Journal of Experimental Botany, Vol. 48, No. 313, pp. 1599–1607, August 1997

Journal of Experimental Botany

# The development of the grape berry cuticle in relation to susceptibility to bunch rot disease

**Pascal Comménil[1,4], Loïc Brunet[2] and Jean-Claude Audran[3]**

[1]*Laboratoire de Cryptogamie-Phytopathologie, Université des Sciences et Technologies de Lille, Bâtiment SN2, F-59655 Villeneuve d'Ascq cedex, France*

[2]*Centre Commun de Microscopie Electronique de Biologie, Université des Sciences et Technologies de Lille, Bâtiment SN3, F-59655 Villeneuve d'Ascq cedex, France*

[3]*Laboratoire de Biologie et Physiologie Végétales, Université de Reims Champagne-Ardenne, UFR des Sciences Exactes et Naturelles, BP 1039, F-51687 Reims cedex 2, France*

Received 15 January 1997; Accepted 18 April 1997

## Abstract

**Some physical and morphological factors of grape berry cuticle were investigated at different developmental stages of three clones of *Vitis vinifera* cv. Pinot noir. The surface morphology of grape berries was examined by scanning electron microscopy and cuticle anatomy was examined by light and transmission electron microscopy. During the period from flowering to maturity, the composition of the cuticular waxes changed, corresponding with an increase of waxy deposits and significant modifications of the wax surface morphology. The content in cutin per unit surface decreased more than 2.5-fold between berry set (16 d after anthesis) and veraison of the grape berries, and might predispose the grape berry to fungal infection. This result was correlated with the differentiation of the cuticle layers and particularly with a decrease in the thickness of the primary cuticle at harvest.**

Key words: *Botrytis cinerea*, cuticle, cutin, epicuticular waxes, *Vitis vinifera L.*

## Introduction

All aerial organs of higher plants are covered by the cuticle at the interface between the plant tissue and the environment. The cuticular membrane serves as protective barrier against fungal pathogens, reduces water loss due to transpiration and contributes to the control of gaseous exchanges (Rosenquist and Morrison, 1989). The cuticle consists of a structural component, the cutin layer, formed from polymer of hydroxy-fatty acid esters, intracuticular waxes, and a thin amorphous epicuticular wax layer consisting of a very complex mixture of long chain lipids. Structure and chemistry of plant cuticles have been extensively investigated (reviews by Baker, 1970; Holloway, 1982). Various factors including physical, chemical and physiological properties of the cuticle have been studied to explain the protective role of the cuticle against pathogenic fungi. The important role played by the epicuticular wax layer in the resistance against fungi is based on the hydrophobic nature of the wax layer (Tewari and Skoropad, 1976), the lipid composition (Marois *et al.*, 1985) as a chemical barrier, and the wax content per unit surface (Rosenquist and Morrison, 1989). Meusel *et al.* (1994) have reported a correlation between the ultrastructure and the chemical composition of various plant waxes.

Insoluble polymeric cutin constitutes the framework of the cuticular membrane and forms a physical barrier against pathogenic fungi. The major component of grapevine cutin are $C_{16}$ and $C_{18}$ fatty acid esters (Walton and Kolattukudy, 1972). As reported by several authors, *Botrytis cinerea* is able to enter through undamaged plant cuticle (McKeen, 1974; Rijkenberg *et al.*, 1980; Pie and de Leeuw, 1991; Salinas, 1992). On the basis of electron microscopic examinations, it has been suggested that enzymatic hydrolysis of the cuticle is involved during penetration of the infection structure into the plant cell (Rijkenberg *et al.*, 1980). Some esterases, cutinase and

[4] To whom correspondence should be addressed. Fax: +33 3 20 43 49 95. E-mail: comménil@univ-lille1.fr

Abbreviations: TLC, thin layer chromatography; SEM, scanning electron microscope; TEM, transmission electron microscope.

lipase, biosynthetized by *Botrytis cinerea* have been shown to be induced by grapevine cutin and are able to hydrolyse cutin (unpublished results). The *Botrytis cinerea* lipase specifically hydrolyses long chain fatty acid esters (Comménil *et al.*, 1995) such as those present in waxes and cutin.

Variations in the susceptibility of grapes to bunch rot disease have been particularly related with arrangement of the pellicular cells (Bernard, 1976), the cuticular thickness (Galet, 1977) and the density of the cuticle layers (Percival *et al.*, 1993) of various cultivars.

Because the susceptibility of grape berries to *Botrytis cinerea* infections has been shown to greatly increase at the onset of ripening (veraison) of grapes (Bulit and Lafon, 1977; Hill *et al.*, 1981; Jeandet and Bessis, 1989), the aim of the present investigation was 2-fold: (a) to study the changes in chemical, physical, and structural factors of the grapevine cuticle during the development of the fruit, from bloom until harvest, (b) to compare three clones of Pinot noir selected for their different susceptibilities to bunch rot disease.

## Materials and methods

### *Plant material*

Bunches of grapes were collected from *Vitis vinifera* cv. Pinot noir located in Champagne vineyards. Three clones of Pinot noir, known for their differences in susceptibility to *Botrytis cinerea*, were selected in adjacent plots: a 17-year-old, tightly clustered, bunch rot disease susceptible *Vitis vinifera* cv. Pinot noir, noted $S_{792}$; a 20-year-old, disease prone one, $MT_{392}$; and a 17-year-old, loose clustered, disease tolerant one, $T_{7613}$. Samples were taken for each clone at different dates spanning the growing season from flowering until harvest. The first three sampling dates were before and the last three sampling dates were at or after the veraison. The differences of susceptibility to bunch rot disease of the three clones was confirmed by the visual estimation of the infection rate. For each sampling date, 12 plants per clone and 8 clusters per plant were selected for a total of about 3000 grape berries. Infection rate was expressed in percentage value of infected grape berries.

