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POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 56/2: 35–48, 2014
DOI: 10.2478/abcsb-2014-0019

PAN POLSKA AKADEMIA NAUK ODDZIAŁ W KRAKOWIE

MORPHOLOGICAL, HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES IN FRUIT EPIDERMIS OF APPLE *MALUS DOMESTICA* CV. LIGOL (ROSACEAE) AT FRUIT SET, MATURITY AND STORAGE

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Received December 11, 2013; revision accepted March 10, 2014

Fruit quality is closely related to the structure of the fruit peel, especially the epidermis and cuticle which form a barrier between its internal and external environments. This study used light, scanning and transmission electron microscopy to examine changes in the epidermal structures of fruits of the Polish apple cultivar *Malus domestica* Borkh. cv. Ligol at three maturity stages. The single-layered epidermis was covered with cuticle and crystalline wax platelets. In the fruit set stage the fruit surface exhibited numerous nonglandular trichomes, stomata, microcracks and crystalline wax platelets. The surface of fruits at harvest and after 6-month controlled-atmosphere storage exhibited lenticels and horizontally or vertically oriented wax platelets. During fruit development there was an increase in epidermal cuticle thickness and a reduction in epidermal cell convexity and number of lenticels. After the storage period fruit weight declined, microcracks deepened and widened, and cuticular waxes accumulated. The cuticle was made up of two layers, lamellar and reticulate. In successive fruit development stages the width of the lamellar layer increased considerably. In the fruit set and harvest maturity stages the epidermal cells contained numerous mitochondria and plastids with starch grains. After the storage period there were fewer amyloplasts and the cell vacuoles contained flocculent residue and dark deposits.

Key words: Epicuticular wax, epidermis and cuticle, fruit, *Malus*, microcracks, micromorphology, plant surface, ultrastructure.

INTRODUCTION

China, the United States, India, Turkey and Poland are the major growers of apples (Dobrzańska, 1995; Dobrzańska and Tymolewska, 1995). For successful commercialization, fruits have to meet a variety of stringent quality criteria such as firmness, attractive color, pleasant smell, juiciness, good flavor, proper size and a nongreasy surface (Tsukamoto, 1981; Kader, 1983; van Woensel et al., 1987; Richardson-Harman et al., 1998). Favorable climatic and cultivation conditions, protection against various pathogens, proper timing of the harvest and proper storage conditions are vital in ensuring high fruit quality. Also important are internal factors associated with fruit structure, which influence the length of the storage period and the amount of water lost by the fruit during storage.

During apple development the fruit intensively grows in volume through an increase in the number and size of cells forming its flesh, and physiological processes protect its interior against mechanical damage, adverse weather conditions, pests, and invasion by bacterial and fungal pathogens throughout its life on the tree as well as during its transport, storage and shelf-life (Jenks et al., 1994; Markstädter et al., 2000). Protection is provided by the external layers forming the peel, composed of an epidermis covered with a cuticle and a multilayered hypodermis (Riederer and Schreiber, 1995). Thick walls and close packing of cells make the hypodermis (collenchyma tissue) strong and resistant to compression and deformation, enabling it to support the growing parts (Taiz and Zeiger, 2002). The cuticle on the external periclinal epidermal cell wall has a protective function. The physical structure, chemical composition and functions of the cuticle have been examined by several authors (e.g., Jeffree, 1996; Riederer and Markstädter, 1996; Barthlott et al., 1998). Its main components include the polymer cutin and soluble cuticular lipids forming cuticular waxes (Kolattukudy, 2001). Cuticular waxes may be

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TABLE 1. Mean values of selected features of different apple cultivars, calculated from published data (A – harvest maturity, B – after storage)

Cultivar	A	B	Source
Thickness of the cuticle (μm)			
Jonathan		4.6	Babos et al., 2004
Starking		4.7	
Gala	19.1		Tessmer et al., 2012
Galaxy	18.4		
Gala	20.6		Ghafir et al., 2009
Starking Delicious	13.8		
Star Cremson	12.3		
Golden Delicious	14.3		
Golden Delicious	15.0		Miller, 1982
Jonathan	13.0		
McIntosh	11.8		
Northern Spy	9.8		
Red Delicious	14.3		
Rome	16.4		
Stayman	14.4		
Summer Rambo	13.0		
York Imperial	18.8		
Lobo		19.3	Konarska, 2012
Boskoop		13.6	
Szampion	14.1	16.0	Konarska, 2013
Jonagold	17.2	17.7	
Height of epidermis cells (μm)			
Jonathan		6.7	Babos et al., 2004
Starking		15.1	
Lobo		9.0	Konarska, 2012
Boskoop		13.6	
Szampion	15.2	14.8	Konarska, 2013
Jonagold	17.4	16.3	

Cultivar	A	B	Source
Loss of fruit weight in storage (%)			
Gala		9.1	Ghafir et al., 2009
Starking Delicious		6.3	
Star Cremson		5.8	
Golden Delicious		7.2	
Delicious		0.6	Link et al., 2004
Fuji		1.1	
Szampion		5.5	Konarska, 2013
Jonagold		3.9	
Wax mass (mg·cm^{-2})			
Dougherty	0.53	0.60	Morice and Shorland, 1973
Granny Smith	0.50	0.64	
Sturmer	0.56	0.68	
Oregon Spur I	0.69		Belding et al., 1998
Red Chief	0.75		
Starkspur Red Rome	0.79		
Silverspur	0.57		
Starkrimson	0.64		
Starkspur Supreme	0.77		
Coop-17	0.58		
Liberty	0.37		
Sundalespur Gold	0.96		Belding et al., 2000
Pure Gold	0.88		
Lys Golden	0.92		
Starkspur Supreme	1.03		
Oregon Spur II	1.04		
Starkrimson	0.88		
Smoothie	0.59		

embedded in the cuticle (intracuticular waxes) or extruded on the surface (epicuticular waxes) (Jenks and Ashworth, 2003). According to Barthlott et al. (1998), two types of epicuticular waxes can be distinguished in the apple fruit: amorphous wax forming a continuous film, and crystalline wax platelets of various dimensions and shapes.

During volume growth the epidermal cuticle is ruptured by the formation of lenticels and microcracks, which lowers the fruit's resistance to adverse external conditions (Riederer and Schreiber, 1995; Maguire et al., 1999; Veraverbake et al., 2003a). These changes accelerate fruit aging, reflected in loss of firmness and reduction of fruit weight due to transpiration (Hõhn, 1990; Lau and Lane, 1998; De Bellie, 2000; Link at al., 2004). The results from a number of authors for some of these changes during fruit development in different apple varieties are summarized in Table 1.

In previous work I showed a significant effect of the genetic background on the fruit peel structure of apple cultivars differing in type of surface and length of storage period (Konarska, 2012, 2013). For consumers the most important criterion for determining the attractiveness of apples is their perceived quality; hence the need for research aimed at understanding the mechanisms and processes that determine fruit quality. The traits that determine the shelf-life, firmness and attractive appearance of fruit develop and change over time. In the available literature there is no detailed, comprehensive information on the characteristics of fruit epidermis structure, although they significantly influence fruit quality and suitability for long-term storage. In this

study I examined quantitative and qualitative changes in fruit micromorphology in the apple cultivar Ligol (*Malus domestica* Borkh.) and investigated its anatomy and the ultrastructure of the epidermal cells, cuticle, cuticular wax, and general surface features during initial growth, full harvest maturity, and after controlled-atmosphere storage. Observations employed bright field and fluorescence microscopy as well as scanning and transmission electron microscopy. Particular attention was paid to cuticle damage (interrupted continuity), which potentially could allow the fruit interior to be entered and attacked by a variety of pathogens both on the tree and during subsequent storage. Also assessed was the effect of several-month controlled-atmosphere storage on fruit firmness and weight and on the quantity of cuticular waxes on the fruit.

MATERIALS AND METHODS

Ligol is a Polish winter apple cultivar favored by consumers and apple growers due to its attractive appearance and taste, and its suitability for long-term storage. Ligol fruits are of a type that does not russet and has a smooth, sticky, greasy surface, like cv. Lobo and cv. Jonagold (Konarska, 2012, 2013).

