydration is that most techniques require the sample to be rehydrated before analysis. In this report, this was overcome by using a low-temperature field emission scanning electron microscope (LTFESEM). This technique allows direct observation of frozen specimens with great resolution without altering their WC or inducing changes in their ultrastructural morphology (Yaklich et al. 1996). Furthermore, much larger freeze-fracture/etched areas can be obtained compared with freeze-fracture procedures in which topographical replicas are observed by transmission electron microscopy. We report that oil bodies in recalcitrant oilseeds contain small amounts or no putative oleosins. Despite the absence or relative dearth of putative oleosins, oil bodies in such seeds appear relatively stable after various drying and storage experiments. However, rehydration of dried material appears to be critical to the stability of the large oil bodies containing low amounts of oleosin.

Materials and methods

Plant materials. Seeds of *Sterculia setigera* Del. were retrieved from frozen storage at Wakehurst Place. Commercial lots of *Coffea arabica* L. cv. Geisen (coffee), *Azadirachta indica* A. Juss. (neem; stage yellow-green) were kept in loosely-tied regularly ventilated polythene bags at 16 °C prior to use. Two additional batches of immature *A. indica* seeds were obtained from drupes that were retrieved from short-term storage at the University of Wageningen, The Netherlands. Seeds of *Brassica napus* L. cv. Tanto were obtained from Twyford Seeds, Banbury, Oxon., UK. Desiccation-intolerant seeds of *Quercus rubra* L. (red oak) and *Theobroma cacao* L. (cocoa) were collected at various locations (Table 1) and transported to Norwich. Upon reception, fruit tissues were removed. Immature seeds and seeds showing signs of deterioration or infection were discarded. Water content and germination of seed materials were assessed immediately on receipt, after storage and prior to oil-body analysis and electron microscopy (Table 1)

Seed drying, storage and germination. For the LTFESEM study, rapid drying was carried out by exposing decoated seeds or embryos to a dry air-flow [approx. 3% relative humidity (RH)] at room temperature for 3–7 d. An additional slow drying treatment was imposed on cocoa embryos by exposure to 26% RH at 23 °C for 7 d. For the desiccation sensitivity experiments, drying was achieved by equilibrating the seed material in various conditions: for coffee and neem, 15% RH at 15 °C followed by 10 d exposure over silica gel; for *S. setigera* and rapeseed, 3% RH at room temperature; for cocoa and red oak, 25% RH at 26 °C. Dry seeds of coffee and neem (11–15% WC) were stored in loosely-tied bags at 4 °C for a month followed by two months at room temperature prior to the electron microscope study. To establish their storage behaviour, dry seeds of coffee and neem (4–9% WC) were stored in foil bags at −20 °C for 3 d. For germination tests, 1 to 5 batches of 5–25 seeds were incubated under optimum conditions selected for each species (Table 1). To assess desiccation sensitivity, seeds were removed at different intervals during drying, weighed and allowed to germinate. For the rehydration treatments prior to electron microscopy, seed tissues were imbibed on damp (not moist) filter papers at room temperature in order to avoid imbibitional damage. The WC of the tissues used for the desiccation-sensitivity measurements; the extraction of oil bodies and the LTFESEM study were determined gravimetrically on three to five replicates of seed tissues following 17 h at 103 °C (ISTA 1985).

Preparation of oil bodies, analysis of oil-body proteins and determination of total lipids. Oil-body samples were isolated from various seed tissues (Table 1) by flotation (Murphy and Cummins 1989). The extraction procedure was carried out at 4 °C for *S. setigera*, red oak and at room temperature for neem, coffee and cocoa in order to maintain the oil in its liquid phase. An aliquot of the oil bodies was further washed twice or thrice with 9 M urea, 50 mM Tris-HCl (pH 7.2) in order to remove protein contaminants as described by Millichip et al. (1996). After isolation and/or washing, oil bodies were resuspended in 50 mM Tris-HCl (pH 7.2) at a concentration of 1–2 μg oil bodies/μl and stored at −20 °C prior to

analysis. Oil bodies were delipidated by three washings with diethylether. The lipid fraction was recovered and dried over N_2 and the oil content determined gravimetrically. The protein fraction was resolved by SDS-PAGE on a 12.5% (w/v) and 6% (w/v) polyacrylamide separating and stacking gel, respectively as described in Schägger and von Jagow (1987). Proteins were either stained with Coomassie blue or electroblotted onto a poly(vinylidene difluoride) membrane according to the manufacturer's instructions (Problott; Applied Biosystems, Warrington, UK). Membranes were either probed with an antiserum raised against the rapeseed oleosin (Murphy and Cummins 1989) or used for amino acid sequencing. Bands containing polypeptides of interest were stained by Coomassie blue, excised, dried under N_2 then submitted to N-terminal amino acid sequencing following Edman degradation using an Applied Biosystems 490 "Procise" Protein Sequencer. Relative amounts of oil-body proteins were quantified by scanning densitometry of the stained gels using a Chromoscan 3 (Joyce Loebl, Germany) densitometer. They were calculated from the peak area underneath each of the stained proteins and are expressed as a percentage of peak area of known amounts of rapeseed oleosins used as standards. Total lipids were extracted from the seed tissues, with chloroform-methanol using a modified Folch's method described in Hamilton et al. (1992), and determined gravimetrically.

Electron microscopy. For transmission electron microscopy, ca. 1 mm^3 of cotyledons from cocoa and red oak were prepared and observed as described in Ross and Murphy (1992). For the LTFESEM study, 1–2 mm^3 of seed tissue from each treatment was dissected from the distal 2–4 mm of the endosperm or cotyledons (Table 1). Samples were either used for WC determination or fixed onto a stub with colloidal carbon adhesive. The stub was plunged into N_2 sludge, then affixed to a stub carrier which was consecutively transferred under vacuum at near liquid-N_2 temperature to the cold stage of a cryo-preparation chamber CT 1500-HF Cryotrans System (Oxford Instruments, UK). After heating to −80 °C, specimens were freeze-fractured with a cold scalpel knife, partially freeze-dried then sputter-coated with Pt. Specimens were then conveyed under high vacuum to the cryostage of a scanning electron microscope equipped with a cold field emission electron gun (JSM 6300F; Jeol, Tokyo, Japan). Freeze-etched surfaces of at least two samples per treatment were observed at −180 °C using a 5- or 10-kV accelerating voltage. Digital images were taken at 850–20 000 × magnification and loaded onto a computer for further analysis. Thirty to 600 oil-body diameters per treatment were measured using a semi-interactive imaging analysis system (TIM Image Processing 3.35 TEA; DIFA, Dordrecht, The Netherlands).

