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Ultrastructure of the cuticle during growth of the grape berry (*Vitis vinifera*)

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The outer surface morphology and the ultrastructure of grape berries during growth were examined by electron microscopy. The cuticle began to form before anthesis as highly organized and tightly appressed cuticular ridges. During the period of rapid expansion, the cuticular material spread out over the grape berry. At the same time, an outer wax layer of about 0.5 μm was indentified. As growth proceeded, the cuticular material flattened out and eventually disappeared. At the final stage of growth, the berry had a smooth, continuous and homogeneous cuticle with a thickness of 3 μm.

Introduction

As a consequence of chemical and biological evolution, living organisms have developed selected biopolymer structures, which serve as protective barriers against the environment. In aerial organs of higher plants, this protective barrier function is carried out by the plant cuticle. From a morphological point of view, the cuticle covers the outer cell wall of the epidermal cells. The plant cuticle is mainly formed by a structural component called cutin, an amorphous biopolyester of hydroxy-fatty acids. Associated with this biopolymer are waxes or soluble cuticular lipids (Holloway 1982). Waxes can be embedded within the cutin, intracuticular waxes, or deposited on the outer surface of the plant cuticle (epicuticular waxes). Ultrastructural and morphological studies carried out in some plants indicated that, during the development of fruits, a rapid deposition of waxes and cutin occurred (Martin and Juniper 1970). Wax biosynthesis and further deposition on the outer surface of aerial parts of plants has been studied (see for example, Baker et al. 1982, Laguna et al. 1999). On the other hand, studies on cutin biosynthesis have been completed by Kolattukudy (1996). Moreover, biosynthesis of cuticular components in leaves and fruits is associated with rapid growth of the tissues (Considine and Knox 1979, Jefree 1996).

The plant cuticle undergoes very large changes in area as cells expand during organ growth. Organs that undergo very rapid area expansion have a very loose and wrinkled cuticle, which allows an increase of the surface area by a factor of two to three (Jefree 1996). This form of wrinkling pre-expansion was described by Considine and Knox (1979) and Rosenquist and Morrison (1988) on the grape ovary, which undergoes a high volume expansion in the 40 days post-anthesis, representing about a 22-fold increase of linear dimensions of the epidermis (Jefree 1996).

The present work reports a detailed study by electron microscopy on the morphology and ultrastructure of the grape berry during cuticle development. Using different physicochemical methodologies, our research group recently documented (Casado and Heredia 1999) that grape berry cuticle is a unique cuticle characterized by a high amount of waxes of a high degree of molecular order. This study pointed out that the wax layer represents a formidable protective molecular barrier.

Materials and methods

Plant material

Bunches of grapes were collected from *Vitis vinifera* L. cv. Palomino fino from Domecq S. A. vineyards located at Jerez de la Frontera, Cádiz, Spain. The material was collected at different growing stages.

Scanning electron microscopy (SEM)

Tangential slices of fresh grapes were taken with a sharp razor blade and fixed in glutaraldehyde (2%, v/v, in phosphate buffer 0.2 *M*, pH 7) overnight at 4°C. The samples

Abbreviations – SEM, scanning electron microscopy; TEM, transmission electron microscopy.

were thoroughly rinsed in fresh phosphate buffer and then dehydrated through an ethanol solution series: 40, 50, 70, 80, 95 and 100% and increasing times, from 15 min to 1 h 30 min. They were placed on a metallic holder using a double-faced adhesive and coated with a 0.05-μm thin film of gold. A JEOL JSM-840 scanning electron microscope operated at 10–20 kV was used for examination of the samples.

Transmission electron microscopy (TEM)

For the analysis by TEM, the samples were cut out in pieces of about 1–2 mm. Material was fixed in glutaraldehyde (2%, v/v, in phosphate buffer 0.2 *M*, pH 7) at 4°C overnight. The tissue was rinsed thoroughly in fresh buffer, post-fixed in OsO_4 (2%, v/v, in the same buffer) for 4 h at 4°C and finally rinsed again thoroughly in phosphate buffer. The samples were dehydrated using different acetone solutions: 40, 50, 60, 70 (this step with uranyl acetate, 1%, w/v), 95 and 100% and increasing times, from 15 min to 1 h 30 min. The samples were kept in a mixture of acetone-resin (1:1, v/v, for 2 h in vacuum) and stored at room temperature overnight. They were then embedded in araldite resin (Araldite 502, DDSA and DMP-30) for 1 h in vacuum and further polymerized at 60°C for 72 h. Transverse sections (70–80 nm) were cut with a diamond knife on an ultramicrotome and the corresponding sections were observed at 80 kV with a Philips CM 100 electron microscope.