Fruits of the cultivar were examined in 2010–2012 at three stages: stage I – fruit set (fruit diameter 1 cm), harvested 21 days after anthesis (May 15–20 2011 and 2012); stage II – harvested at the preclimacteric stage (late September/early October 2010 and 2011); and stage III – after 6-month controlled-atmosphere (CA) storage (late March/early April 2011 and 2012) under the following conditions: 2% O_2, 3% CO_2, +3°C, RH 90–95%.

The fruits were obtained from a commercial orchard in the Lublin region (SE Poland), characterized by temperate continental climate. The orchard employs conventional methods involving the use of standard mineral fertilizers and chemical plant protection products. Medium-sized, similarly colored, intact, healthy apples were collected from the central part of randomly chosen trees in order to eliminate the influence of differences in insolation and moisture, the effects of climatic factors such as wind and rain, and various effects of fertilizers and plant protection products. While picking, transporting and preparing the apples for SEM, special care was taken to avoid touching the fruit surface area intended for observations, to avoid rubbing off and degrading the wax layer.

For microscopic analysis, fruit fragments with peel were sampled from the blushed equatorial part of the fruit. In each of the three analyzed fruit development stages the number of stomata or lenticels within a 1 cm^2 area of the epidermis in ten fruits was counted using morphology software combined with SEM. The individual weight of 20 apples was determined at harvest and after storage.

SCANNING ELECTRON MICROSCOPY (SEM)

Conventional fixation of material for SEM involves dehydration, which can remove or alter the lipids forming the wax coating on the apple surface, and critical-point drying can shrink and distort tissues (Roy et al., 1999). This work did not follow those methods. Samples of the external part of 10 fruits (5 mm × 5 mm × 2 mm) were cut with a razor blade immediately after the fruits were collected from trees or from storage. The samples were not dried. Only the planes of the cuts were gently wiped before carefully mounting them on aluminum stubs with double-sided carbon tape. Thin strips of tape were also affixed to the edges of the samples. After coating with a 15 nm layer of gold, the samples were examined with a TESCAN/VEGA LMU scanning electron microscope at 30 kV accelerating voltage.

LIGHT MICROSCOPY (LM)

For each of the three fruit development stages, cross-sections were hand-cut with a razor blade through fresh epidermis of 10 Ligol fruits. Then the samples were embedded in glycerol gel on a glass slide and observed under a Nikon SE 102 light microscope. Cuticle thickness and epidermal cell height and width were measured at five sites of each sample. Then the samples were stained with Lugol's iodine to disclose starch, and with a saturated ethanol solution of Sudan III to disclose lipophilic substances. Hand-cut samples were observed with a stereoscopic Nikon Eclipse 90i microscope fitted with a UV filter set (wavelength EX 330–380 nm) stimulating autofluorescence of cuticle and chlorophyll in order to analyze the distribution of those substances. Images were obtained with a digital camera (Nikon Fi1) and NIS-Elements Br 2 software, or with a Zeiss AxioImager Z1 fluorescence microscope, AxioCam MR digital camera and NIS-Elements Br 2 software.

Semithin (0.7 μm) transverse sections (perpendicular to the fruit axis) were cut with a Reichert Ultracut-S ultramicrotome and a glass knife and stained with 1% methylene blue and 1% azure II in 1% aqueous solution of sodium tetraborate. Samples were fixed and embedded in synthetic resin by standard TEM methods (see below). The sections were observed with a Nikon Eclipse 90i microscope.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Small samples (2 mm × 2 mm × 2 mm) of 5 Ligol fruits in each stage (I, II, III) were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde buffered at pH 7.4 in 0.1 M cacodylate buffer. Fixation was performed at room temperature for 2 h followed by 12 h at 4°C. When fixed, the samples were rinsed with 0.1 M cacodylate buffer at 4°C for 24 h and then treated with 1% OsO_4. Then the samples were transferred to redistilled water and stained with 0.5 M aqueous solution of uranyl acetate. After passage through increasing concentrations of propylene oxide in ethanol and finally through pure propylene oxide, the samples were embedded for 12 h in Spurr Low Viscosity resin at 70°C (Spurr, 1969). Ultrathin sections (70 nm thick) were treated with 8% solution of uranyl acetate in acetic acid and with lead citrate (Reynolds, 1963). Samples were observed with an FEI Technai G2 Spirit Bio TWIN transmission electron microscope at 120 kV accelerating voltage. Images were captured with a Megaview G2 Olympus Soft Imaging Solutions camera.

CUTICULAR WAX EXTRACTION AND WAX MASS

The method described by Verardo et al. (2003) was used for extracting wax and determining its mass. Using a calipers, the maximum and minimum latitudinal and longitudinal diameter of six fruits of stages II and III was measured in the laboratory. Based on diameter values, the fruit surface area was roughly estimated considering the apple fruit as a perfect sphere. Next, each of the six fruits in stages II and III was immersed in hexane (1.5 L) at room temperature (22°C) for 45 s (Veraverbake et al., 2001). The hexane was redistilled after every fruit of stage II and III and used again for the other fruits of the same stage. The extract obtained was filtered at 50°C, washed with brine (2 × 50 mL) and dried in anhydrous Na_2SO_4. After evaporation of the solvent at 70°C, precipitated and collected yellow-green wax residues were weighed and counted per unit area of the fruit.

STATISTICAL ANALYSES

For all measured parameters the means (±SD) were calculated. Correlation functions were calculated using the statistical package of Excel 7.0 (Microsoft).

RESULTS

FRUIT SURFACE UNDER SCANNING ELECTRON MICROSCOPY

At fruit set (stage I) the Ligol apple surface consisted of tightly packed epidermal cells with polygonal outer periclinal walls producing subulate, twisted, unicellular, nonglandular trichomes 1–2 mm long, which frequently broke and fell off, sometimes leaving their bases visible in the epidermis (Fig. 1a,b). At the sites of broken trichomes (scars), apertures reaching deeper layers, devoid of cuticle, were visible (Fig. 1b–d). At many sites on the surface, epicuticular microcracks 3.9–7.8 μm wide were observed along the epidermal walls and surface cuticle layers (Fig. 1a, b). The epidermis released numerous stomata at various developmental stages (Fig. 1e,f, Tab. 2) and differently shaped crystalline wax platelets reaching 2 × 2 × 0.1 μm maximum size (Fig. 1g).

At harvest maturity (stage II), when the fruits reached the final phase of volume growth, microcracks of the fruit cuticle surface occurred more frequently; they were larger (max. width 37.7 μm), and more irregularly dispersed than in stage I (Fig. 2a,b). Irregularly shaped wax platelets were vertically or obliquely arranged against the fruit surface, concentrated at the edges and inside the microcracks (Fig. 2c,d). Outside the microcracks, wax platelets lay mostly horizontally, parallel to the fruit surface (Fig. 2e). The cuticular cracks were concentrically arranged around the pores (Fig. 2f). On the epidermis were ventilation apertures (lenticels) showing a higher number of stomatal openings than in the fruit set stage (Fig. 2g,h). Due to the increase of fruit volume, in some cases the stomata and trichome fields were stretched along their longer axis or epidermis cells were ruptured, lengthening the stomatal pores (Fig. 2h). On average the total number of both types of lenticels was 95% lower than the number of stomata in the fruit set stage (Tab. 2).

After 6-month storage (stage III), numerous microcracks of varying depth and width were present on the apple surface (Fig. 3a). The width of the widest microcracks increased by 250% versus the harvest maturity stage, exceeding 90 μm. Numerous irregularly shaped wax platelets, arranged vertically and obliquely against the fruit surface, filled the microcracks, forming scars (Fig. 3b,c). Wax platelets arranged horizontally on the fruit surface were visible between the microcracks (Fig. 3d). A few lenticels were bigger and covered a larger area than in stage II (Fig. 3e,f). They were filled with convex or, less frequently, flattened cells. Mycelium hyphae were sometimes visible inside the lenticels (Fig. 3f).

