Deposition, strain, and microcracking of the cuticle in developing 'Riesling' grape berries

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Summary

The objectives of this study were to quantify deposition, strain, and microcracking of the cuticular membrane (CM) in developing 'Riesling' (Vitis vinifera L.) berries. Mass of the CM, the cutin matrix (DCM), and wax increased pre-veraison (26 to 65 days after anthesis, DAA) on a berry (+ 236, + 211, and + 332 %, respectively) and a surface area basis (+ 11, + 3, and + 43 %, respectively). Post-veraison (65 to 138 DAA), CM and DCM mass per berry remained about constant at 3.4 (± 0.16) and 2.4 (± 0.11) mg per berry, respectively, while wax mass continued to increase from 0.8 (± 0.02) to 1.1 (± 0.02) mg per berry. On an area basis, however, CM and cutin mass decreased from 5.0 (± 0.13) to 4.6 (± 0.04) g·m⁻² and from 3.5 (± 0.10) to 3.2 (± 0.03) g·m⁻² between 65 and 138 DAA, respectively, but wax mass remained constant at about 1.5 (± 0.04) g·m⁻². The calculated rate of cutin and wax deposition peaked at about 40 DAA, and declined continuously thereafter. There was no strain and no microcracking of the CM up to veraison. Post-veraison strain of the CM and microcracking in the stylar scar region increased linearly with time. The data suggest that the cessation of cutin deposition in post-veraison berries and the ongoing berry expansion resulted in increased strain of the CM which in turn caused microcracking in the CM.

K e y w o r d s : cutin, fracture, skin, splitting, Vitis vinifera L., wax.

Introduction

Strain of the cuticular membrane (CM) is an important factor in formation of microscopic cracks (microcracks) in the CM of fleshy fruit. Microcracks impair the barrier function of the CM resulting in an increased incidence of fruit rot, uncontrolled water transport, and, possibly, cracking. In sweet cherry, strain of the cuticle is caused by a mismatch of surface expansion and CM deposition during late development (stage III, final swell; LILLELAND and NEWSOME 1934, KNOCHE et al. 2004, PESCHEL and KNOCHE 2005). At this stage CM deposition has essentially ceased, while most growth in surface area still has to occur. Thus, surface expansion distributes an essentially constant amount of CM and its constituents cutin and wax over an enlarging surface. The resulting tangential forces cause strain of and stress in the CM resulting in formation of microcracks. Furthermore, microcracking of the strained CM is aggravated by surface wetness (KNOCHE and PESCHEL 2006). Qualitatively similar relationships were identified in other soft and fleshy fruit, e.g. the European plum (KNOCHE and PESCHEL 2007) and Ribes berries (KHANAL et al. 2011). In fact, across species strain of the CM at maturity and fruit surface expansion following cessation of CM deposition are closely related (KHANAL et al. 2011). These data indicate that the balance between CM deposition and relative growth rate in surface area is a critical determinant in strain which, in turn, represents the driving force for microcracking.

The grape berry is expected to be subjected to the same events, because (1) it has a soft fleshy mesocarp (and endocarp) surrounded by a skinny exocarp, (2) berry growth follows a double sigmoidal pattern with time characterized by rapid expansion in post-veraison development (MULLINS et al. 1992), (3) it has a thin CM that was reported to decrease in thickness post-veraison (CONSIDINE and KNOX 1979, ALLEWELDT et al. 1981, COMMENIL et al. 1997) and (4) microcracks in the CM represent the first event in the macroscopic cracking of berries (CONSIDINE 1982), that often occurs in humid environments. Water uptake through the grape berry surface proceeds along several parallel pathways, i.e., through the surface of its stem and receptacle and the surface of the berry (BECKER and KNOCHE 2011). While uptake through an intact cuticle on the berry surface is by diffusion, uptake through microcracks bypasses the cuticle as a penetration barrier and occurs by viscous flow which is a fast mechanism of transport (BECKER and KNOCHE 2011). Finally, microcracks in the grape berry cuticle facilitate infections by Penicillium expansum or Botrytis cinerea and these may decrease yield and quality of must and wine (MENEGUZZO et al. 2008).