Results

Desiccation sensitivity and storage behaviour. The WC, oil content and initial germination of the seeds varied from 5 to 53%, 17 to 52% and 76 to 100%, respectively (Table 1). Seed desiccation sensitivity is shown in Fig. 1. For the purpose of comparison, percentages of survival are expressed as a function of the embryo WC following drying at room temperature. Recalcitrant seeds such as cocoa and red oak lost their viability at WC between 40 and 20% water, respectively. Intermediate coffee and neem seeds started to lose viability when dried below about 10% and 4% water respectively. Although 50% of the DW of neem embryos is lipid in contrast to only 17% in coffee endosperm (Table 1), the desiccation sensitivity of both intermediate species appeared to be similar when the percentages of desiccation tolerance were expressed as a function of their equilibrium RH determined during drying (data not shown). Because the provenance and genetic background can influence storage behaviour of intermediate species (Ellis et al. 1991), the viability of dry coffee and neem seeds during storage

Table 1. Provenance and physiological characteristics of seeds used in this study. Germination was assessed as apparent radicle outgrowth. Values in parentheses refer to the number of seed batches (*n*) and seeds sown, respectively. Water contents were determined gravimetrically in three to five replicates and expressed on a fresh-weight basis. Lipid contents were determined gravimetrically after extraction from three to five seeds and expressed on a dry-weight basis. When applicable, standard errors are given

	B. napus	*S. setigera*	*C. arabica*	*A. indica*	*Q. rubra*		*T. cacao*
					Lot 1	Lot 2	
Source of material Place and date of collection	Commercial UK, May 95	Collected Angola, April 91	Commercial India	Commercial India, August 95	Commercial Netherlands, November 94	Commercial Netherlands, November 95	Glasshouse Reading (UK), May 95
Water content prior to use (% FW)	2.7 ± 0.7	3.2 ± 1.6	12.3 ± 0.2	10.6 ± 0.3	48 ± 4	37 ± 3	53 ± 5
% of initial germination	99 ± 1 ($n = 3, 25$)	70 ± 14 ($n = 2, 10$)	98 ± 4 ($n = 5, 10$)	62 ± 3 ($n = 2, 25$)	76 ± 11 ($n = 2, 25$)	100 ± 0 ($n = 2, 25$)	100 ($n = 1, 10$)
Germination conditions	26 °C, dark	26 °C, 12 h light	31 °C, dark	26 °C, 12 h light	16 °C, dark	16 °C, dark	25 °C, dark
Material for germination	Seed	Seed	Decoated seed	Seed	Depericarped fruit	Depericarped fruit	Embryo
Storage temperature	4 °C	−20 °C	16 °C	16 °C	2 °C	2 °C	16 °C
Storage behaviour	Orthodox	Orthodox	Intermediate	Intermediate	Recalcitrant	Recalcitrant	Recalcitrant
Material of oil-body analysis	Embryo	Endosperm	Endosperm	Cotyledon	Embryo	Embryo	Cotyledon
Water content (% FW)	3.2 ± 0.5	4.8 ± 0.1	11.1 ± 1.6	10.6 ± 0.3	38.1 ± 2.7	29.5 ± 5.0	52.8 ± 4.7
Lipid content (% DW)	41 ± 2	35 ± 1	17.3 ± 0.2	52.3 ± 1.8	19.9 ± 0.1	20.1 ± 0.5	46.6 ± 2.5

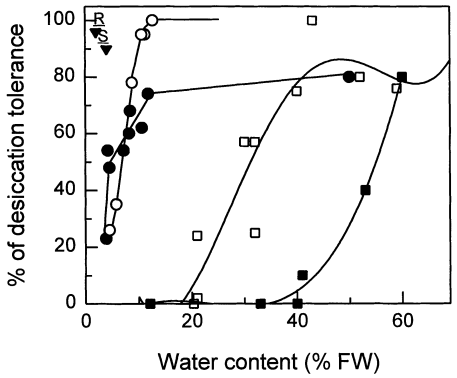

Fig. 1. Relationship between embryo water content during drying and germination in orthodox (rapeseed [▼, R], *Sterculia setigera* [▼, S]), intermediate (coffee [○], neem [●]) and recalcitrant seeds (red oak [□], cocoa [■]). Water contents were determined in triplicate and are expressed as a percentage of FW. Germination data represent the mean of 2 batches of 20–25 seeds. The SE values are around 10% and not shown for clarity. For red oak, additional data are taken from Pritchard (1991) are also shown

at −20 °C was assessed. Neem seeds with 4% water lost ca. 20% germination after 3 d of storage at −20 °C whereas coffee seeds at 9% water lost 75% germination in response to the same storage treatment. *Sterculin setigera*, which is a relative of cocoa and rapeseed, can withstand drying to 3–5% water (Fig. 1) and is freezing-tolerant. Seeds of both species are orthodox and were used as controls.