CUTICLE ON THE FRUIT EPIDERMIS

Cross (radial) sections through Ligol apples at fruit set (state I) showed a basically single-layered epidermis with cells stretched at the anticlinal walls; they were almost 40% larger radially than tangentially (Fig. 4a, Tab. 2). Numerous divisions of the cells of this tissue were observed along the anti- and periclinal walls. A ~8 μm-thick cuticle was observed on the

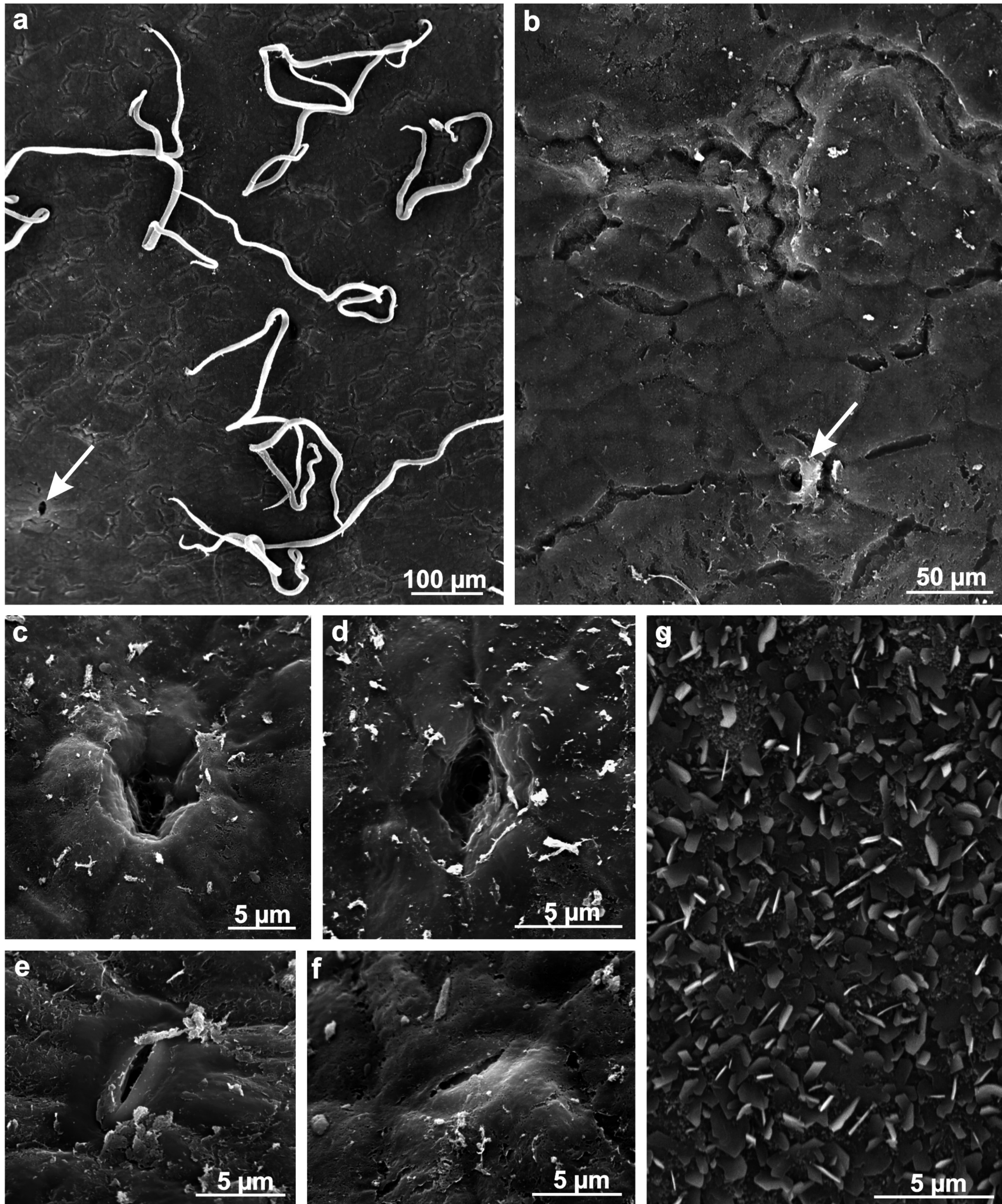


Fig. 1. Epidermis surface of Ligol apple at fruit set. (**a**) Detail of epidermis surface with stomata (arrow), nonglandular trichomes and microcracks, (**b**) Detail of epidermis surface with microcrack varying in width and visible scar of broken-off nonglandular trichome (arrow), (**c, d**) Scars of broken-off nonglandular trichomes, (**e, f**) Stomata, (**g**) Crystalline wax platelets on epidermis surface. SEM images.

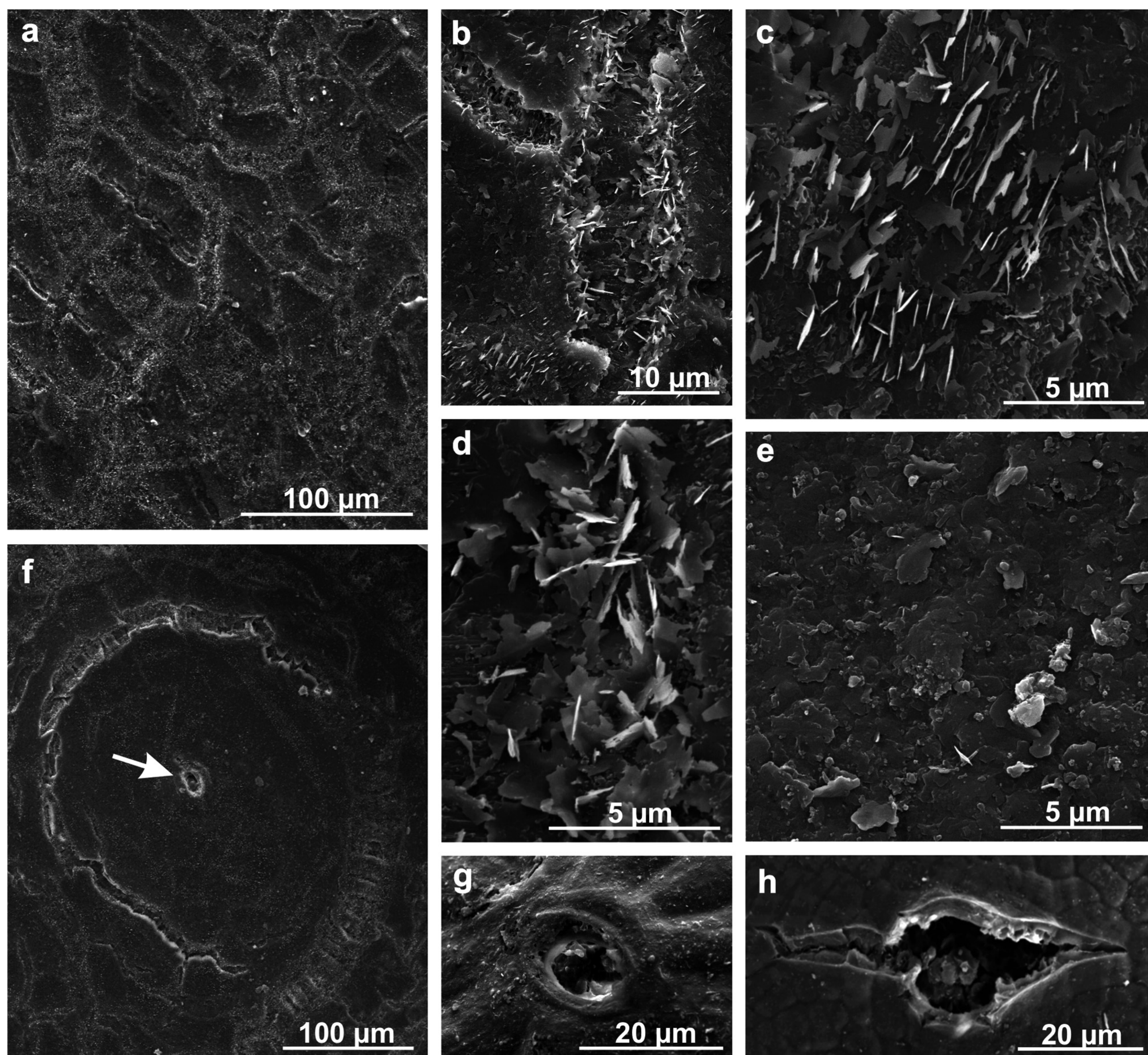


Fig. 2. Epidermis surface of Ligol apple at harvest maturity. (**a**) Detail of epidermis surface structured with numerous microcracks, (**b**) Microcracks with vertical platelets of epicuticular wax at crack edges, (**c, d**) Numerous perpendicularly oriented wax platelets inside microcracks, (**e**) Horizontal epicuticular wax platelets between microcracks, (**f**) Trichome scars (arrow) surrounded by concentrically arranged microcracks, (**g, h**) Lenticels in fruit epidermis. SEM images.

external periclinal wall of epidermal cells (Fig. 4a, Tab. 2), blue under the UV filter (not shown).

