NESTED EFFECTS OF BERRY HALF, BERRY AND BUNCH MICROCLIMATE ON BIOCHEMICAL COMPOSITION IN GRAPE

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Abstract

Aim : Polyphenol composition, an important component of grape quality, is strongly influenced by fruit microclimate. However, information relies exclusively on whole berry data and the underlying response functions to microenvironment variables remain essentially unknown. The aim of this study was therefore to analyze the biochemical composition of grapes at both bunch and berry scales, in relation with microclimate.

Methods and results : Whole berries and berry halves were sampled in mature defoliated bunches from two neighboring Bordeaux vineyards with contrasting row orientations (*Vitis vinifera* cv. Merlot). Flavonoid and amino-acid contents were analyzed by HPLC methods. The main sources of variation were bunch azimuth, berry exposure and, only in South-exposed bunches, berry side. Models were used to estimate radiation at the berry surface and temperature. Intense effects of bunch side and berry side on total flavonol and anthocyanin concentrations were observed. These results were all consistent at both bunch and berry scales. However, the most intense effects were observed at berry scale and mitigated by scaling up from berry to bunch.

Conclusion : Total flavonol concentrations in the berry skin exhibited a clear positive linear relationship with solar radiation. The large heterogeneity of composition at berry scale is consistent with the better known heterogeneity at bunch scale.

Significance and impact of the study : Models and original response functions to microclimate could help optimize vineyard management and grape ripening.

Key words : *Vitis vinifera*, vineyard, berry temperature, solar radiation, anthocyanins, flavonols

manuscript received 17th July 2015 - revised manuscript received 18th January 2016

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OENO One, 2016, **50**, 3, 145-159

INTRODUCTION

Fruit ripening in grapevine is highly sensitive to climatic conditions, which explains the so-called vintage effect (*i.e.*, the year-to-year variations in wine quality). This climate sensitivity may partly be linked to the whole plant response to climate variations (Smart, 1985; Roby *et al.*, 2004; Dai *et al.*, 2011). However, the maturation of grape berries is also strongly influenced by the local microclimate of the fruits (Smart, 1985; Cohen *et al.*, 2008; Dai *et al.*, 2011). Many observations have actually demonstrated that some of the most important components of berry quality exhibit strong variations even if the climate, training system and biological material are all similar (Price *et al.*, 1995; Haselgrove *et al.*, 2000; Bergqvist *et al.*, 2001; Spayd *et al.*, 2002; Downey *et al.*, 2006; Cohen *et al.*, 2008; Tarara *et al.*, 2008; He *et al.*, 2010; Dai *et al.*, 2011). In one case, significant differences in skin composition were observed between the opposite sides of the bunches (Price *et al.*, 1995).

The thermal behavior of fruits is a crucial driving variable for the biochemical and physiological processes involved in the ripening, and it is a key factor for qualitative profiling of primary and secondary metabolites (Jackson and Lombard, 1993; Pereira *et al.*, 2006; Sadras and Moran, 2012; Sweetman *et al.*, 2014). In addition, the bunch microclimate may be deeply modified by the vine grower through the choice of training system and management techniques, including soil surface management (Smart, 1985; Jackson and Lombard, 1993; Schultz, 1995). Since the canopy structure of the vineyard is usually relatively open to exchanges with the atmosphere, the pattern of radiation interception plays a key role in explaining the changes of bunch- and berry-scale microclimate under the influence of training system or management techniques, especially when it comes to fruit's temperature (Spayd *et al.*, 2002; Pieri and Fermaud, 2005; Saudreau *et al.*, 2009). These interactions result in specific and identifiable seasonal and diurnal dynamics of intercepted radiation and temperature of bunches and berries. The position and azimuth of bunches with respect to the row canopy, as well as the position of the berry within the bunch, are essential factors explaining these dynamics (Smart and Sinclair, 1976; Spayd *et al.*, 2002; Pieri and Fermaud, 2005). For instance, in the simple situation of leaf removal around the bunches, (i) South-exposed bunches reach a higher temperature than North-exposed bunches, (ii) external and exposed berries reach a higher temperature than internal or external shaded berries, (iii) the order of magnitude of maximal differences is 10 °C (Smart and Sinclair, 1976; Spayd *et al.*, 2002; Pieri and Fermaud, 2005), and (iv) West-exposed bunches, and particularly external and exposed berries, reach a higher temperature than East-exposed bunches and berries (Smart and Sinclair, 1976; Spayd *et al.*, 2002; Pieri and Fermaud, 2005). All these variations in intercepted solar radiation or radiative balance and fruit temperature can be represented by microclimate models, accurately taking into account the climate variations and the effects of parameters describing the training system, the relative position of the bunches and berries, and the intrinsic properties of the bunches and berries such as albedo and compactness (Smart and Sinclair, 1976; Saudreau *et al.*, 2007; Cola *et al.*, 2009; Pieri, 2010). These models are therefore useful and flexible tools to explore the variability of bunch and berry microclimate and explain the sensitivity of berry composition to factors such as bunch azimuth and berry position.

