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Anatomical Changes and Anthocyanin Contents of the Exocarp by Ethyl Oleate Treatment on 'Merlot' Grapes

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Abstract. Preharvest treatment with 4% ethyl oleate on 'Merlot' (*Vitis vinifera* L.) grape reduced the thickness of the epidermal and hypodermal layers with significantly enhanced pigmentation. Thickness of the skin in treated berries was 90-107 μm, whereas those in control berries were 126-189 μm. Decreases in the thickness of epidermal and hypodermal cell layers seemed to be due to cellular death or dehydration by rapid senescence after the treatment. Immediate change observed in treated berries was the deformation of the wax that appeared melted resulting in color improvement. Total anthocyanin was also increased by ethyl oleate treatment. Separate forms of anthocyanins, acylated and methoxylated anthocyanins increased, whereas hydroxylated anthocyanins tended to decrease.

***Additional key words*:** epidermis, hypodermis, morphology, pigmentation, *Vitis vinifera* L.

Introduction

The primary goal of applying technology in viticulture is to improve berry quality. In practice, some chemicals (ABA, ethylene etc.) are used to improve grape quality (Kataoka et al., 1982; Kim et al., 1998; Lee et al., 1997).

Most popular chemical treatment applied in the raisin industry is the alkaline solution dipping to facilitate raisin production. To obtain raisins, grape clusters are immersed in an alkaline solution to which a great quantity of oil is added ("oil dipped" treatment). The immersion process entails a modification of the wax and cuticle structure of berries (appearance of micro fractures notably), which favors water vapor evaporation of berries thus accelerating drying (Daris, 1977; Exarchos, 1970; Riva and Peri, 1983).

The property of wax on the berries is therefore the major concern. According to reports of Radler (1965) and Radler and Horn (1965), the thickness of wax layer is approximately 2.3 μm and the total quantity of the wax material is 100 to 140 $\mu g \cdot cm^{-2}$. In berry, the wax compounds are grouped into soft wax with complex composition and hard wax composed essentially of oleanolic acid (60-70%).

In the present study, a chemical containing 4% ethyl oleate, the main ingredients of which is comparable to that used for raisin production, was applied before harvest on 'Merlot' grapes for wine to improve berry quality. Anatomical modification of external tissues and changes in anthocyanin concentration as related with enhanced coloration were investigated.

Materials and Methods

Chemical treatment

A chemical containing 40% ethyl oleate was diluted to 4% with water and sprayed over 'Merlot' (*Vitis vinifera* L.) wine grapes 40 days before harvest in 1989. For morphological, histological, and cytological studies, berries were sampled 2 and 6 days after treatment and at harvest from the vineyard of INRA located in Bordeaux region, France.

Light microscopy and transmission electron microscopy

The tissue samples were initially fixed in 2.5% glutaraldehyde for 90 min at 4℃ and then rinsed four or five times with 0.1M phosphate buffer (pH 7.2). The second fixing process was achieved using 1% osmium tetraoxide for 90 min and rinsed again with 0.1 M phosphate buffer five times. The fixed samples were dehydrated in the alcohol series with increasing concentrations. Dehydrated samples were laid in a silicon mold with epon + D.M.P. 30 for 4 days at 60℃. After polymerization, the embedded samples were sectioned into 1 μm thickness using an ultramicrotome (Ultracut R. Leica Co., Austria) and stained with toludine blue for light microscopy.

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At the same time, the samples were sectioned into 60 nm thickness and treated successively with uranyl acetate and lead citrate for 10 min each. After all the preparation processes, the samples were observed under transmission electron microscope (microscope JEOL 100 S).

Scanning electron microscope

Samples were dipped in liquid nitrogen (-196℃) for a few minutes and then immediately transferred to a deep freezer where liquid nitrogen finishes the evaporation process. The lyophilized samples were mounted on sticky-tape and tightly placed on aluminum stubs, coated with gold powder, and observed under scanning electron microscope (JEOL, JSM 840 A).

Anthocyanin analysis

The treatment was applied to 10 vines in 5 replications and 50 berries from 5 replicates were sampled 40 days after treatment. Anthocyanin content of the skin was analyzed according to the method described by Darné and Glories (1988). The analysis of the anthocyanin content was performed using a HPLC system. The separation of the anthocyanins was conducted in a Beckman XL-ODS C18 column, 70 × 4 mm I.D., 3 μm particle size. Five anthocyanin monoglucosides in the free form were delphinidin 3-monoglucoside (Dp-3mG), cyanidin 3-monoglucoside (Cy-3mG), petunidin 3-monoglucoside (Pt-3mG), paeonidin 3-monoglucoside (Pn-3mG), and malvidin 3-monoglucoside (Mv-3mG). Three acylated forms analyzed were acetic esters, caffeic esters, and coumaric esters.