To our knowledge, there is no direct evidence for strain of the grape berry CM or any relationship between strain of the CM and formation of microcracks. The objectives of this study therefore were to quantify cuticle deposition, strain, and formation of microcracks in developing grape berry. 'Riesling' was selected because it is an important variety in European viticulture. To broaden the database, 'Chardonnay' and 'Müller-Thurgau' were included in some experiments.

Material and Methods

P l a n t m a t e r i a l : Entire clusters of grape berries (Vitis vinifera L. 'Chardonnay', 'Müller-Thurgau', and 'Ries-
ling') were sampled randomly from experimental vineyards within a 10 km radius (similar climate) around Neustadt an der Weinstraße, Germany (lat. 49°21'N, long. 8°8'E). All vineyards were cultivated according to current regulations for environmentally sound viticulture (ANONYMOUS 2002). Berries were selected for uniformity of size and color and for freedom from defects by visual inspection.

**Berry development**: Clusters were collected in the mornings at weekly intervals between 13 d after anthesis (DAA; BBCH 71, LORENZ et al. 1995) and maturity and transferred to the laboratory within 30 min. Four samples of 25 berries each were weighed using an analytical balance and the mean mass per berry was calculated.

To establish the relationship between mass and surface area, 25 individual 'Riesling' berries per sampling date were weighed, photographed, and berry diameters determined by digital image analysis (software cell^P; Olympus Soft Imaging Solutions, Münster, Germany). The surface area \( A \) (in \( \text{cm}^2 \)) of a berry was calculated from berry diameter \( d \) (in cm) assuming a spherical shape as a first approximation \( A = \pi d^2 \). Plotting the surface area against mass \( M \) (in g) and fitting a regression line established the equation \( A = 4.4 \pm 0.01 \times M^{0.5}, n = 425, r^2 = 1.000^{**} \). This equation was used to calculate berry surface area from berry mass in all subsequent experiments. Since data for 'Chardonnay' and 'Müller-Thurgau' were well within the variation obtained in 'Riesling', the above equation was also used for these cultivars.

**Isolation of cuticular membranes and extraction of wax**: Cuticles were isolated from four samples of 25 berries per sampling date using standard protocols (ORGELL 1955, YAMADA et al. 1964). Briefly, berries were cut in half and incubated in an enzyme solution containing pectinase (90 ml\(^{-1}\), Panzym Super E flüssig; Novozymes, Bagsvaerd, Denmark) and cellulase (5 ml\(^{-1}\), Cellubrix L; Novozymes) in a 50 mM citric acid buffer at pH 4.0. Sodium azide was added at a final concentration of 30 mM to suppress microbial growth. The enzyme solution was refreshed periodically until CM separated from adhering tissue. When appropriate, the cleaning was supported by carefully removing adhering cellular debris using a fine camel hair brush.

Isolated CMs were rinsed ten times in deionised water, dried at 40 °C, and weighed. Subsequently, wax was extracted by incubating CMs in a chloroform/methanol solution (1:1, v/v) at 38 °C for 30 min. The extraction procedure was repeated ten times. The extracted dewaxed CM (DCM) were dried and weighed and the amount of wax calculated by difference. The DCM reflects primarily the cutin matrix (elsewhere referred to as the polymer matrix or MX; SCHÖNHERR 1982) that remains after solvent extraction of the CM.

Using these procedures, the time courses of cuticle, cutin, and wax deposition were established on an individual berry and a surface area basis in developing 'Riesling' grape berries.

Relationships between berry mass and cuticle, cutin, and wax mass were studied by subjecting post-veraison 'Chardonnay' (99 DAA), 'Müller-Thurgau' (96 DAA) and 'Riesling' (101 DAA) berries to the procedures described above. For these experiments berries were selected for a maximum range in berry mass.