### *Thin layer chromatography analysis*

Fresh plant material was washed in water, dried on filter paper, and epicuticular waxes were removed by immersing grapes in chloroform for 90 s as previously reported by Comménil *et al.* (1996). This method avoids the contamination of epicuticular wax extract by lipids from the cutin matrix or by cellular lipids. The extract was filtered to remove dirt and water on 1 PS phase separator filters (Whatman), and concentrated using a rotary evaporator. A small aliquot (50 $\mu$g) of the chloroform-extracted lipids was spotted on to a TLC silica gel plate (Sil G-25 Macherey-Nagel, HR) which was developed at room temperature in hexane-diethyl ether-acetic acid (78:20:4, by vol.) as mobile solvent. Lipids were detected by spraying with 0.01% (w/v) primulin in water-acetone (80:20, v/v). *n*-Tetracosane, methyl oleate, oleic acid, 1-tetracosanol, and oleanolic acid were used as standards for hydrocarbon, wax ester, fatty acid, fatty alcohol, and triterpenoic acid, respectively. The lipid standards were obtained from Sigma.

### *Cuticle isolation and extraction*

Epidermal discs were removed from the outer layer of untreated berries in the central portion of clusters from different plants using a 5 mm cork borer. From each clone, 30 cuticular membrane discs were treated and all experiments were repeated three times for a total of 90 grape berries selected for each date of sampling. The cuticle and epicuticular wax layers were isolated from the underlying tissues by enzymatic degradation of the cell walls using 0.5% pectinase (Fluka) and 0.1% cellulase (Fluka) at pH 4.5, according to the method of Baker and Bateman (1978). Then, cuticle samples were placed in test tubes and waxes were removed from the cuticle discs by extracting with chloroform overnight according to the method of Percival *et al.* (1993). The wax solution was transferred to a tarred test tube and the chloroform was evaporated under nitrogen flux. The extracted waxes and cuticle were dried in a drying oven at 110 °C to constant weight.

### *Light and electron microscopy*

Tangential slices of fresh grapes were taken with a sharp razor blade. These included the epidermis and a small amount of fleshy tissue. Material was fixed in glutaraldehyde (2%, v/v, in phosphate buffer 100 mM, pH 7.2, containing sucrose 4%, v/v) for 22 h at room temperature. The samples were rinsed three times with fresh buffer, post-fixed in $OsO_4$ (1%, v/v, in the same buffer) for 4 h at room temperature, and finally rinsed three times with distilled water. The tissues were then dehydrated through an ethanol series: 50, 70, 80, 90, 95, and 100% for 1 h, followed by one rinsing with ethanol 100% for 12 h. For the analysis of samples by light and transmission electron microscopy, ethanol was gradually replaced by acetone (1:1, v/v, then 100% for 1 h). The samples were kept in a mixture of acetone-resin (2:1, 1:3, v/v, for 1 h at room temperature). They were then embedded either in araldite resin (araldite, *N-2-dodecen-1-yl-succinic anhydride-dibutyl phthalate-N*-benzyl-dimethylamine, 10:10:1:0.5, by weight) for 1 h, 12 h, and then 1 h at room temperature with polymerization at 60 °C for 72 h. Transverse sections (60 nm) were cut with a diamond knife on a LKB ultramicrotome. Sections were stained with uranyl acetate (2.5% in ethanol 50%) and lead citrate (Reynolds, 1963). Mounted sections were observed with a JEOL 120 CX electron microscope, at 80 kV.

Thick transverse sections of 1 $\mu$m were also made, stained with 1‰ (w/v) toluidine blue, then washed in water.

Samples for scanning electron microscopy were fixed as described before, dehydrated through graded ethanols and amylacetate series, and then dried by the carbon dioxide critical point method. They were placed on a metallic holder using a double-face adhesive and coated with gold in a Balzer SCD 040 sputter unit. A JEOL 120 CX ASID 4-D scanning electron microscope operated at 40 kV was used for examination of the samples.

## Results and discussion

### *Variations in the susceptibility to bunch rot disease during fruit development*

Susceptibility to bunch rot disease was measured, for the three selected clones, during the fruit development, by the rate of infected grape berries. The results presented in Fig. 1 confirmed the fact that young grapes are resistant to *Botrytis cinerea*. With further grape development,

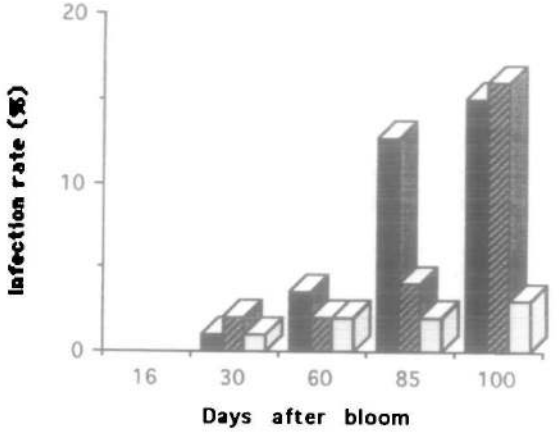


**Fig. 1.** Relative susceptibility of the three clones of Pinot noir cultivar during the fruit development. For each date of sampling, 3000 grape berries were observed and infection rate was expressed as percentage value for $S_{792}$ (■), $MT_{392}$ (▨), and $T_{7613}$ (□) clones.

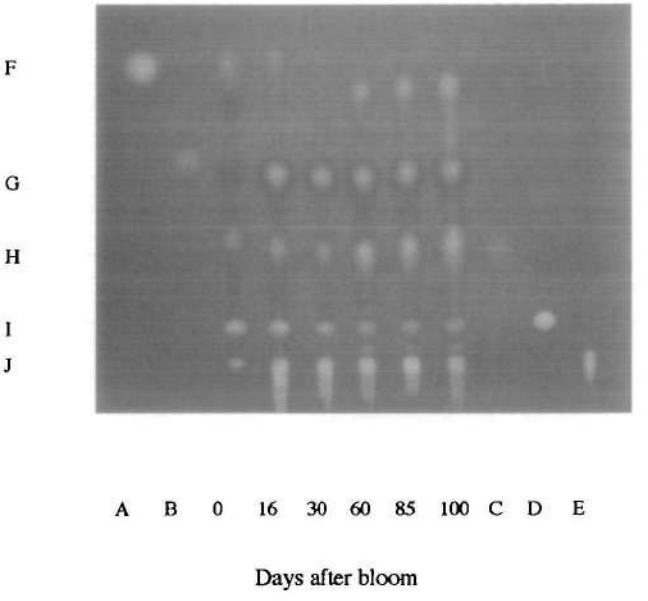


**Plate 1.** Thin layer chromatography of cuticular waxes extracted from grape berries during development, from bloom until harvest. Loaded plate was developed at room temperature in hexane-diethyl ether-acetic acid (78:20:4, by vol.) as mobile solvent. Cuticular wax extracts (50 μg/spot) of grape berries (reference $T_{7613}$) at 0, 16, 30, 60, 85, and 100 d after flowering. (A–E) lipid standards (10 μg/spot). A, *n*-Tetracosan; B, oleic acid; C, methyl oleate; D, 1-tetracosanol; E, oleanolic acid. F, hydrocarbons; G, fatty acid esters, H, free fatty acids; I, primary alcohols; J, oleanolic acid.

