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Anatomical, Histological, and Histochemical Changes in Grape Seeds from *Vitis vinifera* L. cv Cabernet franc during Fruit Development

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Cabernet franc berries were sampled at five stages from berry set to harvest from an experimental vineyard in mid Loire Valley. Seeds were collected from representative berries in term of stage of development. The evolution of seed was followed both macro- and microscopically. For microscopy analysis, seeds were cut, put in a fixation solution, and cut into thin sections with a microtome. Five staining solutions were used for each seed sample: toluidine blue O, phloroglucinol, periodic acid−Schiff's reagent and naphtol blue black, vanillin, and *p*-dimethylaminocinnamaldehyde. Toluidine blue O staining revealed the evolution of tissue structures during grape seed development. We studied the changes in chemical compounds (lignin, polysaccharides, proteins, and tannins) with the other reagents. Seed lignification was achieved at veraison. Proanthocyanidins were localized in epidermis, inner cells of the soft seed coat, and inner cell layer of the inner integument. Finally, the localization of flavan-3-ols was linked with changes in cell walls of the outer integument.

KEYWORDS: Microscopy; histochemistry; *Vitis vinifera*; seeds; phenolic compounds

INTRODUCTION

From a winemaking perspective, the grape berry has four major types of tissue: flesh, skin, seed, and stems. The berry skins initially have greater practical importance because they contribute to easier extraction of polyphenols from musts during maceration than from seeds (*1*, *2*). Even though seeds represent only 0−6% of berry weight, they are a large source of phenolic compounds (*2*−*6*). Studies have investigated the composition of these compounds (*7*−*10*), their changes during ripening (*3*, *11*), and their localization (*12*−*14*). Other studies have indicated that the number and weight of seeds are related to berry weight and berry ripening (*15*−*19*). The relationship between berry size and seed number is quadratic (*20*). Moreover, the composition of wine is linked with berry size; wines made from smaller berries have a higher proportion of compounds derived from skin and seed (*18*). The seeds contribute catechins and procyanidins to red wine, but their flavanol content depends on the maturity of the grape (*4*, *21*, *22*) and maceration practices (*6*, *23*). Most of them are monomeric flavan-3-ols (catechins) and procyanidins or condensed tannins (*24*).

The highest flavanol concentrations are at veraison, after which they declined slowly until near maturity, at which time they remained relatively constant (*22*, *25*, *26*). The decrease is more rapid for flavan-3-ol monomers than for oligomers, so the average degree of polymerization increases with maturity (*12*, *13*, *25*). A study on seed from Cabernet franc showed that the flavanol content in the berries did not evolve during the month before harvest (*3*).

Few studies were done to localize the flavanol content in grape seeds. Thorngate noted a higher amount of flavan-3-ols in outer soft coat than in endosperm and suggested that procyanidins in the seed coat are lost as the seed browns (*13*). Geny showed that the tannins from the cell walls are more polymerized than those from the inner part of the cells and that at maturity, the difference is significant relative to veraison. Geny suggested a slight increase of the mean degree of polimerization of the seed tannins (*12*).

In *Vitis vinifera* varieties, seeds have a specific form. They are pear-shaped and the transverse section is a trigone. The seeds are constituted of a cuticle, an epidermis, and two integuments going round the albumen and the embryo (*27*). Seed color changes during fruit ripening from an initial green to a dark brown at harvest. The change in color and the hardening of the seed are concomitant with oxidation of phenolics (*11*, *28*). Ristic and Iland (*9*) proposed an 11-level diagram to describe changes in grape seeds between 21 and 92 days after flowering (berries reached a maturity of 26−27 °Brix). The relationship between seed growth and fruit maturation differs among varieties and indicate differences in seed polyphenols (*11*, *25*).

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Grape berry growth is characterized by three stages. The first corresponds to the period between the fruit set and the cell division and enlargement, the second to a rapid deceleration of growth and veraison, and the third to maturation. Seed development from fertilization to maturity can also be divided into three phases. Phase I includes fertilization and the period of rapid cell division when all seed structures are formed. During phase II, the seed accumulates mainly reserve materials. The increase in seed water content during this phase reflects the movement of water into the cells, leading to cell expansion. Thus, changes in seed water content and seed volume are closely associated. Cell number does not change during this phase, so the large increase in seed size entirely results from the increased cell volume. In phase III, the accumulation of reserve materials slows and then stops at physiological maturity (*29*).

Although several studies have investigated the development of grape seeds, their structure, and the polyphenol changes during fruit ripening, their histochemistry is not well-known; during ripening, the seeds, which are strongly lignified, become very hard, and few microscopy works are available considering the difficulties to obtain thin sections.

The aim of this investigation was to follow the evolution of the structure of grape seeds between berry set and berry maturity in relation with their compositional changes.

MATERIALS AND METHODS

Plant Material. The vineyard was between Saumur and Bourgueil, in the middle Loire Valley (France), on sedimentary terrains from the secondary and tertiary eras. The vines were *Vitis vinifera* var. Cabernet franc, clone 214 grafted on rootstock 3309C.

Sample Collection. Berries were collected at five stages during berry and seed development, starting when berries were about 3 mm in diameter and finishing when berries reached a maturity of 23 °Brix. These samplings were done 11, 27, 49, 60, and 123 DAF (days after flowering) and corresponded to Baillod and Baggiolini growth stages J, K, L, M, and N, respectively (*30*). To obtain berries at the same stage of development, various methods were used according to the stage. For the sampling of berries before veraison (11, 27, and 49 DAF), flower pedicels were marked when the floral cap fell, corresponding to growth stage I (*30*). At the three dates, 25 of the marked berries were collected randomly. Among these berries, we analyzed only the five most homogeneous berries in terms of size and color and representing the median class. In the field, berries were cut in half to remove the seeds, which were then also cut in half. The parts of seeds were transferred immediately into a fixation solution containing 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. The respective samplings were immediately packed in ice and stored at 4 °C. At the beginning of veraison (60 DAF), 25 green berries were collected for rheologic sampling according to Grotte (*31*). At the end of ripening (123 DAF), berries were collected and immediately stored at 4 °C. These berries were sorted by measurement of the sugar content. Afterward, the seeds of each berry were separated from the skin and the pulp (60 and 123 DAF). One seed from each berry was randomly chosen. We retained three seeds among the 25 that seemed homogeneous in terms of development (color and size). The seeds were transferred immediately into a fixation solution containing 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2.