**Strain of the cuticle**: Strain was established in developing 'Riesling' and, at maturity, in 'Müller-Thurgau' and 'Chardonnay'. Exocarp strips (3.0 mm x 4.2 mm) were excised in vertical (Fig. 1 A, parallel to the stylar scar/pedicel axis) or horizontal (Fig. 1 B, perpendicular to stylar scar/pedicel axis) direction in the equatorial plain of the berries (cheek region) using parallel razor blades (distance between blades 3.0 ± 0.00 mm; Knoch et al. 2004). Following enzymatic isolation, the CM strips were spread in a water droplet on a glass slide, covered by a cover slip, viewed under a binocular (25x, MZ6 microscope; Leica Mikrosysteme, Bensheim, Germany) and photographed (camera DP71; Olympus, Hamburg, Germany). Width of the strips \( w \) was determined by image analysis (software cell^P; Olympus Soft Imaging Solutions). Uniaxial strains in vertical \( (\varepsilon_v, \%) \) or horizontal \( (\varepsilon_h, \%) \) direction were calculated according to equation 1, where \( w \) represented the width of the strip on the berry equivalent to the distance of the razor blades (Fig. 1 C) and \( w_o \), the width of the relaxed CM strip after isolation (Fig. 1 D).

\[
\varepsilon_{x,y} = \frac{w - w_0}{w_0} \times 100
\]

The biaxial area strain \( (\varepsilon_{a,y}, \%) \) was calculated from uniaxial strains according to equation 2 (Knoch et al. 2004).

![Fig. 1: Schematic drawing illustrating the procedure used for determining strain of the cuticular membrane (CM) on grape berries. A, B) Epidermal strip cut in vertical (A) or horizontal direction (B) for assessing uniaxial strain of the CM in equatorial and longitudinal direction of the cheek of a 'Riesling' grape berry. C) Epidermal strip of width \( w \) on the berry surface (prior to excision). D) Same as C, but after excision and isolation and subsequent release of (elastic) strain. The width of the relaxed strip has decreased from \( w \) to \( w_o \).](image-url)
The number of replications per sampling date and orientation was ten.

**Development of microcracks in the CM:** The change in the number of microscopic cracks per unit area was monitored from 43 DAA until maturity in the stylar scar and the cheek region of ‘Riesling’ berries (Fig. 1A). Preliminary observations established that these regions represented those with the highest and lowest frequency of microcracks on the grape berry surface (Becker 2009, unpublished data). Berries were incubated in an aqueous solution of the fluorescent dye acridine orange (0.1 % w/v). After 10 min, berries were removed from the dye solution, rinsed with deionised water for 10 s, and blotted with tissue paper. Exocarp segments (ES) from the stylar scar or the cheek region were excised by razorblade and viewed at 100x using a fluorescence microscope (Ortholux II; Ernst Leitz, Wetzlar, Germany, 390-490 nm excitation wavelength, ≥ 515 nm emission wavelength). The microcracks within a 2.1 mm² window of the microscope were counted on ten randomly selected areas per ES excised from a total of ten berries.

**Data analysis:** Unless individual observations are shown (e.g. Fig. 2), data are presented as means ± standard errors. Data were subjected to linear (Proc REG) and nonlinear regression analysis (Proc NLIN) using SAS (version 9.1.3; SAS Institute, Cary, NC). Significance of coefficients of determination (r²) at the 5, 1 and 0.1 % probability level is indicated by *, ** and ***, respectively.