significant differences appeared between the three clones. For the $S_{792}$ clone, the susceptibility to *Botrytis cinerea* steadily increased at veraison. The $MT_{392}$ clone was marked by an extensive infection only at harvest, while the infection of grape berries from the $T_{7613}$ clone remained at a low rate from bloom until harvest. From these results, the $S_{792}$, $MT_{392}$, and $T_{7613}$ clones were confirmed as disease susceptible, disease prone, and disease tolerant, respectively.

### *Changes in cuticular wax composition during fruit development*

The change in the lipid components during maturation was studied by thin layer chromatography. Lipid classes were identified by comparison of $R_F$ with those of lipid standards. The composition of the chloroform extracts, recovered from the bunch grapes of the disease tolerant clone, changed markedly during fruit development (Plate 1). The extracts obtained from grapes in the bloom stage consisted predominantly of fatty alcohols and oleanolic acid. Cuticular waxes became progressively enriched in wax esters 2 weeks after bloom. During the maturation of grape berries, the cuticular waxes were composed of hydrocarbons, wax esters, fatty acids, primary alcohols, and oleanolic acid. The proportion of primary alcohols decreased steadily throughout fruit development. At veraison, the proportion of hydrocarbons increased and there were trace amounts of one unidentified component ($R_F$ 0.15). Except at flowering stage, oleanolic acid was the dominant component at all stages of fruit development. Similar results were obtained with the epicuticular wax extracts of the two other clones. The composition of cuticular waxes of different grapevine varieties has been studied extensively by several investigators (Radler and Horn, 1965; Radler, 1965*a*, *b*, 1968; Possingham *et al.*, 1967; Yamamura and Naito, 1983; Kolattukudy, 1969).

The grapevine waxes can be separated into two fractions, soft wax generally extracted by petroleum ether and hard wax (petrol-insoluble) formed by oleanolic acid. The latter was present only in fruit waxes and not in leaf waxes (Possingham *et al.*, 1967). At harvest, oleanolic acid ($C_{30}H_{48}O_3$, triterpenoic acid) was the main constituent of the cuticular waxes and could constitute between 50% and 80% of total weight (Radler, 1968; Yamamura and Naito, 1983). The soft wax was a mixture of long chain fatty acids, alcohols, aldehydes, esters and hydrocarbons (Kolattukudy, 1969). Radler (1968) did not correlate the composition of waxes with the susceptibility or the resistance of the grapevine varieties to bunch rot disease, indicating that qualitative differences in the wax composition are not a factor of resistance. The role of the wax as a physical barrier to penetration by the organism was not considered important because most organisms can push their way through the wax (Martin, 1964). Therefore, the role of epicuticular wax layer as chemical barrier has been investigated (Marois *et al.*, 1985, 1986) and notably, the presence of compounds inhibitory to the germination of the *Botrytis cinerea* conidia has been associated with the epicuticular waxes of grape berries from immature stages (Pezet and Pont, 1984; Padgett and Morrison, 1990; Comménil *et al.*, 1996).

*Quantitative changes in cuticular components*

The weight of the cuticular matrix per unit surface was investigated for five stages of berry growth (Fig. 2). No significant variations were found between the three clusters suggesting that this factor does not correlate with the variation of the susceptibility to *Botrytis cinerea* observed in the intravariety clones. Similar observations have been reported by Kerssies and Frinking (1996) on gerbera and rose flowers. On the other hand, a steady decrease in matrix weight per unit surface area occurring during fruit development resulted in a 2.5-fold decrease between the immature-stage and harvest-stage, and particularly between 30 d after flowering until veraison. During ripening, the matrix weight appeared stationary at about 4 $\mu g\,mm^{-2}$. These values are similar to those reported for cuticles isolated from tight clustered cultivars susceptible to bunch rot which range from to 4.2 at 5.2 $\mu g\,mm^{-2}$ (Rosenquist and Morrison, 1989), but are higher than the 0.8 to 1.1 $\mu g\,mm^{-2}$ related by Percival *et al.* (1993) for Cabernet Franc, Optima and Riesling grape berries. The decrease of matrix weight between young grape and veraison stages could be correlated with the susceptibility of grape berries to *Botrytis cinerea*. It is well known that young grapes are resistant to *Botrytis cinerea*, while mature grapes are highly susceptible and that veraison constitutes the critical stage from which the grapes became progressively highly susceptible to bunch rot disease (Bulit and Lafon, 1977; Hill *et al.*, 1981). The resistance of grapes involved various biochemical, morphological, and physical factors (Jeandet and Bessis, 1989). Hill *et al.* (1981), like other authors, considered the cuticle to be primarily a physical barrier and several studies correlated the degree of penetration of *Botrytis cinerea* with the cuticle thickness (Louis, 1963; Martin, 1964; Bernard, 1976; Galet, 1977; Kamoen, 1992). The significant decrease of cuticle content per unit surface at the veraison could reflect the decrease of cuticle thickness or density resulting in a high susceptibility to bunch rot disease.