At harvest maturity the cuticle layer was 83% thicker than at stage I, and the epidermal cells became flattened and stretched tangentially to the fruit surface (Fig. 4b, Tab. 2). They were 80% lower (radial dimension) and 74% wider (tangential dimension) than at fruit set. Epidermal cell divisions were still visible, but fewer, along the tangential walls. The protoplasts of lenticel cells exhibited darker, brown coloration. In some lenticels, phellogen or Sudan III-stained cuticle layers sealing the lenticels were visible under the cells that filled their interior. On the other hand, large intercellular spaces were visible between the cells that filled the more open lenticels (not shown).

After 6-month storage (stage III) the epidermis of Ligol fruits had a cuticle layer 6% thicker than at harvest maturity and almost 94% thicker than at fruit set (Fig. 4c, Tab. 2). Cuticle was sometimes present on the internal periclinal walls of the epidermal cells (not shown), and also on the anticlinal

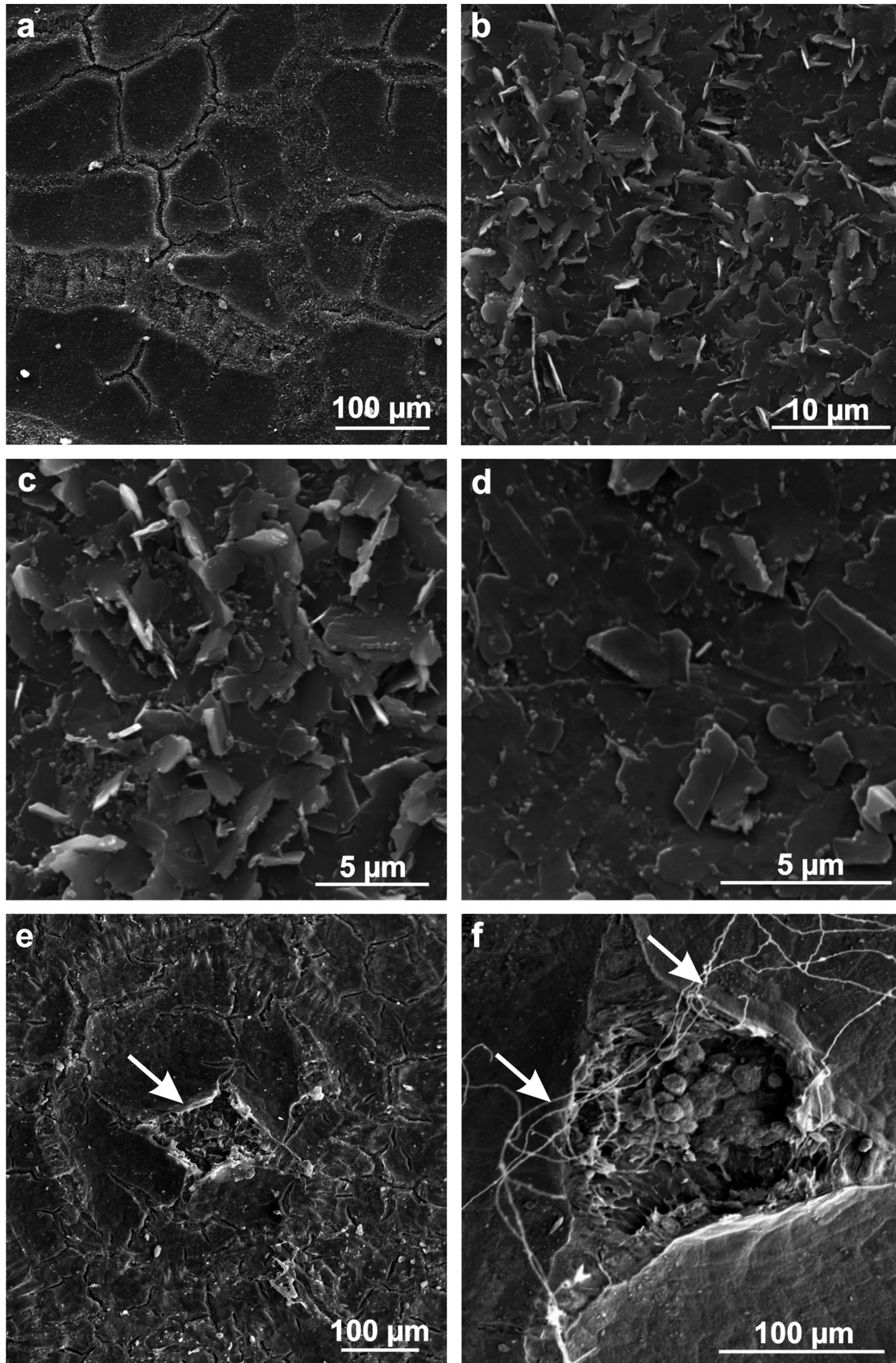


Fig. 3. Epidermis surface of Ligol apple after 6-month CA storage. (**a**) Detail of epidermis surface with microcracks varying in width and depth, (**b, c**) Vertically and obliquely oriented platelets inside microcracks, (**d**) Horizontal platelets covering cuticle interspaces between microcracks, (**e**) Lenticel (arrow) surrounded by concentrically arranged microcracks, (**f**) Lenticel with visible mycelium hyphae (arrows). SEM images.

TABLE 2. Characteristics of Ligol apples at three developmental stages: I – fruit set, II – harvest maturity, III – after storage

Parameter measured	Stage of fruit development	Mean ± standard deviation	Correlation coefficient
Fruit weight (g) (n=20)	II	183.3 ± 12.6	–
	III	175.4 ± 12.9	0.69*
Quantity of wax per cm² of fruit surface area (mg·cm⁻²) (n=6)	II	0.35 ± 0.08	–
	III	0.41 ± 0.12	0.73*
Pooled number of stomata and lenticels per cm² of fruit surface area (n=10)	I	100 ± 2	–
	II	5 ± 2	-0.76*
	III	5 ± 2	-0.76*
Thickness of cuticle (μm) (n=10)	I	8.0 ± 1.2	–
	II	14.7 ± 0.8	-0.83*
	III	15.6 ± 1	-0.33
Height of epidermis cells (μm) (n=10)	I	21.1 ± 1.9	–
	II	16.7 ± 1.5	0.24
	III	16.6 ± 1.3	-0.06
Width of epidermis cells (μm) (n=10)	I	13.1 ± 3.2	–
	II	22.8 ± 3.9	0.21
	III	26.3 ± 5.9	-0.34

*means large correlation

walls; in consequence the lumen of epidermal cells was decreased and the cells had an irregular shape (Fig. 4c). Epidermal cell height after storage was similar to that at harvest maturity and their width was slightly greater (Tab. 2).

Two layers were distinguished in the cuticle at fruit set: an external layer, 0.7–0.9 μm thick and of lamellate structure, forming the cuticle proper, and an internal layer, ~1 μm-thick and reticulate, bordering the epidermal cell wall and forming the cuticular layer (Fig. 5a). The epidermal cells were vacuolized to various degrees and the parietal cytoplasm contained plastids and numerous mitochondria. Numerous electron-dense deposits were observed in vacuoles (Fig. 5b).