The statistical significance was analyzed by t-test using the SAS statistical software (SAS Institute Inc., USA).

Results and Discussion

Appearance of grapes as influenced by the treatment

Treated berries showed a smoother and more colored external aspect than non-treated berries. However, substantial differences in appearance and coloration between the exposed and the hidden parts of the cluster to the chemical spray were observed. Even in individual berries, the extent of surface exposure to the chemical resulted in such differences.

Anatomical studies by light and transmission electron microscope

The ovary wall of the berries at maturity consists of outer epidermis, hypodermis, flesh, and inner epidermis (Fougere-Rifot et al., 1995). Microscopic observation revealed that outer epidermis of non-treated berries was formed as one layer of cells, approximately 6 to 9 μm thick, and hypodermis was formed with 4 to 6 cell layers in 'Merlot' grape. The thickness of the hypodermis, four cell layers beneath the epidermis, ranged from 120 to 180 μm in non-treated berries (Fig. 1A).

Compared to the non-treated berries, treated berries showed decreases in the thickness of epidermis and hypodermis. Epidermal layer of the treated berries was slightly thinner than non-treated berries varying from 5 to 7 μm. The hypodermal layer was notably flattened resulting in reduced thickness of four layers beneath the skin. The thickness varied from 85 to 100 μm (Fig. 1B).

Cellular level observation under transmission electron microscope showed that epidermal cells of non-treated berries were degraded although some organelles were recognizable (Fig. 2A). Those berries exposed to chemical spray collapsed completely with the cytoplasmic plasmolysis, and the organelles were no longer recognizable except the nucleus (Fig. 2B). In contrast, flesh cells did not appear to be affected by the chemical treatment. Flesh cells were still voluminous, very largely vacuolated, and had thin cell wall (Fig. 2C).

Overall microscopic observations suggested that the treatment entailed a decrease in skin thickness. The flattening of the skin might have been caused by the death or by the collapse of the epidermal cells exposed to the chemical that reduced

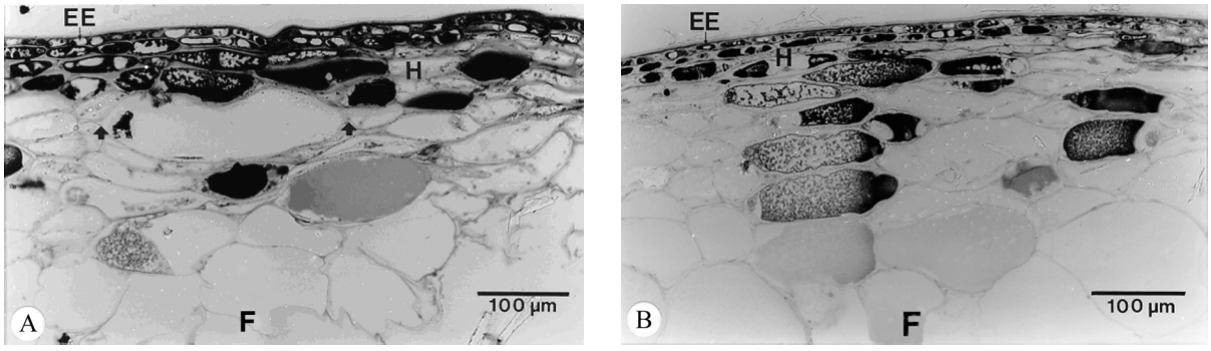

Fig. 1. Anatomical structure of the non-treated (A) and the treated berries (B) with 4% ethyl oleate sampled 6 days after treatment in 'Merlot' grape. A: The thickness of epidermis is 6 to 9 μm and that of the first four cell layers of the hypodermis ranged from 120 to 180 μm. B: Epidermical and hypodermical cells were flattened after treatment. Thickness of epidermis is 5 to 7 μm and that of the first four cell layers of the hypodermis ranged from 85 to 100 μm. EE, external epidermis; F, flesh; H, hypodermis.

turgescence of the vacuole. On the other hand, the fact that the flesh cells remained intact clued that the chemical might not be systemic.

