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Impact of light exposure on fruit composition of white 'Riesling' grape berries (*Vitis vinifera* L.)

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Summary

Microclimate and irradiation have long been known to influence winegrape (*Vitis vinifera*) quality. However, microclimate influence on white grape quality has remained understudied, as most research efforts have focused on red varieties and their anthocyanin content. In this study, we investigated microclimatic effects on the phenolic and amino acid composition of white 'Riesling' grapes using bunch shading and leaf removal to manipulate grape microclimate. Both treatments were applied directly after fruit set (modified E-L 27; (Coombe 1995)) as well as at the onset of veraison (E-L 34), and compared to a non-manipulated control. The concentration of malic acid, amino acids and total nitrogen were decreased by illumination during the berry growth, while content and concentration of phenolics were significantly increased by illumination. Strong negative correlations were observed between accumulation of amino acids and flavonols. Although accumulation of flavonols occurred throughout berry development, the most important phase of accumulation was post-veraison.

K e y w o r d s : Berry composition; leaf removal; light exposure; phenolics.

Introduction

Leaf removal in the bunchzone is one of the most powerful tools for grape producers to influence grape composition and soundness (Smart and Robinson 1991). Modern viticultural management strategies make use of techniques like early leaf removal in order to improve canopy microclimate and grape composition, but also because the removal of the leaves as assimilate source at an early stage of development slows berry growth and leads to a lower susceptibility to bunch rot (Poni *et al.* 2006). The reaction of vines to leaf removal depends on leaf removal severity and timing as well as on the grape variety (Molitor *et al.* 2011, Kotseridis *et al.* 2012, Nicolosi *et al.* 2012). It seems evident that only severe reduction of leaf area before or shortly after flowering will reduce berry size and yield (Ollat and Gaudillere 1998, Poni *et al.* 2006). By applying severe leaf removal during an early stage of berry development, light absorption by the growing berries and, correspondingly, berry temperature are increased. Light interception by grapes has shown to affect the concentration of berry volatiles (Reynolds and Wardle 1989, Bureau *et al.* 2000), phenolics (Price *et al.* 1995, Downey *et al.* 2006) or amino acids (Schultz *et al.* 1998), as well as berry growth (Dokoozlian and Kliewer 1996).

Phenolics display important health benefits and contribute to the sensory perception of foods and beverages (Lesschaeve and Noble 2005). In red wine, phenolics contribute positively to color, taste and shelf-life, while they lead to undesired browning reactions in white wine (Singleton 1987). Furthermore, phenolics are regarded as negative contributors to the sensory properties of white wines associated with bitterness and adringency (Singleton *et al.* 1975, Arnold *et al.* 1980). Phenolics can complex with proteins in wines, leading to haze formation (Ferreira *et al*. 2001).

Amino acids are essential for yeast nutrition and therefore influence the successful fermentation of grape juice (Bell and Henschke 2005). Furthermore, they play a role as wine aroma precursors (Pripis-Nicolau *et al*. 2000, Tominaga *et al.* 1998). An oversupply of amino acids, especially arginine, may lead to the formation of the cancerogenic ethyl carbamate in wine (Ough *et al.* 1988). Little data are available on the effect of light exposure on the composition and growth of white winegrapes, although practices like leaf removal have become increasingly popular in white winegrape production (Poni *et al.* 2006).

The aim of this study was to determine the effects of different irradiation regimes applied at different developmental stages on the accumulation of phenolic compounds, amino acids, sugars and organic acids of white 'Riesling' grape berries.

Material and Methods

E x p e r i m e n t a l s i t e : Field experiments were conducted in the 2011 and 2012 growing season using 'Riesling' (clone Gm 198-25; grafted to rootstock 'SO4 Gm47') in an established vineyard located close to Geisenheim, Germany (49° 59'20" N; 7° 55'56"E). Vines were cane pruned and trained to a vertical shoot positioning (VSP)-type canopy system in a north-south row orientation (Row azimut 164°). Row and vine spacing was 2.10 and 1.05 m, respectively. In order to obtain a homogenous canopy, the shoot number was adjusted to ten shoots per vine.

F i e l d t r i a l **:** A field trial was established in randomized complete block design with three replicates. Be-

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tween each of the blocks, one row of vines was left as a buffer row. Each replicate consisted of four vines. Two treatments were applied: One artificial shading treatment and one leaf removal treatment. In the artificial shading treatment, the effect of light on the grapes was excluded by sheltering whole bunches in boxes made of tetra brick foil, as described by Downey *et al.* (2004). The boxes remained on the clusters from the point of treatment application until harvest. In the leaf removal treatment, all leaves and lateral shoots providing shade to the grapes in the bunch zone were removed. Regrowth in the bunch zone was removed at three-week intervals. The trial conducted in 2011 consisted of two separate experiments on leaf removal and shading. Each experiment had a separate control and both were conducted in randomized complete block design with three replicates for the leaf removal and five replicates for the shading trial. Leaf removal was applied 14 d after flowering ([DAF], 12.06.2011), and shading was applied 33 DAF (01.07.2011). The shading trial was harvested 111 DAF (17.09.2011), the leaf removal trial 114 DAF (20.09.2011).

In the 2012 trial, leaf removal and shading treatments were applied directly after fruit set (E-L 27; 02 July 2012; 16 DAF) and at veraison (E-L 34; 11 August 2012; 57 DAF). Bunches sheltered in the boxes cannot be reached by pesticide spraying. Therefore, the boxes were opened at night and sprayed manually on the same days the bunchzone spraying was applied.