**Results**

**Berry development and cuticle deposition:** Berry growth as indexed by the increase in mass and surface area followed the typical double sigmoidal growth pattern with time. This pattern is characterized by two phases of rapid berry enlargement (stage I and III, Mullins et al. 1992) separated by a lag phase with temporarily decreased growth rate (stage II, Fig. 2 A, Tab. 1). Veraison coincided with the onset of stage III development (BBCH 81, Lorenz et al. 1995) around 65 DAA for ‘Riesling’ and 55 DAA for ‘Chardonnay’ and ‘Müller-Thurgau’. Maximum growth rates in mass during stage I and III were 48 and 28 mg·d⁻¹ in ‘Riesling’, 35 and 40 mg·d⁻¹ in ‘Chardonnay’, and 49 and 75 mg·d⁻¹ in ‘Müller-Thurgau’. The corresponding maximum growth rates in surface area were 17 and 7 mm²·d⁻¹ in ‘Riesling’, 35 and 40 mg·d⁻¹ in ‘Chardonnay’, and 49 and 75 mg·d⁻¹ in ‘Müller-Thurgau’. The corresponding maximum growth rates in surface area were 17 and 7 mm²·d⁻¹, 13 and 10 mm²·d⁻¹, 17 and 18 mm²·d⁻¹ for ‘Riesling’, ‘Chardonnay’, and ‘Müller-Thurgau’, respectively. 

Up until about veraison, the growth of the berries was paralleled by an increase in mass of CM, DCM, and wax on a whole berry basis and also, on a surface area basis (Fig. 2 A, B, C, Tab. 1). In post-veraision berries, however, CM and DCM mass per berry remained constant while mass of wax per berry continued to increase albeit at a reduced rate. On a surface area basis, CM and DCM mass even decreased and the amount of wax remained constant.

![Fig. 2: A) Change in berry mass (main graph) and surface area (Inset) with time in developing 'Riesling'. For regression equation see Tab. 1. B, C) Change in mass of the cuticular membrane (CM), dewaxed CM (DCM), and wax per berry (B) and per unit surface area of the berries (C). D) Rate of deposition of CM, DCM, and wax and the ratio of deposition rates of DCM / wax (inset) in developing berries. The regression equation for the relationship between the ratio of DCM / wax deposition rates and time was: DCM / Wax (ratio) = 3.45 - 0.03 x time, (DAA) r² = 0.54. (DAA = Days after anthesis).](image)
Parameters of regression equations for the relationship between mass (g per berry) and time (DAA = days after anthesis) of ‘Chardonnay’, ‘Müller-Thurgau’, and ‘Riesling’ berries. The segmented regression model was mass = \( a_1 / (1 + b_1 x \exp(-c_1 x \text{time})) \) for time < 0 and mass = \( (a_1 / (1 + b_1 x \exp(-c_1 x \text{time}))) - (a_2 / (1 + b_2 x \exp(-c_2 x (\text{time}-x_0)))) \) for time > 0

**Table 1**

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<td>b1 (g)</td>
<td>c1 (DAA⁻¹)</td>
<td>a2 (g)</td>
<td>b2 (g)</td>
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<td>1.15 (± 0.23)</td>
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<td>0.12 (± 0.04)</td>
<td>1.17 (± 0.23)</td>
<td>7.15 (± 5.19)</td>
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<td>1.48 (± 0.11)</td>
<td>21.80 (± 10.00)</td>
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<td>1.27 (± 0.14)</td>
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**Table 2**

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<td>y₀ ± SE (mg per berry)</td>
<td>r²</td>
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<td>Müller-Thurgau</td>
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**Discussion**

The data presented herein provide direct evidence for strain of the grape berry CM. Furthermore, the data support the hypothesis of a causal relationship between CM deposition, surface expansion, strain, and microcracking. This conclusion is based on the following arguments.

of cutin and wax consistently decreased indicating that cutin deposition decreased more rapidly then wax deposition (Fig. 2 D, inset).

In post-veraison berries, mass of CM, DCM, and wax on a berry and, to a lesser extent, on a surface area basis were positively related to berry mass indicating that larger berries had a higher cuticle mass per area and therefore, slightly thicker cuticles in ‘Riesling’, ‘Chardonnay’, and ‘Müller-Thurgau’ (Fig. 3, Tab. 2).