At harvest maturity the cuticle had a double-layered structure similar to that described in stage I but the lamellate layer was more than 50% thicker, reaching 1.8 μm, and the tangential external epidermal cell wall was 5–6 times thicker (Fig. 5c). A layer of epicuticular wax was observed on the cuticle surface, usually damaged during fixation for TEM (Fig. 5c). The epicuticular wax layer was about half as thick as the entire cuticular membrane and

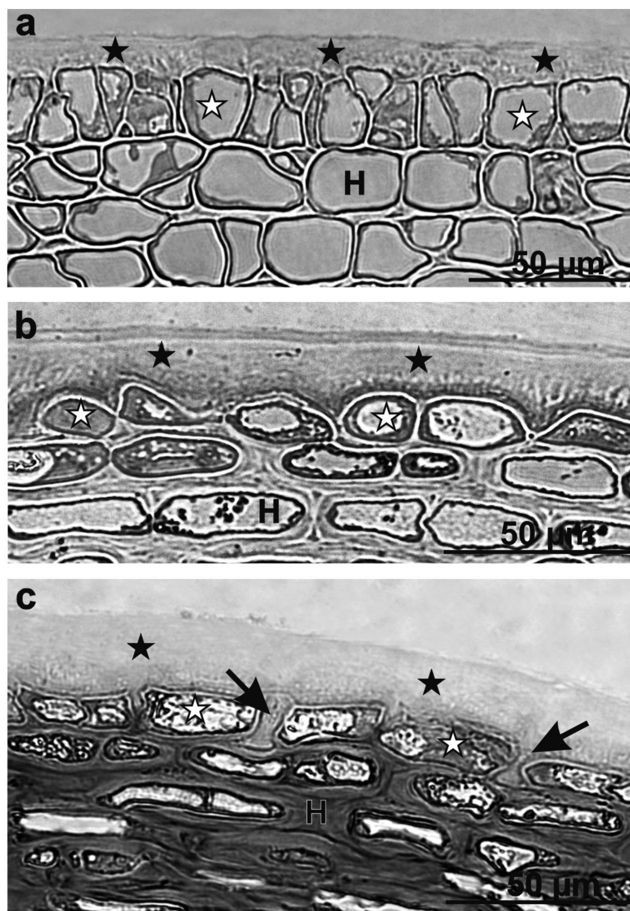


Fig. 4. Details of cross sections through Ligol apple peel. (**a**) At fruit set, visible divisions of epidermal cells, (**b**) At harvest maturity, (**c**) After 6-month CA storage, visible cuticle penetrating anticlinal walls of epidermis (arrows). Black stars – cuticle; white stars – epidermis; H – hypodermis. LM images.

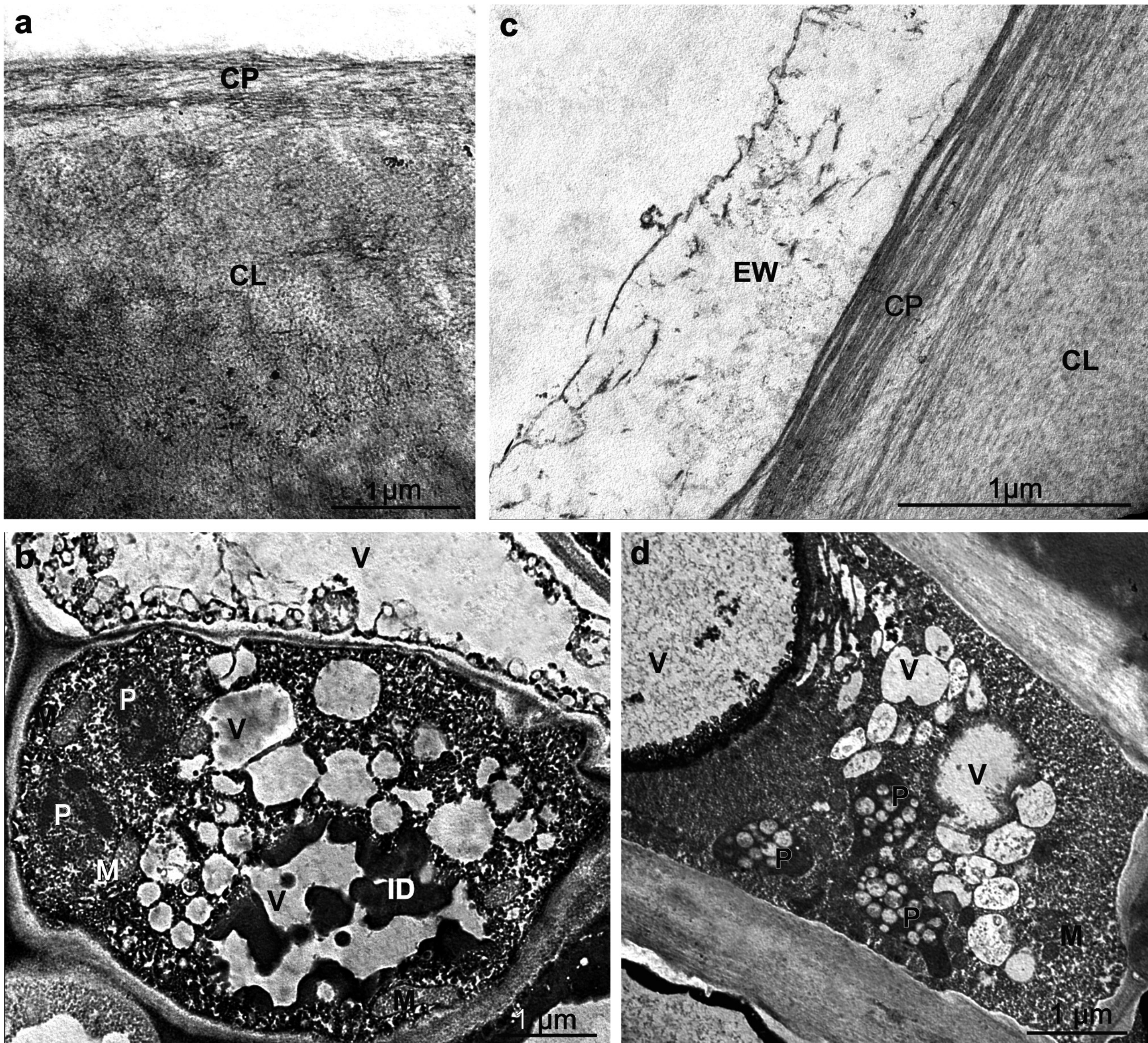


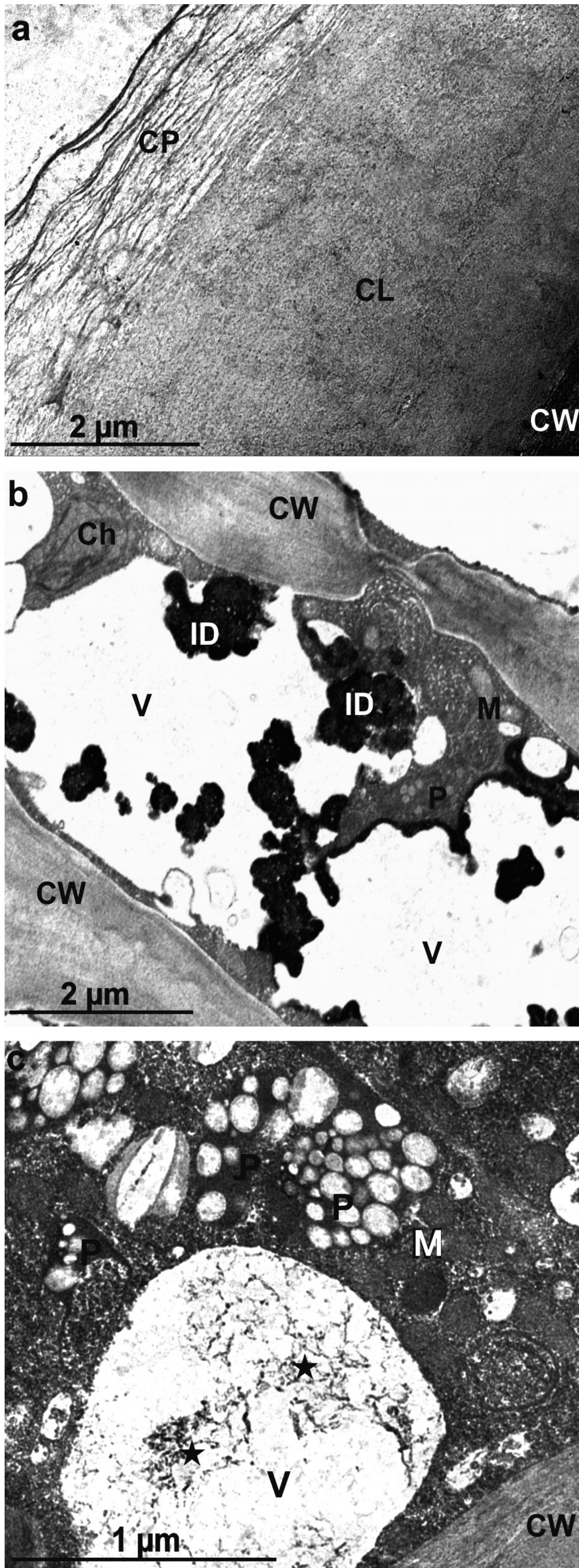
Fig. 5. Ultrastructure of Ligol apple epidermis. (**a, b**) At fruit set, (**a**) Detail of fruit cuticle, (**b**) Epidermis cell, (**c, d**) At harvest maturity, (**c**) Detail of fruit cuticle, (**d**) Detail of epidermis cell. EW – epicuticular wax; CP – cuticle proper; CL – cuticular layer; V – vacuoles; P – plastids with starch grains; ID – intravacuolar deposits; M – mitochondrion. TEM images.