The physiological origins of the relationships between grape temperature or solar radiation and ripening metabolism are still far from being completely understood. Nevertheless, many studies have shown that berry temperature and solar radiation are key factors, even though the underlying relationships are still poorly quantified and the respective effects of solar radiation and temperature seldom distinguished (Spayd *et al.*, 2002). Several studies have highlighted and discussed the link between temperature or solar radiation and (i) sugar and organic acid variations (Smart, 1985; Ollat *et al.*, 2002; Rienth *et al.*, 2014; Sweetman *et al.*, 2014), (ii) amino-acids (Kliewer, 1968; Rienth *et al.*, 2014), (iii) anthocyanin accumulation (Haselgrove *et al.*, 2000; Bergqvist *et al.*, 2001; Spayd *et al.*, 2002; Mori *et al.*, 2005; Cortell and Kennedy, 2006; Downey *et al.*, 2006; Mori *et al.*, 2007; Cohen *et al.*, 2008; Tarara *et al.*, 2008; Matus *et al.*, 2009; Azuma *et al.*, 2012; Sadras and Moran, 2012; Rienth *et al.*, 2014), (iv) flavonol accumulation (Price *et al.*, 1995; Spayd *et al.*, 2002; Downey *et al.*, 2004; Cortell and Kennedy, 2006; Downey *et al.*, 2006; Cohen *et al.*, 2008; Matus *et al.*, 2009; Azuma *et al.*, 2012; Koyama *et al.*, 2012), (v) tannin synthesis (Downey *et al.*, 2004; Cortell and Kennedy, 2006; Cohen *et al.*, 2008; Koyama *et al.*, 2012), (vi) stilbene synthesis (Bavaresco *et al.*, 2008), (vii) terpene synthesis (Macaulay and Morris, 1993; Rienth *et al.*, 2014), and (viii) carotenoid and methoxypyrazine synthesis (Hashizume and Samuta, 1999; Rienth *et al.*, 2014). However, some of the earlier studies considered air temperature rather than berry temperature and some considered stationary controlled conditions; in both

cases, valuable information about the eco-physiological determinism of berry metabolism could be blurred by these shortcomings in the experimental design. In some studies, microclimate variables were neither measured nor estimated, leading only to binary or qualitative conclusions (Cortell and Kennedy, 2006 ; Matus *et al.*, 2009). Moreover, only a few studies truly distinguished the respective effects of these variables, thus overcoming the confusing effect of natural coupling of solar radiation and temperature in outdoor-grown berries (Spayd *et al.*, 2002 ; Cohen *et al.*, 2008 ; Tarara *et al.*, 2008). Some results indicated a complex linkage of primary metabolism in the whole plant and in the fruits, with some influence of microclimate, through the water balance of the berries and water fluxes across berry pedicel and berry skin (Rebucci *et al.*, 1997 ; Martinez-Luscher *et al.*, 2014). However, the direct influence of microclimate on berry secondary metabolism was better demonstrated, particularly when it comes to the phenolic pathway leading to flavonols, anthocyanins, and tannins (Cohen *et al.*, 2008 ; Tarara *et al.*, 2008 ; He *et al.*, 2010 ; Dai *et al.*, 2011 ; Koyama *et al.*, 2012 ; Sadras and Moran, 2012 ; Rienth *et al.*, 2014), with evidence suggesting that, under usual production conditions, the microclimate sensitivity of the maturation process was nearly independent from whole plant function.

In summary, all present microclimate-related knowledge about berry ripening is based on bunch scale/entire berry data analysis and the underlying response functions to microenvironment variables remain essentially unknown. On the other hand, microclimate contrasts also develop at berry scale, for instance between the sunlit and shaded sides of each external berry, and could lead to composition differences within the berry. The aim of this study was therefore to analyze the biochemical composition of field-grown grape bunches and berries and to assess the microclimate influence at both bunch and berry scales. Moreover, new quantified response functions of berry composition to microclimate were explored by linking experimental data to microclimate model outputs.

MATERIALS AND METHODS

1. Experimental sites

The experiment was carried out in two commercial vineyards near Bordeaux, France (Château Figeac and Château Cheval Blanc, in the Saint-Emilion area), on 15-year-old Merlot vines planted on a sandy-gravelly soil at a density of 4525 vines ha^{-1}. The two plots had a surface area of about 1 ha. Vine spacing was 1.70 x 1.10 m with rows orientated East-West (E-W) and North-South (N-S) in the first and second vineyard, respectively. Vines were trained as a Vertical Shoot Positioning system, cane pruned at 8 buds $plant^{-1}$, with total foliage height and width of 1.40 m and 0.50 m, respectively.

The average one-sided leaf area index (LAI) was estimated before veraison, on August 2, 2004, on both sites : LAI was 1.54 and 1.66 (standard deviation 0.32 and 0.41, respectively) for N-S and E-W rows, respectively. In each site, 12 vines where no berry sampling was carried out were randomly selected and all their primary shoots and lateral leaves were counted. The main leaf vein lengths were measured on a total of 60 leaves randomly sampled over the 12 vines but equally distributed along the shoots (basal, middle and apical). A correlation of one-sided area of leaves with the main vein length, calibrated beforehand for the Merlot variety, was used to calculate total leaf area.

