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Use of Metabolic Profiling To Study Grape Skin Polyphenol Behavior as a Result of Canopy Microclimate Manipulation in a 'Pinot noir' Vineyard

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Supporting Information

ABSTRACT: Canopy microclimate manipulation can have a significant effect on grapevine gene expression and can thus affect the yield of many important berry compounds. Focusing on only a few targeted phenolics in the past, advanced multimethod analytical approaches are opening up much wider possibilities to fill in the gaps of missing knowledge about plant secondary metabolism. Different leaf removal timings, leading to different microclimate scenarios, were thus introduced in a 'Pinot noir' vineyard to reveal related alterations of multiple classes of skin phenolics, including some rarely studied to date. Different accumulation trends during cluster development were detected not only between groups but also between individual compounds within groups. Although many significant changes were observed early in the season, these were later often less significant. However, at harvest, 31 of 72 detected compounds showed significant differences in comparison to control for at least one of three leaf removal approaches.

KEYWORDS: *metabolic profiling, canopy microclimate manipulation, leaf removal, preflowering leaf removal, secondary metabolism, phenolics, 'Pinot noir' (Vitis vinifera L.)*

INTRODUCTION

Grapevine constituents that are not directly involved in the primary biochemical pathways of cell development are commonly classified as secondary metabolites. Even though they are not considered as essential for normal plant functioning, many of them have important roles such as signaling and attraction, as well as protecting the plant from different biotic and/or abiotic stresses.[1] This concerns compounds involved in plant−pathogen (or insect) interactions, compounds preventing UV damage to different plant tissues, and compounds implicated in hormone homeostasis.[2] Consequently, secondary metabolites are normally present in higher concentrations when a plant is subjected to different stresses or elicitors.[3] However, not only are they important for plant adaptation and survival, but their occurrence and chemical diversity can also account for significant diversity in the quality of different agricultural crops.

In general, secondary metabolites consist of a wide array of species-specific chemicals, belonging to different phytochemical groups, such as alkaloids, terpenes, antibiotics, volatile oils, resins, cardiac glycosides, sterols, saponins, and phenolic compounds.[4] Phenolics in particular are currently under special research interest, not only due to crop plant quality issues but also due to various associations with human health-promoting effects.

Apart from variety specifics, the occurrence of vine secondary metabolites, including phenolics, is largely determined by the geoclimatic conditions (terroir) in which the plant is grown.[5] Although regional macroclimate or site mesoclimate cannot be influenced, grapevine canopy microclimate conditions can, on the other hand, be manipulated by implementing some viticultural practices in the vineyard environment. Leaf removal is one of the techniques that can be employed to maneuver the microclimate in the cluster area; however, its performance is particularly related to the phenological stages of grape berry development at which the practice is adopted.[6] It is commonly performed in the postflowering period (between phenological growth stages BBCH 69 and BBCH 83), whereas earlier implementation before flowering (at BBCH 57) and the effects on the secondary metabolism due to such early alterations in microclimate conditions have not yet been extensively explored.

When actually manipulating canopy microclimate in the field, temperature is one of the factors to be considered carefully. Apart from many effects on vine performance, a trend toward a reduction in total acidity in particular is confirmed also in Slovenia, as a result of higher temperatures during the growing season nowadays.[7] Temperature has also been shown to significantly affect flavonoid biosynthesis. Several experiments have revealed the inhibitory effect of (overly) high temperature on anthocyanin accumulation in berry skins,[8−10] whereas the critical temperature leading to the inhibition of anthocyanin synthesis is reported to be between 30 and 35 °C, varying according to different authors. Moreover, cool night temperatures and day/night temperature regimes can also lead to modifications in red grape color characteristics.[8,11,12] Recent studies on the biosynthesis of grape flavonoids as affected by temperature have indeed mainly focused on anthocyanins;

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however, ref 13 also highlighted high temperatures causing a moderate reduction in proanthocyanidin and quercetin concentrations in berry skins. Furthermore, the temperature in the last phase of ripening played an important role in the observed *cis*-piceid levels in 'Barbera', as described in ref 14. Few research projects have focused on other skin phenylpropanoids and their responses to changes in temperature; thus, little is still known about accumulation trends related to such climatic issues.

Sunlight exposure is another important factor with a known impact on the phenolic composition of grapes.[10,15] There is much contradictory data about the impact of light on red grape color; however, apart from concentration, ultraviolet light exposure has been reported to alter the anthocyanin composition of different grape varieties.[10,16] On the other hand, exposure to UV light has a very evident effect on flavonols.[10,15,16] UV light has also been reported to stimulate the production of the stilbene resveratrol,[17] whereas the accumulation of tannins in grape berries was shown to be relatively unaffected, with some changes being detected only early in the season.[18,19] Once again, little research has yet been done on many other grape skin phenolics in relation to light exposure or further single/multiple microclimate parameters.

Environmental metabolomics and related advanced multimethod approaches initially focused mainly on the whole fruit/crop metabolome as affected by environmental conditions. However, for its successful implementation into standard practice, an understanding of environmental effects on a range of compounds in a single plant tissue is also very important. Many grape phenolics are known to be synthesized exclusively in (*Vitis vinifera*) grape berry skins (e.g., anthocyanins, flavonols, stilbenes). Some others, such as flavan-3-ols, are also present in the seeds, whereas those from skins seem to be more affected by environmental factors and have a higher tendency for polymerization[19,20] as well are essentially more likely to be extracted in must/wine. Grape berry skins thus undoubtedly represent a very important tissue to be considered separately using new analytical possibilities to improve knowledge and enable more successful grapevine canopy management.

Whereas scientific studies manipulating a single controlled factor may indeed help to improve the understanding of the direct effects on plant biosynthesis, studies in realistic conditions may be more helpful for viticultural practice.[21] However, in the case of actual field observations, multiple factors should be considered together due to their known synergic effects. A field trial was thus designed to employ new advanced analytical possibilities to improve knowledge of grapevine phenylpropanoid biosynthetic behavior in the case of different "real case" (and up to a certain level defined) microclimate scenarios, created by carrying out leaf removal at different phenological stages within 'Pinot noir' cluster development.

MATERIALS AND METHODS

Experimental Vineyard and Plant Material. The experiment was carried out in 2010, observing in total 320 'Pinot noir' (*V. vinifera* L.) grapevines in a vineyard located in Potoce (Vipava Valley, Slovenia). The vineyard was planted in 2004; its rows are oriented east−west, with a plant density of 5682 plants/ha (0.8 m vine spacing × 2.2 m row spacing). It is situated at an altitude of 95 m above sea level, and the training system adopted is the single Guyot. A completely randomized experimental design was set up in the middle of the vineyard, with four treatments and three replicates (12 plots of 20 plants). The treatments were applied as follow: PF (preflowering), leaf removal (LR) performed before flowering, at phenological stage BBCH 57;[22] BS (berry set), LR applied at BBCH 71; VE (veraison), LR performed at BBCH 83; UN (control treatment), LR not applied (untreated vines/leaves retained). Leaf removal was performed manually, removing the basal four-to-six leaves of all the shoots as normally carried out for prebloom treatments;[23] thus, for each treatment the same leaf removal severity was applied (at preflowering (PF), berry set (BS), and veraison (VE) time, respectively). After initial performance of experimental defoliations, the leaf removed zones were not maintained leaf free.