Recalcitrant seeds contain low amounts or no putative oleosins. The polypeptide profiles of oil-body preparations and the effects of urea washings are shown in Fig. 2. To assist further in assessing the presence or absence of oleosins, polypeptides of molecular masses in a range equivalent to that of oleosins were submitted to N-terminal sequencing (Table 2). The two major oil-body proteins of *S. setigera* were 18-kDa and 26-kDa polypeptides, both of which were urea-resistant and had N-terminal sequences that gave no significant database matches (Table 2). However, the N-terminal regions of seed oleosins are extremely diverse and it cannot be ruled out that either or both of these polypeptides are oleosins. In coffee, the 16-kDa polypeptide was confirmed to be an oleosin of which the central domain was partially sequenced (Table 2). In neem and all three recalcitrant species, oil bodies contained very low amounts of oil-body proteins after urea washing (Fig. 2). Furthermore, despite urea washing to remove artefactually-bound proteins from oil bodies, it was found that most of the remaining oil-body proteins sequenced in neem and recalcitrant species originated from elsewhere in the cell. In neem and red oak cotyledons, the sequences of these oil-body-associated proteins corresponded to known storage proteins which are located in protein bodies. In cocoa, the only oil-body-associated protein, a 20-kDa polypeptide, was found to be a trypsin inhibitor, which is normally a soluble protein (Table 2). The artefactual association of relatively abundant proteins, such as proteases and storage proteins with oil bodies has been

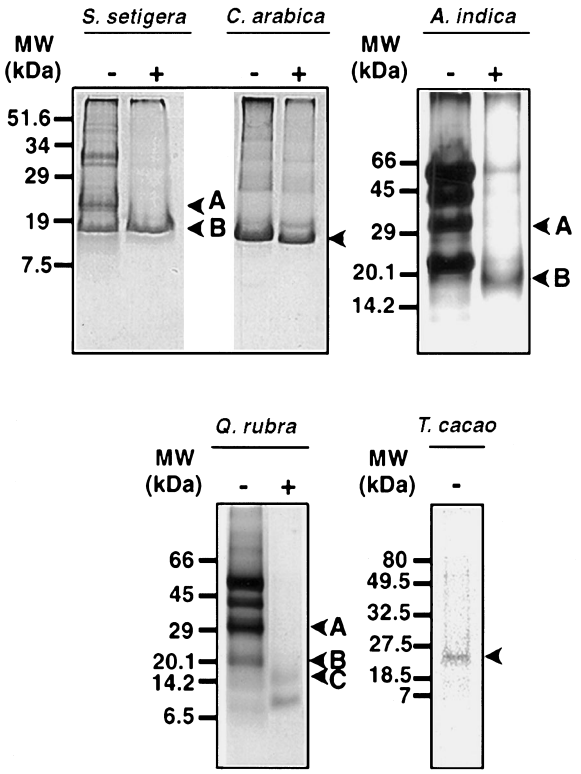

Fig. 2. Analysis by SDS-PAGE of oil-body proteins isolated from the seed of various species and submitted to urea washing (+) or not (–). Labels on the *left* indicate molecular masses (kDa) of standards. Labels on the *right* indicate the position of polypeptides which were N-terminally sequenced. Various quantities of oil body were loaded for every species but the same amounts were used before and after urea washings

reported previously (Kalinski et al. 1992; Millichip et al. 1996). However, Fig. 2 shows that the urea washing is still an unsatisfactory method for removing specifically all protein contaminants and thence ascertain the presence of oleosin. It has been shown that antibodies raised against the oleosin isoform from one seed species may recognize a similar isoform from another species (Tzen et al. 1990). In this study, the antibodies raised against the 19-kDa rapeseed oleosin did not recognize the oil-body proteins from any of our samples following immunoblotting. In neem, polypeptide B isolated after SDS-PAGE was found to be resistant to tryptic digestion (data not shown). This feature is characteristic of the presence of the central domain of oleosins. Because of the low amount of oil-body proteins and the restricted availability of the seed material, it was not possible to extend further a tryptic-digestion study to other species. As a result of these observations, it is likely therefore that in neem and recalcitrant seeds, the oil bodies contain either small amounts of oleosins, or even none at all.

Without confirmation of the presence of oleosins in neem and red oak, it was important at least to estimate the ratios of oil:protein in intermediate and recalcitrant species and compare them with their desiccation-tolerant counterparts. Table 3 indicates clearly that the

Table 2. Amino acid sequence analysis of putative oleosins separated on SDS-PAGE and comparison with published sequences. Labels refer to polypeptides shown in Fig. 2

Species	Polypeptide		Amino acid sequence	Protein from protein data base with similar amino acid sequences	Highest scores
	Label	Mass (kDa)			
S. setigera	A	26	SEQRHLDQQQKIARFSFRHDLIA	None	No significant match
	B	18	RLXE(+R)[a]SKSHTFQ(+K)[a]GLXLSN	None	No significant match
C. arabica	–	16	PLLVIFSPVLV	Oleosin, central domain	100% identity with thale cress and almond
A. indica	A	30	LEETFCTMKLREN	Glutelin (Type 2)	78% identity with lupin
	B	19	SEQYQLTLAIYLLLTQAG	None	No significant match
Q. rubra (lot 1)	A	33	GIEETLXTLRLRENIHDPSR	Legumin	95% identity with *Quercus robur*
	B	22	RITXTQVSKSLTPXLTYXKS	Non-specific lipid-transfer protein	50% identity with *Amaranthus candatus*
Q. rubra (lot 2)	C	15.5	YQ/[b]LTL/[b]QAI	None	No significant match
T. cacao	–	20	ANSPVLDTDGDELQTGVQYYVLSSIIGAAGG GLALXRA	Trypsin inhibitor	100% identity with *T. cacao*

[a]Presence of two isomers
[b]Unsettled alternative at the sequencing HPLC output

oil-body protein levels in neem and the recalcitrant seeds were less than 10% of the amount of equivalent rapeseed oleosin.

Putative oleosins may stabilize oil bodies during rehydration rather then dehydration. Oil-body stability during drying and rehydration was studied in situ using LTFESEM. In conjunction with the qualitative observations (Figs. 3–6), the oil-body diameters after each drying or storage treatment were measured (see Fig. 7). In mature orthodox seeds, numerous oil bodies were observed at the freeze-etched surface as small round organelles trapped in the dense and compact cytoplasm (Fig. 3a,b). In *S. setigera* endosperm cells, the morphology of oil bodies or their size did not change significantly after drying (Fig. 7a). Figure 3d shows the extent and location of water following partial rehydration of control (i.e. on receipt of seed material) and dried endosperm cells, respectively. Water was evenly distributed both in the cytoplasm and storage-protein vacuoles. The intercellular space was also partially filled with ice. The oil bodies still appeared as discrete organelles of around 2.5 μm although their morphology was not homogeneous (Fig. 3d). In endosperm cells of coffee seeds, oil bodies were less numerous with a diameter of 2 μm (Fig. 4, Fig. 7b). Following partial rehydration of control seeds or seeds that were either dried to 9% water or stored for a month at 4 °C, the size and morphology of oil bodies in such material remained unchanged (Fig. 4b–e, Fig. 7b). Following either desiccation to this WC, or one month in cold storage, 75–100% germination was recorded.