S a m p l i n g : To ensure that bunches sampled in the experiment were influenced by a similar light climate, only bunches exposed to the western side of the canopy in a height of 80-110 cm above ground were sampled. Sampling took place at the beginning of the trial (07/02/2012, 16 DAF, only berry skin phenolics), at veraison (08/10/2012, 57 DAF) and at harvest (10/16/2012, 123 DAF). In the 2011 trial, sampling was only conducted at harvest. Sample size for the analysis of berry skin phenols was 20 berries per replicate from four bunches of different vines (five berries per bunch) at veraison and harvest, and 50 berries from four bunches at the first sampling date. The berries were cut off with their pedicel and stored immediately under CO_2 atmosphere and frozen at -20 °C. Berries were peeled whilst frozen. Skins were then freeze dried, ground and stored in an exsiccator until analysis. Skin water content was calculated as (skin fresh weight - skin dry weight) / skin fresh weight. Sample size for the analysis of grape juice parameters was 100 randomly selected berries per replicate from four bunches (25 berries per bunch). The samples were pressed for 5 minutes and filtered through a 16 µ Munktell 33/N folded filter (90 g m^{-2}; Ahlstrom, Helsinki, Finland) prior to analysis.

M i c r o c l i m a t i c m e a s u r e m e n t s : Temperature and humidity were monitored by placing three temperature probes (LASCAR, UK) inside the boxes and in the surrounding canopy respectively for the duration of the experiment. Incident radiation in the boxes was measured by inserting three LI-190 SA50 Quantum Sensors (Li-Cor, Lincoln, USA) connected to a LI-1400 data logger inside the boxes and on the western side of the canopy. These data were compared to ambient photon flux density measured by a weather station of the German Meteorological Service approximately 1 km from the experimental vineyard. Bunch surface temperatures were measured by infrared thermography (H2640, NEC Avio Infrared Technologies, Tokyo, Japan) on three days (17.08.; 30.08.; 31.08.2011). Measurements were taken in the morning (8:00-9:00), at noon (12:30-13:30) and in the afternoon (15:30-16:30) on exposed bunches, bunches sheltered in boxes, and bunches under one and two leaf layers on the western side of the canopy. Mean temperature for control bunches was calculated from point quadrat data and bunch temperatures. Point quadrat analysis (Smart and Robinson 1991) with three replicates of 50 insertions each was utilized to describe canopy conditions at veraison in the central bunch zone. Spacing between insertions was 20 cm.

A n a l y t i c a l a p p r o a c h e s : Grape juice was analyzed for pH, titratable acidity, malic acid, relative density and the concentration of glucose and fructose by Fourier-Transform Infrared Spectroscopy using an inhouse calibration on a FT2 Winescan Instrument (Foss Electric, Denmark). Berry amino acids (only 2012) were analyzed with an amino acid analyzer S433 (Sykam, Eresing, Germany). Chromatographic separation was achieved on a 4.6 x 150 mm LCA K 07/Li cation-exchange column (Sykam) with post-column ninhydrin derivatisation and photometric detection at 570 and 440 nm for primary and secondary amino acids. α-Amino acid concentration was also analyzed by the N-OPA method, following the protocol of Dukes and Butzke (1998).

For HPLC analysis of phenolics, phenolic compounds were extracted from the freeze dried grape skin powder in acidified acetonitrile under SO_2 protection followed by vacuum distillation of the extracts. The extracts were analyzed by an ACCELA HPLC/DAD system coupled to a LXQ mass spectrometer (ThermoFisher, Dreieich, Germany). Chromatographic separation was achieved on a 150 x 2 mm i.d., 3 µm Luna 3u C18 100A column (Phenomenex, Aschaffenburg, Germany) protected with a guard column of the same material. Injection volume was 3 µL, at a flow rate of 250 µL·min^{-1}. Elution conditions were: solvent A was 2 % acetic acid; solvent B was acetonitrile/Water/acetic acid (50:50:0,5; v/v/v). Gradient elution was applied: 0-20 min from 96-50 % solvent A, 4-50 % solvent B, 20-23.1 min to 100 % B; washing with 100 % B for 2 min before re-equilibrating the column. Detection wavelengths were 280 nm for flavanols, 320 nm for phenolcarbonic acids and 360 nm for flavonols. The following mass spec conditions were used: ESI source voltage -3.00 kV during negative and +5.00 kV during positive ionization mode; capillary temperature 275 °C; collision energy for MS^n-experiments 35 % (arbitrary units). Peak identification was based on a combination of HPLC retention time and UV spectra as well as mass spectral data. Quantification was carried out using peak areas from external calibration curves. A table containing all standard sources is presented as supplemental Table. Where no standards were available, substances were quantified using the calibration for the closest phenolic relatives (caftaric acid as caffeic acid; fertaric acid as ferulic acid, coutaric acid and p-CGT as coumaric acid). Total nitrogen in grape juice and grape

skin powder was analyzed by a modified Kjeldahl-method with ammonia determination by flow injection analysis (FIAstar 5000, Foss, Denmark) with photometric detection at 720 nm (Persson *et al*. 2008).

Data analysis: Experimental results were evaluated using a generalized linear model (GLM) for normally distributed data with treatment, year and sampling date as factors. Post-hoc pairwise comparisons were performed by a Fisher's LSD test. Statistical testing was performed with SPSS 15.0 Software (IBM, Armonk, U.S.). Principal component analysis (PCA) was applied on the harvest data of 2011 and 2012, using autoscaling as data standardisation method. PCA was calculated using MatLab (The Mathworks, Natick, U.S.) software with PLS toolbox (Eigenvector Inc., Eaglerock, U.S.).

Results

Experimental conditions: Point quadrat analysis showed that canopy conditions (number of leaf layers, number of shaded leaves) in the three control blocks and the two experimental years were not significantly different (Tab. 1). The average number of leaf layers in both years was two, with homogenous distribution along the VSP trellis. About 45 % of the clusters were exposed to direct sunlight in the control. Monitoring of PAR showed that bunches sheltered in the boxes were only exposed to approximately 1.6 % of total PAR averaged over a day, compared to 60.3 % for exposed clusters. Temperature and humidity in the boxes were only slightly elevated compared to the canopy environment on a sunny day. This is in accordance with data published by Downey *et al.* (2004), who developed this method of bunch shading. Bunch temperatures were lowest in the shading treatment as direct solar heating of the bunches did not occur. However, the temperature difference to the control bunches was negligible. Exposed clusters showed the highest temperatures, up to 6 °C higher than bunches in boxes and 8 °C higher than bunches shaded by two leaf layers when exposed to peak radiation.