Monitoring of Microclimate Conditions. The temperature and relative humidity in the cluster area of all the treatments were monitored during the hottest period (from August 14 to September 11, 2010) via DS1923 i-Button sensors (Maxim Integrated Products, San Jose, CA, USA), collecting and storing data on an hourly basis.

Sampling and Sample Preparation. Grape berry samples from all plots were collected separately during maturation (from June to September 2010). Sampling was essentially carried out at 10 day intervals (with some adjustments due to rain events) and at harvest time (based on the maturity level recorded in the control grapes: 22 °Brix and 5.6 g L^{-1} titratable acidity on average). The first sampling was done when the berries were formed enough for successful separation of the skins; thus, it was done after the performance of the first two leaf removal strategies (at preflowering and berry set). Berry samples were carefully collected together with their pedicels to avoid any damage and/or oxidation risks. Samples were then frozen immediately and methanol extracts (MeOH extracts hereinafter) of berry skins were prepared as previously described.[24] Briefly, the skins of previously weighed berries were carefully separated from flesh and seeds (while the berries were still frozen) and put directly into a dark glass container with 100 mL of MeOH. After 24 h of initial extraction, the extract was separated and the second extraction for 2 h in 50 mL of MeOH was performed. Both methanolic extracts were then combined, and the MeOH extracts were kept at −20 °C until the analyses were carried out.

Before UHPLC-QqQ-MS/MS analysis of phenolic compounds, an aliquot of 10 mL of MeOH extract was first evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd., UK) under reduced pressure at 45 °C. The sample was then reconstructed in a quantitative flask up to 1 mL of the final volume with methanol (Fluka Sigma-Aldrich) and filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA, USA). Additional dilution with MeOH was carried out if needed for the compounds present in higher concentrations.

Before HPLC analysis of grape anthocyanins, the MeOH extracts were first filtered (0.45 μm Millipore HPLC filter) and thereafter diluted with 1% trifluoroacetic acid (TFA, Sigma, Germany) in water using a 1:1 (v/v) ratio to maintain the symmetry of chromatographic peaks.

UHPLC-QqQ-MS/MS (Targeted Metabolomics) Analysis. A comprehensive targeted metabolomic analytical approach according to ref 25 was applied. The method was developed with the potential to perform qualification and quantification of 135 phenolics belonging to different chemical groups present in fruit, such as benzoates, phenylpropanoids, coumarins, stilbenes, and flavonoids (flavones, isoflavones, flavanones, flavan-3-ols, flavonols, and dihydrochalcones). Ultrahigh-performance liquid chromatography (UHPLC-MS/MS) was performed using a Waters Acquity UHPLC system (Milford, MA, USA). Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm, kept at 40 °C.[25] All analyses were performed in biological triplicates.

HPLC Determination of Anthocyanins. The analytical method as previously presented by ref 6 was applied for the detection of anthocyanins. Separation and quantification of delphinidin-3-glucoside (Del-3-Glu), cyanidin-3-glucoside (Cy-3-Glu), petunidin-3-glucoside (Pet-3-Glu), peonidin-3-glucoside (Peo-3-Glu), and malvidin-3-glucoside (Mal-3-Glu) were performed using gradient high-performance liquid chromatography with UV−vis detection at 520 nm (Waters chromatographic system). Individual anthocyanins were separated on

the Atlantis column C18, 3.9 × 150 mm, 3 μm (Waters, USA), and quantified as malvidin-3-glucoside (mg/L) equivalent. All analyses were performed in biological triplicates.

Data Processing and Statistical Analysis. Processing of phenolic raw data sets was performed with the help of Mass Lynx Target Lynx Application Manager (Waters), except for anthocyanins, which were processed with the help of Empower software (Waters). Multivariate principal component analysis (PCA) on the autoscaled data was performed to visualize the effects of the different leaf removal strategies. To get further insight on the metabolic effects of the different leaf removal strategies, separate ANOVA models for the different metabolites were performed. Because many tests were done, some form of multiple-testing correction was necessary. Here, we have used the false-discovery rate (FDR) correction.[26] In those cases when the corrected p values were below 0.05, Tukey's Honest Significant Difference (HSD) test was used to find which factor levels actually differ. All of these statistical tests were performed with R.[27]

RESULTS AND DISCUSSION

The vintage 2010 in Vipava Valley (macroclimate) was quite warm and sunny earlier in the season (June, July), but colder during late season (September), mainly due to frequent rainfall events (Supporting Information Table S1). However, different leaf removal treatments led to four different canopy microclimate scenarios (sun exposure, temperature, humidity) in the cluster area. Earlier leaf removal (LR) treatments (preflowering (PF) and berry set (BS)) caused clusters to be more exposed to the sun during June and July (open canopies), whereas clusters of late veraison (VE) LR treatment were initially covered by leaves (closed canopy) and then opened in mid August. On the other hand, untreated control (UN) remained closed until harvest. Differences in temperature and humidity within cluster areas were thus recorded in critical, hot August days, with basically lower relative humidity and higher temperature in the case of LR treatments when compared to control and furthermore with considerable differences also between early and late leaf removal treatments (Supporting Information Figures S2 and S3). As the present field experiment is supported by measurements of microclimate conditions and thus four different purposely induced and, to certain level, defined microclimate scenarios were compared, this work is of great value on a more global level even if the statistical significance and reproduction of the results over more harvest seasons have not been completed.

Within viticultural parameters (Supporting Information Table S2) a reduced cluster weight and yield were observed for PF, as previously observed in the case of cvs. 'Barbera' and 'Lambrusco salamino' (*V. vinifera* L.).[23]

Phenolic Compounds. To adapt to ongoing changes in the environment, plants can use "their enormous metabolic capacity to produce a large variety of secondary metabolites",[28] including phenolics. Of 140 phenolic compounds under observation, in total 72 different phenolics were detected in skin samples in the case of at least one sampling point during the maturation period (June−September 2010). The detected compounds were the group representatives of flavonols (22), stilbenes (18), flavan-3-ols (10), benzoates (7), anthocyanins (5), hydroxycinnamates (5), flavones (2), flavanones (2), and dihydrochalcones (1). Different accumulation trends (peaking behavior) during cluster berry development were detected, not only between groups but interestingly also between individual compounds within the same chemical group, signifying that the timing of leaf removal may play an important role in targeted promoting of specific compounds with either early or late biosynthetic behavior.