At the onset of maturation drying, developing cotyledons from neem seeds contained numerous flat oil bodies trapped in non-etchable water (Fig. 5a). During maturation drying, oil bodies remained as discrete organelles (Fig. 5b) but their size progressively increased from 3.4 μm in fresh material to 4.7 μm in dry tissues (Fig. 7c). During imbibition, the hydration pattern in neem cotyledons was similar to that observed in *S. setigera* endosperm cells. Neither oil-body size or morphology changed appreciably. The exposure of mature seeds to 3% RH reduced the WC from ca. 11 to 6% but did not greatly alter the oil-body size or morphology (Figs. 5d, 7d). A similar result was obtained when control neem seeds were exposed to storage for one month at 4 °C followed by two months at room temperature (Fig. 7d). When neem seeds which had been subjected to extreme drying (3% RH) or stored were rehydrated, two symptoms of structural damage were observed in the cotyledons (Fig. 5e and f, respectively). On the one hand, the cytoplasm and various organelles were not uniformly hydrated, unlike the situation observed in coffee and *S. setigera* endosperm. Most of the non-etchable water crystallised in large areas on both cytoplasmic and exoplasmic faces of the plasmalemma. On the other hand, oil bodies underwent fusion and occupied the remaining cellular volume left by ice (Fig. 5e–f). About a four-fold increase in oil-body diameters was measured following partial rehydration of both dried and stored material (Fig. 7d). Both drying

Table 3. Relative amounts of oil-body proteins (excluding identified contaminants and proteins removed following urea washing) in orthodox (O), intermediate (I) and recalcitrant (R) seeds of various species. Relative amounts of putative oleosin were determined by scanning densitometry of stained gels and expressed as a percentage of rapeseed oleosin used as standard for the purpose of comparison. OB, oil body, na, not applicable, nd, not determined

Species	Desiccation sensitivity	Polypeptide see Fig 2	Confirmation of the presence (P) or absence (A) of an oleosin	% of equivalent rapeseed oleosin/ μg OB
B. napus	O	19 kDa	P	100
S. setigera	O	B, 18 kDa	nd	73
C. arabica	I	16 kDa	P	83
A. indica	I	B, 21 kDa	nd	7
Q. rubra	R	B, 22 kDa	nd	4.1
		C, 15.5 kDa	nd	2.5
T. cacao	R	na	A	0