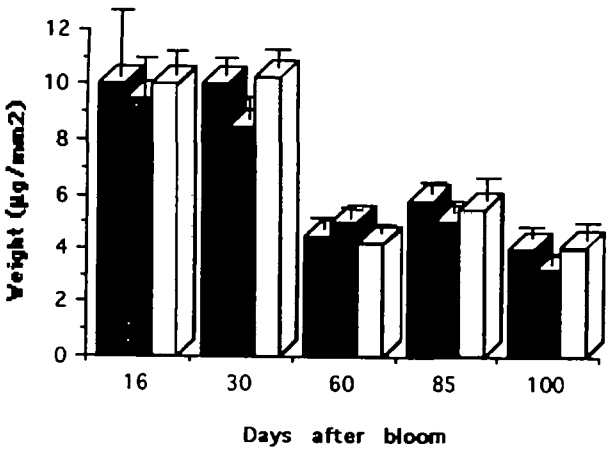


**Fig. 2.** Grape berry cuticle deposition during fruit development of $S_{792}$ (■), $MT_{392}$ (▨), and $T_{7613}$ (□) clones of *Vitis vinifera* cv. Pinot noir. Error bars represent SE of the mean of three experiments (30 replicates per treatment).

In contrast, a measurable increase of wax weight per unit surface was noted between early season sample (30 d after bloom) and harvest sample from about 1.2 to 2.2 $\mu g\,mm^{-2}$. No significant differences were observed between the three clones. At harvest, the amount of cuticular wax per unit surface was similar to those related by Percival *et al.* (1993) for disease tolerant cultivar as Cabernet franc (1.9 $\mu g\,mm^{-2}$), and higher to those measured in disease susceptible cultivars as Optima (1.3 $\mu g\,mm^{-2}$). Several studies have shown that the wax content per unit surface was highly correlated with the resistance against *Botrytis cinerea* (Marois *et al.*, 1986; Rosenquist and Morrison, 1989) and especially with the effects of cluster exposure and berry contact on the occurrence of bunch rot (Percival *et al.*, 1993).

*Scanning electron microscopy*

Development of the cuticular surface has been studied from flowering until harvest. At anthesis, an epicuticular wax layer covered the entire surface of the ovary (Plate 2A). The morphology of epicuticular wax consisted in a tangle of vermicular structures which formed a continuous layer (Plate 2B, C) and were called 'primary waxes' (Bessis, 1972*a*). The central portion of the epidermal cells was higher than the adjacent cell region. Outlines of the underlying epidermal cells were clearly visible and the vermicular structures were radially oriented upon the cells. At berry set, about 16 d after bloom, the highly ordered pattern of primary waxes seen at bloom became rapidly and strongly disorganized. The vermicular structures of primary waxes were very flattened and spread on the berry surface and the epicuticular wax layer consisted predominantly of small, individual upright wax platelets (Plate 2D, E, F), called 'secondary waxes' (Bessis, 1972*a*). The epicuticular wax structure did not show significant modifications until harvest. At maturity, this layer was formed by the secondary waxes and only remnants of primary wax structures were still visible. The wax platelets were more rough and densely distributed at harvest than the berry set period. Contrary to Hammer and Evensen (1994) who reported some differences in cuticle surface morphology between susceptible and resistant rose cultivars, no differences in cuticle surface morphology were observed between the three clones of Pinot noir cultivar.

Evolution of the grape berry surface has been correlated with the berry growth (Bessis, 1972*a*; Considine and Knox, 1979; Rosenquist and Morrison, 1989), changes of chemical composition of the wax (Radler and Horn, 1965), and variations of the amount of berry wax per unit surface during fruit development (Yamamura and Naito, 1983). These authors reported conflicting results

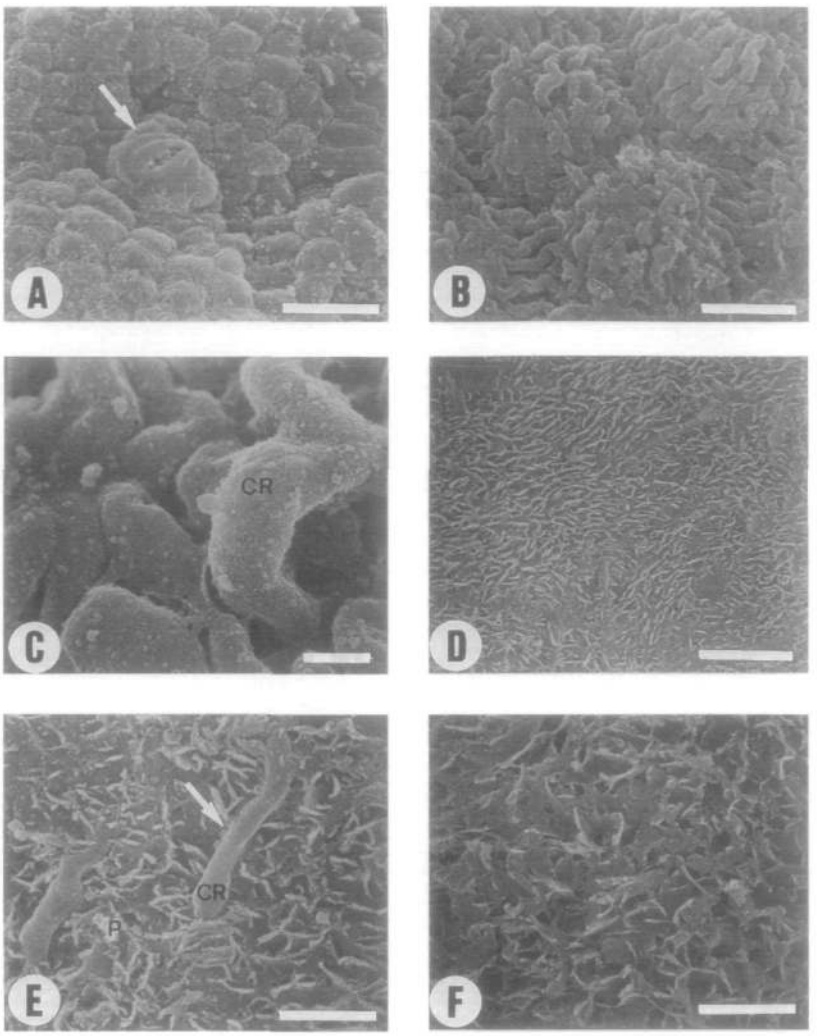


**Plate 2.** Scanning electron micrographs of the epicuticular wax development on grape berries. All samples were fixed in glutaraldehyde and post-fixed with osmium tetroxide, then dried with the carbon dioxide critical point method. (A, B, C): The surface of the ovary at anthesis covered by cuticular (vermicular) ridges of primary waxes. The stomata were functional (arrow). (D, E): The berry surface at berry set, 16 d after flowering, showing remnants of primary waxes (arrow) and the development of the wax platelets of secondary waxes. (F): The berry surface at maturity, showing an increase of secondary wax deposits. CR = cuticular ridge (vermicular ridge), P = wax platelet, (A) Bar = 20 $\mu$m; (B, D) bar = 5 $\mu$m, (E, F) bar = 2 $\mu$m; (C) bar = 0.5 $\mu$m.