Five adjacent vines of apparent homogeneous vigor were chosen in each site, far enough (>30 m) from the field borders. On each of these 5 plants, one bunch was selected for both azimuthal directions ; therefore, two bunches of opposite azimuth were chosen from the same vine, but never from the same shoot. The bunch selection was operated on the basis of homogeneous morphology and dimensions, thus avoiding smaller and larger bunches. Moreover, in order to get a true azimuthal contrast, bunches were also selected according to their relative position, near the bottom outside of the row, thus avoiding central, near row axis positions. Selected bunches were all ranked #1 or #2 on the shoot. A total amount of 20 bunches (4 azimuth x 5 replicate bunches) was therefore available for berry composition analysis. Previously chosen, labeled and defoliated bunches were harvested manually on September 30, at full maturity. Harvest time was determined by the actual harvest of the two vineyards by the viticulturists in the following days.

2. Leaf removal treatment

Since the study focused on the ripening period, the leaf removal treatment was implemented on the chosen bunches on August 19, soon after 100 % veraison (which was estimated visually as % of fully colored berries in all bunches of 10 vines around the two plots ; 50 % veraison was estimated to have occurred on August 14, with very little difference between the two sites). Leaf removal was applied around bunches considered individually, either on the East- or West- or North- or South-facing sides and on

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one side only of each bunch. Leaf removal was designed to get maximal solar irradiation in one direction but minimize disturbance to the whole plant function and leaf-to-fruit ratio. All the leaves in front of a bunch and situated within a radius of about 30 cm were removed manually. In that way, East-exposed bunches were illuminated directly by the solar radiation nearly all morning long and the West-exposed bunches nearly all afternoon long.

3. Experimental design

Since the row orientation differed in the two spots (N-S and E-W, respectively), North-, South-, East- and West-exposed bunches were compared (labeled N, S, E and W, respectively). Within each bunch, external exposed (*X*) and external opposite (*O*, usually shaded) berries were sampled, as a mean to evaluate bunch side effects. These berries were chosen at mid-height within the bunch and near an orthogonal to the row-axis horizontal direction. Therefore, selected berries all came from the central middle area of the outside- (*X*) or inside- (*O*) looking bunch side. In each bunch, 5 *O* replicate berries and 5 *X* replicate berries were sampled and pooled together to make up representative bunch side samples. This experimental design led to 40 different bunch side samples that were submitted to biochemical composition analysis: 4 bunch azimuth x 5 replicate bunches x 2 bunch sides.

In South-exposed bunches exclusively, a more detailed analysis was done by sampling 5 additional *X* and *O* berries and cutting them into two halves of near-hemispherical shape, one external (*ext*) outside-looking and one internal (*int*) inside-looking. The *int* berry halves included the pedicel attachment point and were nearly symmetrical around this point, but the pedicel itself was removed before analysis. Therefore, 100 half berry samples were submitted to biochemical composition analysis: 1 bunch azimuth x 2 bunch sides x 2 berry sides x 5 replicate bunches x 5 replicate berries. Skin and pulp were separated afterward, prior to the HPLC analysis.

4. Radiation and berry temperature models

Since the experiment was conducted in a natural, non-controlled vineyard environment, and since there was no easy way to accurately track the radiation and temperature of the sampled bunches and berries without disturbing berry integrity or berry microenvironment, models were used to assess solar radiation and temperature. The models chosen were based on classical assumptions about the simplified row geometry and the canopy architecture, essentially described as a porous rectangular shaped hedgerow (Smart and Sinclair, 1976; Cola *et al.*, 2009; Pieri, 2010). All parameters related to row azimuth, canopy structure, bunch position, and soil surface albedo were adapted to the specific situation of both sites. For temperature, only a simplified version of the model of Cola *et al.* (2009) was used – air convection terms in the model were averaged since no *in situ* wind velocity measurement was available; however, the main traits of berry temperature response to solar radiation illumination were considered realistic enough, especially since the investigated system was also simplified, due to the defoliation around bunches. In order to estimate the integrated effect of the potential driving variables, a large part of the maturation period was considered, from the end of veraison to maturity. Therefore, simulations of intercepted radiation and temperature were applied to the 30 days ending with the day before sampling date, and averaged. Air temperature and incoming solar radiation data used as input variables were taken from a nearby meteorological station (at Château Cheval Blanc, < 1 km away from both fields).

5. Biochemical analysis

Sample preparation: Berries were weighed and the skins were separated from the pulp, frozen at -80 °C and freeze-dried. Fresh and dried skins, pulps and seeds were weighted. Pulps were crushed to determine mineral, sugar and amino-acid content. The dried skins were powdered in a ball grinder MM200 (Retsch, Haan, Germany) and extracted in 50 % methanol in water (v/v). After sampling an aliquot for the analysis of amino-acid content, the extract was adjusted to 0.1 % HCl (v/v). Acidified extracts were filtered through a 0.45-µm polypropylene syringe filter (Pall Corporation, Ann Arbor, USA) for HPLC analysis of anthocyanins and flavonols.

Maturation parameters: Total soluble solids (°Brix) of berries were determined using a hand-held refractometer with temperature compensation (model RF233, Merck Eurolab, Fontenay-sous-Bois, France). Sugar, malic acid and tartaric acid were determined by an automated colorimetric method using the autoanalyzer TRAACS 800 (Bran & Luebbe, Plaisir, France).