ranged from 1.2 to 9.8 μm thick. Variously shaped plastids containing starch grains and mitochondria were visible in the epidermal cells, which were variously vacuolized (Fig. 5d).

After storage the lamellate cuticular layer was more than twice as thick as the cuticle of stage II, reaching 3.4–4.1 μm thick; the thickness of the tangential epidermal cell wall did not change (Fig. 6a). Flocculent residue and dark electron-dense deposits were observed in the vacuoles of the epidermal cells (Fig. 6b,c). As before the storage period, the cells contained one large or several smaller vacuoles, a few plastids with starch grains, and mitochondria.

CUTICULAR WAX AND FRUIT WEIGHT

The wax mass obtained from individual fruits was directly proportional to their surface area (i.e., the surface area of the cuticle, containing cuticular waxes). After half-year storage the weight of the Ligol fruits was lower by an average 4.5% (2.2–5.7%), a decrease of almost 9 g (4–10 g) per fruit (Tab. 2).



At harvest maturity the amount of wax per 1 cm^2 fruit surface area ranged between 0.26 and 0.44 mg; per single fruit the amount of wax was ~60 mg. After storage the quantity of wax increased by more than 17%, reaching 0.32–0.59 mg per cm^2 surface area and 70.5 mg per fruit (Tab. 2).

DISCUSSION

Throughout the life of fruits on the tree, in storage or on the shop shelf, changes associated with protection of the fruit interior from adverse environmental conditions take place in the external layer covering the fruit. Besides climatic and storage conditions, fruit health, and microenvironmental conditions, the genetic background determines changes occurring in apple peel, which in turn affect attributes associated with the quality of the fruit. The present study examined in detail the development of traits responsible for the firmness and freshness of the Ligol apple cultivar at fruit set, harvest maturity and after storage for sale. The results on apple fruit set, obtained by several types of microscopy, supplement the body of knowledge about apple fruit development.

In the early stage of fruit set the epidermis of Ligol fruits was densely covered with nonglandular trichomes 1–2 mm long. Their function probably involves protecting young dividing cells of the receptacle and fruit buds against infection and pests. A similar role for trichomes on the surface of young or ripening fruits has been reported in different species (Miller, 1984; Harrison and Beveridge, 2002; Celano et al., 2009). Trichomes may also form a barrier against harmful excess solar radiation (UV), which is then unable to penetrate the deeper layers of the pericarp as the rays are dispersed and reflected from its surface (Barnes et al., 1996). The ephemeral trichomes on the Ligol fruits fell off, leaving scars which potentially are penetration sites for bacterial or fungal pathogens during subsequent development stages. Similar infection sites are offered by stomata, which occur abundantly on Ligol apples at fruit set, and by the lenticels which cover an increasing area of the fruit surface during volume growth and maturation. Some lenticels were filled with cork cells or else comprised a cuticle layer among the filling cells (closed lenticels), which probably substantially restricted gas exchange. However, many of

Fig. 6. Ultrastructure of Ligol apple epidermis after 6-month CA storage. (**a**) Detail of fruit cuticle, (**b, c**) Epidermis cells with plastids and spherical deposits, and fibrous residue (asterisks) in vacuoles. CP – cuticle proper; CL – cuticular layer; V – vacuoles; P – plastids with starch grains; Ch – chloroplast; ID – intravacuolar deposits; M – mitochondrion. TEM images.

them were further enlarged by rupture (open lenticels), which increased the transpiration area. Other authors have described this role and structure of lenticels (Miller, 1984; Maguire et al., 1999; Veraverbake et al., 2003a). Lenticel cells in the Ligol fruits exhibited dark brown coloration of protoplasts, probably due to the presence of tannin compounds in these cells. Such compounds were identified by Miller (1984) in lenticel cells of *Mespilus germanica* (Rosaceae) fruits.

At maturation and after storage, microcracks on the fruit surface were more abundant, deeper and wider than at fruit set. Maguire et al. (1999) and Curry (2009) reported similar observations. Roy et al. (1994) and Knoche et al. (2004) suggested that the microcracks occur on apple fruits as a result of their intensive growth and in particular the enlargement of their volume and surface area, while the mass of the cuticular membrane per fruit remains constant. The deepening of microcracks results from shrinkage of the fruits during storage, associated with water loss from their cells. According to Maguire et al. (1999) the presence of microcracks with depth equal to half the thickness of the cuticle results in 12–15-fold higher permeability than in intact cuticle. Veraverbake et al. (2003a) state that microcracks in different apple varieties may cover even up to 10% of the fruit surface area. Microcracks have also been found in the cuticle of nectarine, sweet cherry and plum fruits; they developed either along with fruit development, as in the apple (Nguyen-the, 1991), or through intensive water uptake by maturing fruits during rainfall (Storey and Price, 1999; Peschel and Knoche, 2005).

Six-month storage of Ligol fruits resulted in loss of fruit mass by several percent, related to the development of ventilation apertures and microcracks. Similar changes in stored apple fruits have been described in other work (Hatfield and Knee, 1988; Jenks et al., 1994; Fellman et al., 2003; Link et al., 2004, Konarska, 2013). According to Maguire et al. (1999) the total number of lenticels is not correlated with the quantity of water lost by fruits, as transpiration proceeds only and to a small extent through open lenticels. Most water transpires through the cuticle and its microcracks. This has been controversially discussed by Veraverbake et al. (2003a), who attributed the major role in water transpiration to lenticels. Veraverbake et al. (2001) and Ghafir et al. (2009) stated that the decline in the weight of stored apple fruit increased with length of the storage period, the dryness of the fruit peel, and the thickness of the epicuticular wax layer.

In this study the growth, maturation and storage of Ligol fruits was accompanied by thickening of cuticle layer and an increase in the total mass of cuticular waxes (intra- and epicuticular). Many researchers have argued that these waxes, and particularly epicuticular waxes, have a function opposite to that of ventilation apertures and microcracks, as they inhibit transpiration and isolate the fruit interior from the external environment (Faust and Shear, 1972; Roy et al., 1994; Belding et al., 1998; Veraverbake et al., 2001, 2003b). When raindrops fall on such a waxy surface they neither adhere to it nor wet it; this limits the surface moisture fungal diseases require for sporulation (Schreiber and Schönherr, 1992; Kerstiens, 1996). According to Al Bitar et al. (2014) these epicuticular waxes render the apple fruit surface hydrophobic, which can help insects grasp it and attach their eggs. Riederer and Schreiber (1995) and Knoche et al. (2000) suggest that the amount of wax but not cuticle thickness is correlated with the quantity of water transpired from fruits, although, as demonstrated by Veraverbake et al. (2003a), the diffusion coefficient of cutin and cuticle is 4-fold higher than that of wax. Changes in the amount of wax during growth and storage have been demonstrated in many apple varieties (Morice and Shortland, 1973; Belding et al., 1998, 2000; Veraverbake et al., 2001; Curry, 2005; Tab. 1) and in this study.