on the increase or decrease of cuticular wax density during fruit development which could be cultivar-dependent. Our observations by SEM suggested an increase of wax density between veraison and harvest. This result was confirmed by the estimation of the amount of wax per unit surface as described before. Similar microscopic observations were made by Bessis (1972*a*) in the Pinot cultivar with untreated samples, suggesting that organic solvents used for sample preparation have not altered the cuticular waxes.

Evolution of the stomata occurred a few days after flowering. The functional stomata (Plate 2A) were modified into lenticels and some microfissures appeared in the peristomatic areas (Plate 3A). Bessis (1972*b*) expressed the view that fungal penetration could occur mainly through lesions or lenticels, but no correlation has been shown between the number of lenticels on grapes and the susceptibility of different cultivars (Bernard *et al.*, 1981). Hill *et al.* (1981) reported a higher degree of penetration through the intact cuticle than through lenticels.

At maturity, cracks in the skin of grapes were found (Plate 3B, C) in higher frequency in the disease susceptible clone than in the disease tolerant clone. Cracks could make a preferential entry point for the penetration hyphae of *Botrytis cinerea* conidia (Plate 3D). The formation of cracks has been studied by Considine and Knox (1979) and could be related with the thickness of the cuticle or the grape morphology (Marois *et al.*, 1986).

### *Light and transmission electron microscopy*

The pellicular structure of the grape berries of the three clones, observed at harvest by light microscopy, consisted

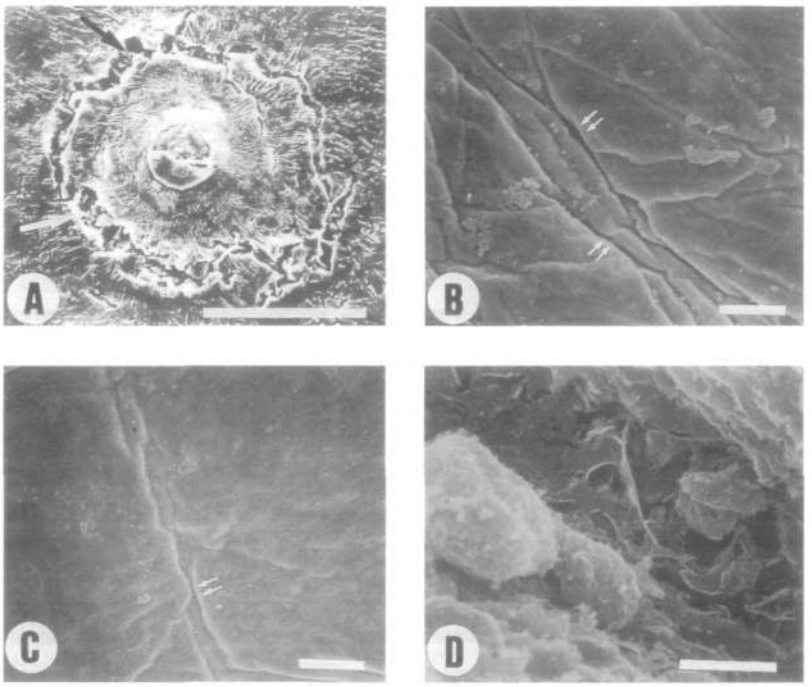


**Plate 3.** Scanning electron micrographs of a lenticel and the appearance of cracks in the skin of grapes at maturity. (A) Lenticel with microfissures in the peristomatic area (arrow). (B, C) Cracks in the disease-susceptible and disease-tolerant clone skin grapes, respectively (double arrow) (D) Germination of a *Botrytis cinerea* conidia near a crack. (A, B, C) Bar = 100 $\mu$m; (D) bar = 2 $\mu$m.

of two outer epidermal cell layers covered by a thick cuticle, and 6 or 8 inner collenchymatous hypodermal cell layers (Plate 4A). Bernard (1976) proposed that the susceptibility of grape berries to *Botrytis cinerea* was correlated with the histological arrangement of the epidermal layers. Disease-tolerant cultivars showed 3 to 10 epidermal cell layers. These consisted, however, only of 1 or 2 layers in disease susceptible cultivars like the Pinot noir cultivar.

The ultrastructure of the cuticular membrane has been studied by transmission electron microscopy at different developmental stages of grape berries (Plate 4B–F). From 30 d after anthesis, the cuticle was clearly differentiated in three distinct zones (Plate 4B, C). The outermost zone, constituted by a convoluted amorphous layer with rugose appearance and variable thickness, corresponded to the epicuticular wax. Beneath this layer, the cuticle proper (Von Mohl, 1847) or primary cuticle (Sargent, 1976) was found, defined as the region of the cuticular membrane containing no cellulose or cell wall materials and which could be completely dissolved after treatment with alkali. This layer was formed by the apposition of cutin (Holloway, 1982) and does not show a structural organization. Primary cuticle formed about two-thirds (1.4 $\mu$m) of the cuticle thickness. Below the cuticle proper, the inner layer was formed by a 0.7 $\mu$m thick reticulate region corresponding to the cuticular layer or secondary cuticle; these terms were used for the inner region of the cuticle with incrustations of cellulose or cell wall materials. The separation between these two layers was hardly distinguishable.