Binocular Analysis. The seeds were cut at the median level with a strip razor. Photographs were taken with a digital camera, OLYMPUS C-3030, coupled to a zoom stereo microscope, OLYMPUS SZ-60.

Microscopic Analysis. Seeds were transferred into a fixation solution for 2 h, under vacuum. Thereafter, samples were dehydrated in an increasing gradient of ethanol and embedded in a Technovit 7100 [2-hydroxyethyl methacrylate] resin (Kulzer, Germany) according to Kroes (*32*). Specimens were stored at 37 °C. The sections, from 2 μm, were obtained by a LEICA RM 2165 microtome.

Five staining solutions were used for each seed sample.

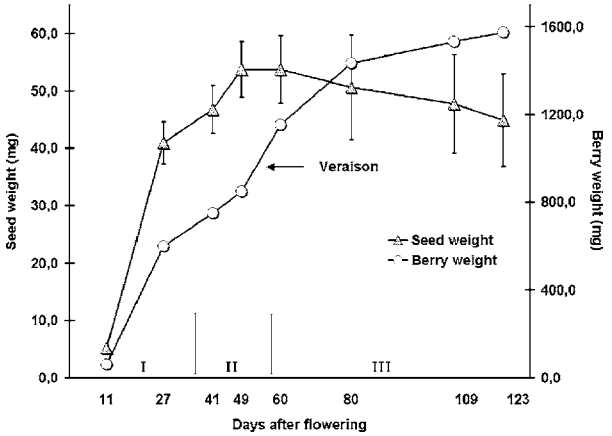

Figure 1. Growth of the grape seeds throughout berry development. The development of a grape berry follows a double sigmoid, which can be divided into three growth stages. Seed weight increased proportionally with the increase in mass of berries during phase I, corresponding to the first two samples.

Toluidine blue O (Réactifs-RAL, France), a metachromatic reagent, was used to determine the seed structure. The different structures of grape seed are colored in several blues (*33*). The sections were stained for 1.5 min in 0.5% toluidine blue and rinsed with distilled water.

DMACA (*p*-dimethylaminocinnamaldehyde), purchased from Acros Organics (Morris Plains, NJ), was utilized to stain flavan-3-ols by Gutmann and Feucht (*34*) and in our laboratory by Chevalier (*35*). The staining procedure was modified as follows. The slides with sections were placed on a hot plate at 45 °C, and the reagent was added (0.5 DMACA in H_2SO_4 98% 0.5 M in butan-1-ol). After 15 min, they were rinsed with 95% ethanol and dried at ambient temperature. Flavan-3-ols are colored in blue; cell walls and cytoplasm are not stained.

Vanillin−HCl (Merck, Darmstadt, Germany) was used to identify the catechins and condensed tannins according to Dai (*36*). Sections were stained for 30 min (vanillin 10% in HCl−ethanol (v/v)), regressed with ethanol 95%, and dried on hot plate.

Phloroglucinol−HCl (1,3,5-trihydroxybenzene from Sigma, St. Louis, MO) was used to label lignin (*37*). The sections were stained for 30 min with phloroglucinol reagent (phloroglucinol 10 g, ethanol 100, 95 mL), regressed with HCl, and air-dried.

Periodic acid−Schiff's reagent (Merck, Darmstadt, Germany) and naphtol blue black (Sigma) were utilized to stain insoluble carbohydrates and proteins, respectively (*38*). The staining procedure was modified as follows. After hydrolysis with periodic acid (1% in distilled water) for 20 min, the sections were rinsed with distilled water. In the dark, sections were stained with Schiff's reagent for 30 min and then rinsed with distilled water. Staining with naphtol blue black lasted for 30 min (naphtol blue back 1 g, acetic acid 7 mL, distilled water 100 mL) and then the sections were rinsed with distilled water. The samples were regressed with acetic acid at 7% and air-dried.

All the sections were mounted on Isomount and observed with an OLYMPUS BH-RFC microscope coupled to a camera 3CCD SONY.

RESULTS

Macroscopic Observation. Seed growth was linked with the increase in berry mass during phase I. Seed weight increased up to 27 DAF and then slowed. Maximum seed weight was reached 1 week before veraison (49 DAF) and then declined by about 16% until harvest (123 DAF, **Figure 1**). Relative to berry development, the first two samples corresponded to stage I, the next two to stage II, and the last one to stage III (*25*).

Seed size increased at stage I but did not change between the beginning and the end of veraison (**Figures 1** and **2A**). Seed size declined little from veraison to harvest (**Figures 1** and **2B**).

Seed color changed during development. Eleven DAF, the seed was translucent white-green. After 27 days, the seed was

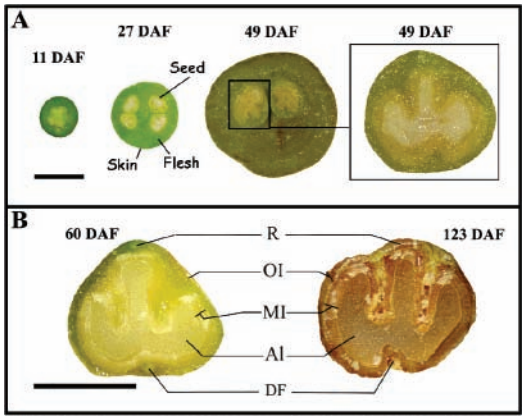

Figure 2. Cross section of berries and seeds from berry set to harvest. The color of the grape seed changed from translucent white-green to dark brown: (**A**) size increased among the first three samples; there was no evolution in seed size between the beginning and end of veraison; (**B**) the size did not change from veraison to harvest; the cross section in the median area of the seed served to distinguish the outer integument (OI), medium integument (MI), and albumen (AI). The raphe (R) is visible. DF = dorsal face. Bar = 2.5 mm.