**Strain of the CM**: Uniaxial and biaxial elastic strain of the cuticle increased with time in post-veraison ‘Riesling’ (Fig. 4). There was no difference in uniaxial strains parallel or perpendicular to the stylar scar/pedicel axis (Fig. 4, inset). At maturity, biaxial strain averaged 18.4 (± 1.2) %. Quantitatively similar data were obtained for post-veraison ‘Müller-Thurgau’ (20.7 ± 0.7 %), but strain of the CM of ‘Chardonnay’ berries was markedly lower (4.6 ± 0.8 %, data not shown).

**Formation of microcracks**: The number of microcracks per unit surface area increased linearly with time in the stylar scar region of post-veraison ‘Riesling’ berries. In the cheek region, the frequency of microcracks was markedly lower and there was no consistent and significant change with time (Fig. 5).

First, rates of cutin and wax deposition peaked pre-veraison and rapidly declined post-veraison. Growth of the berries followed a double sigmoidal pattern with time characterized by a pre- and post-veraison maximum in surface expansion (Fig. 2). Second, there was no detectable elastic strain in pre-veraison berries when deposition rates of cuticle constituents were at a maximum. However, post-veraison development of (elastic) strain coincided with decreased cutin deposition and increased surface expansion (Figs 2, 3, and 4). Thus, in pre-veraison but not in post-veraison berry CM deposition apparently “fixed” any strain present in older CM layers deposited earlier in develop-

**Table 1**

**Table 2**

First, rates of cutin and wax deposition peaked pre-veraison and rapidly declined post-veraison. Growth of the berries followed a double sigmoidal pattern with time characterized by a pre- and post-veraison maximum in surface expansion (Fig. 2). Second, there was no detectable elastic strain in pre-veraison berries when deposition rates of cuticle constituents were at a maximum. However, post-veraison development of (elastic) strain coincided with decreased cutin deposition and increased surface expansion (Figs 2, 3, and 4). Thus, in pre-veraison but not in post-veraison berry CM deposition apparently “fixed” any strain present in older CM layers deposited earlier in develop-
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Quantitatively similar relationships between surface expansion, strain, and microcracking as in ‘Riesling’ were obtained for ‘Müller-Thurgau’. However, for post-veraison ‘Chardonnay’ elastic strain was much lower than expected based on the higher rate of post-veraison surface expansion as compared to ‘Riesling’. Since surface areas of berries at maturity were similar, total strain must have been similar and the lower elastic strain of the CM in ‘Chardonnay’ implies a higher proportion of plastic (irreversible) strain. The reason for the higher plasticity of the ‘Chardonnay’ CM as compared to those of ‘Riesling’ and ‘Müller-Thurgau’ is not known. Potential explanations include differences in CM composition that possibly result in differing cross-linking of CM constituents.

Cuticle thickness in post-veraison berries as indexed by cuticle mass per unit area was positively correlated with berry size (Fig. 3 B). Hence, larger berries had thicker cuticles. Thus, the effect of berry size on cuticle thickness differed from that of berry development, where the young (and smaller) berry had a thicker CM than the mature larger berry (Fig. 2 C).

Comparing CM thickness in ‘Riesling’ to other cultivars: The pattern of CM deposition in ‘Riesling’ as indexed by the change in CM mass of developing berries is in general agreement with published data, particularly those reported for post-veraison development. In the present study CM thickness in post-veraison ‘Riesling’ decreased by -22 % which is consistent with published data ranging from -20 % in ‘Gordo’ (CONSIDINE and KNOX 1979) to -29 % in ‘Pinot Noir’ (COMMENIL et al. 1997). Also, CM mass per unit area at maturity (4.6 ± 0.04 g·m⁻² in ‘Riesling’) was within the range of CM thickness published for other cultivars (4.2 ± 0.2 to 11.9 ± 0.5 g·m⁻² in ‘Grenache’ to ‘Cabernet Sauvignon’; ROSENQUIST and MORRISON 1989). For pre-veraison berries, cuticle deposition was more variable ranging from +11 % in CM thickness in ‘Riesling’ (Fig. 1 C) to +55 % in ‘Gordo’ (CONSIDINE and KNOX 1979). Whether these differences are related to the stage of development at sampling, to genotype or methodology that ranged from microscopical observations to gravimetry of isolated CM, is currently unknown.