At veraison, the differentiation of the cuticle was greatly marked (Plate 4D, E). Epicuticular wax layer appeared strongly contrasted, continuous, and thicker than in young grapes in relation with the increase of wax content described before. Primary and secondary cuticles have approximatively the same thickness (1.5 $\mu$m) with good demarcation between these two layers. At this developmental stage of grapes, changes in the thickness of the cuticle (3.1 $\mu$m) occurred, corresponding to the increase of the thickness of the secondary cuticle. An additional zone with intense staining was resolved. This third layer was very thin and laid at the interface of the cell wall with the cuticle.

Some changes of the cuticle ultrastructure occurred at fruit maturity (Plate 4F), especially the emergence of a new intermediate region between the amorphous and the reticulate zones of the cuticule. At harvest, the cuticle of grape berry looked almost like the type 3 cuticular membrane defined by Holloway (1982), with an amorphous outer region and an mainly reticulate inner region. The cuticle thickness decreased between veraison (3.1 $\mu$m) and maturity (2.2 $\mu$m), corresponding to the variation of the cuticle proper. The cuticle thickness of grape berry cv. Pinot noir was similar to that reported by Bernard (1976) for susceptible cultivars, i.e. Grenache or Cinsaut

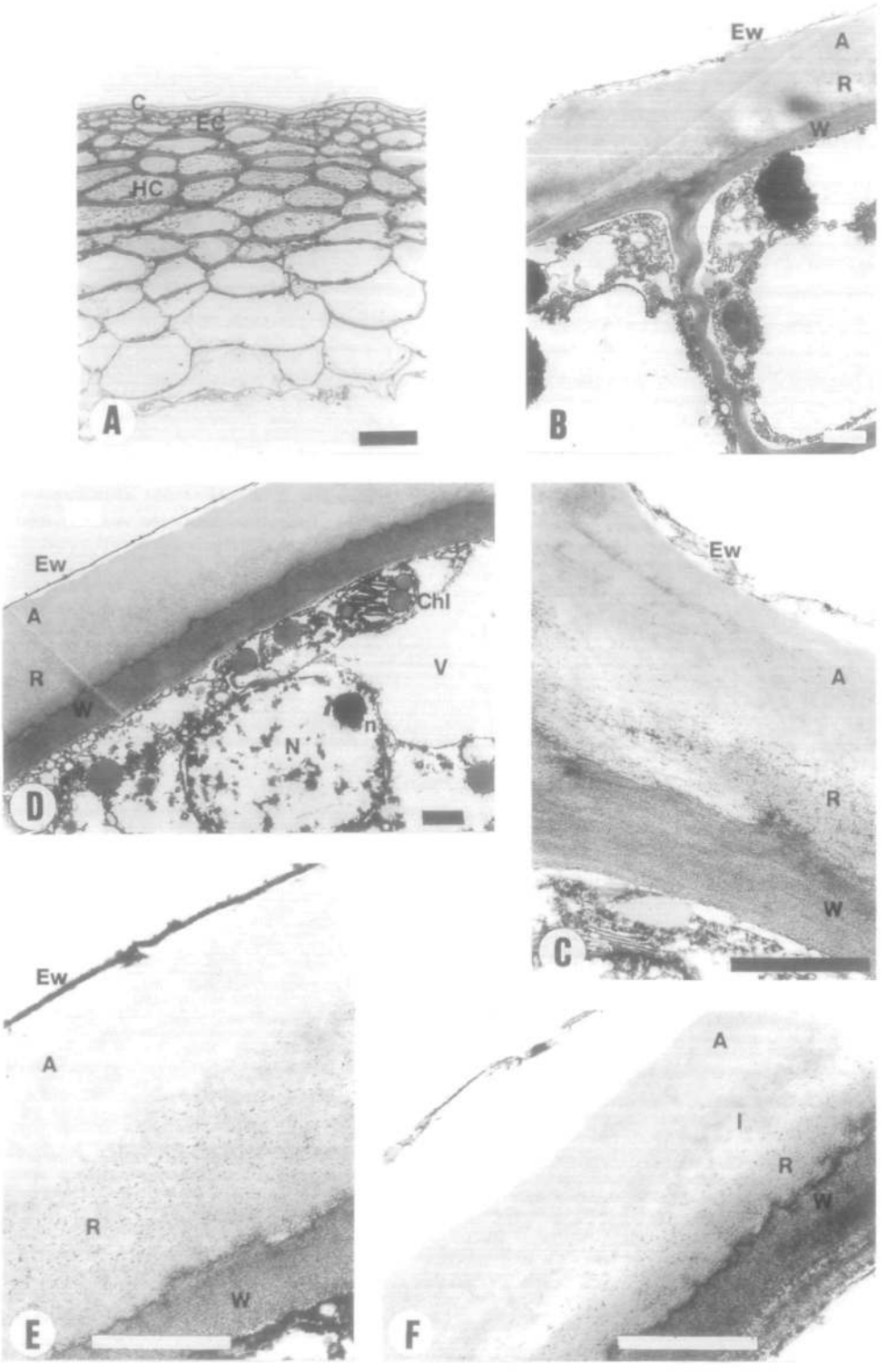


**Plate 4.** Light and transmission electron micrographs of transverse sections of grapevine cuticular membrane at different developmental stages of fruit. Plant materials fixed in glutaraldehyde, post-stained with osmium tetroxide ($OsO_4$), dehydrated through an ethanol series and embedded in araldite resin, sections stained with toluidine blue (A) or uranyl acetate and Reynold's lead citrate (B–F). (A) Light micrograph of the pellicular structure of the bunch grape at harvest with the cuticle (C), two outer epidermal cell layers (EC) and 6 or 8 inner collenchymatous hypodermal cell layers (HC). (B–F): Transmission electron micrographs of the cuticle. At 30 d after anthesis (B, C), the cuticle was clearly differentiated in three zones: an epicuticular wax layer (EW), an amorphous region (A) occupying about two-thirds (1.4 $\mu$m) of the cuticular membrane thickness, and a reticulate region (R). At veraison (D, E), the junction between the reticulate region and the epidermal cell wall (W) appeared strongly contrasted. The thickness of the reticulate region was increased and epicuticular wax layer was more defined and thicker than those in young grapes. At harvest (F), the epicuticular wax layer seems detached from the cuticular membrane. A new intermediate region (I) appeared between the amorphous and reticulate region. V, vacuole, N, nucleus; n, nucleolus; Chl, chloroplast. (A) Bar = 400 $\mu$m; (B–F) bar = 1 $\mu$m.

cultivars (1.5–2.2 $\mu$m) and lower than the 2.5–3.7 $\mu$m reported for tolerant cultivars (Cabernet-Sauvignon, Ribol, Servant).