PCA was performed to visualize the effects of different leaf removal strategies and to highlight the evolution of the metabolic profiles over time. Figure 1 shows the projection of the data set on the PC1 × PC2 plane accounting for 65% of the total variance. The effects of the different treatments over time segments are graphically shown by connecting the median of the replicates. Figure 1 highlights that at the earlier stage, the control was different from PF and BS, indicating that both types of early leaf removal (performed before first sampling, see Sampling) caused substantial changes in plant biosynthetic behavior soon after they were implemented. The global view obtained by PCA, however, does not allow clear separation of the treatments at the latest time point. To get further insight on the metabolic effects of the different leaf removal strategies, separate ANOVA models for the different metabolites were performed. In the following they are discussed in details.

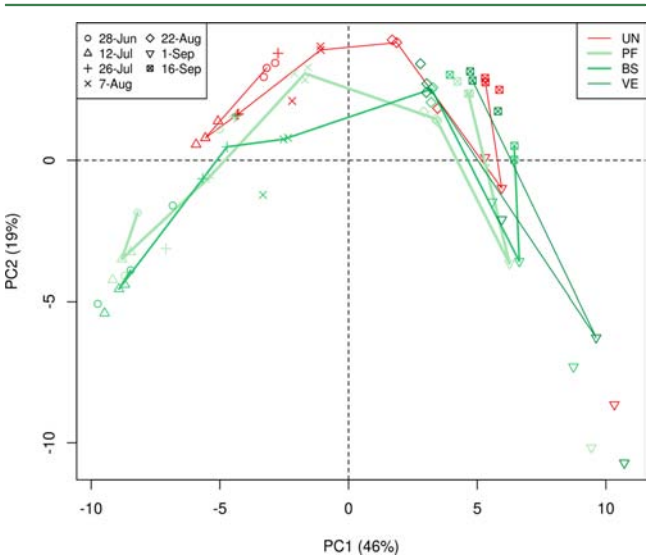

Figure 1. Multivariate principal component analysis (PCA) on the autoscaled data showing the projection of the data set in the PC1 × PC2 plane as affected by different leaf removal strategies highlighting the evolution of the metabolic profiles over time (PF, preflowering; BS, at berry set; VE, at veraison; UN, control with no leaf removal). The segments connect the median of the biological replicates.

Flavonoids. *Flavonols.* Flavonols are known to be the products of the flavonoid biosynthetic pathway, which in red grapevine cultivars also engenders anthocyanins and condensed tannins. In *V. vinifera* grapes they are mostly present as glycosides and are synthesized only in grape skins.[15,20] Different physiological functions of flavonols are reported in plants; however, their most widespread role still appears to be protection from excessive UV damages. The biosynthesis of total flavonols in our experiment was extensively triggered by leaf removal treatments, because the light environment within the grapevine canopy was considerably enhanced. A clear increase in flavonols following an increase in cluster sun exposure has already been reported.[10,15,16] As they can be almost entirely absent in the case of shaded bunches[19] and with a prompt increase after later exposure, flavonols can be considered as a biomarker for the sun exposure regime achieved in a bunch area following canopy microclimate manipulation.[29]

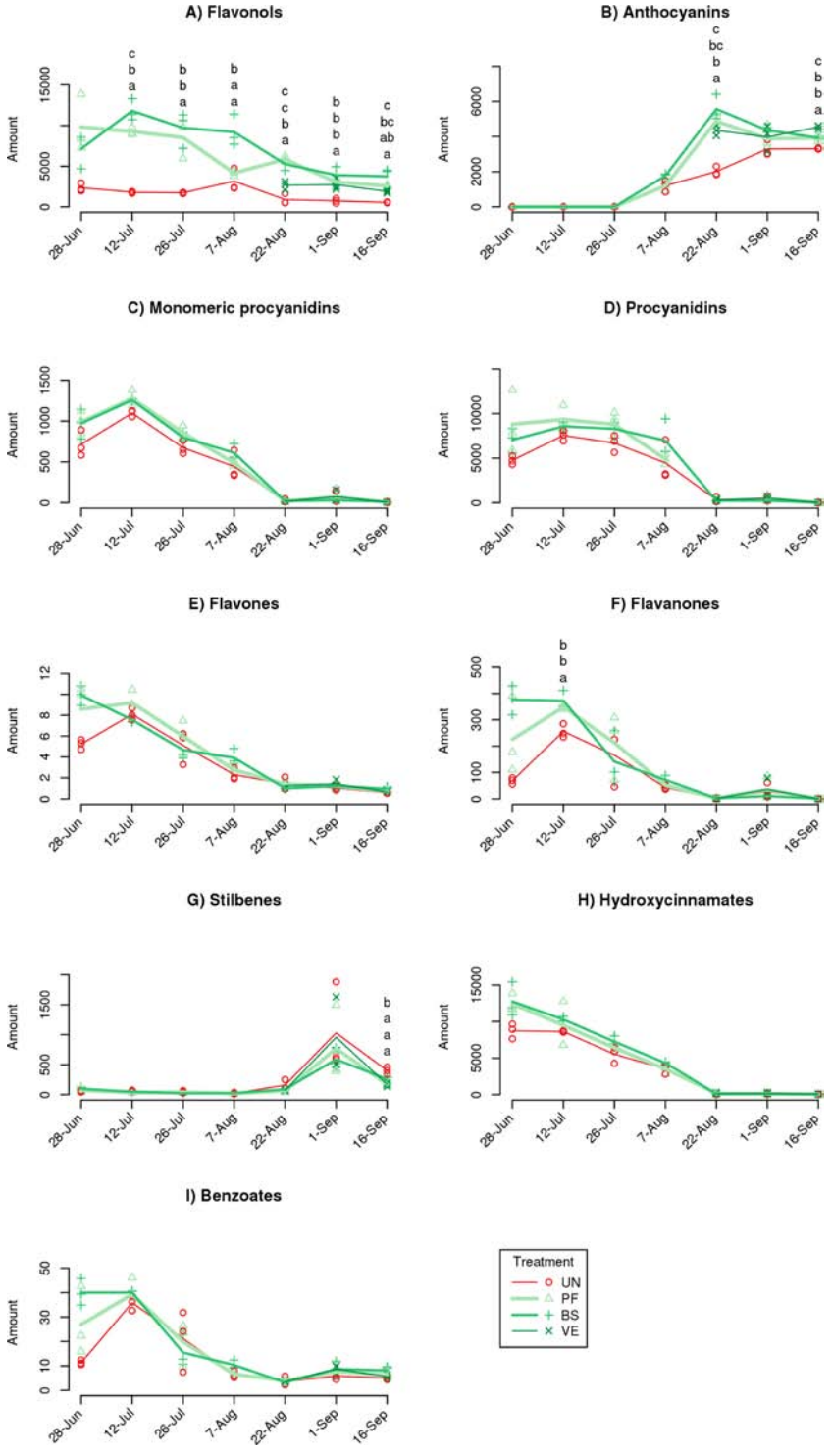

Figure 2. Total phenolics within different phenolic groups (μg/g skins) as affected by canopy microclimate manipulation through leaf removal at different phenological stages (PF, preflowering; BS, at berry set; VE, at veraison; UN, control with no leaf removal).