Table 1

Description of cluster environment. [†]Values ± standard deviation calculated from point quadrat analysis (3 replicates, 50 insertions); [‡]Box and leaf removal: mean of nine IR-thermographic measurements on two bunches during 3 days; control: mean of nine measurements on five clusters under different shading levels during three days; [§] mean of two hot and sunny days (18.-19.08.2012)

		Control	Box	Leaf removal
2011	Leaf layers[†]	1.99 ± 0.13	n.d.	0
	% exposed clusters[†]	43.7 ± 12.9	0	100
	% interior leaves[†]	20.06 ± 1.73	n.d.	0
	Bunch Temperature[‡]	21.1	20.9	22.7
2012	Leaf layers[†]	1.98 ± 0.11	n.d.	0
	% exposed clusters[†]	47.8 ± 18.8	0	100
	% interior leaves[†]	19.63 ± 0.51	n.d.	0
	PAR (% of Ambient)	n.d.	1.62	60.23
	Air Temperature[§]	27.21	27.7	n.d.

Grape compounds: In 2011, berry weight was higher than in 2012, but berries showed lower sugar concentration, titratable acidity, malic acid and N-OPA. The effects of shading and leaf removal were similar in both seasons (Tab. 2). Berry weight, total soluble solids and berry skin nitrogen were not affected by the treatments. The pH-value was decreased by leaf removal at E-L 27 and increased by shading at E-L 27 when compared to the control, but remained unaffected when the treatments were applied at veraison. Malic acid was increased by shading, but remained unaffected by leaf removal. N-OPA and total juice nitrogen were strongly affected by the treatments, with leaf removal decreasing and shading increasing the concentration and content of nitrogen compounds in the berries. Although these effects were observed already at veraison in 2012, treatments applied at E-L 27 were not different to treatments applied at E-L 34 when sampled at harvest. The increased concentration of titratable acidity in the shade E-L 34 treatment may be related to the elevated concentration of malic acid observed in this treatment in 2012.

Amino acids: Analysis of the single amino acids in 2012 showed that the amino acid profile at veraison was dominated by glutamic acid, glutamine, aspartic acid and arginine (Tab. 3). Ammonia nitrogen was about twice as abundant as amino acid nitrogen at veraison (data not shown). The ratio of ammonia nitrogen to amino acid nitrogen was significantly elevated in leaf removal bunches compared to shaded bunches. Generally, leaf removal showed a larger effect than shading before veraison, decreasing the amino acid concentration by more than 25 % compared to the control. The fact that NH_4-nitrogen and transport/storage amino acids like glutamine and arginine, as well as glutamic acid, the key amino acid in transamination, were dominating the amino acid profile at veraison corresponds well with this early stage of fruit compositional development.

At harvest, the differences between treatments increased and all amino acids differed significantly between treatments (Tab. 3). However, there was never a difference between the two leaf removal treatments. Surprisingly, the concentration of some amino acids in the late (E-L 34) shading treatment was significantly higher than in the early shading treatment. In general, berries from the shading treatments had a significantly higher concentration of amino acids than control and leaf removal treatments, while control and leaf removal treatments differed significantly only for some amino acids. Amino acid nitrogen was more than four times as abundant as ammonia nitrogen at harvest due to a decrease in ammonia and an increase in amino acid nitrogen concentration. The ratio of ammonia nitrogen to amino acid nitrogen was not influenced by the irradiation regimes at harvest.

The amino acids most strongly affected by the treatments at harvest were arginine, tryptophan, methionine, glutamine and asparagine, while at veraison alanine, asparagine, glutamine and arginine were most severely changed by the treatments. During ripening, the most notable change observed in the amino acid profile was the concentration of proline, which increased 54-fold. Of other quantitatively

Table 2

Ripening parameters of all treatments ± standard deviation at veraison (E-L 34) and harvest, experimental years 2011 and 2012. Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Titratable acidity is expressed as tartaric acid. Treatment, sampling date and year effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Date	Harvest 2011		Harvest 2011		Veraison 2012		
Treatment	Leaf removal E-L 27	Control	Shade E-L 29-31	Control	Shade E-L 27	Control	Leaf removal E-L 27
Berry weight [g]	1.54 ± 0.08	1.59 ± 0.01	1.42 ± 0.15	1.49 ± 0.14	0.60 ± 0.01	0.63 ± 0.05	0.68 ± 0.05
TSS [°Brix]	18.29 ± 0.24	17.56 ± 0.65	19.64 ± 1.46	18.79 ± 0.74	6.03 ± 0.21	5.94 ± 0.17	5.82 ± 0.17
TA [g L^{-1}]	8.21 ± 0.08	8.36 ± 0.54	9.71 ± 1.89	8.11 ± 0.99	37.69 ± 0.8	39.47 ± 0.82	39.47 ± 1.46
pH	2.99 ± 0.02	3 ± 0.02	2.98 ± 0.05	3 ± 0.04	n.d.	n.d.	n.d.
Malic acid [g L^{-1}]	1.97 ± 0.21	2.29 ± 0.21	3.29 ± 1.1	1.67 ± 0.39	23.25 ± 0.69	23.00 ± 0.79	22.22 ± 0.66
Total N Juice [mg L^{-1}]	n.d.	n.d.	n.d.	n.d.	257.82 ± 60.07	215.47 ± 7.51	184.31 ± 46.01
Total N Skin [%]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-OPA juice [mg L^{-1}]	61.66 ± 7.02	73.33 ± 4.73	98.33 ± 10.69	74.33 ± 13.05	51.00 ± 3.46	50.67 ± 3.21	46.67 ± 8.08
Date	Harvest 2012						
Treatment	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	Sign year	Sign date
Berry weight [g]	1.21 ± 0.16	1.20 ± 0.12	1.28 ± 0.06	1.19 ± 0.07	1.09 ± 0.17	***	***
TSS [°Brix]	20.77 ± 1.30	20.81 ± 0.17	20.69 ± 0.52	19.35 ± 0.58	21.62 ± 2.11	***	***
TA [g L^{-1}]	10.02 ± 0.09 b	9.66 ± 0.13 b	9.61 ± 0.29 b	11.00 ± 0.91 a	9.70 ± 1.04 b	***	***
pH	2.87 ± 0.06 c	2.92 ± 0.03 bc	2.93 ± 0.02 b	2.96 ± 0.04 bc	3.01 ± 0.04 a	*	n.t.
Malic acid [g L^{-1}]	2.60 ± 0.35 b	2.55 ± 0.15 b	2.94 ± 0.20 b	4.15 ± 0.53 a	3.68 ± 0.63 a	***	***
Total N Juice [mg L^{-1}]	171.00 ± 43.42 c	186.70 ± 10.84 bc	213.03 ± 27.12 b	301.80 ± 15.64 a	294.13 ± 61.42 a	n.t.	-
Total N Skin [%]	0.65 ± 0.05	0.63 ± 0.06	0.61 ± 0.06	0.66 ± 0.02	0.67 ± 0.02	n.t.	n.t.
N-OPA juice [mg L^{-1}]	69.7 ± 6.7 c	74.7 ± 4.0 c	93.3 ± 3.8 b	137.3 ± 9.1 a	131.0 ± 20.9 a	***	***