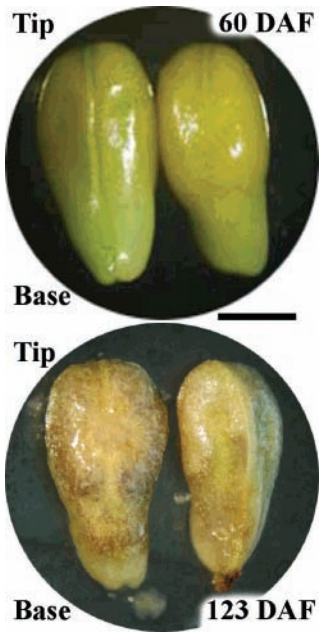

Figure 3. Macroscopic observation of seeds at two stages: 60 DAF and 123 DAF. The tip had a deeper color than the base. Bar = 2.5 mm.

yellow-green. Before veraison, 49 DAF, the seed was green and then turned brown until veraison. From veraison to harvest the brown darkened. At the beginning of veraison, 60 DAF, seeds were green-brown and at the end, 123 DAF, brown.

In addition, the color was not uniform. Seeds could be divided into two parts: the tip and the base. Visually, the tip had a deeper color than the base, because the tip was covered by a soft mass thicker than that of the base (**Figure 3**).

Seed Structure (Figure 4). Only the samples corresponding to 11, 27, 60, and 123 DAF are described in this section.

The evolution of the structure was studied with toluidine blue O staining, which allowed us to visualize the whole tissue, since it is a metachromatic reagent.

The cross section in the median area of the seed revealed five zones (**Figure 4**): (i) cuticle and epidermis; (ii) outer integument or soft seed coat, composed of large parenchymatous cells; (iii) medium integument or hard seed coat, composed of two layers of cells; (iv) inner integument with three layers of cells; (v) endosperm and embryo.

Evolution from Berry Set to Harvest (Figures 5A−D). Each zone underwent drastic modifications from berry set to maturity. At 11 DAF, the transversal section of seed was circular, but the trigonal form started to appear (**Figure 5A**). The different layers were composed of cells organized in concentric tissues; fossettes or seed folds began to differentiate; the ridge under the raphe appeared. At 27 DAF, the seed reached its definitive structure, but these tissues continued to differentiate (**Figure 5B**).

Cuticle and Epidermis. At the first observation, the cuticle was thin and deeply colored. At the first stage, the epidermis was constituted of cubical cells, and after the cells were rectangular (**Figure 5A,B**). The epidermis stopped growing at veraison; cell size evolved little (**Figure 5C,D**). At the first stage, the cytoplasm was very dense. The vacuoles were occupied either by many spherical structures or by structures forming a network occupying the entire vacuole or placed side by side along the tonoplast. These structures were intensively colored by toluidine blue O (**Figure 5A−D**).

Outer Integument. The outer integument is constituted of parenchymatous cells. The thickness of the cell layers was greater in the ventral face than in the dorsal face. The difference between both sides increased during ripening; the dorsal face became thinner. The volume of the parenchyma cells increased until veraison; thereafter only the form of the cells was modified; the deeper cells were often round after veraison. The toluidine blue O reagent distinguished two parts in the outer integument. On the ventral face, the external cells had vacuoles without precipitates, whereas the cells near the medium integument contained many precipitates from 11 DAF (**Figure 5A**). For this latter zone, all the cells were fully and intensively colored at maturity (**Figure 5D**). In the dorsal face, 11 DAF, the aspect of the outer integument was similar to that of the ventral face. At maturity, vacuoles of cells were entirely and intensively colored, and the cell size had increased. From veraison until harvest, the cells of the outer cellular tissue became deformed and plasmolyzed, but the cells of the inner cellular tissue remained turgescent. The epidermis and the outer integument were made of a soft layer of cells, which covered the seed. The raphe was located in the dorsal face of this tissue after the first stage.

Medium Integument. At 11 DAF, one layer of rectangular cells with thin walls constituted the medium tissue (**Figure 5A**). After 27 days, there were two layers of cells. The cell walls became thicker, whereas the cell lumen decreased. Wall thickening was achieved before ripening. From veraison, the histological structure of the medium tissue was completely set with cells strongly sclerified (**Figure 5C**).

Inner Integument. The inner integument is between the medium integument and the albumen. Three layers of rectangular cells constituted this integument (**Figure 5A**). At 11 DAF, this tissue was well differentiated. Very small cells with a dense cytoplasm constituted the first layer; the second showed cells with a higher diameter, thin cell wall, and no precipitate in the vacuoles; the third had cells with an intermediate diameter and with a strongly colored precipitate in the vacuoles. At 27 DAF, the first and the third layer of cells were visible, but the second layer was rarely visible, because it was torn (**Figure 5B** and **Figure 6**). At veraison, the cells of the inner layer were intensively colored, but no variation in staining was observed until harvest (**Figures 5C,D** and **6**).