Results and discussion

Grape fruit development of the variety Palomino fino occurred in two distinct periods of growth, shown as transverse and longitudinal diameter (Fig. 1). The first one commenced from the anthesis and continued for about 95 days. The onset of the second phase was marked by a decrease in the rate of both transverse and longitudinal fruit diameters. Maturity was attained during the next 40 days. Grape berry weight followed a similar developmental pattern to berry diameter (data not shown).

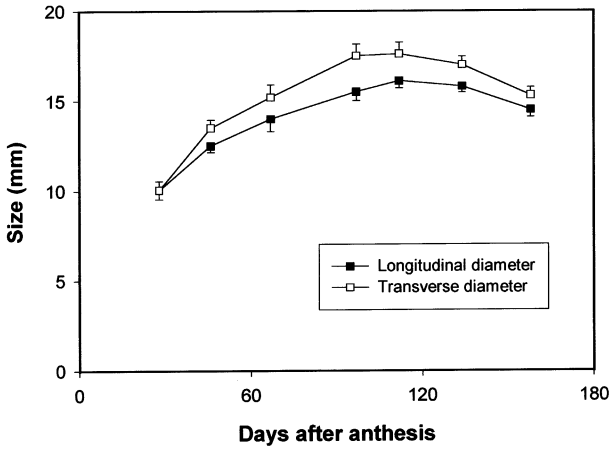

Fig. 1. Grape berry transverse and longitudinal diameters (*V. vinifera* cv. Palomino fino) during fruit development.

SEM

The outer surface of the cuticle of Palomino fino grape berries during growth has been firstly studied by SEM.

The formation of the cuticle began at the pre-anthesis about 1 week before bloom. Cuticle development began with the formation of numerous ridges, tightly appressed and highly organized, which covered the wall cell of the ovary epidermal cells (Fig. 2A). At anthesis, the ovary surface also had cuticular ridges, which were less tightly appressed (Fig. 2B), showing a variability in orientation: some ridges were orientated forming parallel rows to the longitudinal axis of epidermal cells and other ridges appeared orientated perpendicular to the longitudinal axis between cells. This particular cuticular ridge orientation has been previously reported in the excellent paper of Considine and Knox (1979) and by Rosenquist and Morrison (1988) for the cultivar Thompson seedless.

Fig. 2C shows the post-anthesis grape berry surface (0.2–0.3-cm transversal diameter), whose cuticular ridge density was lower, due to the ridges seeming to fuse, probably as a consequence of the rapid berry growth. According to Rosenquist and Morrison (1988), the ridges represent a form of stored cuticular material, which later spreads out, giving a continuous protective layer over the developing berry during periods of rapid berry expansion. Just at this stage, it was possible to distinguish some small and individual globules over the cuticular ridges (Fig. 2D), which can be assigned to macromolecular aggregates of the different epicuticular wax components.

At the same time as grape berry expansion, the cuticle formed a thin, continuous and smooth layer, underlying epicuticular wax, with only scattered remnants of cuticular ridges (Fig. 2E). The wax grains fused and the waxes appeared in the form of platelets densely distributed over the surface of the cuticle, increasing in size and complexity during berry development (Fig. 2F). This epicuticular wax morphology and ultrastructure has been also observed by Commenil et al. (1997) for the cultivar Pinot. Finally, at maturity, the cuticle appeared to be a smooth and uniform layer and the wax platelets spreads out over the berry surface (Fig. 2G).

TEM

The ultrastructure of the grape grain cuticle was also examined using TEM. At pre-anthesis, cuticle development began with the formation of numerous cuticular ridges (Fig. 3A). According to Jefree (1996), one could apply the term procuticle to the outer cuticular membrane that appears on the ovary. At early stages of epidermal development, rapidly dividing cells are covered by a highly water-repellent and impermeable osmiophilic film, which forms a dense, amorphous and superficial layer called procuticle (Jefree 1996). In this case, the procuticle appeared as a thin and uniform electro-dense layer (Fig. 3B).