important amino acids, the concentration of GABA and alanine increased 11.5 and 5.5-fold, respectively. The concentration of aspartatic acid, asparagine and glutamic acid decreased by 79, 32 and 47 % respectively between veraison and harvest. The amount of free amino acids in grape juice correlated strongly with the amount of total nitrogen ($r^2 = 0.72$).

P h e n o l i c s : The content of phenols in the berry skin increased from 0.04 mg $berry^{-1}$ at E-L 27 to 0.24 mg $berry^{-1}$ at harvest in the control treatment in 2012. The content of total phenols increased significantly for all sampling dates and in all treatments. In parallel, the water content of the berry skins decreased from 82 % at E-L 27 to 72 % at E-L 34 and to 56 % at harvest (54 % in 2011). No significant differences in skin weight or skin water content were detected between treatments or years.

The content (not shown) and concentration of most skin flavanols and hydroxycinnamic acids rose from E-L 27 to veraison and stagnated or decreased (flavanol concentration) after veraison, with the exception of caftaric, coutaric and fertaric acid, which also increased significantly post-veraison. In contrast, quercetin glycoside content and concentration remained rather stable before veraison, but increased drastically post-veraison. No increase in berry quercetin glycoside content took place in the shading treatments over time, while concentration decreased (Tab. 4). The increase in total phenolic content in these treatments was mainly due to an increasing content of hydroxycinnamic acids, while their concentration stagnated. Before veraison, leaf removal increased the concentration of all detected quercetin glycosides, while concentration in control and shading treatments stagnated. At harvest, leaf removal at E-L 27 showed higher flavonol concentration than leaf removal at E-L 34 due to elevated concentrations of que-3-rutinoside and que-3-glucuronide. Concentration of all flavonols was increased by leaf removal and decreased by shading. Shading or leaf removal effects on non-flavonol-phenols were less clear. The concentration of catechin, fertaric acid, caftaric acid and caffeic acid were increased by leaf removal or decreased by shading, while coutaric acid was increased by shading. No treatment had an effect on total hydroxycinnamic acid or flavanol concentration. Changes in the quercetin glycoside profile were observed throughout fruit development. At berry set and veraison, the main quercetin glycosides present in berry skins were que-3-glucuronide, que-3-glucoside and que-3-rutinoside. These were also the main quercetin glycosides found in shaded bunches in both experimental years. The increase in flavonols after veraison was due to an increase in que-3-glucoside, que-3-galactoside, que-3-arabinoside, que-3-glucuronide and que-3-rhamnoside content.

Mean quercetin glycoside content of the early leaf removal treatment was 0.345 mg $berry^{-1}$ in 2011 and 0.341 mg $berry^{-1}$ in 2012, however, quercetin glycoside concentration in the berry skins of the control treatment was about 20 % higher in 2011 than in 2012 (Tab. 4 and supplemental Table). Berries shaded before veraison also showed higher concentration of quercetin glycosides in 2011 than in 2012 (0.651 mg g^{-1} berry skin fresh mass and 0.188 mg mg g^{-1} berry skin fresh mass, respectively). This difference may

Table 3

Concentration of amino acids given as mg L^{-1} ± standard deviation at veraison (E-L 34) and harvest, experimental year 2012; Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Treatment and sampling date effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$. "n.d." = not detected; "+" = values are between limit of detection and limit of quantification