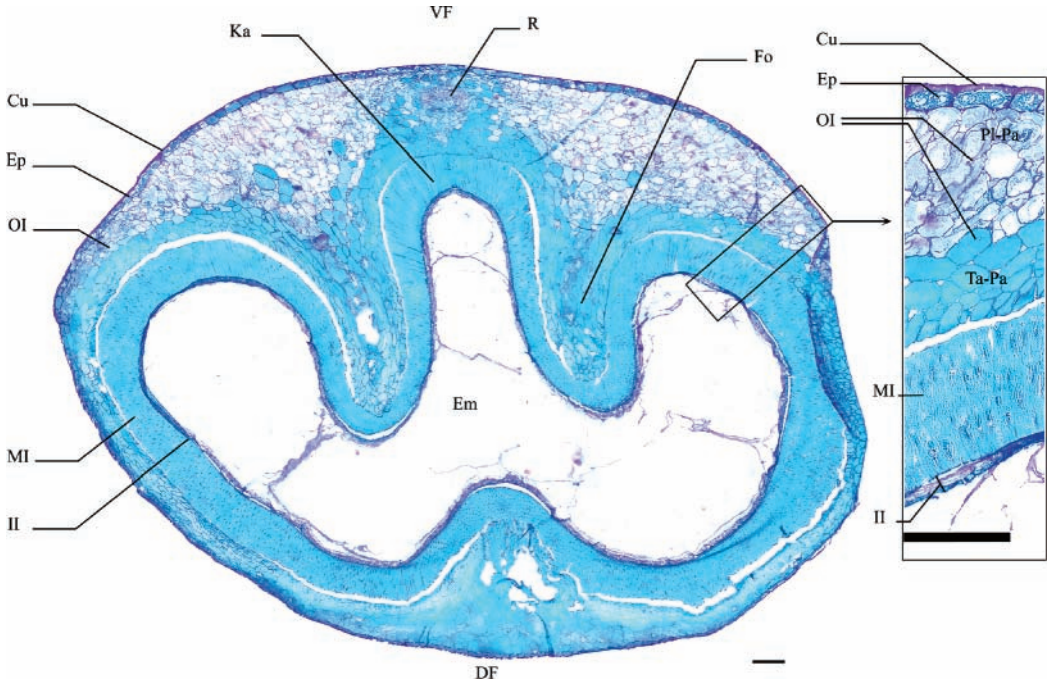

Figure 4. Seed structure at harvest. Light micrograph of cells of grape seeds at 123 DAF with toluidine blue O staining. The cross section in the median area of the seed served to distinguish five zones: cuticle (Cu) and epidermis (Ep), outer integument (OI) composed of large parenchymatous cells; medium integument (MI) composed of two layers of cells; inner integument (II) with three layers of cells; endosperm and embryo. As in some grape seeds, in this section, the endosperm was replaced by an empty space (Em). The thickness of the cell layers of the OI was larger in the ventral face (VF) than in the dorsal face (DF). The cells of the outer cellular tissue of the OI became deformed and plasmolyzed (Pl-Pa); the cells of the inner cellular tissue were still turgescent, and vacuoles of these cells were entirely and intensively colored (Ta-Pa). Fo = Fossettes; R = Raphe; Ka = Karina. Bar = 10 μm.

Endosperm and Embryo. The center of the seed was occupied by the nucellus from the first stage. The nucellus appeared as a parenchymatous tissue, surrounded by integuments and containing the embryo sac. The center of the section was empty. At 27 DAF, the albumen was differentiated and delimited by an epidermis; the cells were numerous, with a narrow form, which became cubical. At veraison, the cells of this layer were deformed, and at maturity, they disappeared. The cells of the albumen were polygonal. Between 27 DAF and veraison, the cytoplasm was occupied by vacuoles with a diffuse content. At veraison, many cells with small vacuoles but with a dense content constituted the albumen. During fruit ripening, the cytoplasm was occupied by a large vacuole, but there were also some little ones with dense content. In some seeds, the endosperm was replaced by an empty space; no cells were observed (**Figures 4** and **5D**).

Histochemical Results. *Lignin (**Figure 7A,B**).* At 11 DAF, no coloring was observed in the seed (**Figure 7A**). A light pink was visible 27 DAF on the cell walls of the medium integument; at this date, the walls were thin and the cells were large. At the beginning of veraison, the cell walls of medium tissue were colored. At this time, the cell wall was very thick, and the cellular volume was strongly reduced. The color intensity of the wall increased up to the end of veraison. The color intensity was not modified between veraison and harvest.

*Polysaccharides (**Figure 7C,D**).* The cuticle was intensively colored from flowering to harvest. The cell walls of all tissues except the medium tissue were colored at the four dates studied. At the first date, the walls were slightly colored, and after veraison, they were more stained. In the epidermal cells and the internal cell layers of the outer integument, the precipitates or the vacuole volumes were colored. This color increased before veraison and then remained constant until harvest. The cell content of the last layer of the inner integument was strongly colored. The medium integument was distinctly stained at 11 DAF (**Figure 7C**). At harvest, the medium integument was light pink at the cellular lumen level (**Figure 7D**).

*Proteins (**Figure 7C,D**).* Proteins stained in blue were largely present in the endosperm. In this tissue, the proteins were contained in the vacuoles. Before veraison, the coloration was diffuse (**Figure 7C**), and after veraison it was concentrated in globoid structures. The proteins were strongly condensed at harvest (**Figure 7D**). Their size and number varied according to the cells. The cytoplasm of the parenchymatous cells was lighter blue at 11 DAF. This staining did not appear thereafter.

*Catechin and Condensed Tannins (**Figure 8A−D** and **Figure 9**).* DMACA (**Figure 8A,B**) and vanillin−HCl (**Figure 8C,D**) colored flavan-3-ols in blue and pink respectively. The same tissues were colored by the two staining solutions (**Figure 8A−D**). **Table 1** shows the time course of flavan-3-ols in the grape seed. From 11 DAF, three tissues were intensively colored by the DMACA and vanillin−HCl reagents: the epidermis, the outer integument, and the inner integument. In the outer integument, the cells stained were the parenchymatous ones of the internal zone and the cells around the raphe. In the inner integument, it was the last cell layer (**Figure 9a**).

On the first date, the cellular content appeared very granulous; the granulations were dispersed in the vacuole volume or stuck to the inner face of the tonoplast. At 27 DAF, the precipitates in the vacuole were strongly colored and coalesced into large globules. After this date, the precipitate density and the coloring intensity showed few modifications in epidermal cells.