At post-anthesis (Fig. 3C), the thickness of the cuticle corresponding to a grape grain of 0.4–0.5 cm diameter was about 0.7 μm. The outermost zone, constituted by a convoluted and electron-light amorphous layer of variable

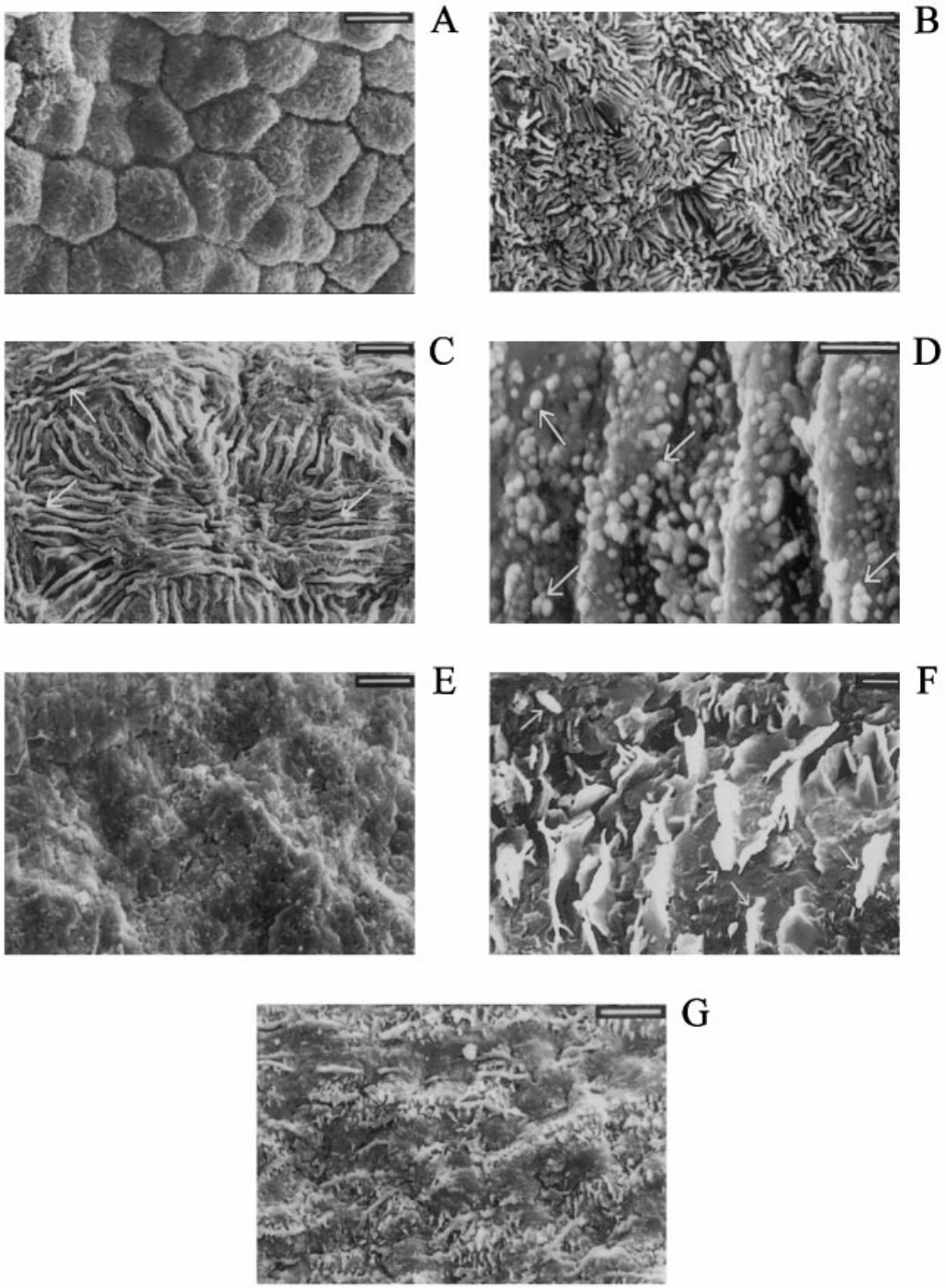

Fig. 2. Scanning electron micrographs of grape berry outer surface during growth. All samples were fixed in glutaraldehyde: (A) The surface of the ovary approximately 1 week before anthesis, showing cuticular ridges covering the outer epidermal cell walls. (B) The ovary at anthesis covered by cuticular ridges showing variability in orientation (arrows). (C) The berry surface after anthesis, transverse diameter about 0.2–0.3 cm. The cuticular ridges (arrows) are less tightly appressed. (D) Detail of C, showing development of epicuticular wax: small grains of epicuticular waxes (arrows). (E) The berry surface, grain transverse diameter about 0.7 cm, showing remnants of cuticular ridges and epicuticular wax in the form of platelets. (F) Detail of E, showing wax platelets (arrows) spread out over the outer berry surface. (G) The surface of the berry at the final growth stage, showing a smooth, continuous and homogeneous cuticle. (A–G) Bar, 10 μm; (D,F) bar, 1 μm.