Crystalline epicuticular wax platelets were observed in consecutive developmental stages of Ligol fruits. They occurred more abundantly after storage, when vertically and obliquely oriented wax platelets merged and filled the microcracks. The shapes of epicuticular wax crystals found in the present study are similar to those reported previously (Glenn et al., 1990; Roy et al., 1994; Koch et al., 2004; Curry, 2005). Lurie et al. (1996) and Curry (2001) suggested that a tear-and-repair mechanism operates in apple fruits, thanks to which microcracks on the apple surface are gradually cicatrized and covered by newly crystallized wax platelets produced during storage. This mechanism was confirmed in the apple cultivar Ligol after 6-month CA storage in my study. According to Morice and Shorland (1973) and Belding et al. (1998), synthesis of cuticular waxes is inhibited at the time of depletion of wax components in epidermal cells or as a result of necrosis of tissue cells.

The changes in epidermal cell ultrastructure induced by maturation and storage primarily involved an increase in the thickness of the lamellate cuticle layer forming the cuticle proper. Reticulate-lamellate structure of the cuticle in apple was also described by de Vries (1968), Holloway (1982) and Kerstiens (1996). Synthesis of cuticle components takes place in plastids and the endoplasmic reticulum of epidermal cells, from where the components are transported through cell membranes and cell walls onto the cuticle surface (Peng and Zhang, 2000; Samuels et al., 2008). According to Neinhuis et al. (2001) these compounds are extruded by permeating through the cuticle, a partially permeable

layer. Miller (1982) and Jeffree (1996) state that this transport proceeds through microchannels in the cuticle. My results rather support Neinhuis et al. (2001), since the numerous TEM micrographs did not show microchannels in the Ligol fruit cuticle.

In the successive stages of Ligol fruit development, an ongoing process of epidermal cell vacuolization was accompanied by deposition of electron-dense deposits and flocculent residue inside the vacuoles, which may evidence progressive degradation of cell structures or membranes. Peng and Zhang (2000) and Bae et al. (2006) observed similar electron-dense globular deposits in the vacuoles of apples at harvest maturity. According to Bae et al. (2006) these may be deposits of phenolic compounds with anthocyanin, which is abundant in the apple epidermis.

CONCLUSIONS

1. The epidermal cells of the Ligol apple cultivar retain the characteristics of meristematic tissue and, by dividing and extending along the periclinal walls, contribute to expansion of the epidermis surface area as the fruit increases in volume.
2. Trichomes covering the apple surface at fruit set (stage I) form a mechanical defense and protect against abiotic and biotic factors; after their degeneration this function is performed by the cuticle and epicuticular wax.
3. Cuticle thickness and the quantity of epicuticular waxes increase during fruit development and storage, but fruit weight and firmness decrease during storage.
4. The stomata, lenticels and microcracks in the fruit epidermis are weak sites where the peel is often torn by tensile forces, contributing to transpiration and increasing the fruit's susceptibility to pathogens.
5. Changes in the ultrastructure of epidermal cells, particularly in the vacuoles, indicate progressive processes of degradation and disorganization, that is, cell aging.

ACKNOWLEDGEMENTS

This work was supported by the Polish Ministry of Science and Higher Education of Poland from statutory funds of the Department of Botany, University of Life Sciences in Lublin.

REFERENCES

Al Bitar L, Gorb, SN, Zebitz CP, and Voigt D. 2014. Egg adhesion of the codling moth *Cydia pomonella* L. (Lepidoptera, Tortricidae) to various substrates: II. Fruit surfaces of different apple cultivars. *Arthropod – Plant Interactions* 8,1: 57–77.

Babos K, Sass P, and Mohácsy P. 1984. Relationship between the peel structure and storability of apples. *Acta Agronomica Hungarica* 33: 41–50.

Bae RN, Kim KW, Kim TC, and Lee SK. 2006. Anatomical observations of anthocyanin rich cells in apple skins. *HortScience* 41: 733–736.

Barnes JD, Percy KE, Paul ND, Jones P, McLaughlin CK, Mullineaux PM, Creissen G, and Wellburn AR. 1996. The influence of UV-B radiation on the physiochemical nature of tobacco (*Nicotiana tabacum* L.) leaf surfaces. *Journal of Experimental Botany* 47: 99–109.

Barthlott W, Neinhuis C, Cutler D, Ditsch F, Meusel I, Theisen I, and Wilhelmi H. 1998. Classification and terminology of plant epicuticular waxes. *Botanical Journal of the Linnean Society* 126: 237–260.

Belding RD, Blankenship SM, Young E, and Leidy RB. 1998. Composition and variability of epicuticular waxes in apple cultivars. *Journal of the American Society for Horticultural Science* 123: 348–356.

Belding RD, Sutton TB, Blankenship SM, and Young E. 2000. Relationship between apple fruit epicuticular wax and growth of *Peltaster fructicola and Leptodontidium elatius*, two fungi that cause sooty blotch disease. *Plant Disease* 84: 767–772.

Celano G, Minnocci A, Sebastiani L, d'Auria M, and Xiloyannis C. 2009. Changes in the structure of the skin of kiwifruit in a relation to water. *Journal of Horticultural Science and Biotechnology* 84: 41–46.

Curry EA. 2001. Lenticel and cuticle disorders: a survey. *Proceedings of the Washington Tree Fruit Postharvest Conference*, 13–14 March 2001, 1–4. Wenatchee, WA, USA.

Curry EA. 2005. Ultrastructure of epicuticular wax aggregates during fruit development in apple (*Malus domestica* Borkh). *Journal of Horticultural Science and Biotechnology* 80: 668–676.

Curry EA. 2009. Growth-induced microcracking and repair mechanisms of fruit cuticles. Plant Physiology. *Proceedings of the SEM Annual Conference*, 1–4 June 2009. Albuquereque New Mexico, USA.

de Bellie N, Schotte S, Soucke P, and de Baerdemaeker J. 2000. Development of an automated monitoring device to quantify changes in firmness of apples during storage. *Postharvest Biology and Technology* 18: 1–8.

de Vries HAMA. 1968. Development of the structure of the normal, smooth cuticle of the apple 'Golden Delicious'. *Acta Botanica Neerlandica* 17: 229–241.

Dobrzańska A, and Tymolewska B. 1995. The comparison of level and structure of nutrition of Austria and Poland in the years 1960–1990. *Seventh European Nutrition Conference*, 24–28 May 1995. Vienna, Austria.

Dobrzańska A. 1995. Changes in levels of nutrition and the structure of the Polish population in the years 1958–1990 on the basis of household budgets. *Materia Medica Polona* 27: 78.

Faust M, and Shear CB. 1972. Fine structure of the fruit surface of three apple cultivars. *Journal of the American Society for Horticultural Science* 97: 351–355.

Fellman JK, Rudell DR, Mattinson DS, and Mattheis JP. 2003. Relationship of harvest maturity to flavor regener-

ation after CA storage of 'Delicious' apples. *Postharvest Biology and Technology* 27: 39–51.

GHAFIR SAM, GADALLA SO, MURAJEI BN, and EL-NADY MF. 2009. Physiological and anatomical comparison between four different apple cultivars under cold-storage conditions. *African Journal of Plant Science* 3: 133–138.

GLENN GM, ROM CR, RASMUSSEN HP, and POOVAIAH BW. 1990. Influence of cuticular structure on the appearance of artificially waxed 'Delicious' apple fruit. *Scientia Horticulturae* 42: 89–297.

HARRISON JE, and BEVERIDGE T. 2002. Fruit structure of *Hippophae rhamnoides* cv. Indian Summer (sea buckhorn). *Canadian Journal of Botany* 80: 399–409.

HATFIELD SGS, and KNEE M. 1988. Effects of water loss on apples in storage. *International Journal of Food Science and Technology* 23: 575–583.

HÖHN E. 1990. Quality of apples. *Acta Horticulturae* 285: 111–118.

HOLLOWAY PJ. 1982. Structure and histochemistry of plant epicuticular membranes an overview. In: Cutler DF, Alvin KL, Price CE [eds.], *The Plant Cuticle*, 39–53. Academic Press, London.