Two distinct periods in flavonol synthesis have been already reported in grapes, the first around flowering and the second during ripening of developing berries.[18] This "two peak" biosynthetic behavior in relation to total flavonols was also detected in our experiment (Figure 2A), although the increase in total flavonols (particularly in the case of earlier BS and PF leaf removal) was much higher early in the season and was followed by only a minor increase (in the case of VE/UN and PF) later in the season.

However, observing only total flavonol content seriously masks the interesting behavior of individual compounds. By focusing on the accumulation trends of individual flavonols, it was indeed revealed that their concentration peaks differ considerably. Although most of them showed the greatest synthesis later in the season, therefore signifying late peaking behavior, others, on the contrary, showed the greatest synthesis early in the season (early peaking behavior), whereas some of flavonols showed no specific behavior at all (Table 1). Finally,

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Table 1. Grouping of Detected Skin Phenolic Compounds According to Their Biosynthetic Behavior (Their Concentration Peak within Berry Development Period): Early Peak Behavior (Peak before Veraison); Late Peak Behavior (Peak after Veraison)

PEAK BEHAVIOUR	Early peak behaviour	Two peaks behaviour	Late peak behaviour	Mainly increasing behaviour	Mainly decreasing behaviour	No specific peak behaviour
SKIN PHENOLIC COMPOUNDS	•4-hydroxybenzoic acid •syringaldehyde •caffeic acid •caffeic acid+catechin con. •phlorizin •luteolin-7-glucoside •naringenin-7-glucoside •catechin •gallocatechin •procyanidin B1 •procyanidin B2+B4 •rutin •quercetin-3-glucunoride •kaempferol-3-rutinoside •quercetin-3-Glc-Ara •taxifolin	•quercetin-3-rhamnoside •kaempferol-3-glucunoride	•methyl gallate •syringic acid •*trans*-resveratrol •*cis*-resveratrol •piceatannol •pterostilbene •*trans*-piceid •*cis*-piceid •astringin •isorhapontin •*cis*-ε-viniferin •*cis*-ω-viniferin •*trans*-ω-viniferin •ampelopsin D+ quadrangularin A •pallidol •isorhamnetin •myricetin •laricitrin •syringetin •kaempferol-3-glucoside •myricetin-3-rhamnoside •isorhamnetin-3-glucoside •isorhamnetin-3-rutinoside •quercetin-3,4′-diglucoside •delphinidin-3-glucoside •cyanidin-3-glucoside •petunidin-3-glucoside •malvidin-3-glucoside	•vanillic acid •*trans*-ε-viniferin •α-viniferin •Z-miyabenol C •isohopeaphenol •ampelopsin H + vaticanol C-like isomer •syringetin-3-glucoside+ syringetin-3-galactoside •peonidin-3-glucoside	•caftaric acid •fertaric acid •*trans*-coutaric acid •epicatechin gallate •epigallocatechin gallate •procyanidin B3	•vanillin •gallic acid •ferulic acid •apigenin-7-glucoside •naringenin •epicatechin •epigallocatechin •quercetin-4′-glucoside •kaempferol •quercetin •quercetin-3-glucoside •quercetin-3-galactoside

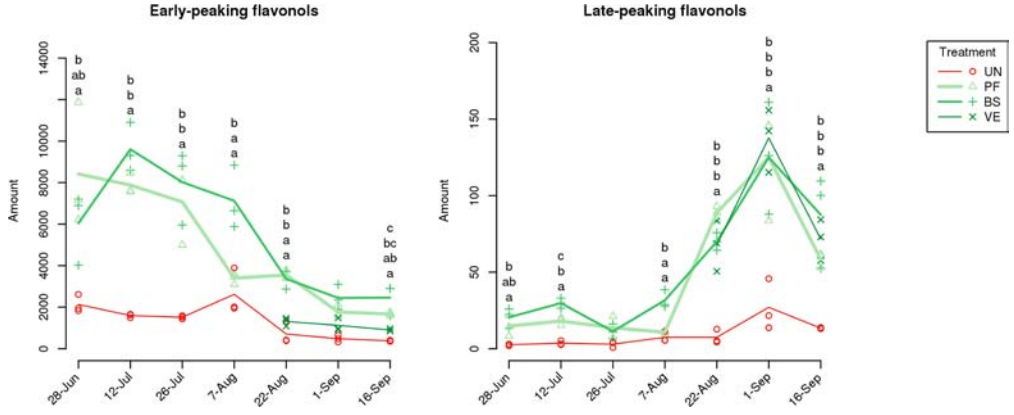

Figure 3. Total flavonols (μg/g skins) with early peak behavior and late peak behavior as affected by canopy microclimate manipulation through leaf removal at different phenological stages (PF, preflowering; BS, at berry set; VE, at veraison; UN, control with no leaf removal).

only two flavonols showed two-peak behavior during berry development (Table 1). If the "two-hump" curve of total flavonols is occurring during grape berry development, this is most likely due to a combined reality of differently behaved individuals.

Despite the fact that most flavonols peaked relatively late, those same flavonols were present in relatively small concentrations (all ranging on average from 125 to 138 μg/g skins at the highest point in the case of LR treatments) (Figure 3). On the other hand, there were fewer peaking earlier, but they occurred in much higher concentrations (on average from 7878 to 9604 μg/g skins at the highest point in the case of LR treatments). Moreover, early peaking flavonols in total were still present at harvest with a 12−28 times higher concentration than in total late peaking flavonols and represented 65% of total flavonols in the case PF leaf removal, the same (65%) in the case of BS leaf removal treatment, but only 47% in the case of late leaf removal (VE).

Considering different leaf removal treatments, we could also observe that earlier leaf removal treatments (PF and BS) fundamentally affected the occurrence of flavonols with early peaking behavior and that the related improvement in total flavonol content was also retained at harvest (3755 ± 1174 and 2563 ± 105 μg/g skins for BS and PF, respectively; as compared to 1902 ± 212 and 540 ± 51 μg/g skins for VE and UN, respectively). Late (veraison) leaf removal, on the other hand, caused only a minor increase in total flavonol concentration as compared to untreated control and with no significant results at harvest (Figure 2A). This clearly indicates that early peaking flavonols can only be promoted by early leaf removal intervention. On the contrary, the accumulation of late peaking flavonols as compared to the control was similar at harvest (with no significant differences) for all of the leaf removal treatments, regardless of the timing at which it was carried out (Figure 3).