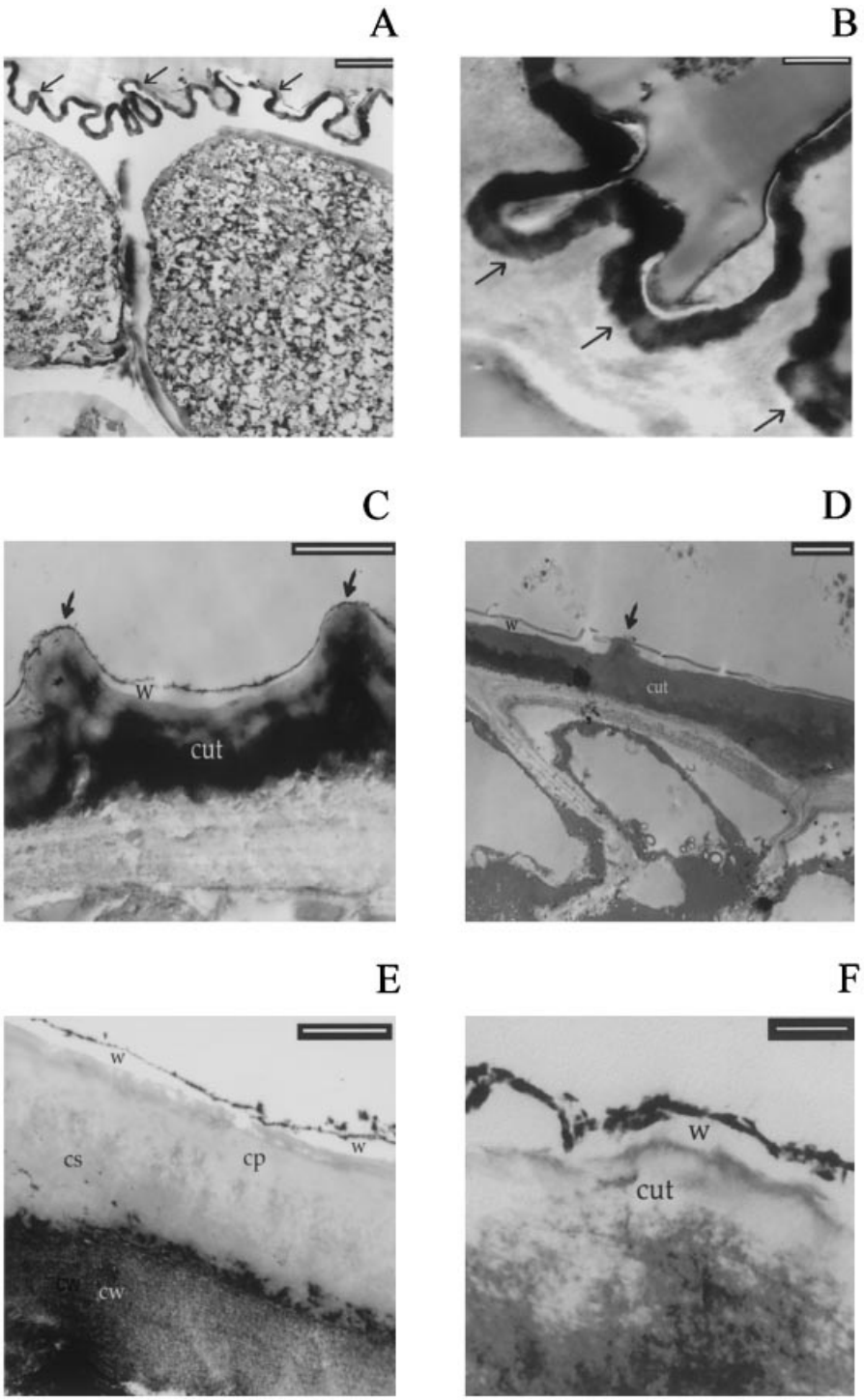

Fig. 3. Transmission electron micrographs of transverse sections of grape berries at different growth stages. All samples were fixed in glutaraldehyde, post-fixed with osmium tetroxide, dehydrated through acetone series and embedded in araldite resin (see Methods). (A) Tranverse section of the ovary, approximately 1 week before anthesis, showing an electro-dense zone corresponding to the procuticle (arrows). (B) Detail of A showing a thin and uniform procuticle. (C) Transverse section of grape berry after anthesis, transverse diameter about 0.4–0.5 cm. The cuticle appears strongly contrasted (0.7 μm thick) and the outermost zone is assigned to epicuticular waxes (0.15–0.2 μm). Arrows show cuticular ridges. (D) Transverse section of grape berry of 0.7-cm transverse diameter. Epicuticular waxes form a continuous layer with a thickness of 0.35–0.4 μm. (E) Cuticle of grape berry of 1-cm transversal diameter. The cuticle appears formed by a 2.5-μm thick layer. It is differentiated in 3 zones: an outer epicuticular wax layer, an amorphous region and an inner reticulate region The junction between the reticulate region and the epidermal cell wall appears strongly contrasted. (F) Grape cuticle with a transverse diameter of about 1.6–1.7 cm. The cuticle appears developed completely with a thickness of about 3 μm and the epicuticular wax layer of a thickness of 0.5 μm. Nomenclature: cs, secondary cuticle; cp, primary cuticle, cut, cutin; cw, cell wall; w, epicuticular wax layer. (A,D) Bar, 2 μm; (B,F) bar, 0.5 μm; (C,E) bar, 1 μm.

thickness (0.15–0.2 μm) corresponded to the epicuticular waxes. At this stage, some cuticular ridges still remain on the cuticle surface (Fig. 3C). Next changes in the cuticle thickness are closely related to the growth of grape berry. Thus, epicuticular wax layer appeared to be constituted of a continuous and smooth layer of a thickness of approximately 0.35–0.40 μm (Fig. 3D,E). Beneath this wax layer, differentiation between the cuticle proper, or primary cuticle, and the cuticle layer, or secondary