Amino-acid content: After derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (Cohen and Michaud, 1993), amino-acids in pulp were analyzed using a Waters 2695 HPLC system equipped with Waters 474 fluorescence detector (Waters, Milford, MA, USA). Separation was

performed on a Nova-Pak C18 AccQ-Tag column (Waters, Milford, MA, USA) at 37 °C with elution at 1 mL min^{-1} with a 67-min linear gradient (eluent A, sodium acetate buffer, 140 mM at pH 5.7; eluent B, acetonitrile 60 % in water (v/v)). Chromatograms corresponding to excitation at 250 nm and emission at 395 nm were recorded. Individual amino-acids were quantified by their peak area with Chromeleon software, version 6.60 (Dionex Corporation, Sunnyvale, CA, USA) using external standards. Chemical standards were purchased from Sigma (St Louis, MO, USA). Ultrapurified 18 MW water (ELGA, Bucks, UK) and analytical grade reagents were used. Eluents were filtered through a 0.45-µm polypropylene GHP membrane (Pall Corporation, Ann Arbor, USA). Twenty amino-acids were identified and quantified as described by Pereira *et al.* (2006). The results were expressed as concentrations of amine N in mmol L^{-1} juice.

Anthocyanin and flavonol analysis: Individual anthocyanin and flavonol analysis in skin extracts was performed by using a Summit HPLC System consisting of a P680 pump, ASI-100T™ autosampler and UVD 340U UV-Vis detector operating at 520 nm and 360 nm (Dionex Corporation, Sunnyvale, CA, USA). After injecting 20 µL, separation was achieved on a reverse-phase Ultrasphere ODS column 25 cm x 4.6 mm, 5-µm particle size, with an Ultrasphere ODS guard column 4.5 cm x 4.6 mm obtained from Beckman Instruments Inc. (Fullerton, CA, USA), at ambient temperature. All reagents were of analytical grade. Water was purified (18 MW) with an ELGA (Bucks, UK) UHQ water purification system. Acetonitrile (HPLC grade) was obtained from Baker (Mallinckrodt Baker, Noisy-Le-Sec, France) and formic acid (99 %) from Merck (Merck Eurolab, Fontenay-sous-Bois, France). Binary gradient elution with a 0.6 mL min^{-1} flow rate started with 80 % eluent A (10 % formic acid in water (v/v)) and ended with 85 % eluent B (10 % formic acid and 30 % acetonitrile in water (v/v)) in 70 min. The column temperature was 25 °C. External standards were used for quantification on the basis of peak area. Chromeleon software, version 6.60 (Dionex Corporation, Sunnyvale, CA, USA) was used to calculate peak area. Identification and peak assignment of phenolic compounds were based on comparison of their retention times and UV-Vis spectrometric data with that of pure standards. Malvidin-3-glucoside (Oenin) was used as common standard for all the quantified anthocyanins (at 520 nm) and quercetin-3-glucoside was used for all the quantified flavonols (at 360 nm). All standards were purchased from Extrasynthese (Genay, France). Nineteen different anthocyanin forms and seven different flavonol forms were identified and quantified. Since only strictly local effects were investigated, without any need to estimate global production by berry, by bunch or by plant, all berry composition data were expressed as concentrations, either by volume in the flesh, or by mass ratio in dry weight of skin.

Data processing: The variability in all the amino-acid, flavonol and anthocyanin data was analyzed by means of classical analysis of variance (R software, "aov()" instruction). This analysis was performed on both experimental set-ups (pooled entire berries vs. half berries), on either total content by metabolite class (total amino-acids in pulp and total amino-acids, flavonols and anthocyanins in skin) or all available data representing individual components. The experimental design was balanced in all cases; the only missing data was one berry half sample for amino-acids in pulp (namely replicate #4 of S, X, ext).

RESULTS

1. Modelling of berry radiation and temperature

The effects of bunch azimuth and berry position within the bunch on berry radiation and temperature could be easily modelled since the canopy geometry was simple: the vineyard rows were trained as a well cared vertical plane hedgerows and the chosen bunches were located near the external outline of row volume. Additionally, the experimental set-up was simplified further by leaf removal, removing potential local effects of radiation interception by neighboring leaves. Therefore, solar radiation intercepted by the berries could easily be estimated for the different microclimate categories tested (Fig. 1), in agreement with already observed dynamics (Schultz, 1995; Tarara *et al.*, 2008).

Berry temperature primarily responds to the dynamics of illumination by direct solar radiation (Fig. 1C). Thus, direct solar radiation caused a marked increase of berry temperature above air temperature (measured as dT = Tberry – Tbunch zone air) in exposed berries (*X*); up to 10 °C for clear-sky days and 5 °C on average over the period. To a lesser extent, this temperature increase also appeared for opposite berries because some solar radiation was transmitted through gaps in the foliage and/or by reflection on the leaves.