Date	10.08.2012			16.10.2012					
Treatment	Leaf removal E-L 27	Control	Shade E-L 27	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	date sig.
Aspartate									
aspartic acid	41.74 ± 6.08	52.1 ± 2.3	52.69 ± 2.00	7.16 ± 2.3 c	7.13 ± 2.28 c	8.53 ± 1.88 bc	14.34 ± 1.19 a	14.02 ± 3.01 ab	***
asparagine	3.48 ± 0.88	4.51 ± 0.59	7.05 ± 1.99	1.68 ± 1.03 c	1.98 ± 0.18 c	2.6 ± 0.85 c	6.26 ± 0.99 a	4.49 ± 0.96 b	***
methionine	n.d.	n.d.	+	2.52 ± 1.28 c	2.06 ± 0.17 c	3.45 ± 1.65 c	9.25 ± 2.13 a	8.13 ± 1.87 b	***
threonine	7.37 ± 1.35	10.06 ± 0.57	9.35 ± 1.34	21.8 ± 5.82 d	22.36 ± 1.52 cd	30.73 ± 5.07 c	53.64 ± 6.87 a	48.23 ± 5.24 b	***
lysine	+	+	+	+ c	+ c	0.95 ± 0.19 b	1.47 ± 0.03 a	1.2 ± 0.13 b	***
isoleucine	1.13 ± 0.25	1.31 ± 0.03	1.07 ± 0.29	17.08 ± 3.44 b	16.55 ± 1.61 b	20.69 ± 7.13 b	39.34 ± 9.63 a	38.72 ± 4.95 a	***
3-phosphoglycerate									
serine	10.99 ± 2.02	10.98 ± 0.13	7.78 ± 1.23	20.98 ± 2.25 c	23.03 ± 1.26 c	25.76 ± 6.53 bc	39.83 ± 4.98 a	39.42 ± 6.71 ab	***
glycine	+	+	+	1.14 ± 0.1 c	1.25 ± 0.11 c	1.35 ± 0.33 bc	2.11 ± 0.25 a	2 ± 0.17 ab	***
Pyruvate									
alanine	5.17 ± 1.37	8.23 ± 0.54	12.28 ± 3.32	33.12 ± 6.78 c	34.28 ± 2.49 c	39.92 ± 9.25 c	67.28 ± 7.08 a	60.01 ± 12.37 b	***
valine	10.21 ± 2.08	12.08 ± 0.1	11.8 ± 1.67	17.9 ± 4.2 b	17.47 ± 1.44 b	21.22 ± 6.6 b	37.19 ± 8.95 a	35.09 ± 5.31 a	***
leucine	1.73 ± 0.25	2.17 ± 0.21	1.84 ± 0.54	20.72 ± 5.45 b	20.02 ± 2.45 b	26.01 ± 9.92 b	51.8 ± 13.64 a	52.27 ± 7.04 a	***
Shikimate									
tyrosine	3.43 ± 0.47	4.15 ± 0.63	4.53 ± 2.02	2.68 ± 0.38 b	2.88 ± 0.62 b	3.49 ± 0.8 b	6.22 ± 1.46 a	5.6 ± 0.48 a	-
phenylalanine	2.29 ± 0.32	3.24 ± 0.7	2.48 ± 0.51	18.75 ± 4.93 b	17.73 ± 1.52 b	24.26 ± 7.53 b	51.2 ± 11.37 a	46.78 ± 5.49 a	***
tryptophan	10.89 ± 3.93	10.32 ± 0.09	14.5 ± 2.5	5.45 ± 1.2 c	4.5 ± 0.42 c	7.17 ± 2.9 c	19.25 ± 3.95 a	15.62 ± 1.57 b	*
α-Ketoglutarate									
glutamic acid	42.18 ± 3.17	47.41 ± 2.4	50.96 ± 3.04	18.93 ± 5.66 b	23.76 ± 6.32 ab	25.94 ± 5.12 a	28.53 ± 4.01 a	28.00 ± 4.36 a	***
glutamine	30.68 ± 9.66	49.6 ± 1.95	68.82 ± 21.52	33.32 ± 15.36 c	36.57 ± 2.15 c	47.5 ± 15.33 c	132.29 ± 20.23 a	114.96 ± 19.1 b	*
arginine	18.53 ± 7.63	40.13 ± 4.12	34.46 ± 7	28.48 ± 20.94 d	32.27 ± 3.46 d	65.93 ± 8.92 c	164.11 ± 15.39 a	108.66 ± 24.68 b	***
proline	2.51 ± 0.5	3.85 ± 0.19	4.68 ± 0.43	132.55 ± 37.04 b	156.38 ± 26.27 b	190.91 ± 56.85 ab	246.38 ± 57.04 a	260.38 ± 87.73 a	***
histidine	8.91 ± 1.88	10.85 ± 0.64	12.53 ± 2.95	5.82 ± 1.64 b	6.19 ± 0.76 b	8.35 ± 1.81 b	17.4 ± 3.83 a	14.84 ± 1.4 a	-
GABA	6.88 ± 2.66	9.64 ± 1.27	8.99 ± 2.04	83.17 ± 20.84 b	78.61 ± 9.73 b	91.55 ± 16.64 b	139.55 ± 5.12 a	95.96 ± 25.39 b	***
ornithine	+	+	+	+ c	+ c	+ c	1.25 ± 0.17 a	0.74 ± 0.12 b	***
Total amino acids	209.61 ± 41.79	282.36 ± 3.22	307.59 ± 44.17	474.37 ± 135.93 d	506.21 ± 22.78 d	646.85 ± 140.59 c	1128.69 ± 135.68 a	995.1 ± 159.42 b	***
Other									
ammonia	96.82 ± 19.99	112.23 ± 2.26	122.04 ± 18.29	18.35 ± 9.32 d	25.03 ± 1.44 cd	32.69 ± 10.21 bc	53.26 ± 5.56 a	39.17 ± 4.46 ab	***

be attributed to the fact that the shading treatment was applied in a later growth stage in 2011 than in 2012 (E-L 27 in 2012 and E-L 29-31 in 2011) or to higher radiation during the flowering phase (mean PAR during flowering: 258 µmol m^{-2} s^{-1} in 2012 and 428 µmol m^{-2} s^{-1} in 2011).

PCA: A PCA model was constructed with two latent variables explaining 56.29 % of x-block variation. From the scores-plot (Figure, a) it is clearly visible that treatments were effectively separated by PC1 and to a minor extent PC2, while the differences between experimental years were modeled exclusively on the second principal component. The loadings plot (Figure, b) showed that treatments were mainly separated by malic acid and N-OPA, which were more abundant in shaded samples, and quercetin glu-

Table 4

Concentration of phenolics given as µg g^{-1} berry skin fresh weight ± standard deviation at E-L 27, E-L 34 (veraison) and harvest, experimental year 2012. Leaf removal: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. Treatment, year and sampling date effects were evaluated using a generalized linear model (GLM). Different letters indicate significant differences for treatments of all sampling dates according to Fisher's LSD test ($p < 0.05$). Year and sampling date differences are given as asterisks on the right hand side of the table. *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$. "n.d." = not detected; "+" = values are between limit of detection and limit of quantification. GRP = grape reaction product; p-CGT = p-coumaroylglycosyltartrate; Que = quercetin