The outer integument evolved until harvest. On the dorsal face of the seed, the coloration of the cell progressed toward the epidermis. At the end of veraison, the cell vacuolar volume of the parenchyma was completely empty (**Figure 9b**). As harvest approached, the colored cells were closer to the

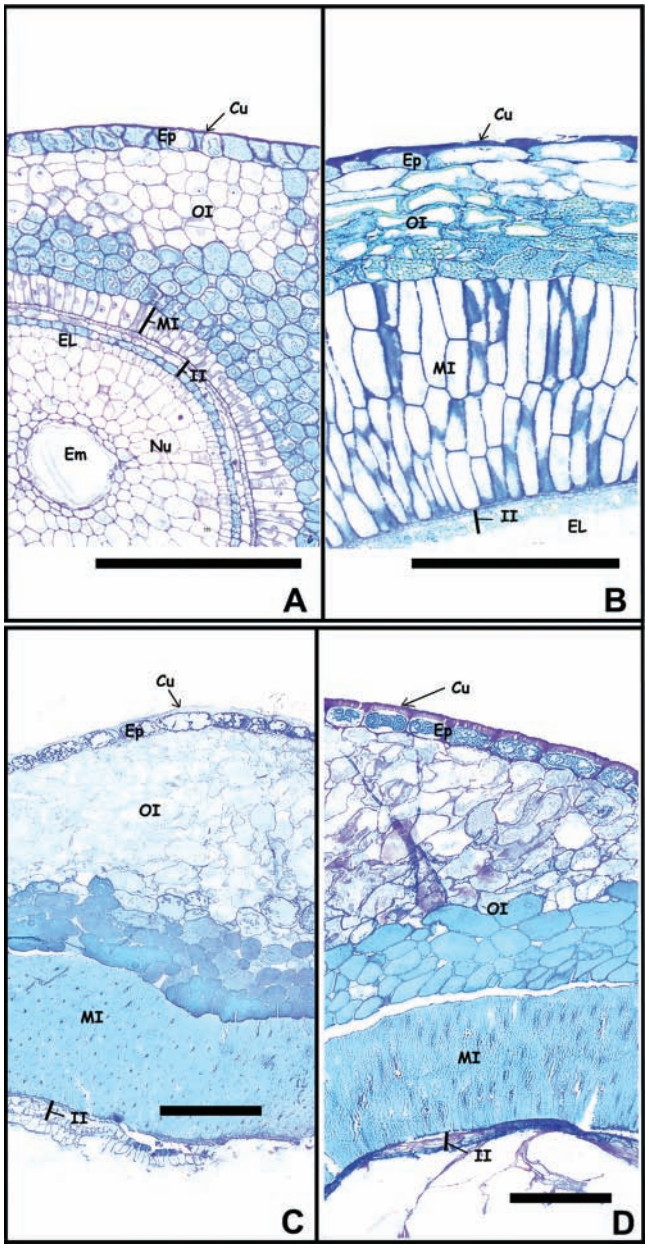

Figure 5. Evolution of the seed structure from berry set to harvest. Light micrograph of cells of grape seeds (**A**) 11, (**B**) 27, (**C**) 60, and (**D**) 123 DAF with toluidine blue O staining. The cross section in the median area of the seed served to distinguish the cuticle (Cu), epidermis (Ep), outer integument (OI), medium integument (MI), inner integument (II), nucellus (Nu), and embryo (Em). In **panel A**: the cytoplasm of the cells of Ep was very dense. The vacuoles were occupied by structures placed side by side to the tonoplast or forming a network occupying the whole of the vacuole. One layer of rectangular cells constituted the medium integument. The center of the seed was occupied by the nucellus surrounded by integuments and containing the embryo sac. The center of the section was occupied by an empty space. An epidermis layer limited the nucellus, with a narrow form (El). In **panel B**, two layers of rectangular cells constituted the medium integument. The albumen was differentiated, and an epidermis layer (El) limited the perisperm, with a cubical form. In **panel C**, The vacuoles of the cells of Ep were occupied by many spherical structures intensively stained. The volume of the parenchyma cells (OI) increased. The cell walls of MI became thick, whereas the cell lumen decreased. In **panel D**, The external cells of the OI were plasmolyzed; the cells of the inner cellular tissue were still turgescent and intensively stained. The thickness of the cell walls of the MI did not change. Bar = 10 μm.

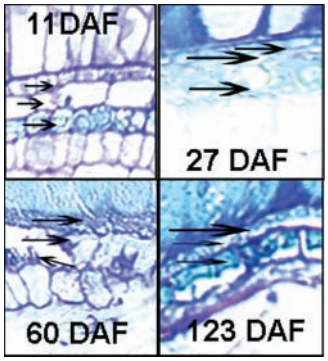

Figure 6. Evolution of the three layers of cells of the internal integument (a shadow per layer).

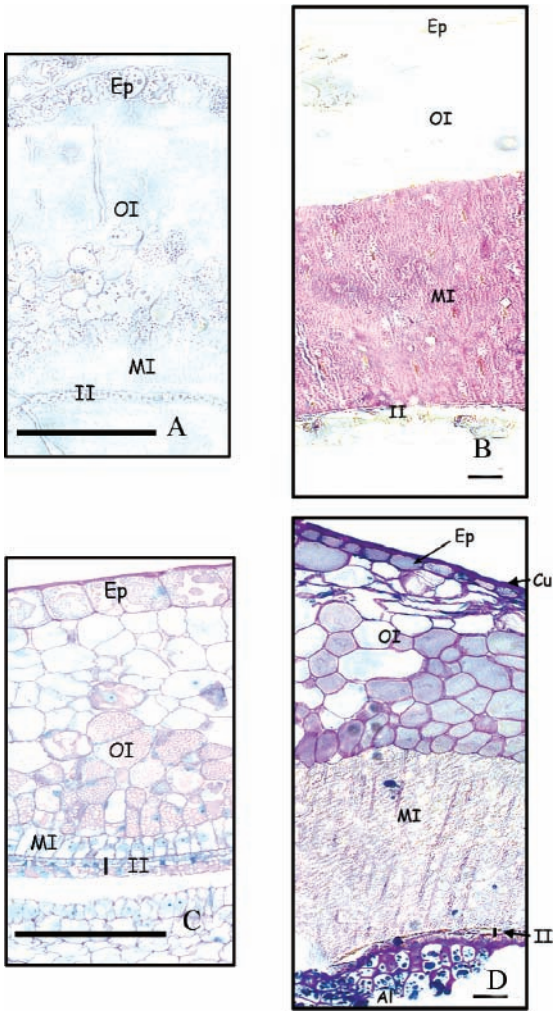

Figure 7. Histochemistry of the seed. Light micrograph of cells of grape seeds at berry set and harvest (11 DAF and 123 DAF). **Panels A** and **B** show the lignin stained by the phloroglucinol reagent in pink. In **panel A**, at 11 DAF, the cell walls of medium integument (MI) were not colored. In **panel B**, at 123 DAF, the cell walls of medium integument (MI) were colored. The cell wall was very thick and the cellular volume was strongly reduced. In **panels C** (11 DAF) and **D** (123 DAF), the polysaccharides were stained in pink by periodic acid–Schiff reagent (PAS). In **panel C**, the proteins were stained in blue by the naphtol blue black. The cell walls of all tissues were colored by PAS; however, the medium integument (MI) was light pink. Proteins were concentrated in the endosperm (Al).