Surface observation under scanning electronic microscope

Scanning electron microscopy revealed that wax layer morphology was modified by the chemical treatment. In non-treated berries, the epidermal surface was covered with platelet-shaped epicuticular waxes (Fig. 3A). The platelet-shaped wax was attached to the surface in different angles with different width/height ratios as reported by Barthlott et al. (1998).

Epicuticular wax layer seemed to be affected very rapidly by the treatment. After 2 days of treatment, epicuticular waxes appeared to be melting. The platelets disappeared and the surface seemed to be covered with a uniform flat wax layer (Fig. 3B). Even in treated berries, the epicuticular surface morphology showed difference between the exposed and nonexposed portions to the treatment. On the nonexposed portion, unchanged platelet-shaped wax existed with partially melted waxes on the limited area.

Biochemical modifications

Average anthocyanin composition of the non-treated and the treated berries were analyzed and compared in separate forms and in total (Table 1). Methylated free form Mv-3mG, acylated acetic ester, and coumaric ester anthocyanins increased, whereas the hydroxylated free form anthocyanins (Cy-3mG and Pt-3mG) tended to decrease. On the quantitative basis, total anthocyanin content increased by 8% in treated berries. Although the percentage increases were higher in acylated forms (21%) than in free forms (2%), the amount of increases in a large quantity was the highest in Mv-3mG (1.0 $mg \cdot g^{-1}$), the major pigment in grape. The result suggested that enhanced coloration of treated berries might be mainly the result of the increase in Mv-3mG and partly because of increases in acylated anthocyanins.

The application of ethyl oleate-base chemicals seemed to modify relative proportions of anthocyanin forms. The chemical could induce methylation and esterification of the hydroxylated free anthocyanins with acetic acids and p-coumaric acid converting some free form anthocyanins to methoxylated and acylated forms. The methylation of hydroxylated anthocyanins

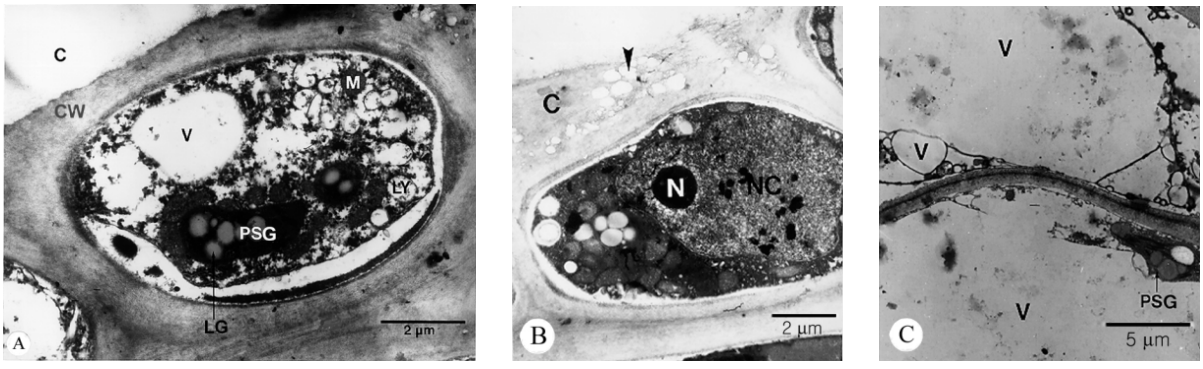

Fig. 2. Transmission electron micrographs of the non-treated (A) and the treated berries (B and C) with 4% ethyl oleate sampled 6 days after treatment in 'Merlot' grape. A: The cell was degraded but some organelles were still recognizable. B: Epidermal cells touched by the product are dead. The cytoplasm is precipitate. C: Flesh cells were not affected by the treatment. C, cuticle; CW, cell wall; LG, lipid globule; LY, lysosome; M, mitochondria; N, nucleus; PSG, plastid without starch grain; V, vacuole.