Date	02.07.2012		10.08.2012		16.10.2012						
Treatment	Control	Leaf removal E-L 27	Control	Shade E-L 27	Leaf removal E-L 27	Leaf removal E-L 34	Control	Shade E-L 34	Shade E-L 27	sign year	sign date
Flavanols											
Procyanidin B1	0.017 ± 0.006	0.054 ± 0.008	0.057 ± 0.03	0.033 ± 0.007	0.05 ± 0.045	0.051 ± 0.016	0.066 ± 0.023	0.057 ± 0.02	0.052 ± 0.025	-	***
Catechin	0.076 ± 0.011	0.119 ± 0.03	0.1 ± 0.015	0.102 ± 0.025	0.097 ± 0.033 a	0.067 ± 0.006 ab	0.074 ± 0.004 ab	0.057 ± 0.004 ab	0.048 ± 0.012 b	-	***
Procyanidin B2	0.048 ± 0.007	0.074 ± 0.021	0.061 ± 0.012	0.056 ± 0.02	0.037 ± 0.006	0.037 ± 0.004	0.034 ± 0.008	0.027 ± 0.005	0.03 ± 0.016	-	***
Epicatechin	0.013 ± 0.004	0.025 ± 0.008	0.034 ± 0.007	0.024 ± 0.007	0.017 ± 0.008	0.009 ± 0	0.015 ± 0.002	0.01 ± 0.002	0.013 ± 0.006	-	***
Total Flavanols	0.154 ± 0.013	0.271 ± 0.064	0.251 ± 0.013	0.215 ± 0.058	0.2 ± 0.051	0.164 ± 0.015	0.188 ± 0.023	0.152 ± 0.012	0.143 ± 0.054	-	***
Hydroxycinnamic acids											
Coumaroylglucose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	***	-
Caftaric Acid	0.53 ± 0.057	0.597 ± 0.051	0.577 ± 0.064	0.583 ± 0.042	0.519 ± 0.064 a	0.386 ± 0.043 b	0.495 ± 0.039 b	0.477 ± 0.027 b	0.508 ± 0.094 b	-	*
GRP	0.003 ± 0	0.002 ± 0	n.d.	0.001 ± 0.001	0.003 ± 0.002 b	0.004 ± 0.001 a	0.001 ± 0 b	n.d. b	n.d. b	*	-
p-CGT	0.009 ± 0.003	0.014 ± 0.002	0.013 ± 0.003	0.006 ± 0.001	0.009 ± 0.008 b	0.017 ± 0.001 a	0.008 ± 0.001 b	0.007 ± 0.001 c	0.008 ± 0.006 bc	-	-
Coutaric acid	0.322 ± 0.036	0.382 ± 0.036	0.351 ± 0.056	0.307 ± 0.025	0.256 ± 0.037 b	0.177 ± 0.041 b	0.229 ± 0.021 b	0.229 ± 0.016 a	0.182 ± 0.042 b	-	***
Fertaric acid	0.016 ± 0.002	0.026 ± 0.002	0.033 ± 0.003	0.039 ± 0.001	0.038 ± 0.005 b	0.048 ± 0.005 a	0.03 ± 0.002 cd	0.024 ± 0.004 d	0.035 ± 0.003 c	***	***
Caffeic acid	0.004 ± 0.002	0.006 ± 0.001	0.004 ± 0.001	0.005 ± 0.002	0.004 ± 0.003 a	0.007 ± 0.001 a	0.006 ± 0.002 a	0.004 ± 0.001 a	0.003 ± 0.001 b	*	-
Cumaric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	***	-
Total Hydroxycin. acids	0.883 ± 0.096	1.027 ± 0.088	0.979 ± 0.124	0.941 ± 0.069	0.83 ± 0.097	0.64 ± 0.079	0.769 ± 0.057	0.742 ± 0.043	0.736 ± 0.14	***	-
Flavonols											
Que-3-rutinoside	0.058 ± 0.006	0.176 ± 0.052	0.026 ± 0.018	0.034 ± 0.013	0.271 ± 0.023 a	0.134 ± 0.048 b	0.083 ± 0.01 b	0.072 ± 0.023 c	0.015 ± 0.004 c	***	*
Que-3-galactoside	0.015 ± 0.002	0.037 ± 0.011	0.005 ± 0.004	0.009 ± 0.003	0.237 ± 0.032 b	0.286 ± 0.058 a	0.127 ± 0.016 c	0.016 ± 0.006 d	0.006 ± 0.001 e	-	***
Que-3-glucoside	0.033 ± 0.004	0.067 ± 0.018	0.011 ± 0.007	0.02 ± 0.006	0.783 ± 0.055 b	0.906 ± 0.112 a	0.476 ± 0.056 c	0.039 ± 0.012 d	0.032 ± 0.005 e	-	***
Que-3-glucuronide	0.384 ± 0.018	0.61 ± 0.113	0.188 ± 0.102	0.227 ± 0.034	0.884 ± 0.084 a	0.492 ± 0.132 b	0.375 ± 0.01 bc	0.343 ± 0.037 c	0.127 ± 0.025 c	**	*
Que-3-xyloside	0.003 ± 0	0.005 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0.012 ± 0.02 b	0.03 ± 0.004 a	0.014 ± 0.001 bc	0.004 ± 0.001 cd	0.003 ± 0.001 d	-	*
Que-3-arabinoside	0.01 ± 0.002	0.036 ± 0.011	0.003 ± 0.003	0.004 ± 0.002	0.262 ± 0.013 a	0.259 ± 0.07 a	0.099 ± 0.013 b	0.013 ± 0.007 c	0.002 ± 0.001 d	-	***
Que-3-rhamnoside	0.005 ± 0.001	0.014 ± 0.003	0.002 ± 0.002	0.004 ± 0.001	0.652 ± 0.056 b	0.765 ± 0.176 a	0.312 ± 0.052 c	0.007 ± 0.003 d	0.002 ± 0.001 d	-	***
Total Flavonols	0.508 ± 0.015	0.945 ± 0.208	0.237 ± 0.138	0.301 ± 0.058	3.101 ± 0.093 a	2.871 ± 0.551 b	1.485 ± 0.141 c	0.495 ± 0.086 d	0.188 ± 0.033 d	-	***
Total Phenolics	1.544 ± 0.115	2.243 ± 0.339	1.467 ± 0.248	1.457 ± 0.068	4.131 ± 0.131 a	3.675 ± 0.54 a	2.442 ± 0.073 b	1.388 ± 0.12 c	1.068 ± 0.166 c	*	***