2. Berry composition analysis – sugars and organic acids

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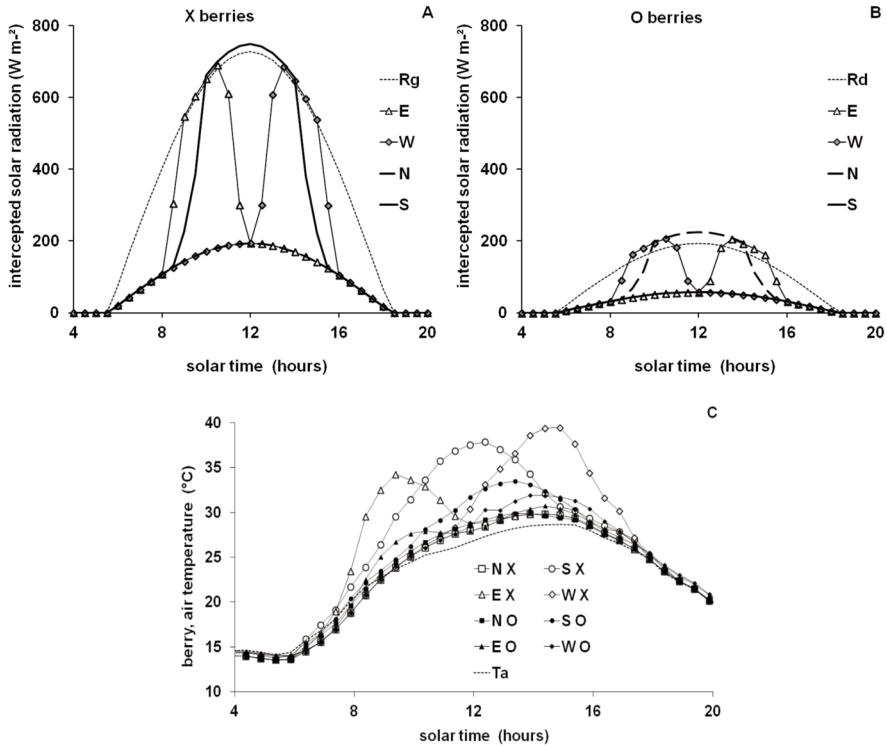

Figure 1 - Daily time courses of incoming global solar radiation (Rg), diffuse solar radiation (Rd) and air temperature (Ta), modelled solar radiation intercepted by the berries (A, B) and modelled berry temperature (C) along an average clear-sky day. Variations with the microclimate category: bunch azimuth (N, S, E, W) and outer berry exposure (X - exposed (A, C) vs. O - opposite (B, C)).

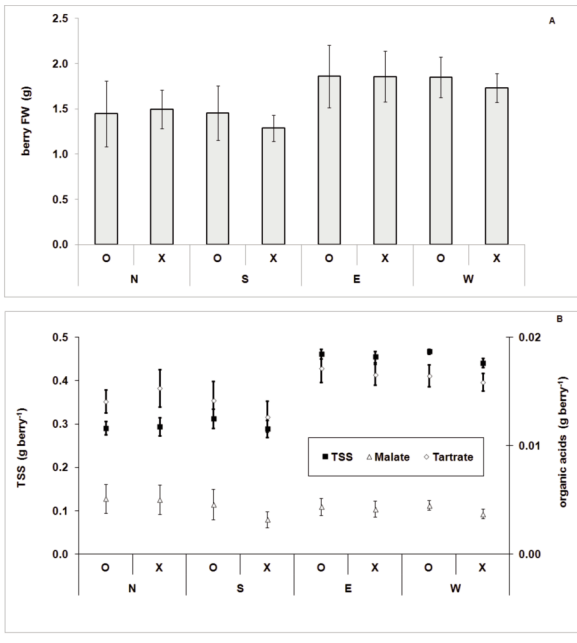

Figure 2 - (A) Berry fresh weight and (B) total soluble sugars (TSS) and organic acid (malate, tartrate) contents of berries and variations with bunch azimuth (N, S, E, W) and outer berry position (exposed – X and opposite – O). All data: means ± 1 sd bars.

Berries were harvested at technological maturity, as confirmed by weight and sugar and main organic acid concentrations (Fig. 2). The sugar/acids ratio was around 30. Little variation was observed with respect to the microclimate experienced by the bunches, whereas some differences linked to berry growth appeared between the two vineyards. This result confirmed the *a priori* assumption that the primary metabolism was very similar in all berry categories investigated, and therefore that the differences observed in the berry composition of secondary metabolites could be related exclusively to microclimatic factors.

3. Variability in amino-acid and polyphenol results – ranking of factors

Analysis of variance: The experimental design was set up in order to test the nested effects linked to the microclimate experienced by bunches and berries. Therefore, a variance analysis was a straightforward way to confirm these effects and eventually distinguish and quantify them separately. However, part of the experimental design involved entire berries from two different sites. In this case, the effect of bunch azimuth could be confused at least partly by the site effect since obviously the row direction and

bunch azimuth differed in the two plots (N, S vs. E, W), leading us to wonder about uncontrolled side effects like different water or N uptake by the plants. Therefore, a block effect was introduced and tested, each block representing one of the two plots.

Here, only the results of analysis of variance applied to totals by family are shown (Tables 1 and 2) and discussed.

In pooled entire berries, a strong bunch side effect was found to influence flavonol content, with neither bunch azimuth nor block effect. Only weak non significant bunch azimuth and bunch side effects were observed in anthocyanins. In amino-acids in the pulp or in the skin, no bunch side effect was observed; a bunch azimuth effect was significant in the flesh but a much stronger block effect was dominant. These results suggested that both sites experienced different conditions leading to distinct amino-acid contents in the berries; since the soils were structurally similar and since the training system and the climate were the same, this effect was most likely due to nitrogen feeding. However, no significant block effect was observed for secondary metabolites (flavonols and anthocyanins), indicating that the difference in amino-acid content did not affect this branch of secondary metabolism. Thus, the observed differences in berry anthocyanin and flavonol content were clearly due to a microclimate effect. For instance, the clear-cut influence of bunch side on flavonols could only be linked to radiation or temperature, with no significant influence of N uptake or metabolism.