Mattivi et al.[24] have already reported certain correlations between the metabolic pathways of anthocyanins and flavonols, implying that any attempt to optimize the pattern of one might also affect the patterns of others. However, both groups are important for grape and wine quality. Anthocyanins are important mostly in terms of color intensity, whereas anthocyanins and flavonols are both involved into copigmentation reactions,[30] crucial for the development of wine color stability (a known problem for 'Pinot noir' wines).[31] Therefore, if the biosynthesis of flavonols would be strongly promoted early in the season, even before anthocyanins start to accumulate, there would probably be a chance of improving the yield of both at harvest. This could/should indeed be one of the tasks of successful canopy microclimate manipulation.

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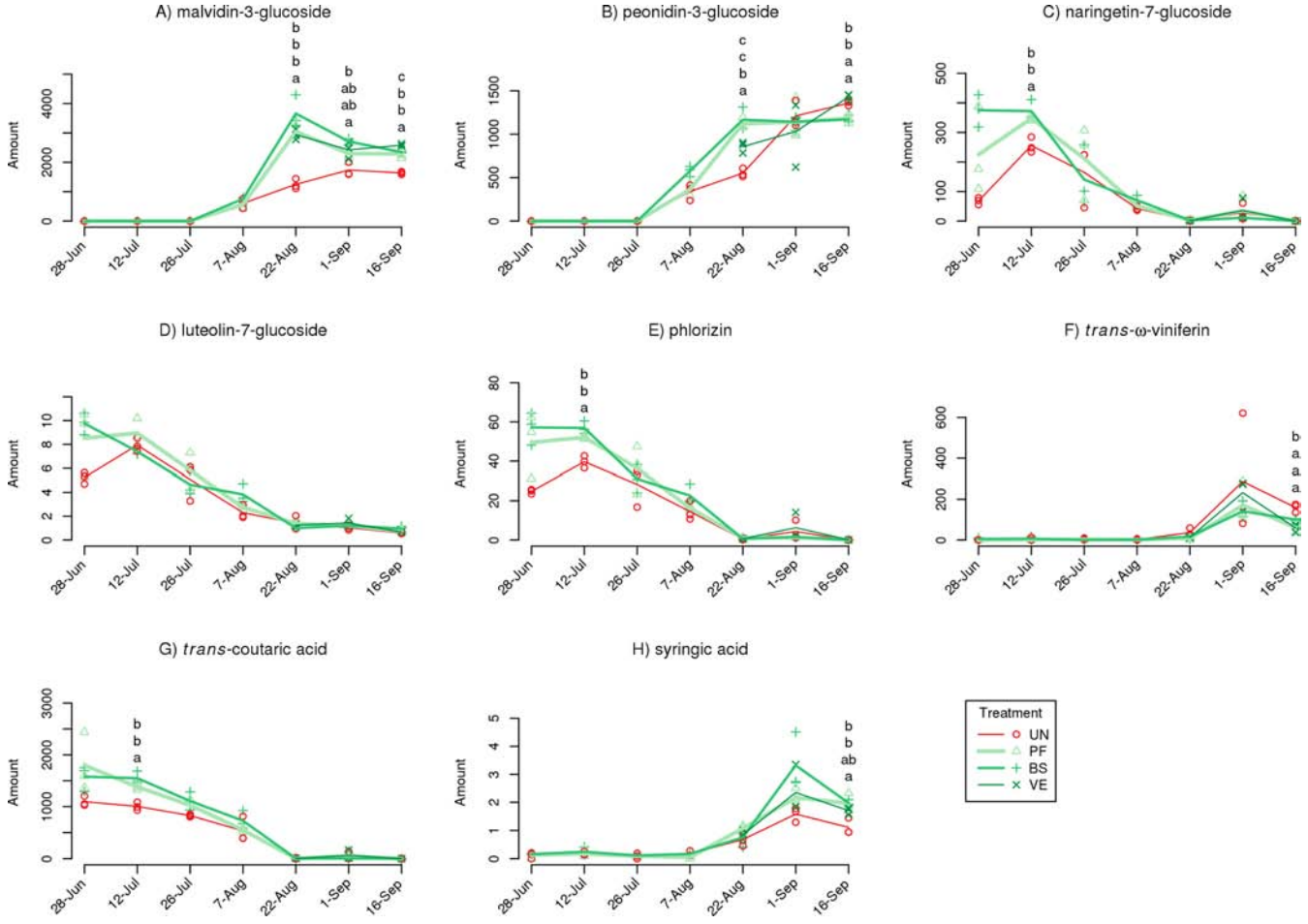

Figure 4. Accumulation dynamics (μg/g skins) of selected typical individual representatives from different phenolic groups as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF, preflowering leaf removal; BS, berry-set leaf removal; VE, veraison leaf removal; UN, control with no leaf removal). Accumulation dynamics of the rest of the detected individual representatives can be seen within the Supporting Information.

Anthocyanins. Anthocyanins are synthesized from phenylalanine through an anthocyanin biosynthetic pathway regulated by enzyme activities and gene expressions.[32] In addition to the enzymes required for the synthesis of flavan-3-ols, two additional enzymes (LDOX and UFGT) are required for anthocyanin biosynthesis.[32] They are expressed mainly in the skins and typically from the onset of ripening (veraison), as confirmed again in our experiment. The role of anthocyanins in plants has been investigated in many studies, but compelling evidence is still lacking. However, they are mainly associated with protection from solar ultraviolet rays and attack by herbivores or pathogens.[33]

In the samples, anthocyanins were first detected on August 7 (Figure 2B). From that sampling date, total anthocyanins increased rapidly until they became stable (or even declined) in late August (between samplings on August 22 and September 1). However, when looking at total anthocyanins, we observed that the accumulation in the case of the untreated control (in contrast to all of the leaf removal treatments) did not experience the "late August decline". This raises the question of what could briefly inhibit synthesis in the case of open canopy (leaf removal treatment) but not in the case of shaded grapes (untreated control UN); even the total amount in the case of UN was still lower than in LR treatments at the same time. This last fact is, however, consistent with the literature, stating that light exposure is required in addition to optimal temperature to promote anthocyanin synthesis.[16] On the other hand, with regard to temperature, it has previously been reported that the average daily temperature on the berry surface is higher in the case of leaf removal treatments than in the untreated control[29] and that too high temperature can inhibit anthocyanin synthesis,[8] which may be one of the reasons for a brief slowing biosynthetic behavior. It seems that higher temperature within the cluster area of PF, BS, and VE (Supporting Information Figure S2) did briefly inhibit anthocyanin synthesis; but, on the other hand, light exposure still favored open canopies. This finally led to a situation at harvest that was clearly in favor of leaf removal treatments, with VE showing the best results (4545 ± 99 μg/g skins), followed by BS and PF (3927 ± 81 and 3921 ± 197 μg/g skins, respectively), whereas the lowest concentration was detected in UN (3313 ± 33 μg/g skins). As 2010 was rather cold and rainy in the Vipava Valley, the results differ slightly from our previous observations.[6] In the warmer and drier 2009, the BS treatment (PF not performed) led to higher total anthocyanins than VE. This was probably due to higher berry surface temperatures after veraison leaf removal, which exposed the grapes directly to the sun in the hottest period, as August was very hot in 2009.[29] BS (earlier treated) grapes, on the other hand, were partially protected by the regrowth of lateral leaves by that time.