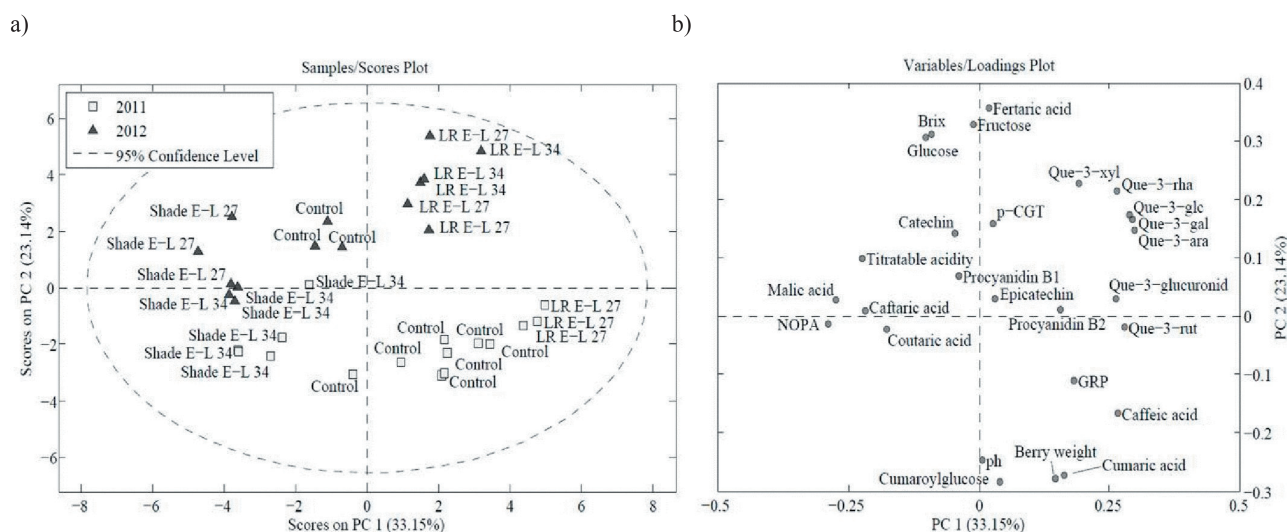

Figure: Scores and loadings plots of the principal component analysis (PCA) conducted on 2011 and 2012 measurements at harvest. LR: all leaves in the bunch zone removed; Shade: Complete shading by covering bunches with boxes impermeable to light. E-L numbers given after the treatment indicate the developmental stage in which the treatment was applied. GRP = grape reaction product ; p-CGT = p-coumaroylglycosyltartrate ; Que = quercetin.

cosides, which were more concentrated in leaf removal samples. Flavanols had the smallest influence on sample separation, while hydroxycinnamic acids, except coutaric and caftaric acid, showed strong loadings on PC2, which separates years, but not treatments. Malic acid and N-OPA were strongly positively correlated. Both correlated negatively with quercetin-glycosides, mainly quercetin-3-glucuronide and quercetin-3-runtinoside. Berry weight correlated positively with coumaric acid and negatively with sugars. Univariate correlation analysis confirmed these results.

Discussion

Several studies have investigated the effects of microclimate manipulation on berry quality traits, mainly focusing on the effects of the qualitatively important phenolics in red winegrape production. Some of these studies have found an effect of microclimate manipulation on grape ripeness (°Brix), while others did not find a significant effect. The results obtained in our study are in accordance with some studies published on post-flowering leaf removal (e.g. (Molitor *et al.* 2011)) or using an artificial shading methodology after anthesis (Spayd *et al.* 2002, Downey *et al.* 2004), while standing in contrast to others (Dokoozlian and Kliewer 1996, Koyama *et al.* 2012). In the latter studies, significant changes in berry weight and sugar concentration have been observed after artificially shading berries at the beginning of flowering and directly after berry set, respectively. In our study, however, the shading treatment was only applied about 14 d after flowering. Therefore, the treatments in our study might have been applied at a developmental stage in which berry size had been determined already. Although temperatures in the boxes and ambient temperatures were similar (Downey *et al.* 2004, Koyama *et al.* 2012), it has to be stressed that berry temperatures in the boxes are different to the temperature of exposed berries, as the shaded berries are not heated up by solar radiation. Berries grown in boxes are therefore exposed to a compressed diurnal temperature range and diminished light and temperature stress, which may hasten berry development (Spayd *et al.* 2002, Cohen *et al.* 2012) and therefore compensate growth deficits induced by bunch shading.

Similar to the shaded bunches, no differences were found in sugar accumulation of bunches from leaf removal vines. Vines can compensate the reduction in leaf area caused by leaf removal by mobilization of reserve carbohydrates, an increase in photosynthetic activity and stronger growth of lateral shoots (Poni *et al.* 2006). The leaf removal intensity applied in our study might not have been severe enough to overcome these compensatory effects and influence berry size and sugar content of the leaf removal treatment significantly.

Elevated malic acid concentrations were detected in shading treatments in both years. Malic acid is respired at a higher rate at high berry temperatures (Lakso and Kliewer 1975), which explains the differences found between shaded and control or exposed berries. Only in samples shaded at veraison the differences in malic acid led to a significantly elevated level of titratable acidity. In contrast to malic acid concentration, pH values appeared to decline with increasing sun exposure, a fact that has previously been reported for Spanish vineyards (Martinez de Toda and Balda 2014) and may be related to decreased potassium concentrations in exposed berries, as reported by Smart *et al.* (1985).

In general, shaded samples showed a higher concentration of amino acids and total nitrogen than control or defoliated samples, which is in accordance with other studies (Schultz *et al.* 1998, Kliewer and Ough 1970). Although berry skins and juice have been analyzed in this study, the grape seeds, as one of the largest nitrogen depots of the berry (about 500 µg N berry^{-1}, calculated using seed

N concentrations from Castrotta and Canella (1978) and Fantozzi (1981)), were not analyzed. More research will therefore be necessary to clarify if equal amounts of N-containing compounds are transferred to the berries and the N-compounds undergo a different fate, e.g. accelerated transport to the seeds, or if the N transport into the berry is modified by grape microclimate. Although some microclimatic effects on single amino acids could be shown before veraison, the changes induced by microclimatic differences were more pronounced after veraison, when significant differences were measured for all amino acids except glutamic acid and proline. The standard deviations for field replicates of amino acids were rather large when compared to the ones obtained for berry phenolics, indicating that factors other than light play a stronger role in amino acid than in phenolic accumulation.