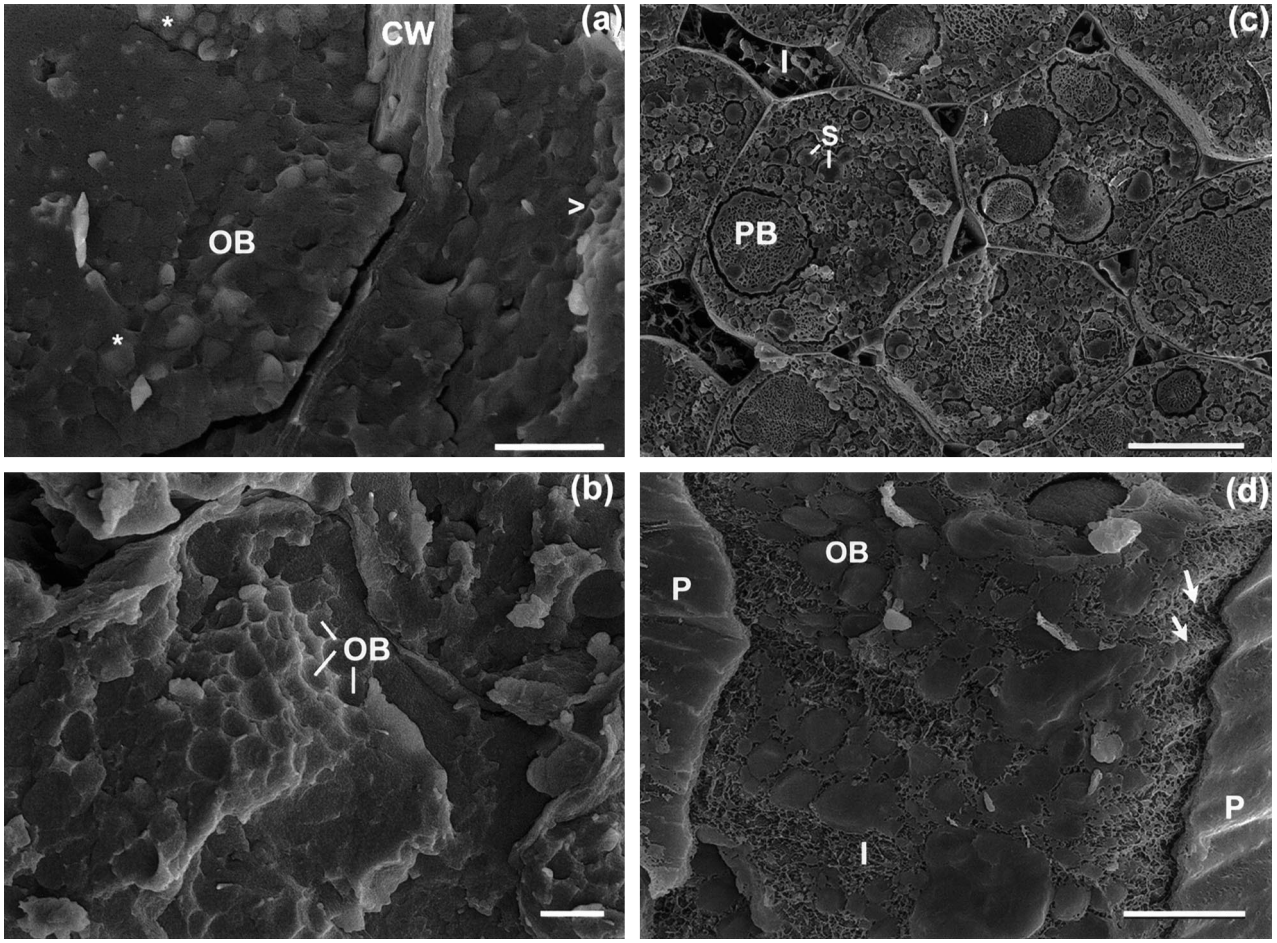

Fig. 3a–d. LTFESEM micrographs of parenchyma cells of cotyledons of rapeseed (**a**) and endosperm of *S. setigera* (**b–d**). **a** Freeze-fracture and partially freeze-dried face of cytoplasm along the cell wall of cotyledon cells at seed maturity (3% WC). Oil bodies appear as small round-shaped organelles emerging out of the dry compact cytoplasm (*) or as imprints (>). ×7100; bar = 2 μm. **b** Detail of an endosperm cell at seed maturity (5% water). ×4250; bar = 2 μm. **c** Cells from mature endosperm rehydrated to 52–58% water. ×1600; bar = 10 μm. **d** Details of the endosperm dried at 3% RH then rehydrated to 48% water showing intact small oil bodies trapped in water. *Arrows* show the undulating plasmalemma leaving an imprint in the ice crystals. ×8250; bar = 2 μm. *CW*, cell wall; *N*, nucleus; *OB*, oil body; *P*, plasmalemma; *PB*, protein body; *S*, starch

and cold-warm storage regimes killed the seeds upon imbibition

Cells of mature cotyledons of cocoa contained discrete oil bodies with a rough, irregular surface and a diameter of around 7 μm (Fig. 6a). Desiccation sensitivity in seeds can be dependent on the drying rate (Berjak et al. 1989; Leprince et al. 1993). The effects of two drying rates on the oil-body stability were investigated in mature cocoa cotyledons. Following a fast drying regime during which water was lost within 24 h, oil bodies were observed as discrete organelles, pressed against one another in groups of two to four (Fig. 6b). After a slow drying which took place over a week, oil bodies were observed as discrete organelles of 6–10 μm in diameter which were packed together but not fused or

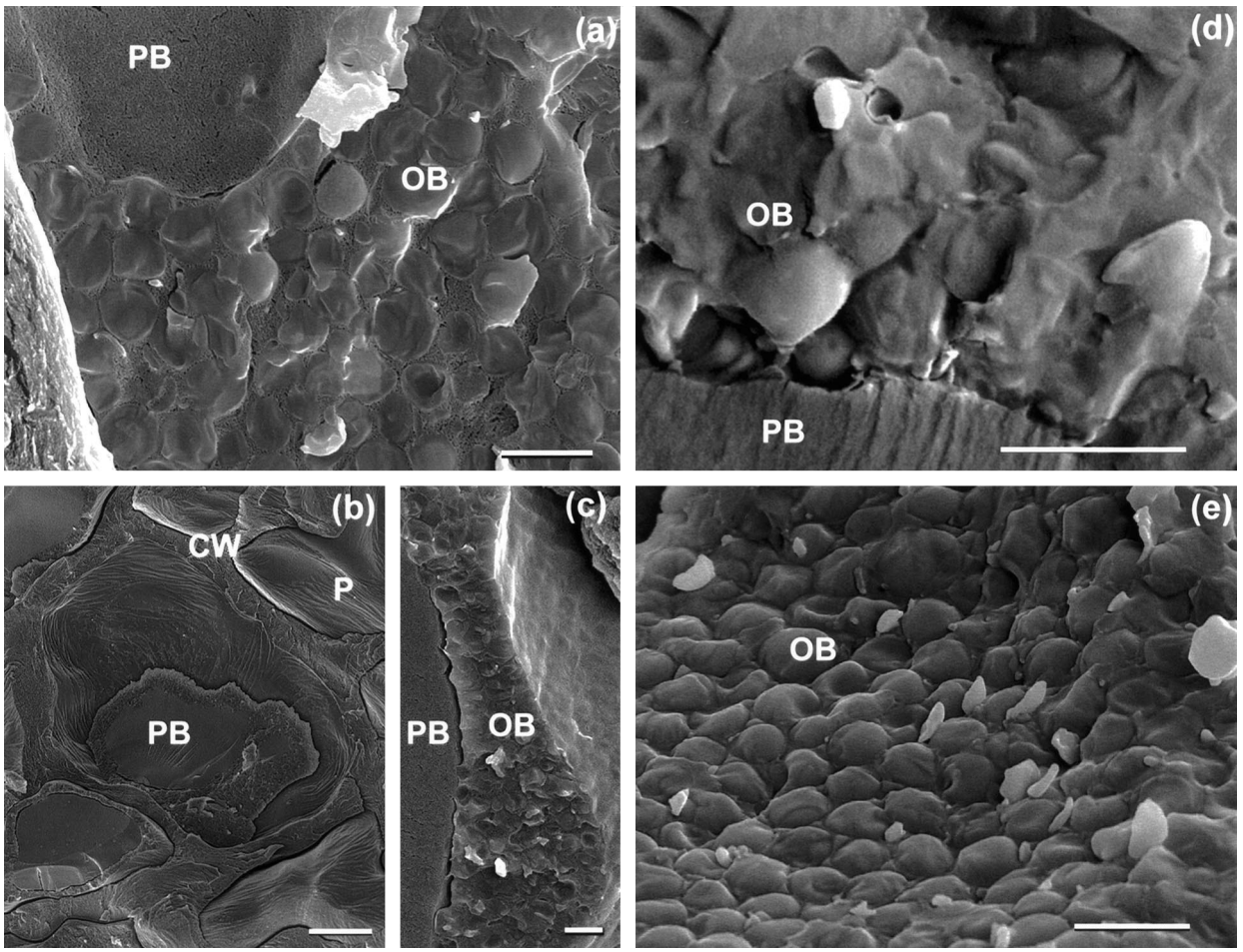

Fig. 4a–e. LTFESEM micrographs of freeze-fractured, partially freeze-dried parenchyma cells from endosperm of coffee. **a** Details of a cell at seed maturity (11% water). ×12200; bar = 1 μm. **b** Endosperm cells of mature seeds rehydrated to 33% water showing folds in the thick cell wall. ×850; bar = 10 μm. **c** Higher magnification of **b** showing individual oil body packed between a protein body and the plasmalemma. ×5000; bar = 1 μm. **d** Endosperm cells from mature seeds dried to 9% water showing dense amorphous cytoplasm containing discrete oil bodies. ×19800; bar = 1 μm. **e** Detailed view of a layer of intact, discrete oil bodies from a partially rehydrated endosperm previously dried to 25% water. ×15500; bar = 1 μm. *CW*, cell wall; *OB*, oil body; *P*, plasmalemma; *PB*, protein body