The changes in the cuticle thickness which appeared during the fruit development with a thickening between flowering and veraison, and a thinning between veraison and harvest, could be correlated with the growth of grape berry. Development of the fruit occurred in two distinct phases of growth (Considine and Knox, 1979), the first between anthesis and veraison and the second corresponded to the veraison until harvest. The second phase was marked by an extensive growth of the fruit associated with an increase of fruit plasticity. Rajaei (1987) has shown an increase in the cuticle thickness at veraison but did not observe variations of the whole cuticle until fruit maturity. Considine and Knox (1979) reported an identical thickness of the cuticle throughout the life of the fruit. Because the cuticle is a heterogeneous and dynamic structure, it was difficult to consider the entire cuticular membrane without distinction of the different layers.

## Conclusion

In this study, the first objective was to correlate the observations on physical and morphological modifications of grape berry cuticle during fruit development, with the susceptibility of Pinot noir cultivar to bunch rot disease. As fruits expanded, the density of cuticle decreased, related to a thinning of the cuticle proper. It seems that the biosynthesis of the polymeric cutin does not keep up with the development of the fruit. Modifications of thickness or density of the cuticle were correlated with the susceptibility of grape to bunch rot at veraison. A weak cuticle could predispose the fruit to fungal attack involving mechanical or enzymatical means. On the basis of our results in comparison with other reports on the pellicular histology, the thickness and the cutin content of the cuticle (Bernard, 1976; Rosenquist and Morrison, 1989; Percival *et al.*, 1993), the Pinot noir cultivar was classed with the disease-susceptible cultivars.

Modifications of the morphology, content, and composition of cuticular waxes were shown. It was difficult to explain to which degree these changes could play a role in the evolution of susceptibility of grape berries to *Botrytis cinerea* during fruit development. More attention should be payed to interactions between conidia and host cuticle, and particularly the influence of composition and structure of epicuticular waxes upon attachment or adhesion of conidia, infection hyphae, or development of appressoria on the aerial surface.

If the development of the cuticle (ultrastructure, cutin content) led to enhance the susceptibility to bunch rot disease at veraison, this susceptibility was expressed differently according to the Pinot noir clones. The second objective was therefore to compare the development of the cuticle of the three clones of Pinot noir. As described by Marois *et al.* (1986), the differences in susceptibility to bunch rot disease could be attributed to the grape cluster morphology. The cluster morphology (or canopy) forms a particular micro-environment which may affect the development of cuticle. Except for the higher frequency of cracks in the disease susceptible clone, no physiological or morphological modifications of cuticle development were detected between the clones. The present study was based on the observations of clones of the same cultivar and differences in susceptibility were not related with differences in cuticle development. In a comparative study on the susceptibility of rose flowers to infection by *Botrytis cinerea*, Hammer and Evensen (1994) have detected some differences in cuticle thickness between two rose cultivars. However, the differences in cuticle thickness were not always related with the differences in susceptibility. These results, as well as the present results, suggest that other physiological or biochemical factors are involved in the variations of susceptibility to *Botrytis cinerea*. The micro-environment may also affect the fungal growth. For example, the occurrence of bunch rot disease should greatly be enhanced by the presence of a favourable microclimate in the tightly clustered grapes.

## Acknowledgements

This research was supported by a grant from the Europol'Agro of Champagne-Ardenne. The experimental plant material was given by Mumm–Perrier-Jouët, Vignobles et Recherches.

## References

**Baker EA.** 1970. The morphology and composition of isolated plant cuticles. *New Phytologist* **69,** 1053–8.

**Baker CJ, Bateman DF.** 1978. Cutin degradation by plant pathogenic fungi. *Phytopathology* **68,** 1577–84.

**Bernard AC.** 1976. Résistance mécanique des baies de *Vitis vinifera* au *Botrytis cinerea* Pers. *France viticole* **8,** 301–7.

**Bernard AC, Dallas JP, Adheran F.** 1981. Observations sur le nombre de stomates des baies de variétés de *Vitis vinifera* L. Relation avec leur comportement à l'égard de la pourriture grise (*Botrytis cinerea* Pers.). *Le Progrès Agricole et Viticole* **8,** 230–2.

**Bessis R.** 1972*a*. Etude de l'évolution des caractères morphologiques des cires cuticulaires au cours de la vie du fruit de la vigne. *Comptes Rendus de l'Académie des Sciences de Paris, Série D* **274,** 1911–14.

**Bessis R.** 1972*b*. Etude de l'évolution des stomates et des tissus péristomatiques du fruit de la vigne. *Comptes Rendus de l'Académie des Sciences de Paris, Série D* **274,** 2158–61.

**Bulit J, Lafon R.** 1977. Observations sur la contamination des raisins par le *Botrytis cinerea* Pers. Travaux dédiés à Georges Viennot-Bourgin. *Société Française de Phytopathologie*, 61–9.

**Comménil P, Belingheri L, Sancholle M, Dehorter B.** 1995. Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. *Lipids* **30,** 351–6.

**Comménil P, Belingheri L, Audran JC, Collas A., Dehorter B.** 1996. Mise en évidence d'une activité anti-*Botrytis* dans les

cires épicuticulaires de jeunes baies de *Vitis vinifera*, variété Pinot noir. *Journal International des Sciences de la Vigne et du Vin* **30,** 7–13.

**Considine JA, Knox RB.** 1979. Development and histochemistry of the cells, cell walls, and cuticle of the dermal system of fruit of the grape, *Vitis vinifera* L. *Protoplasma* **99,** 347–68.

**Galet P.** 1977. *Pourriture grise. Les maladies et parasites de la vigne*. Imprimerie du Paysan du Midi, Montpellier, 313–60.