epidermis. On the ventral face of the seed, only the deeper cellular layers of the outer integument fixed the coloring. The fossette cells were completely colored (**Figure 9a,b**). From

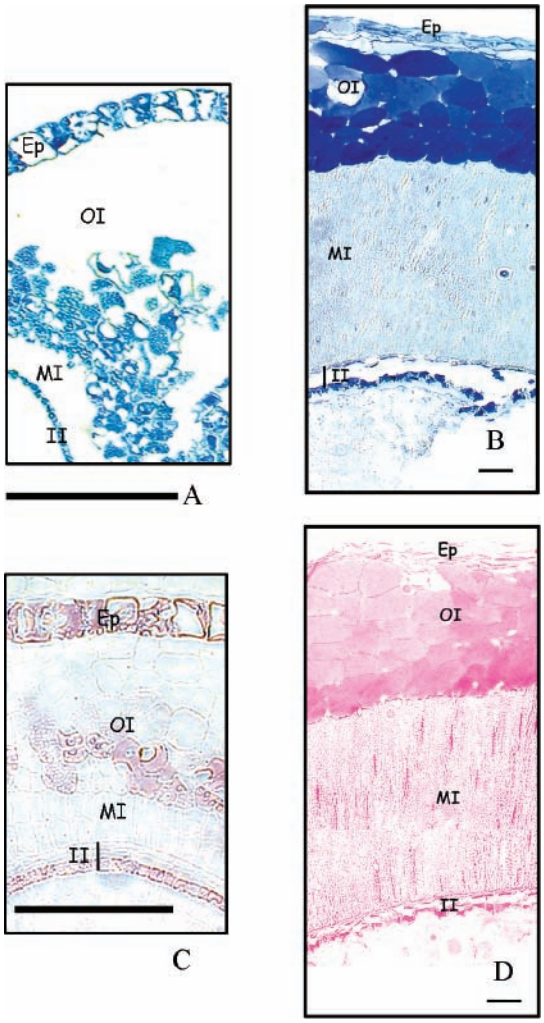

Figure 8. Histochemistry of the seed. Light micrograph of cells of grape seeds at berry set and harvest (11 DAF and 123 DAF). In **panels A** (11 DAF) and **B** (123 DAF), the flavan-3-ols were stained with DMACA in blue. The epidermis (Ep), the outer integument (OI), and the last cell layer of the inner integument (II) were intensively colored. In **panels C** and **D**, the flavan-3-ols were stained with vanillin in pink. The same tissues were colored by DMACA and vanillin. Cu = cuticle. Bar = 10 μm.

veraison to harvest, the color intensity increased strongly and the cells remained round.

There were no tannins in the medium integument; sometimes the narrow lumen of the cells was slightly colored. At ripening, the last layer of the inner integument was intensively colored by the DMACA and vanillin−HCl reagents (**Figure 8B,D**). Whatever the date studied, there was no flavan-3-ol in the endosperm.

DISCUSSION

Macroscopic Observations. Four of the five samples could be placed in the linear diagram of developmental changes in the appearance of grape seeds of Ristic (*9*): 27, 49, 60 and 127 DAF (days after flowering corresponds to 30, 45, 70, or 75 and 92 DAF in Ristic's diagram (Table 2). Size, shape, hardness, and color evolved during seed development, and our observations agree with other works (*9*, *11*, *25*).

During ripening, the tissues evolved; their hardness and color changed. In agreement with Ristic and Iland (*9*), at nouaison the seeds were soft, and they were hard at veraison.

Seed Structure. Ristic and Iland distinguish two integuments: outer and inner (*9*). In line with Galet and Riberau-Gayon and Peynaud (*15*, *27*), we propose three integuments: outer integument, medium integument, and inner integument. The outer integument is formed by the cuticle, epidermis, and parenchymatous tissue. Hard cell layers constitute the medium integument. Three cellular layers before endosperm form the inner integument.

Evolution of the Seed Structure. The inner structure of grape seed evolves in time. From anthesis to veraison, the outer integument with epidermis forms the soft tissues. This period corresponds to intensive cellular division and differentiation, which reaches a maximum at 20−25 DAF (*9*, *39*). The seed increases in size during this period. The thickness of the integument increases initially and then decreases at veraison, when seeds reach their full shape and size (*40*). At ripening there is a rapid dehydration of outer integument, the seed surface becomes hard with a rough surface and browner appearance (*41*). From a histological point of view, this phenomenon is characterized by cell plasmolysis of external zone, and the epidermal cells remain thin-walled. Cells in the fossettes may become dry sooner than cells in the chalazal area. The dehydration of outer integument may be associated with seed-coat waterproof qualities and seed dormancy (*41*). The external integument is completely dehydrated at maturity, takes a "fibrous aspect", and forms a fine skin around the seed. Nevertheless, the cells of the inner tissue remain turgescent with a rounded form.

In the medium tissue, the cell wall becomes thick and impregnated with lignin. These cells are strongly lignified, and the cell lumen is reduced. The differentiation of this tissue is effected before the veraison and hardening during the ripening. Hardening of the grape seed is due to lignification of this tissue. The cells have a structure similar to that of sclereids. Our observations agree with those of Galet (*15*). Yet, there was no localization of raphides in the medium tissue of Cabernet franc. Lignin in cell walls gives the seed its hardness and the definitive form and strengthens seed coat cells; these mechanisms render them less digestible by animals.