as much larger ones (10–20 μm in diameter, Fig. 6c). On average a 1.5- and 2-fold increase in oil-body diameter was found after fast and slow drying respectively (Fig. 7e). Rehydration induced major structural damage which was similar regardless of the drying rate (Fig. 6d–f). The oil bodies were fused into one single oil droplet which occupied most of the cellular volume. This process is referred to here as "cytoplasmic phase inversion" because organelles and small hydrated cytoplasmic droplets become completely embedded in oil (Fig 6d–f). Water consequently formed large droplets within the oil and was randomly distributed on both sides of the plasmalemma and cell walls (Fig. 6e).

In red oak cotyledons, oil bodies were in low number and had a diameter of 4–5 μm (Fig. 7f). They were scattered in the cytoplasm along numerous starch granules and protein bodies (data not shown). Neither drying nor the combination of drying and rehydration induced major changes in their size (Fig. 7f) or morphology (data not shown).

Discussion

In-vitro reconstitution experiments have shown that oleosins stabilise oil bodies as small discrete structures in aqueous solutions (Tzen and Huang 1992; Cummins et al. 1993). It is assumed that this is also the case in vivo and that oleosins are particularly important in preventing oil-body coalescence during maturation drying in seeds (Huang 1992, 1996; Murphy 1993). In order to test these assumptions we studied the effects of low moisture conditions on the stability of the oil bodies of different oilseed species. To avoid the artefacts introduced during the preparation of specimens for transmission electron microscopy, we used LTFESEM which preserves the ultrastructural integrity of specimens even at low WC. We also analysed oil bodies isolated from such seeds for the presence of oleosin proteins. Using rapeseed as a reference, we showed that the orthodox oilseed, *S. setigera*, and the intermediate oilseed, coffee, each contain approximately the same ratio of oil-body protein: oil as rapeseed (Table 2). In coffee, the major 16-kDa oil-body protein was confirmed by microsequencing to be an