JEFFREE CE. 1996. Structure and ontogeny of plant cuticles. In: Kerstiens G [ed.], *Plant cuticles: An Integrated Functional Approach*, 33–82. BIOS Scientific Publishers LTD, Oxford.

JENKS MA, and ASHWORTH EN. 2003. Plant epicuticular waxes: function, production, and genetics. *Horticultural Reviews* 23: 1–68.

JENKS MA, RICH PJ, and ASHWORTH EN. 1994. Involvement of cork cells in the secretion of epicuticular wax filaments on *Sorghum bicolor* (L.) Moench. *International Journal of Plant Sciences* 155: 506–518.

KADER AA. 1983. Postharvest quality maintenance of fruits and vegetables in developing countries. In Lieberman M [ed.], *Postharvest Physiology and Crop Preservation*, 455–469. Plenum Press, New York.

KERSTIENS G. 1996. Cuticular water permeability and its physiological significance. *Journal of Experimental Botany* 74: 1260–1264.

KNOCHE M, BEYER M, PESCHEL S, OPARLAKOV B, and BUKOVAC MJ. 2004. Changes in strain and deposition of cuticle in developing sweet cherry fruit. *Physiologia Plantarum* 120: 667–677.

KNOCHE M, PESCHEL S, HINZ M, and BUKOVAC MJ. 2000. Studies on water transport through the sweet cherry fruit surface: Characterizing conductance of the cuticular membrane using pericarp segments. *Planta* 212: 127–135.

KOCH K, NEINHUIS C, ENSIKAT HJ, and BARTHLOTT W. 2004. Self assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). *Journal of Experimental Botany* 55: 711–718.

KOLATTUKUDY PE. 2001. Polyesters in higher plants. In Scheper T [ed.], *Biochemical Engineering/Biotechnology*, 4–49. Springer, Berlin Heidelberg, New York.

KONARSKA A. 2012. Differences in the fruit peel structures between two apple cultivars during storage. *Acta Scientiarum Polonorum – Hortorum Cultus* 11: 3–13.

KONARSKA A. 2013. The structure of the fruit peel in two varieties of *Malus domestica* Borkh. (Rosaceae) before and after storage. *Protoplasma* 250: 701–714.

LAU OL, and LANE WD. 1998. Harvest indices, storability, and poststorage refrigeration requirements of 'Sunrise' apple. *HortScience* 33: 302–304.

LINK SO, DRAKE SR, and THIEDE ME. 2004. Prediction of apple firmness from mass loss and shrinkage. *Journal of Food Quality* 27: 13–26.

LURIE S, FALLIK E, and KLEIN JD. 1996. The effect of heat treatment of apple epicuticular wax and calcium uptake. *Postharvest Biology and Technology* 8: 271–277.

MAGUIRE KM, LANG A, BANKS NH, HALL A, HOPCROFT D, and BENNETI R. 1999. Relationship between water vapour permeance of apples and micro-cracking of the cuticle. *Postharvest Biology and Technology* 17: 89–96.

MARKSTÄDTER C, FEDERLE W, JETTER R, RIEDERER M, and HÖLLDOBLER B. 2000. Chemical composition of the slippery epicuticular wax blooms on *Macaranga* (Euphorbiaceae) ant-plants. *Chemoecology* 10: 33–40.

MILLER RH. 1982. Apple fruit cuticles and the occurrence of pores and transcuticular canals. *Annals of Botany* 50: 355–371.

MILLER RH. 1984. The multiple epidermis-cuticle complex of Medlar fruit *Mespilus germanica* L. (Rosaceae). *Annals of Botany* 53: 779–792.

MORICE IM, and SHORLAND FB. 1973. Composition of the surface waxes of apple fruits and changes during storage. *Journal of the Science of Food and Agriculture* 24: 1331–1339.

NEINHUIS C, KOCH K, and BARTHLOTT W. 2001. Movement and regeneration of epicuticular waxes through plant cuticles. *Planta* 213: 427–34.

NGUYEN-THE C. 1991. Structure of epidermis wall, cuticle and cuticular microcracks in nectarine fruit. *Agronomie* 11: 902–920.

PENG YB, and ZHANG DP. 2000. Ultrastructure of epidermis and flesh of the developing apple fruit. *Acta Phytotaxonomica Sinica* 42: 794–802.

PESCHEL S, and KNOCHE M. 2005. Characterization of microcracks in the cuticle of developing sweet cherry fruit. *Journal of the American Society for Horticultural Science* 130: 487–495.

REYNOLDS ES. 1963. The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. *Journal of Cell Biology* 17: 208–212.

RICHARDSON-HARMAN N, PHELPS T, MCDERMOTT S, and GUNSON A. 1998. Use of tactile and visual cues in consumer judgments of apple ripeness. *Journal of Sensory Studies* 13: 121–126.

RIEDERER M, and MARKSTÄDTER C. 1996. Cutical waxes: a critical assessment of current knowledge. In Kerstiens G [ed.], *Plant Cuticles: An Integrated Functional Approach*, 189–200. BIOS Scientific Publishers, Oxford.

RIEDERER M, SCHREIBER L. 1995. Waxes – the transport barriers of plant cuticles. In: Hamilton RJ [ed.], *Waxes: Chemistry, Molecular Biology and Functions*, 131–156. The Oily Press, Dundee, Scotland.

ROY S, CONWAY WS, WATADA AE, SAMS CE, ERBE EF, and WERGIN WP. 1994. Heat treatment affects epicuticular wax structure and postharvest calcium uptake in 'Golden Delicious' apples. *HortScience* 29: 1056–1058.

ROY S, CONWAY WS, WATADA AE, SAMS CE, ERBE EF, and WERGIN WP. 1999. Changes in the ultrastructure of the epicuticular wax and postharvest calcium uptake in apples. *HortScience* 34: 121–124.

Samuels L, Kunst L, and Jetter R. 2008. Scaling plant surfaces: cuticular wax formation by epidermal cells. *Annual Review of Plant Soil* 59: 683–707.

Schreiber L, and Schönherr J. 1992. Uptake of organic chemicals in conifer needles: surface adsorption and permeability of cuticles. *Environmental Science and Technology* 26: 153–159.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* 26: 31–43.

Storey R, and Price WE. 1999. Microstructure of the skin of d'Agen plums. *Scientia Horticulturae* 81: 279–286.

Taiz L, and Zeiger E. 2002. *Plant Physiology*, Sinauer Associates, Inc., Sunderland, MA.

Tessmer MA, Antoniolli LR, and Appezzato-da-Gloria B. 2012. Cuticle of 'Gala' and 'Galaxy' apple cultivars under different environment al conditions. *Brazilian Archives of Biology and Technology* 55: 709–714.

Tsukamoto M. 1981. Studies on the mechanical injury of fruit: II. Susceptibility to impact and compression in apple fruit as related to storage periods and fruit portions. *Journal of the Japanese Society for Horticultural Science* 49: 571–575.

van Woensel G, Wouters A, and de Baerdemaker J. 1987. Relation between mechanical properties of apple fruit and sensory quality. *Journal of Food Process Engineering* 9: 173–189.

Verardo G, Pagani E, Geatti P, and Martinuzzi P. 2003. A thorough study of the surface wax of apple fruits. *Analytical and Bioanalytical Chemistry* 376: 659–667.

Veraverbake EA, Lammertyn J, Saevels S, and Nicolai BM. 2001. Changes in chemical wax composition of three different apple (*Malus domestica* Borkh.) cultivars during storage. *Postharvest Biology and Technology* 23: 197–208.

Veraverbake EA, Verboven P, van Oostveldt P, and Nicolai BM. 2003a. Prediction of moisture loss across the cuticle of apple (*Malus sylvestris* subsp. *mitis* (Wallr.)) during storage: part 1. Model development and determination of diffusion coefficients. *Postharvest Biology and Technology* 30: 75–88.

Veraverbake EA, Verboven P, van Oostveldt P, and Nicolai BM. 2003b. Prediction of moisture loss across the cuticle of apple (*Malus sylvestris* subsp. *mitis* (Wallr.)) during storage: part 2. Model simulations and practical applications. *Postharvest Biology and Technology* 30: 89–97.