The epidermis and inner and medium integuments correspond to the transformation of the external integument of the ovule (primine). The inner integument of the grape seed corresponds to the internal integument of the ovule (secondine).

At 11 DAF, only nucellus was observed. The endosperm grows and replaces the nucellus from 35 days after anthesis (*9*). In our study, the endosperm was clearly differentiated at 27 DAF. The endosperm cells contained globoids in the vacuole and contained proteins but no crystalloids. The endosperm is a soft tissue in the inner integument. In mature seeds, the seed coat offers mechanical protection to the endosperm and embryo and contributes to maintaining dormancy (*9*). Numerous grape seeds are empty and the percentage of empty grape seeds varies with the cultivar and year; this aspect may be due to a nutritional disorder (*15*). The endosperm is degenerative; the microscopic observations sometimes revealed traces of endosperm. Quickly after fecundation, the endosperm tissue became disorganized.

The color in the seeds was not uniform; the tip was more deeply colored than the base because the tip was covered by a soft mass thicker than that of the base.

The change of color of the seed during fruit ripening was accompanied by a hardening of the medium integument. The dehydration of the outer integument, the intensive lignification of medium integument, and the phenolic compounds in the inner integument suggest several mechanisms. (i) An explanation for these concomitant events is the lignification of the medium tissue just before veraison and a change in seed development, which

Table 1. The Evolution of Flavan-3-ols in the Grape Seed[a]

tannins (DMACA/vanillin–HCl)	epidermis	outer integument		medium integument	inner integument	albumen
		outer layer	inner layer			
11 DAF	3	2[b]	5	0	4[c]	0
27 DAF	3	3[b]	5	0	4[c]	0
60 DAF; veraison	4	5[b]	5	1	5[c]	0
123 DAF; harvest	4	5[b]	5	1	5[c]	0

[a] Layers are scored from 0 (lack of coloring) to 5 (highly colored). DAF refers to days after flowering. [b] On the ventral face, only the inner cells were colored. [c] Only the last cell layer.

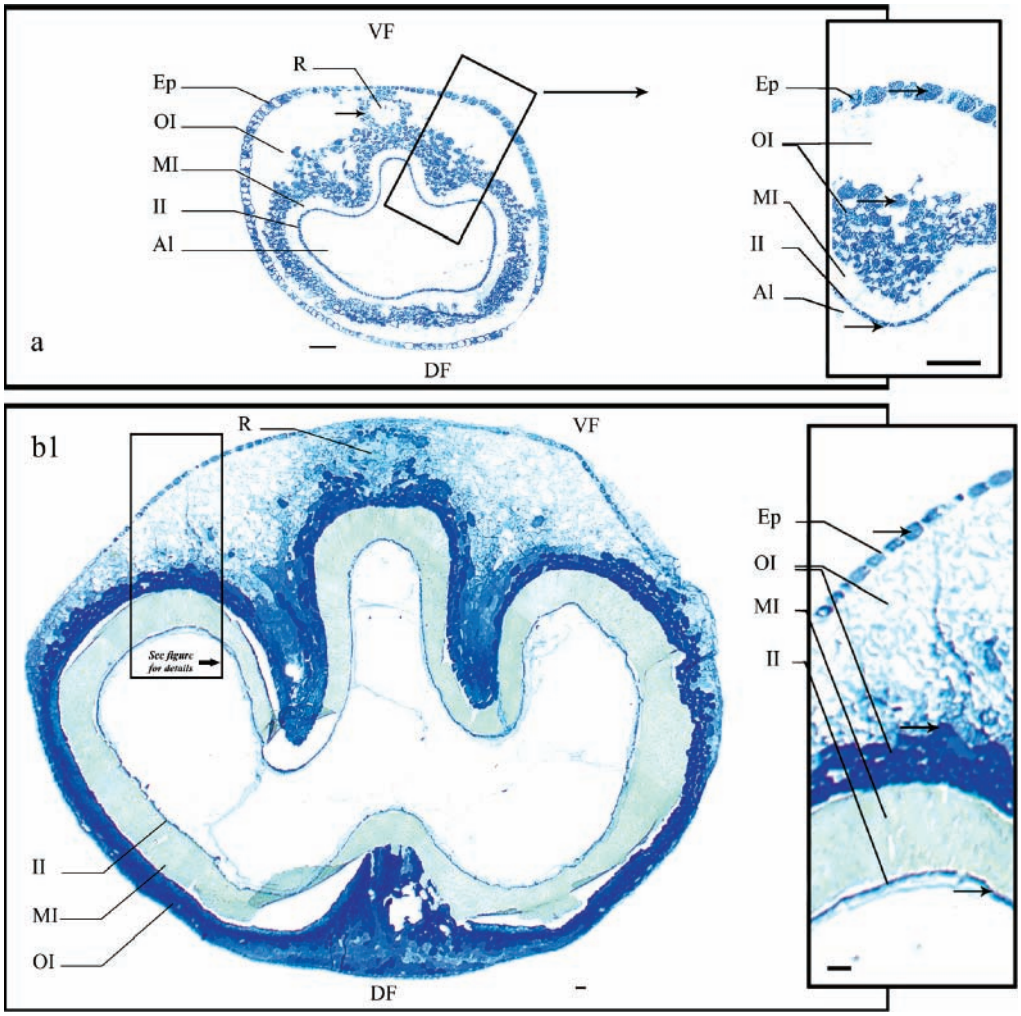

Figure 9. Evolution of the flavan-3-ol contents of the seed. Light micrograph of cells of grape seeds at berry set and at harvest (11 and 123 DAF) with DMACA reagent. **Panel a** shows a cross section of a seed 11 days after flowering. Three tissues were stained with DMACA (arrows): the epidermis (Ep); cells in the internal zone and cells around the raphe (R) in the outer integument (OI); the last cell layer in the inner integument (II). In **panel b**, the same tissues were stained but more intensively. On the dorsal face (DF), the OI was entirely stained. On the ventral face (VF), the deeper cells of the OI were still turgescent and intensively stained. Cu = cuticle. Bar = 10 μm.

led to an oxidative process of flavan-3-ol monomers and procyanidins in the external tissue during ripening. This oxidation process fits with that reported in a previous study (*11*, *28*). The products of oxidation would be flavan-3-ols cross-linked with carbohydrates and proteins. Wall-bound phenolic groups appear to act as a nucleation site allowing oxidative coupling to cross-link wall components as part of maturation events, in defense responses, and in the formation of specialized cell walls (*42*). Moreover, this oxidation has important implications in wine-making (*43*, *44*). (ii) Hardness of the medium integument and the dehydration of the outer integument provided mechanical protection. They constitute a physical barrier to entry of oxygen. Presence of phenolics restricts exchange of gases between the embryo and environment. (iii) The phenolic compounds and the hardening of cell walls could serve in protection (role in limiting insect predation or fungi attack) and may also cause cells to be waterproof. This is especially the case for the third layer of the inner integument. (iv) The biosynthesis of seed polyphenols in the seed coat is consistent with their supposed role in seed dormancy and longevity (*45*).