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However, the decrease in anthocyanins in grape skins with higher temperatures could be caused by many factors, such as chemical factors (pH, temperature, light, oxygen) and/or enzymatic degradation and not only due to inhibition of anthocyanin biosynthesis.[34]

Finally, by looking more closely into the accumulation trends for individual anthocyanins, in the case of LR treatments as compared to untreated grapes (UN) we observed an increase in delphinidin, cyanidin, petunidin, and malvidin glucosides (95, 34, 71, and 58%, respectively, at harvest in the case of VE, reaching the highest values). In the case of early LR samples (BS and PF) a slight decrease in peonidin-3-glucoside (14 and 13%) as compared to UN was shown, but again an increase (although slight and not significant) as compared to VE (5%). Malvidin and petunidin glucosides can be seen in Figure 4A,B; others with behavior similar to malvidin-3-glucoside can be found in the Supporting Information Figure S1. These trends are similar to the observations of ref 20 in their light exclusion trial, so such behavior is probably more a consequence of light than of temperature.

Flavan-3-ol Monomers and Proanthocyanidins. Flavan-3-ols consist of both monomers and polymers with different degrees of polymerization (proanthocyanidins) and share a biosynthetic pathway similar to that of flavonols and anthocyanins. In contrast to anthocyanins and flavonols, flavan-3-ols can be found not only in the skins but also in the seeds of the grape berry.[35] The generally accepted biological role of flavan-3-ols in plants is linked to protection against microbes, fungi, insects, and herbivorous animals,[36] whereas it is also believed that polymers, tannins, play a certain structural role in plants.

Flavan-3-ols were present with the highest concentrations around berry set, but started to decrease before veraison, finally remaining more or less stable in the last stages of maturation (Figure 2C,D). This is in agreement with the observations of ref 18 highlighting that the synthesis of procyanidins in skins occurs early in berry development and reaches a maximum around veraison, in our case, just before the veraison. Procyanidin B1, the most abundant flavan-3-ol in our samples (>6000 μg/g skins at the highest point for LR treatments) actually dictated the trend in terms of totals, whereas B3 showed a slightly more decreasing trend, in the case of PF already starting from the first sampling point. It is hard to arrive at conclusions regarding the exact trends shown for procyanidins B2 and B4, because it was not possible to separate them analytically due to their coelution. However, their sum (B2 + B4) showed early peaking behavior, similar to B1, but with a much lower total concentration (68−80 μg/g skins on average at the highest point for LR treatments) (Supporting Information Figure S1).

Many other authors have also observed skin and seed flavan-3-ols accumulation during berry development,[20,35,37] although their findings regarding total amounts and accumulation trends are not always consistent. Furthermore, the specific effects of grape cultivar[35] and vintage[37] seem to be significant. On the other hand, it has been reported that seed tannins are normally made up of monomeric flavan-3-ols (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate, whereas skin tannins can also accumulate (−)-epigallocatechin, (+)-gallocatechin, and (−)-epigallocatechin gallate,[38] which is in accordance with our results for monomeric flavan-3-ols. If we compare total monomeric flavan-3-ols and total procyanidins from our trial (Figure 2C,D) separately, we can observe that their (early) peaking behavior is similar, although procyanidins are present in much higher concentrations. In general, it has been reported that skin tannins tend to have a much higher degree of polymerization than tannins present in seeds.[38] In the case of individual flavan-3-ol monomers, peaking behavior similar to that of total flavan-3-ols was observed for (+)-catechin, (+)-gallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate. (−)-Epicatechin and (−)-epigallocatechin, on the other hand, showed completely random behavior (Supporting Information Figure S1). Furthermore, our study of canopy microclimate manipulation (through leaf removal treatments) revealed that the different trends between untreated (shaded) control and (sun exposed) leaf removal treatments could be observed only up to the point in which total flavan-3-ol concentrations become lowest but stable (last 3 weeks of maturation) (Figure 2C,D). After that point (and at harvest) no significant differences were observed between the treatments or with the control. Light exposure in relation to skin flavan-3-ols has been previously studied in 'Shiraz' and 'Pinot noir'.[19,20] In their study, the authors observed a change in the abundance and/or polymerization level of flavan-3-ols in the skins of the shaded fruit at veraison in both 'Shiraz' and 'Pinot noir', whereas in the study of ref 20 (on 'Pinot noir') the differences also remained evident at harvest. However, both of the studies were done through artificial manipulation of light exposure, using boxes over the grapes to achieve very low exposure environment, probably much lower than in an actual situation in the case of vine canopies with no leaf removal. In addition to the research carried out on the effect of light, ref 12 also reported (in their experiment with controlled sun exposure) some changes in skin proanthocyanidin concentration at harvest as a result of berry temperature.

Focusing further on individual compounds affected by leaf removal, all procyanidins and some flavan-3-ol monomers, (+)-catechin, (+)-gallocatechin, and particulary (−)-epicatechin gallate, showed lower accumulation trends in the case of untreated vines as compared to leaf removal treatments. In any case, the difference could again be seen only early in the season and had actually disappeared by harvest time. In the case of (−)-epicatechin and (−)-epigallocatechin no specific trends were generally observed in favor of any treatment (Supporting Information Figure S1).

Flavones and Flavanones. Flavanones can be formed from the chalcone structure, whereas flavones are synthesized at a branch point of the anthocyanidin/proanthocyanidin pathway from flavanones as direct biosynthetical precursors.[39] Flavone formation in various tissues of a wide range of plant species is catalyzed by the flavone synthase (mainly FSNII).[39] Apart from other biological roles linked to them to date (e.g., flavone glycosides acting as copigments), they may also act as UV protectants.[40]

Two representatives of flavones (luteolin-7-glucoside and apigenin-7-glucoside) and two members of flavanones (naringetin and naringetin-7-glucoside) were detected in our skin samples. They have previously been reported in grapes;[25] however, to our knowledge, flavones and flavanones have never been studied with the scope of showing changes during berry development and studying biosynthetic behavior resulting from canopy microclimate manipulation. The most abundantly present naringetin-7-glucoside (on average, 375 μg/g skins at highest point for BS) showed typical early peaking behavior (Figure 4C), whereas luteolin-7-glucoside (Figure 4D) showed similar behavior, but was present in lower concentrations (up to

10 μg/g skins at highest point). Together they are mainly responsible for the trends observed in total amounts (Figure 2E,F). On the other hand, naringetin and apigenin-7-glucoside accumulated in very low concentrations (below 1.0 and 0.25 μg/g skins on average, respectively) and consequently did not show particularly clear (trustable) trends during the observation period (Supporting Information Figure S1). For all four compounds we detected the lowest concentration trends at the beginning of observation (June 28) for the control grapes, but the differences later disappeared, reaching similar values for all treatments at harvest (on average, between 0.3 and 0.4 μg/g skins for naringetin; between 0.6 and 0.9 μg/g skins for luteolin-7-glucoside; between 0.2 and 0.6 μg/g skins for naringetin-7-glucoside; and below the detection limit for apigenin-7-glucoside). As flavones share common precursors with anthocyanins, their levels are generally negatively correlated, which basically means that a reduction in flavones will probably cause "an increase in anthocyanins due to the precursor flowing in only one direction".[39] Although flavones and flavanones are both present in relatively small concentrations, this is probably also something to be considered in future detailed canopy microclimate manipulation research.