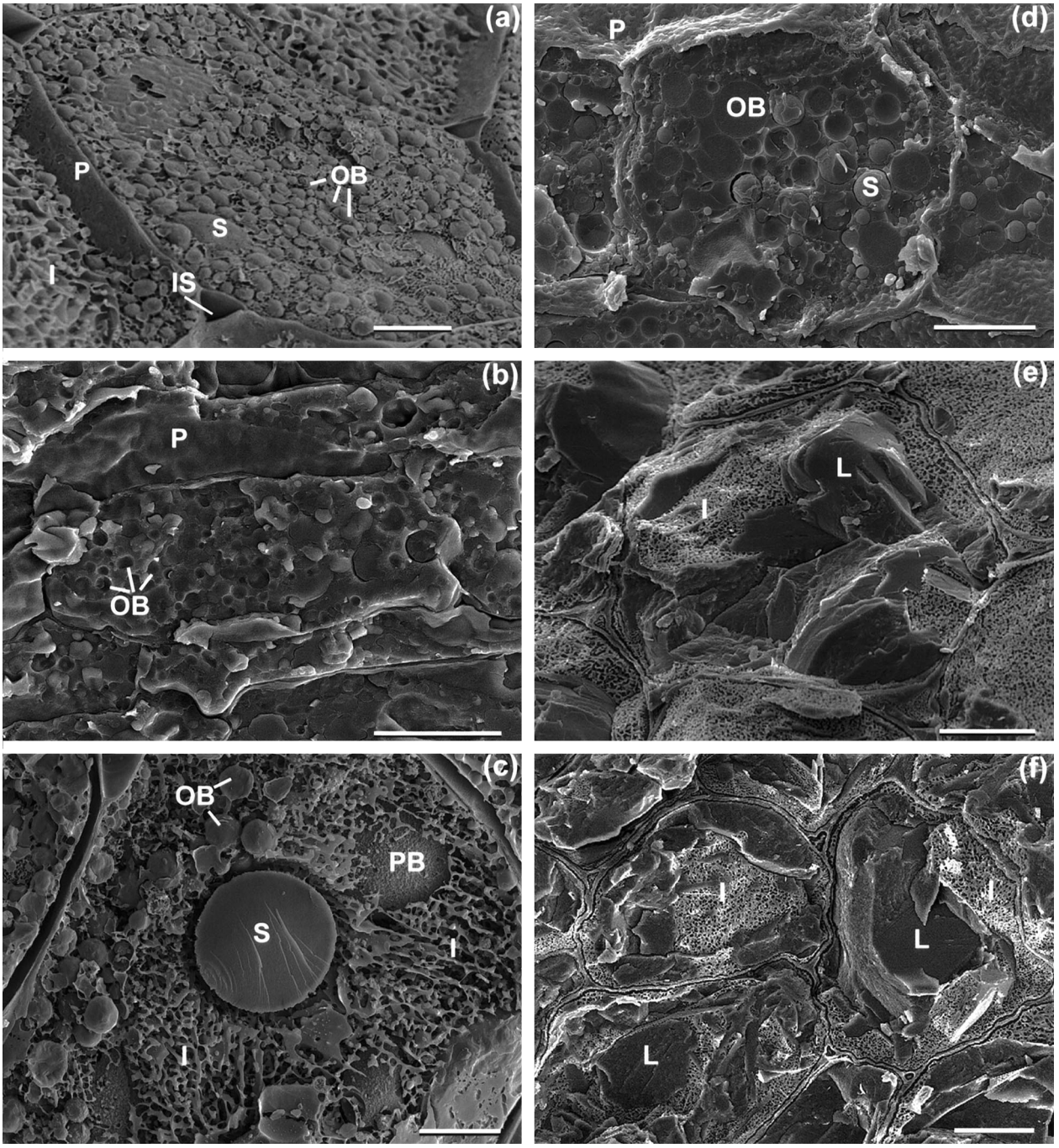

Fig. 5a–f. LTFESEM micrographs of freeze-fractured, partially freeze-dried parenchyma cells from cotyledons of neem. **a** Cells of cotyledons of immature, green seed (73% water). The void in the intercellular space indicates the absence of etchable water. ×2500; bar = 5 μm. **b** Cells of cotyledon of mature, yellow-green seed (18% water). ×4100; bar = 5 μm. **c** Cell of cotyledon of mature seed that was rehydrated to 34% WC. ×2550; bar = 5 μm. **d** Cell of cotyledon of mature seed artificially dried to 6% water. Oil bodies can still be observed as discrete organelles trapped in the dense amorphous cytoplasm. ×3240; bar = 5 μm. **e** Cells of cotyledons of mature seed dried as above then rehydrated to 57% water. In contrast with **c**, water is confined to certain areas of the cell due to non-uniform rehydration. ×1550; bar = 10 μm. **f** Cells of mature seed that was rehydrated to 44% water after storage for a month at 4 °C followed by two months at ambient conditions. Storage and rehydration have also caused coalescence of oil bodies. ×1350; bar = 10 μm. *CW*, cell wall; *I*, ice; *L*, lipid oil; *P*, plasmalemma; *OB*, oil bodies; *S*, starch

oleosin. In neem and both recalcitrant species, we showed that in contrast to rapeseed, coffee and *S. setigera*, the oil bodies contain either very small amounts of oleosins, or even none at all. This confirms earlier observations on oil bodies isolated from recalcitrant seeds of other species (Murphy et al. 1995). Regardless of the oleosin:oil ratio, the oil bodies remained stable during drying of all the species studied. This indicates that factors other than the presence of oleosins are involved in the prevention of oil-body coalescence during maturation drying in seeds. Our data, together with other published evidence, point to two factors which are likely to interact in preventing oil-body

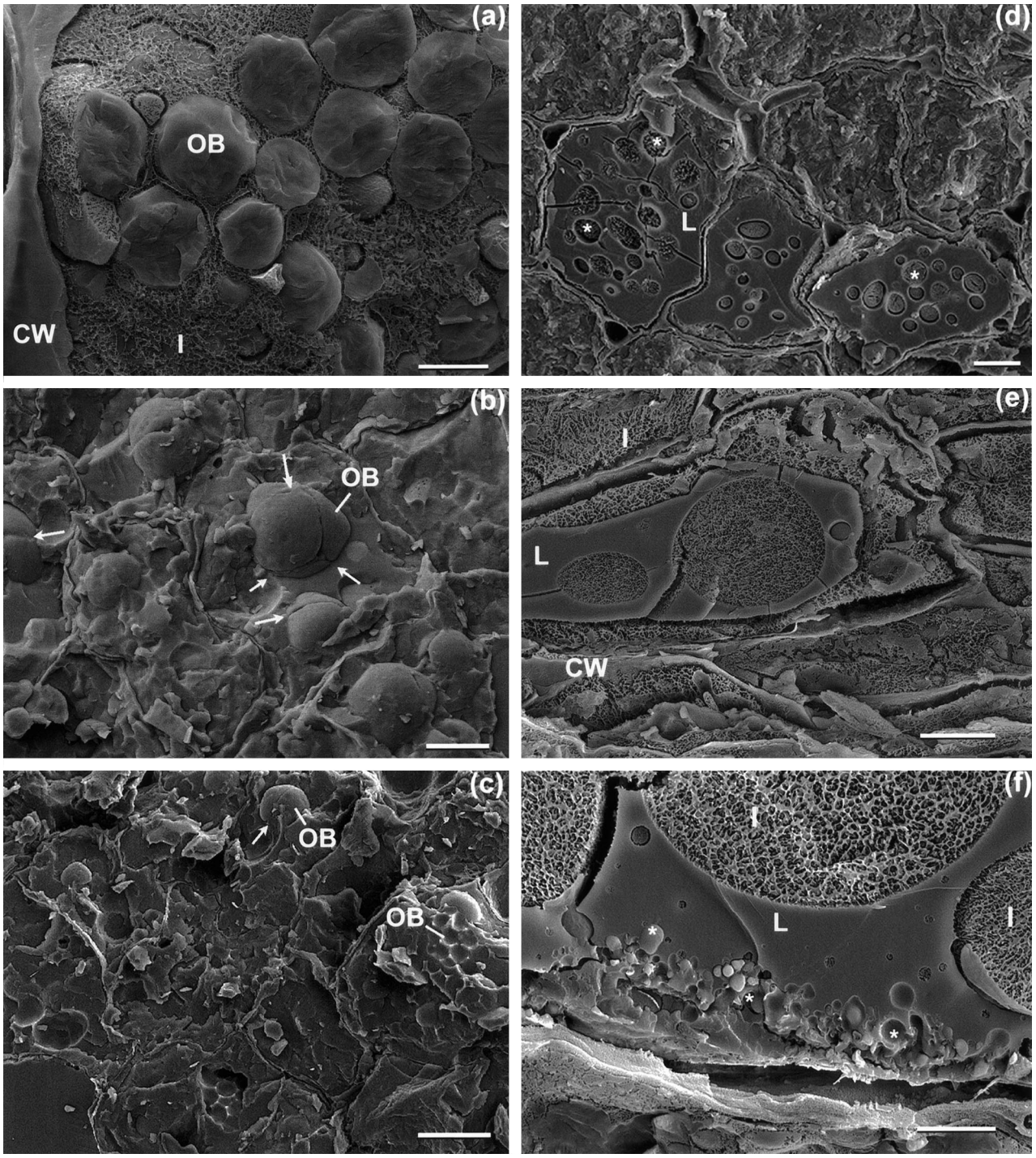

Fig. 6a–f. LTFESEM micrographs of freeze-fractured, partially freeze-dried parenchyma cells from cotyledons of cocoa. **a** Cells of mature cotyledons (60% WC) showing oil bodies trapped in non-etchable water. ×1120; bar=10 μm. **b** Cells from cotyledons after fast drying (6% WC). Oil bodies are pressed against one another but not totally fused (*arrows*). ×1150; bar=10 μm. **c** Cells from a cotyledon that was slowly dried to 12% WC. Note that oil bodies are not fused. ×1250; bar=10 μm. **d** Cells from slowly dried cotyledons that were rehydrated to 48% WC. The oil bodies have coalesced in a single oil droplet within which the cytoplasm has formed inclusions. ×800; bar=10 μm. **e, f** Cells from fast-dried cotyledons that were rehydrated to 56% WC. Note also the abnormal distribution of non-etchable water and the formation of cytoplasmic and ice droplets. ×1250; bar=10 μm (**e**); ×2600 bar=10 μm (**f**). *CW*, cell wall; *I*, ice; *L*, lipid; *OB*, oil body; (*) unidentifiable organelles