Phenolic compounds were observed in three tissues: epidermis, a wide part of the outer integument, and the inner layer of

Table 2. Relationship between Appearance of Grape Seeds Sampled and Linear Diagram of Developmental Changes in the Appearance of Shiraz Seeds from Ristic and Iland (*9*)

seeds samples (DAF)	appearance description	Ristic and Iland diagram (DAF)
27	Seeds were green-yellow and soft. Chalaza appeared as a slight light circle	stage 2 (30)
49	Seeds appeared to be green and soft. They reached 90% of their full length. Chalaza was a light yellow smooth circle. Keel appeared as a light colored narrow line	stage 4 (45)
60	Seeds were yellow brown to brown (dorsal side). The light brown chalaza was sunken and wrinkled. Seed became harder and rougher in texture	stage 7 and 8 (70 and 75)
123	Seeds were fully formed. Chalaza was located toward the notch, sunken and dark. Raphe was distinctly noticeable. Fossetes were dark and hard.	stage 11 (92)

inner integument. Flavan-3-ols were concentrated in the outer integument; from a winemaking perspective, this observation is important because the compounds from this region would be partly extracted during the wine process. Histological studies revealed no flavan-3-ol compounds in endosperm. This observation disagrees with that of Thorngate and Singleton (*13*) who found flavan-3-ols in the endosperm but in quantities lower than those in the outer soft seed coat. It might be argued that phenolic compounds found in the endosperm by biochemical studies could be attributed simply to contamination of the endosperm with material from seed coat, especially the flavan-3-ols from the last cell layer of the inner integument.

From 11 DAF, the amount of phenolics was large. Biosynthesis of procyanidins appears to coincide with the initial rapid period of berry growth (*19*). In small green seeds, the concentration in tannins was at maximum, indicating that the accumulation of seed tannins began during the early stage of seed development. The level of seed tannins is highest at the beginning of veraison (*46*) and declines during fruit ripening (*11*, *25*). Nevertheless, the intensity of DMACA coloring increased strongly after veraison; concomitantly the outer layers of parenchymatous cell on the ventral face were plamolyzed, whereas the inner layers kept their round form. These observations may be explained by a solidification of the cell content. This solidification could be correlated with the strong dehydration of the outer integument during ripening and the abundance of polymerized phenolic compounds. Pratt noted that flavan-3-ol concentration in cells is roughly accounted for by the concentration found in the outer seed coat (*39*). The solidification of the cells rich in tannins before harvest could affect the aptitude for extraction of these compounds. The reduced amount of seed tannins was attributed to their reduced extractability (*11*). The placement side by side of the tannins and the tonoplast could lead to an irreversible adsorption between the galloylated tannins and the cell wall (*12*).

The presence of the medium integument, which is waterproof and very hard, could prevent the phenolic compounds of the inner integument from being extracted during winemaking.

Moreover, the increased intensity of DMACA coloring during ripening that we found seems to disagree with previous studies. Several explanations may be proposed. (i) In these studies, after the seeds were crushed, the flavonoids were extracted with acetone, methyl alcohol, or both. Then, the identification and the quantification of monomeric and polymeric flavanols were achieved using RP-HPLC-UV. UV detection is well correlated with the amount of flavanol units. Because UV (280 nm) is not specific to flavanols and a high concentration of anthocyanins could interfere, fractionation is generally used to remove these flavonoids (*23*). This fractionation could lead to a loss of catechin. (ii) Using histochemistry/DMACA could lead to differences with RPLC−UV detection (*47*). DMACA is very specific to flavanols, in particular, epicatechin (*48*). As pointed out by Errea, staining with DMACA provides selective demonstration of monomeric and oligomeric flavanols with a characteristic bluish color; polymeric flavanols are not stained but are known to give a yellowish tint when present in sufficient amounts (*49*). Vivas et al. (*50*) showed that the high number of units in a fraction of procyanidins led to a probable clutter of the molecule, and the response of the chemical reaction decreased with increasing polymerization. The type of polymerization, "C4−C8" or "C4−C6", or the type of condensate could modify the yield of the reaction with DMACA. Moreover, aggregation without polymerization could strengthen the staining of these compounds. Because of the amplitude of polymerization in the seed, the reaction may prevent precise quantification of these compounds (*47*). (iii) In our study, the procyanidins were colored whatever their extractability, because it was an *in situ* reaction. The aggregation could lead to a modification of the solubility of these compounds and could explain differences with the RPLC methods. Because extractability could be modified during ripening (*22*), this could decrease the extraction yield before measurement by RP-HPLC. (iv) Cabernet franc could have specific quantity and quality of procyanidins, as was shown for other varieties (*8*, *51*).

Our results suggest that evolution of the tannins in the seeds is related to evolution of cell wall, but more investigations should be done, in particular, about the composition and structure of the grape seed cell wall and the evolution of the cellular structures, the oxidation of phenolic compounds and their implication in the changes in cells walls, and the impact of cellular death on the extractability of procyanidins. Cytological and cytochemical studies should be undertaken on relationships between cellular walls and phenolic compounds during the drying phase of the outer integument. The link between histochemical/cytochemical and biochemical analyses of these compounds, particularly after fractionation and thiolysis, could improve knowledge.

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