Consequences of strain of the CM - Formation of microcracks and water transport: The increase in strain in post-veraison grape berries resulted in increased formation of microcracks in the stylar scar region, but not in the cheek region. The question arises as to why a relationship between strain and failure was limited to the stylar scar region. The ‘Riesling’ berry is approximately spherical (height (mm) = 0.94 (± 0.01) * diameter (mm) + 0.51 (± 0.06); r² = 0.98***, n = 426) and, in an homogenous sphere, stress and hence, microcracking is expected to be uniform (CONSIDINE and BROWN 1981). This, however, was clearly not the case. Microcracks in the stylar scar region occurred more frequently than in the cheek region. Also, in the stylar scar region microcracks developed in a concentric manner around the stylar scar. Several factors may be involved. First, stiffness of the periderm of the stylar scar and the surrounding exocarp is likely to differ. According to BROWN and CONSIDINE (1982) a rigid plug such as a lenticel or, in analogy, the stylar scar surrounded by an elastic skin acts as a stress concentrator and microcracks will develop preferentially in the immediate vicinity of the plug. If then the berry skin was strained close to the limit of failure, this limit may be exceeded only in the stylar scar region as a result of stress concentration. Second, a higher density of microcracks in the stylar scar region may result from the absence of a network of peripheral vascular bundles that envelopes the skin in the cheek, but not the stylar scar region (CHATLET et al. 1997). Third, the cuticle and the underlying dermal system in the stylar scar region may be weaker per se as compared to that in the cheek region. For example, the cell walls at the poles of ‘Sultana’ berries were thinner compared to those of the cheek (CONSIDINE 1982). At present, there is no evidence for the latter in ‘Riesling’. Finally, surface wetness duration...
will differ between the stylar scar region, where a pending droplet collects after precipitation. In the cheek region water droplets are more likely to run-off. In cherries water on the fruit surface of a strained CM induced microcracking (Knoch and Peschel 2006). Whether this applies also to grape berries is currently unknown.

The change in deposition of the cuticular membrane in developing 'Riesling' berries apparently also affected the permeability of the berry surface for transpiration, but not that for water uptake. Recent investigations established that the permeability of the 'Riesling' grape berry surface decreased between 34 and 129 DAA from 5.6 (± 0.2) to 1.6 (± 0.0) mm·s⁻¹ in transpiration and from 61.0 (± 13.8) to 4.1 (± 1.2) mm·s⁻¹ in water uptake, respectively (Becker and Knoch 2011). Correlating the changes in permeability with cuticle deposition revealed a significant negative relationship between the wax mass per unit area and the permeability of the berry surface for transpiration ($r = -0.81$), but not for water uptake ($r = -0.55$). This observation is consistent with the view that transpiration occurs by diffusion through the berry surface, while water uptake is by diffusion plus some contribution of viscous flow most likely through microcracks that bypass the cuticle as the primary barrier to water uptake (Becker and Knoch 2011).

Conclusion

The data presented herein established that cutin deposition in developing 'Riesling' does not keep pace with post-veraison berry expansion resulting in (1) a continuous increase in elastic strain of the CM and (2) an increase in microcracking of the CM in the stylar scar, but not the cheek region. Furthermore, the change in wax deposition in the course of pre- and post-veraison development was significantly correlated with the change in permeability in transpiration, but not in water uptake. The increase in frequency of microcracks is likely to contribute to an increased incidence of bunch rots and possibly berry cracking in humid environments (Vail and Marois 1990, Pieri and Fermaud 2005).

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