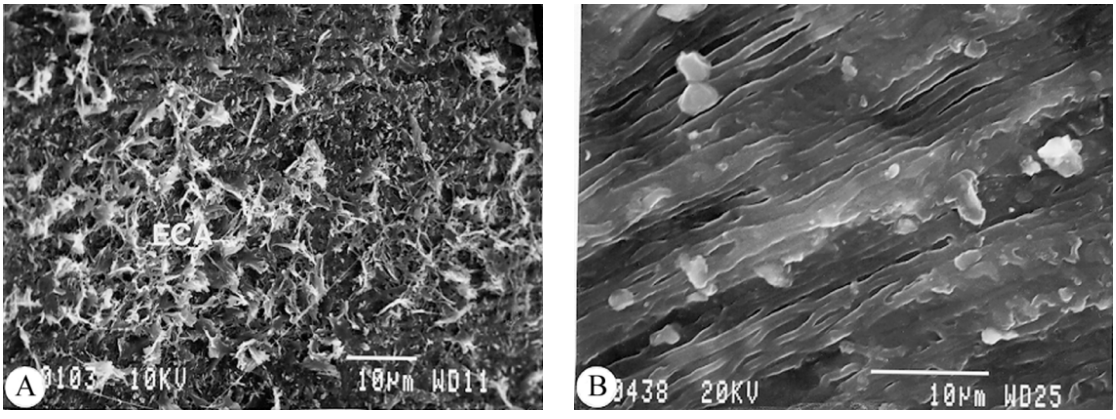

Fig. 3. Scanning electron micrograph of the non-treated (A) and the treated berries (B) with 4% ethyl oleate sampled 2 days after treatment in 'Merlot' grape. A: Epidermal surface covered epicuticula waxes formed platelet. B: Epicuticular waxes are melted and the surface is covered with one layer of uniformed waxed. ECA, epicuticular waxes.

Table 1. Anthocyanin contents of the skin according to 4% ethyl oleate treatment in harvested 'Merlot' grape.

Treatment	Anthocyanin contents ($mg \cdot g^{-1}$ fw)								
	Dp-3mG[z]	Cy-3mG	Pt-3mG	Pn-3mG	Mv-3mG	Acetic esters	Caffeic esters	Coumaric esrers	Total anthocyanins
Control	2.90 NS[y]	0.51 *	0.84 **	1.98 NS	4.93 *	3.20 *	0.31 NS	1.31 **	15.98 *
Treated berries	2.65	0.37	0.50	2.01	5.84	3.57	0.26	1.99	17.19

[z]Dp-3mG, delphinidin 3-monoglucoside; Cy-3mG, cyanidin 3-monoglucoside; Pt-3mG, petunidin 3-monoglucoside; Pn-3mG, paeonidin 3-monoglucoside; Mv-3mG, malvidin 3-monoglucoside. Three acylated forms were acetic esters, caffeic esters, and coumaric esters.
[y]NS,*,** indicate nonsignificant, significantly different at p=0.05 and p=0.01, according to the t-test, respectively.

might enhance deep black coloration of the treated berries. Therefore, besides the modification of epicuticular wax structure, changes in absolute pigment concentrations and relative proportion of individual anthocyanin forms could have altered the visual perception of the skin color.

Finally, the study showed morphological, histological, and cytological modification in the epidermal and hypodermal layers by using 4% ethyl oleate spray. Epidermal cells in berries exposed to the spray were dead and collapsed. Flattening of hypodermal layer also occurred by the cellular breakdown and the loss of turgescence of the vacuole resulting in the decrease in thickness. At the same time, treated berries rapidly experienced the deformation of the cuticular wax layer showing melting appearance. From biochemical aspects, modification of the relative proportion of individual anthocyanin forms was might be caused by low water content in the skin tissue.

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Ethyl oleate 처리에 의한 'Merlot' 포도 과피의 안토시아닌 함량과 해부학적 변화

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초 록. 'Merlot' 포도 품종의 수확전 ethyl oleate 처리는 외표피와 아표피의 두께를 감소시키며 착색을 현저히 향상시킨다. 무처리구의 과피 두께는 126-189μm인데 반하여 처리 과실의 과피두께는 90-107μm로 조사되었으며, 이러한 외표피와 아표피층의 두께 감소는 처리 후 외표피와 아표피를 구성하는 세포의 빠른 노화에 의하여 세포가 죽거나 탈수가 나타나기 때문인 것으로 조사되었다. 처리 후에 빠르게 과피 표면의 왁스층이 녹은 듯한 형태를 보이며 이는 착색을 증진시키는 것으로 관찰되었으며, 전체적인 안토시아닌 함량 역시 증가하는 것으로 조사되었다. 반면에 각각의 안토시아닌 함량에 있어서는 메톡시기와 결합하거나 유기산과 결합한 안토시아닌이 증가한 반면 수산기와 결합한 안토시아닌은 감소하는 경향이었다.

추가 주요어 : 외표피, 아표피, 형태학, 착색, *Vitis vinifera* L.