**Hammer PE, Evensen KB.** 1994. Differences between rose cultivars in susceptibility to infection by *Botrytis cinerea*. *Phytopathology* **84,** 1305–12.

**Hill G, Stellwaag-Kittler F, Huth G, Schlosser E.** 1981. Resistance of grapes in different developmental stages to *Botrytis cinerea*. *Phytopathologische Zeitschrift* **102,** 328–38.

**Holloway PJ.** 1982. Structure and histochemistry of plant cuticular membranes: an overview. In: Cutler DF, Alvin KL, Price CE, eds. *Plant cuticle*. New York: Academic Press, 1–32.

**Jeandet P, Bessis R.** 1989. Une réflexion sur les mécanismes morphologiques et biochimiques de l'interaction vigne-*Botrytis*. *Bulletin de l'Office International de la Vigne et du Vin*, 637–57.

**Kamoen O.** 1992. *Botrytis cinerea*: host-pathogen interactions. In: Verhoeff K, Malathrakis NE, Williamson B, eds. *Recent advances in* Botrytis *research*. Wageningen: Pudoc Scientific Publishers, 39–47.

**Kerssies A, Frinking HD.** 1996. Relations between glasshouse climate and dry weight of petals, epicuticular wax, cuticle, pre-harvest flowering period and susceptibility to *Botrytis cinerea* of gerbera and rose flowers. *European Journal of Plant Pathology* **102,** 257–63.

**Kolattukudy PE.** 1969. Plant waxes. *Lipids* **5,** 259–75.

**Louis D.** 1963. Les modalités de la pénétration du *Botrytis cinerea* Pers. dans les plantes. *Annals of Epiphyties* **14,** 57–72.

**Marois JJ, Bledsoe AM, Gubler WD.** 1985. Effect of surfactants on epicutilar wax and infection of grape berries by *Botrytis cinerea*. *Phytopathology* **75,** 1329.

**Marois JJ, Nelson JK, Morrison JC, Lile LS, Bledsoe AM.** 1986. The influence of berry contact within grape clusters on the development of *Botrytis cinerea* and epicuticular wax. *American Journal of Enology and Viticulture* **37,** 293–6.

**Martin JT.** 1964. Role of cuticle in the defense against plant disease. *Annual Review of Phytopathology* **2,** 81–100.

**Meusel I, Leistner E, Barthlott W.** 1994. Chemistry and micromorphology of compound epicuticular wax crystalloids (*Strelitzia* type). *Plant Systematics and Evolution* **193,** 115–23.

**McKeen WE.** 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. *Phytopathology* **64,** 461–7.

**Padgett M, Morrison JC.** 1990. Changes in grape berry exudates during fruit development and their effect on mycelial growth of *Botrytis cinerea*. *Journal of the American Society for Horticultural Science* **115,** 269–73.

**Percival DC, Sullivan JA, Fisher KH.** 1993. Effect ot cluster exposure, berry contact and cultivar on cuticular membrane formation and occurence of bunch rot (*Botrytis cinerea*) with three *Vitis vinifera* L. cultivars. *Vitis* **32,** 87–99.

**Pezet R, Pont V.** 1984. *Botrytis cinerea*: activité antifongique dans les jeunes grappes de *Vitis vinifera*, variété gamay. *Phytopathologische Zeitschrift* **111,** 73–81.

**Pie K, de Leeuw GTN.** 1991. Histopathology of the initial stages of the interaction between rose flowers and *Botrytis cinerea*. *Netherland Journal of Plant Pathology* **97,** 335–44.

**Possingham JV, Chambers TC, Radler F, Grncarevic M.** 1967. Cuticular transpiration and wax structure and composition of leaves and fruit of *Vitis vinifera*. *Australian Journal of Biological Sciences* **20,** 1149–53.

**Radler F.** 1965*a*. The main constituents of the surface waxes of varieties and species of the genus *Vitis*. *American Journal of Enology and Viticulture* **16,** 159–67.

**Radler F.** 1965*b*. The surface waxes of the sultana vine (*Vitis vinifera* cv. Thompson seedless). *Australian Journal of Biological Sciences* **18,** 1045–56.

**Radler F.** 1968. La cire cuticulaire des grains de raisin et des feuilles de la vigne. *Connaissances de la Vigne et du Vin* **3,** 271–94.

**Radler F, Horn DHS.** 1965. The composition of grape cuticle wax. *Australian Journal of Chemistry* **18,** 1059–69.

**Rajaei H.** 1987. Changements cytochimiques et ultrastructuraux des parois cellulaires de la pellicule du raisin, *Vitis vinifera*, durant la croissance et la maturation de la baie.*Canadian Journal of Botany* **65,** 1343–55.

**Reynolds ES.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17,** 208–12.

**Rijkenberg FHJ, de Leeuw GTN, Verhoeff K.** 1980. Light and electron microscopy studies on the infection of tomato fruits by *Botrytis cinerea*.*Canadian Journal of Botany* **58,** 1394–1404.

**Rosenquist JK, Morrison JC.** 1989. Some factors affecting cuticle and wax accumulation on grape berries. *American Journal of Enology and Viticulture* **40,** 241–4.

**Salinas J.** 1992. Function of cutinolytic enzymes in the infection of Gerbera flowers by *Botrytis cinerea*. PhD thesis. Utrecht University.

**Sargent C.** 1976. Studies on the ultrastructure and development of the plant cuticle. PhD thesis, London University.

**Tewari JP, Skoropad, WP.** 1976. Relationship between epicuticular wax and blackspot caused by *Alternaria brassicae* in three lines of rapeseed. *Canadian Journal of Plant Science* **56,** 781–5.

**Von Mohl H.** 1847. Untersuchung der Frage: Bildet die Cellulose die Grundlage sämmtlicher vegetabilischen Membranen? *Botanische Zeitung* **5,** 497–505.

**Walton TJ, Kolattukudy PE.** 1972. Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. *Biochemistry* **11,** 1885–96.

**Yamamura H, Naito R.** 1983. The surface wax of several grapes in Japan. *Journal of the Japanese Society for Horticultural Science* **52,** 266–72.