Dihydrochalcones. Chalcones are of great significance biosynthetically as they are the immediate precursors of all other classes of flavonoids. However, very little is known about the biosynthesis of dihydrochalcones from chalcones.[41]

Phlorizin, as the only detected representative of dihydrochalcones, is a natural product and dietary constituent found in several fruits,[42] mainly in apples but also in grapes.[25]

The phlorizin concentration was highest (between ≈20 and 60 μg/g skins on average) at the first two sampling points during the observations (Figure 4E). It decreased steadily thereafter, being hardly present at harvest time (<0.2 μg/g skins). Indeed, untreated grapes early in the season showed a lower accumulation of phlorizin than those subjected to leaf removal treatments, but later the difference was no longer significant.

Nonflavonoids. *Stilbenes.* Stilbene and flavonoid syntheses have a common origin, as both derive from the general phenylpropanoid metabolism, although stilbenes are synthesized by stilbene synthase from coumaroyl-coenzyme A (CoA) and three molecules of malonyl-CoA via cleavage of four carbon dioxide molecules.[43] In grapes, the synthesis of stilbenes takes place in berry skins,[44] and it is known that they play an important role in plant and environment interactions. Resveratrol, as the most studied stilbene-type compound, and some of its derivatives, such as viniferins, pterostilbene, and piceid, have already been reported to be involved in plant defense mechanisms against abiotic stress, such as UV light, and biotic stress.[43,45] The level of stilbenes produced has also been found to be cultivar specific,[14] and in their study ref 46 defined 'Pinot noir' as a high producer of resveratrol, reaching the highest content among 78 observed varieties. The total amount of stilbenes in our samples increased significantly and reached a peak during the berry coloration (veraison) period (589, 769, 958, and 1030 μg/g skins on average in BS, PF, VE, and UN, respectively), but later decreased on approaching harvest, reaching 257, 170, 171, and 402 μg/g skins on average in BS, PF, VE, and UN, respectively, at harvest (Figure 2G). The increase and peak within the last stages of development (from veraison until harvest) are consistent with the findings of ref 47, although their work was done on 'Corvina'. Moreover, even if they did not focus their observations on the stages before veraison, it is evident that the detected amounts of observed stilbenes were very low at veraison, as we found in our study. On the other hand, ref 44 also reported the presence of resveratrol earlier in the season (before veraison), but in their research they artificially applied UV radiation to detached 'Pinot noir' grapes. Their findings can, however, explain some low concentration trends of several individual stilbenes (hardly seen, if only the totals are observed) around the time of early leaf removal (PF, BS), as the treatments opened up the developing berries more directly to the sun.

Then again, the biggest increase in total stilbenes in our case was undoubtedly observed in late August and early September, with the highest values being shown for untreated grapes (even though these grapes were the least exposed to UV light) and VE (where the clusters were highly exposed to light, because leaf removal in this case had just recently been performed). Some authors[14] already discussed a possible role of stilbene accumulation in responding to the changes in microclimate. In 2010 the Vipava Valley was basically warm and sunny in early summer (June and July), but cold later with extensive rainfall in August and September (Supporting Information Table S1). In the present experiment the highest relative humidity within cluster area was detected in the case of UN (Supporting Information Figure S3) leading to the increased probability of *Botrytis cinerea* infection.[48] Luczka[49] already pointed out that UV light exposure induces similar amounts of resveratrol as mold *B. cinerea*; thus, in our experiment the occurrence of mold (due to wet conditions) could account for the higher content of stilbenes in UN grapes, even more than UV exposure did.

Apart from resveratrol (*trans* isomer between 120 and 143 μg/g skins on average at the highest point), many other stilbene representatives were also detected in relatively large amounts in our 'Pinot noir' samples, such as *trans-* and *cis*-piceid with ranges from 157 to 244 μg/g skins and from 28–154 μg/g skins on average, respectively, at the highest points, as well as *trans*-ω-viniferin with ranges from 142 to 287 μg/g skins on average at the highest point (Figure 4F). Furthermore, resveratrol can be transformed by *B. cinerea* into resveratrol (*E*)-dehydrodimer, pallidol, leachinol F, and restrytisols A–C[50] which in our case can also explain the relatively high pallidol occurrence, especially in the case of untreated grapes (117 μg/g skins on average). Finally, regardless of the abundance of other individually detected representatives (Supporting Information Figure S1), all of them showed typical late peaking behavior (Table 1).

Hydroxycinnamates. Hydroxycinnamic acids (HCAs) are precursors for the synthesis of many other molecules, such as flavonoids and lignin,[51] and are known to be located in the vacuoles of the skin and pulp cells. HCAs have important functions in maturation processes and in plant defense and can also improve fruit flavor quality.[52] The principal hydroxycinnamic acids occurring in *V. vinifera* grapes are caftaric, coutaric, and fertaric acids in *trans* form, although small quantities of the *cis* isomers can also be detected.[53] Furthermore, they are reported to be involved in the browning reactions of must and wine and carry out antimicrobial and antioxidant activities.[54] As can be seen for total HCAs (Figure 2H) as well as for individual HCAs (Figure 4G and Supporting Information Figure S1), neither total nor any of the individual HCAs showed significantly higher values for any of the treatments at harvest time, although the trends were higher for early leaf removal at the beginning of the observations, which is similar to that reported previously.[29] In general, a decreasing trend was shown

for HCAs. It appears that hydroxycinnamic acids in the skin cannot be easily manipulated through different timing of leaf removal treatments.