cuticle, was also possible (Fig. 3E). The cuticle proper is an amorphous layer, which does not contain cellulose or cell wall material. On the other hand, the cuticle layer is the most inner region of the cuticle appearing reticulate and more electro-dense, probably due to the incrustations of polysaccharides or other cell wall materials. Overall thickness of these two layers was about 2.5 μm (Fig. 3E).

At maturity, the development of the grape berry cuticle was almost complete (Fig. 3F). Cuticle thickness was about

3 μm and the epicuticular wax layer wax formed by a 0.5-μm layer. This overall cuticle thickness remained unchanged throughout the process of grape fruit maturation. The results presented here are similar to those reported by Considine and Knox (1979), who carried out a detailed histochemical study of grape cuticle during growth. According to these authors, the cuticle thickness is maintained through the life of the fruit, implying continuous synthesis and degradation of this lipophilic material and a physiological role as mechanical support. On the other hand, the secondary cuticle appears more osmiophilic than young grape tissue due to the incrustations of polysaccharide material as a consequence of fruit maturation processes (Fig. 3F).

Taken together, our results obtained by SEM and TEM confirm that a cuticle is present at very early stages of grape berry development and that its development takes place by a continuous and rapid synthesis of cuticular material, which fully covers the ovary. This cuticular material, named procuticle, constitutes the first protective membrane against the environment. During the period of grape berry expansion, the cuticular material spreads out over the berry, which undergoes a very rapid area expansion. An outer wax layer of about 0.5 μm was also formed. As growth proceeds, the cuticular material flattens out and eventually disappears. At the end stage of growth, the berry has a smooth, continuous and homogeneous cuticle with an overall thickness about 3 μm.

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