coalescence: the high viscosity of the cytoplasm in dried seeds and the rate of drying. During the maturation drying of seeds, the viscosity of the cytoplasm rises by several orders of magnitude as soluble sugars and cytoplasmic proteins become more concentrated. Consequently a glassy state is formed in which all organelles are trapped and protected by an extremely viscous cytoplasm (Williams and Leopold 1989; Leprince and Walter-Vertucci 1995; Wolkers and Hoekstra 1997). In emulsion models, a high viscosity generated by high concentrations of polysaccharides is a key factor in the stabilization of small oil droplets (Mangino 1989). Therefore, in dehydrated seeds, the high viscosity of

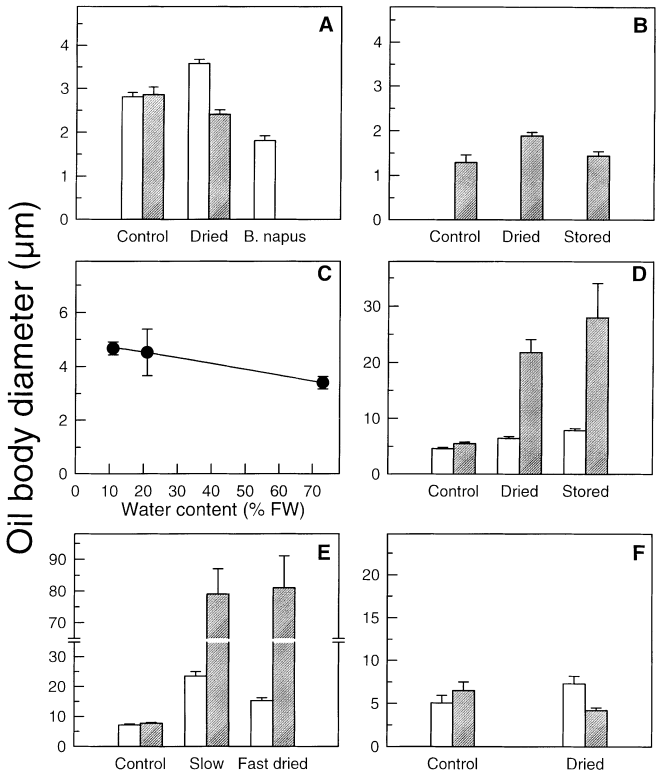

Fig. 7A–F. Oil-body diameters of various species of seeds upon receipt prior to (*Control*) and after various drying or storage treatments, both before (□) and after rehydration (▨). **A** *Sterculia setigera* endosperm and rapeseed cotyledons; **B** coffee endosperm; **C** developing neem cotyledons (stages of development expressed on a WC basis); **D** neem cotyledons (stage yellow-green); **E** cocoa cotyledons; **F** red oak cotyledons. Bars indicate SE. Note the different scales of y-axes

the cytoplasm surrounding oil bodies may prevent their coalescence, even in the absence of oleosins. In addition, the hydrophobic domains of cytoplasmic proteins may also provide an interface between the oil and the cytoplasm and act as a barrier to coalescence as suggested by studies on stabilization of emulsions systems (Barford et al. 1989; Mangino 1989). The drying rates can also influence the oil-body stability, as observed in dried cocoa cotyledons. After fast drying, the oil bodies were still observed as discrete organelles, whereas after slow drying, some coalescence had occurred. Since the rate of drying indirectly controls the increase in viscosity, high viscosities are likely to be generated rapidly during fast drying, hence effectively trapping the oil bodies and preventing their coalescence. In contrast, during slow drying, some of the oil bodies may be appressed together and hence have the opportunity to fuse before the cytoplasm enters a solid-like state.

Rehydration of dried and/or stored oilseeds does not affect the stability of oil bodies which contain large amounts of oleosins such as in coffee or orthodox material. In neem and recalcitrant seeds, the influence of rehydration on the oil-body stability varied greatly according to the species and oil contents. Oil-body coalescence following inhibition was not observed in red oak, which has a lower oil content than neem and cocoa seeds. The relatively low number of oil bodies in red oak cotyledons ensures that they are more dispersed and hence less likely to undergo fusion following imbibition. In seeds containing large amounts of storage oil bodies, but little, if any oleosins, the oil bodies of artificially dried seeds showed a strong tendency to coalesce during imbibition, indicating that oleosins may have a stabilising function during rehydration. Our structural observations indicate that water may have entered the tissues as a stream of liquid. Therefore, during seed imbibition, the flow of water into cells containing large numbers of oil bodies unprotected by oleosins would result in their coalescence in order to reduce the free energy associated with the interfacial tension of the oil at the water interface. Coalescence of the oil droplets into progressively larger oil bodies will disrupt the cellular structure as shown, for example, in rehydrating cocoa cotyledons (Fig. 6d–f). The cytoplasmic phase inversion observed in this material is likely to jeopardise the survival of seed tissues. In emulsion models, phase inversion has been described as a mechanism accounting for emulsion destabilization (Barford et al. 1989; Manginon 1989). In the presence of oleosins, electrostatic repulsion caused by their negative charge and steric hinderance would prevent oil-body coalescence (Tzen and Huang 1992; Cummins et al. 1993). We therefore propose that the major role for oleosins in oilseeds is not directly concerned with protecting oil bodies from the effects of desiccation, but rather with the stabilisation of oil bodies following seed imbibition prior to germination. Therefore, in being important for normal rehydration, oleosins may be indirectly contribute to desiccation tolerance because any desiccation-tolerance test must incorporate a rehydration phase which allows the dried seeds to germinate.

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