Benzoates. Hydroxybenzoic acids are synthesized in numerous plants from the corresponding hydroxycinnamic acids. The four most common plant hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids, whereas the corresponding hydroxybenzoic acids are *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids.[55] In our experiment, seven compounds classified as benzoates were detected. By observing the total amount (Figure 2I) we can see that they are more abundant earlier in the season, with a peak in the middle of July, but later decreasing until they became stable with a very low concentration during the last 3 weeks of maturation. Leaf removal did not cause any stable or clear accumulation trends; therefore, from the totals alone we could not reach any conclusions in favor of any treatment. Individual representatives, vanillic, *p*-hydroxybenzoic, syringic, and gallic acid, have previously been reported in grapes with comparable values at harvest.[25,55] However, they were analyzed only in ripe grapes, whereas our results also show their behavior during grape berry development. Whereas vanillic acid generally increased (reaching 1.6 (UN)−2.2 (BS) μg/g skins on average at harvest), *p*-hydroxybenzoic acid, on the other hand, showed early peaking behavior, but decreased later, being hardly detectable at harvest time. Neither compound showed any important differences between leaf removal treatments. On the contrary, syringic acid (Figure 4H) (peaking late in the season with 1.6−3.3 μg/g skins on average at the highest point) was the only benzoate showing significant differences at harvest as compared with UN, essentially in favor of early leaf removal (BS and PF) (both around 2 μg/g skins on average). Vanillin was already detected in traces[56] in 'Pinot noir' grapes during berry development. Also, in our grape skins vanillin (<0.5 μg/g skins on average and with no specific behavior) as well as syringaldehyde were found, generally early in the season, with later up to 2 μg/g skins on average, decreasing later until harvest to <0.5 μg/g skins on average. Finally, an ester of gallic acid, methyl gallate, was also detected in low amounts in the second half of our observation period.

When summarizing all of the results, we can see that changes in microclimate conditions in the cluster area affected many of the compounds observed. However, some were affected only early in the season, whereas many others retained significant alterations until harvest (Table 2). Canopy microclimate manipulation had a big effect in the case of flavonols, particularly early peaking flavonols. Higher synthesis of anthocyanins in the case of veraison leaf removal was detected, whereas early leaf removals showed a reduction in peonidin glucoside but an increase in all other individual compounds. Despite the fact that flavonols and anthocyanins are on the same biosynthetic pathway, it seems possible to positively affect both classes by taking into account their (different) peaking behaviors. Furthermore, stilbenes showed late peaking behavior and were generally highest in the case of no leaf removal (closed canopy of control vines), most probably due to better conditions for *B. cinerea* development. Within benzoates, syringic acid was the only representative still showing significant changes in favor of early leaf removal at harvest. Many representatives of other classes of polyphenols, such as flavones, flavan-3-ols, flavanones, and hydroxycinnamic acids, essentially reduced their concentration from the time of veraison, with different trends between treatments often shown only in the first stages of maturation. One question was raised: Is the higher concentration obtained with early leaf removal at the beginning of maturation nevertheless still important within the complex pattern of physiological changes of the berry and finally for overall grape quality at harvest? In the future further research should be carried out, aiming to understand how early peaking could affect late peaking compounds in different canopy microclimate scenarios and climatic conditions.

Table 2. Detected Phenolic Compounds (Micrograms per Gram Skins) Showing Significant Alterations at Harvest Point in Comparison to the Control, as a Result of Canopy Microclimate Manipulation through Leaf Removal at Different Phenological Stages (PF, Preflowering; BS, at Berry Set; VE, at Veraison; UN, Control with No Leaf Removal)

at harvest	UN	PF	BS	VE	*p* value	F^a
syringic acid	a[b]	b	b	b	0.0276	*
cis-resveratrol	a	b	b	b	0.0400	*
cis-ε-viniferin	a	b	b	b	0.0345	*
trans-ω-viniferin	a	b	b	b	0.0041	**
pallidol	a	b	b	b	0.0024	**
ampelopsin D + quadrangularin A	a	b	b	b	0.0055	**
isohopeaphenol	a	b	b	b	0.0343	*
ampelopsin H + vaticanol C-like isomer	a	b	b	b	0.0095	**
naringenin	a	b	b	b	0.0262	*
kaempferol-3-rutinoside	a	b	b	c	0.0072	**
quercetin-Glc-Ara	a	a	a	b	0.0459	*
rutin	a	b	c	d	0.0024	**
quercetin-3-glucuronide	a	b	b	c	0.0071	**
kaempferol	a	b	b	b	0.0194	*
myricetin	a	ab	b	b	0.0306	*
kaempferol-3-glucoside	a	b	b	b	0.0170	*
myricetin-3-rhamnoside	a	ab	bc	c	0.0083	**
isorhamnetin-3-glucoside	a	ab	b	b	0.0136	*
syringetin-3-glucoside + syr-3-galactoside	a	ab	ab	b	0.0262	*
isorhamnetin-3-rutinoside	a	b	b	c	0.0170	*
taxifolin	a	ab	ab	b	0.0412	*
quercetin-3-rhamnoside	a	ab	b	b	0.0337	*
kaempferol-3-glucuronide	a	ab	ab	b	0.0369	*
quercetin	a	ab	b	b	0.0412	*
quercetin-3-glucoside	a	b	b	b	0.0092	**
quercetin-3-galatoside	a	b	b	b	0.0120	*
delphinidin-3-glucoside	a	b	c	d	0.0006	***
cyanidin-3-glucoside	a	ab	b	c	0.0197	*
petunidin-3-glucoside	a	b	c	d	0.0001	***
peonidin-3-glucoside	a	b	c	d	0.0019	**
malvidin-3-glucoside	a	b	c	d	0.0004	***

[a]Data were processed through ANOVA and means separated using Tukey's Honest Significant Difference (HSD) test ($P < 0.05$) (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). [b]Means followed by the same letter are not significantly different.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S1: Basic Seasonal (Monthly) Characteristics of the Observed Vintage (2010).[57] Table S2: Basic Viticultural Parameters As Affected by Canopy Microclimate Manipulation through Leaf Removal at Different Phenological Stages of

Grape Berry Development (PF, Preflowering Leaf Removal; BS, Berry-Set Leaf Removal; VE, Veraison Leaf Removal; UN, Control with No Leaf Removal) (Relative Values). Figure S1: Accumulation dynamics (μg/g skins) of 64 (data not shown) individual representatives from different phenolic groups as affected by canopy microclimate manipulation through leaf removal at different phenological stages of grape berry development (PF, preflowering leaf removal; BS, berry-set leaf removal; VE, veraison leaf removal; UN, control with no leaf removal). Figure S2: Hourly temperatures (°C) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Top panel shows the comparison between control (UN) and preflowering leaf removal (PF, green line). Middle panel shows the comparison between control (UN) and berry set leaf removal (BS, green line). Bottom panel shows the comparison between control (UN) and veraison leaf removal (VE, green line). Figure S3: Hourly relative humidity (%) within cluster area as affected by different leaf removal timing as compared with control (UN, red line). Top panel shows the comparison between control (UN) and preflowering leaf removal (PF, green line). Middle panel shows the comparison between control (UN) and berry set leaf removal (BS, green line). Bottom panel shows the comparison between control (UN) and veraison leaf removal (VE, green line). This material is available free of charge via the Internet at http://pubs.acs.org.

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