In berry halves from South-exposed bunches, extremely strong effects of bunch side and berry side were observed in both anthocyanins and flavonols, with significant interactions for the latter (Table 2). The variability of amino-acids only exhibited a berry side effect that was very significant in the pulp.

Principal components analysis - evidence of a microclimate effect: The same data set was analyzed by principal components analysis (PCA) (R software, "amap::acp()" instruction) in order to check correlations and internal structure of the data set. An identical analysis was performed separately on entire berries from North-, South-, East- and West-exposed bunches and on half berries from South-exposed bunches, either on total amino-acid content in flesh and skin, flavanols in skin and anthocyanins in skin (4 variables) or on data distinguishing each species (63 variables: 18 forms of anthocyanins, 7 forms of flavonols, 19 amino-acids (*2 for pulp and skin)). Only the simplest results from total contents by family are presented (Fig. 3) since the more complete data set exhibited nearly the same structure (the two first components explained 45 % of the variance with 63 variables, against 81 % with 4 variables).

The results confirmed that the flavonol and anthocyanin contents in the berries were correlated and that both were uncorrelated to amino-acids (Fig. 3). The data also indicated a strong microclimate effect since different categories of berries or half berries were separated by their coordinates on principal component axis, mostly component #1 (Fig. 3). For instance, external half berries (*ext*) isolated from exposed berries (*X*) were well distinguished from both internal half berries

Table 1 - Analysis of variance – pooled entire berries from North-, South-, East- and West-exposed bunches from two plots with block effect (N, S vs. E, W).

Component	Effect (interactions ":")	Df	F value	Pr (>F)
total anthocyanins				
	block	1	3.38	0.075
	bunch azimuth	2	2.05	0.145
	bunch side	1	3.21	0.083
	block:bunch side	1	1.88	0.180
	bunch azimuth:bunch side	2	0.14	0.873
	residuals	32		
total flavonols				
	block	1	2.55	0.120
	bunch azimuth	2	0.35	0.709
	bunch side	1	30.62	P<0.0001
	block:bunch side	1	2.10	0.157
	bunch azimuth:bunch side	2	1.76	0.188
	residuals	32		
total amino-acids in skin				
	block	1	16.64	P<0.0001
	bunch azimuth	2	1.33	0.279
	bunch side	1	0.34	0.566
	block:bunch side	1	0.36	0.550
	bunch azimuth:bunch side	2	1.80	0.181
	residuals	32		
total amino-acids in flesh				
	block	1	13.51	P<0.0001
	bunch azimuth	2	3.33	0.049
	bunch side	1	0.32	0.577
	block:bunch side	1	0.74	0.395
	bunch azimuth:bunch side	2	0.71	0.498
	residuals	32		

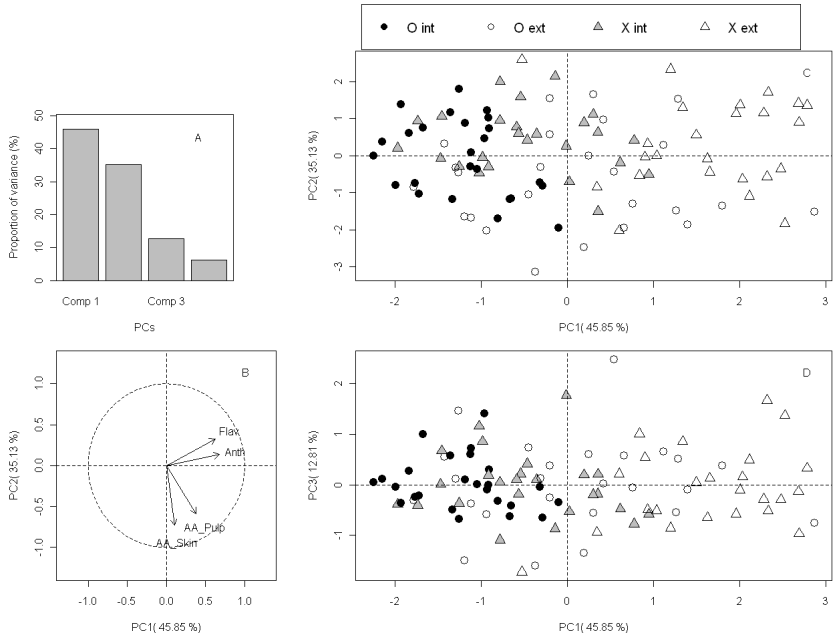

Figure 3 - Results of principal components analysis (PCA) applied to global composition of half berries from South-exposed bunches (4 variables).
Weights of principal components (A), variable loadings (B),
and sample scores in PC1-PC2 plane (C) and PC1-PC3 plane (D). Effects of bunch side
(exposed "X" vs. opposite "O") and berry side (internal "int" vs. external "ext").

(*int*) isolated from either exposed (*X*) or opposite (*O*) berries, whereas external half berries (*ext*) from opposite (*O*) berries were found at an intermediate position (Fig. 3). This without *a priori* data clustering was mainly driven by the first principal component axis, which was strongly linked to flavonol and anthocyanin composition and therefore to the flavonoid biosynthetic pathway of secondary metabolism.