On average, amino acid concentration of control samples and shaded samples was 30 % and 120 %, respectively, elevated as compared to fully exposed samples. Differences between the timing of treatment application were only marginal. Thus, the post-veraison period seems to be crucial for light influence on amino acid synthesis. Both amino acid and ammonia concentration in fully exposed samples of our experiment can be regarded as insufficient for yeast nutrition (Ribereau-Gayon *et al*. 2006).

A clear temporal pattern was observed in the accumulation of the various classes of phenolics. Flavanols and most hydroxycinnamic acids accumulated mainly before veraison, while the main querctin glycoside accumulation occurred post-veraison. The synthesis of quercetin glycosides seemed to follow the interception of direct radiation of the grapes in an almost linear way. At harvest, total phenolic content of all treatments differed significantly, with the exception that there was no significant difference between the two shading treatments. From these results it can be concluded that the timing of leaf removal treatments does influence the content of phenolics of the grapes at harvest. This effect can almost exclusively (to about 95 % on average) be explained by the accumulation of quercetin glycosides induced by excess light, which has been observed pre- and post veraison.

Shading and leaf removal did not influence the level of flavanol accumulation, except for catechin, the content of which was moderately increased by light interception at harvest in 2012. Other authors have shown light-induced effects on flavanol accumulation when treatments were applied directly at the beginning of flowering (Koyama *et al.* 2012). Therefore, it appears likely that the enzymatic setup for flavonol synthesis takes place during flowering and shortly afterwards, and can be influenced by light only then. Flavanol content of the berries then continues to increase, but is no longer subject to light influence. The concentration of flavanols was similar in both experimental years. Compared to flavonol accumulation, light influence on flavanol accumulation is relatively weak in red grapes (Koyama *et al.* 2012), which is in accordance with our results. Similar to flavanol accumulation, little light influence was measured on the accumulation of hydroxycinnamic acids. Although it has been shown that hydroxycinnamic acid synthesis is influenced by light in other species, like *Echinacea purpurea* (Abbasi *et al*. 2007) little such data are available for *Vitis vinifera*. The content of hydroxycinnamic acids was decreased by shading before veraison in 2012. However, the effects of light exposure and shading remained inconsistent during the experimental years, as no light influence was detected in 2011. Just as flavanol synthesis, hydroxycinnamic acid synthesis occurs mainly pre-veraison, and an earlier onset of the experiment may have revealed light influence on the synthesis of these compounds at earlier developmental stages.

Flavonol accumulation in control and leaf removal samples occurred during the entire experimental period, the main phase of accumulation being post-veraison. While other authors (Downey *et al.* 2004, Koyama *et al.* 2012) observed a decreasing content and concentration of flavonols in shaded bunches of 'Shiraz' and 'Cabernet Sauvignon', the quercetin glycoside content of the berries in our study was not decreased by shading, but remained remarkably stable. Shading virtually "froze" the content of the respective glycosides, giving an exact picture of the flavonol profile at the time the shading was applied. For example, almost no quercetin glycosides except que-3-glucuronide and que-3-rutinoside were present in the early shading treatments of both experimental years as well as in the berries sampled at the beginning of the experiment and at veraison in 2012. While que-3-glucuronide and que-3-rutinoside were already present at the beginning of the experiment, leaf removal or shading after veraison did not significantly change the levels of these flavonols at harvest. On the other hand, our data suggest that the accumulation of que-3-rhamnoside occurs almost exclusively after veraison, and was little influenced even by leaf removal before veraison. A similar pattern was also shown for que-3-arabinoside and que-3-galactoside. Hence, it is highly likely that the accumulation of specific flavonol glycosides underlies strong developmental regulation, in accordance with data published by Ono *et al.* (2010), who show the developmental regulation of two flavonol glycosyltransferases. Nevertheless, the function of the various quercetin glycosides in the berry is yet to be clarified and deserves further research.

The strong negative correlation between the accumulation of phenolics and amino acids underlines the tight relation of both metabolic pathways. However, as in this study the light-induced flavonols are the main contributor to the phenolic profile of 'Riesling' and juice amino acids are decreased by radiation, the strong correlation between light, phenolics and amino acids is not surprising. It has been shown that reactions to oxidative stress and nitrogen deficiency are similar (Keller and Hrazdina 1998, Lea *et al.* 2007), and share, at least partially, a common signaling pathway (Harding *et al.* 2003). Further, nitrate inhibits the synthesis of phenolics in grape tissue cultures (Pirie and Mullins 1976). At least at veraison, NH_4-Nitrogen contribution to the nitrogen pool was elevated in berries exposed to high levels of radiation by leaf removal. This may be a hint that there but metabolic pathways may compete for carbon skeletons, which are limiting for ammonia

integration into the amino acid metabolism. Nevertheless, more research is needed to clarify whether there is indeed a common control of both pathways, or if the regulation of both pathways occurs independently of each other.

Conclusion

Compositional changes in white 'Riesling' induced by leaf removal were observed for the flavonoids, amino acids and malic acid. These changes can be attributed to the effect of increased light interception by the grapes. The changes in leaf-fruit ratio showed no significant effects on sugar accumulation, nor did shading of the bunches. Early (E-L 27) leaf removal was shown to increase the skin content of quercetin glycosides and some hydroxycinnamic acids already before veraison. The differences in skin quercetin glycoside content between early and late leaf removal were still measurable at harvest. Early leaf removal of 'Riesling' grapes may therefore increase the bitter perception in the resulting wine, especially when there are long skin contact times during processing. By excluding the influence of light from an early developmental stadium, the synthesis of quercetin glycosides was inhibited completely. Manipulation of the grape microclimate affected the concentration of some amino acids already at veraison, however much stronger effects were observed post-veraison. Leaf removal before or at veraison may lead to low yeast available nitrogen and therefore increase the risk of stuck or sluggish fermentations.

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