4. Berry composition – amino-acids

The results were investigated by comparing samples exposed to different microclimates. In entire berries, no clear bunch azimuth or bunch side influence on amino-acid content was noticeable ; any apparent bunch azimuth difference was rather actually a block effect (N, S vs. E, W) explained by the site. In berry halves, however, amino-acid contents in external berry parts (*ext*) were significantly higher than in internal halves (*int*). The weak trend towards lower amino-acid content in *X* berries vs. O berries was not significant at 0.05 level. Similar results were observed for phenylalanine variations.

5. Berry composition – flavonols

In data sets from both entire berries and half berries, total flavonol contents in outside exposed berries (*X*) were significantly higher than in mostly shaded opposite berries (*O*) (Fig. 4). Neither bunch azimuth effect nor any site effect was noticeable. In berry halves, flavonol contents in external berry sides (*ext*) were significantly higher than in internal halves (*int*) (Fig. 4B) and the significant bunch side-berry side interaction (Table 2) was confirmed since the *ext-int* difference was much higher in *X* berries than in *O* berries (Fig. 4B).

6. Berry composition – anthocyanins

In the data set from entire berries, total anthocyanin content was usually higher in *X* berries than in *O* berries, with the exception of West-exposed bunches, leading to a weak non-significant effect of bunch side (Fig. 5A). The same effect was significant for half berries (Fig. 5B). As with flavonols, neither bunch azimuth nor any site effect was noticeable. In berry halves, anthocyanin contents were significantly higher in external (*ext*) than in internal (*int*) berry sides (Fig. 5B). Here, no significant bunch side-berry side interaction was visible.

7. Berry composition : amino-acids - flavonols - anthocyanins relationships

In the secondary metabolism pathway of phenolics that takes place in the berry skin, phenylalanine is the initial common substrate and flavonols and anthocyanins are the terminal products of diverging

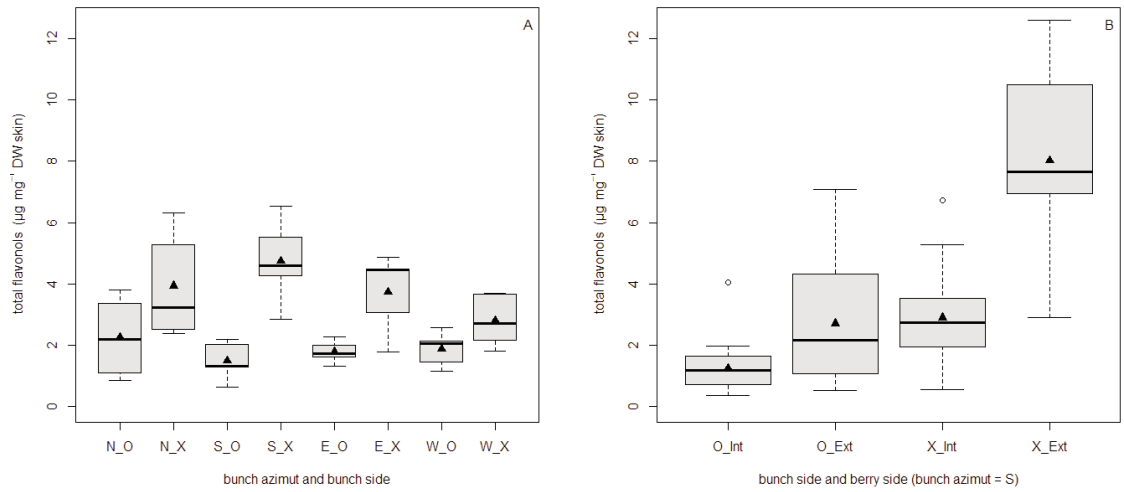

Figure 4 - Total flavonol content of berry skin and its variations with microclimate.
Pooled entire berries (A) and separate half berries from South-exposed bunches (B). n = 5 (A) and n = 25 (B), respectively, for each category represented. Effects of bunch azimuth (N, S, E, W), bunch side (exposed "X" vs. opposite "O") and berry side (internal "int" vs. external "ext"). Boxplots with common conventions [boxes limited by 1st and 3rd quartiles (Q1 and Q3); the upper (resp. lower) whisker located at the smaller (resp. larger) of the maximum (resp. minimum) value and Q3 + 1.5 IQR (resp. Q1-1.5 IQR), with IQR = Q3-Q1]; middle horizontal line: median; filled triangle: mean. None of the differences is significant in entire berries (A). Of all combinations in berry halves, only O_ext vs. X_int are not significantly different (B). (t-tests at 0.05 level).

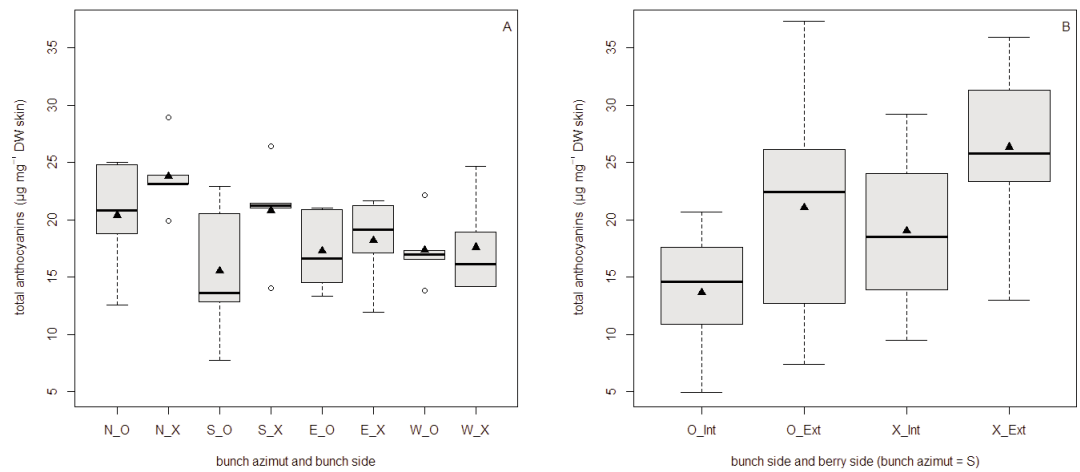

Figure 5 - Total anthocyanin content of berry skin and its variations with microclimate.

Pooled entire berries (A) and separate half berries from South-exposed bunches (B). n = 5 (A) and n = 25 (B), respectively. Effects of bunch azimuth (N, S, E, W), bunch side (exposed "X" vs. opposite "O") and berry side (internal "int" vs. external "ext"). Boxplots with common conventions (as Fig. 4); horizontal line: median; filled triangle: mean. None of the differences is significant in entire berries (A). Of all combinations in berry-halves, only O_ext vs. X_int are not significantly different (B). (t-tests at 0.05 level).

sub-pathways (Cohen *et al.*, 2008; Matus *et al.*, 2009; He *et al.*, 2010; Cohen *et al.*, 2012). Therefore, all correlations between these product classes and with phenylalanine were checked in the same data sets (Fig. 6).

As already shown by PCA, anthocyanin and flavonol contents were both mostly uncoupled from amino-acids, including phenylalanine (Fig. 6). The anthocyanins-flavonols relationship was the most consistent in all the categories investigated. This relationship was approximately linear and positive; higher levels of anthocyanins were clearly correlated to higher levels of flavonols. The different microclimatic conditions had little impact on this relationship and merely altered the anthocyanin and flavonol levels and their variability (Fig. 6).

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DISCUSSION

The experiment was explicitly designed to study the influence of microclimate on the main components of berry composition within the framework of a few basic assumptions. It was assumed that C and N from primary metabolism were supplied in non-limiting amounts to the bunches, or, since these factors were not controlled, that at least the same levels of water, sugar and N feeding were provided to each sampled bunch. The data showed these assumptions were mostly met; only N feeding was probably different in the two sites, leading to a significant block effect and a doubtful azimuth influence on amino-acid content at bunch scale. However, this difference had no apparent consequence on secondary metabolism of phenolics, as demonstrated by analysis of variance and correlation patterns of total anthocyanins and flavonols (Tables 1 and 2, Figs. 3 and 6).

The positive relationship between anthocyanins and flavonols in different microclimate conditions (Fig. 6) was an indication that anthocyanins and flavonols, as terminal products of divergent sub-pathways (Cohen *et al.*, 2008; Matus *et al.*, 2009; He *et al.*, 2010; Cohen *et al.*, 2012; Koyama *et al.*, 2012), did not compete for their common substrate phenylalanine. Therefore, it is likely that N uptake, transport and metabolism produced more than enough amino-acids in the berry, and more specifically phenylalanine, for the phenylpropanoid metabolism to carry on undisturbed. Thus, at least for the conditions of this experiment, it may be concluded that the main control on the secondary metabolism of phenolic pathway was exerted by the very local microclimate factors.

According to the experimental design, two main factors were involved: berry temperature and light, with variations produced by bunch azimuth, bunch side and berry side. However, an additional potentially disturbing anatomical factor was also introduced since splitting berries in South-exposed bunches lead to two structurally slightly different halves, one (proximal) attached to the pedicel and including xylem and phloem vessels, and one (distal or stylar) without pedicel attachment and including much less vessel-related anatomical structures. This effect of anatomical differences was therefore likely to affect pulp composition (Castellarin *et al.*, 2011) but unlikely to affect skin composition, especially in fully mature berries (Castellarin *et al.*, 2011); therefore, it was neglected. Another potential effect of berry height position within the bunch (Pagay and Cheng, 2010) was minimized by sampling only the middle parts of the bunch. Therefore, the effect of lateral position within the bunch was assumed to be entirely due to microclimate. The effects of other intrinsic properties or status of the berries like berry size, number of seeds, presence of cuticular waxes, and time elapsed since veraison (Pagay and Cheng, 2010; Dai *et al.*, 2011) were also neglected.

Table 2 - Analysis of variance – berry-halves from South-exposed bunches.

Component	Effect (interactions ":")	Df	F value	Pr(>F)
total anthocyanins				
	bunch side	1	17.64	P<0.0001
	berry side	1	33.45	P<0.0001
	bunch side:berry side	1	0.00	0.959
	residuals	96		
total flavonols				
	bunch side	1	86.20	P<0.0001
	berry side	1	76.84	P<0.0001
	bunch side:berry side	1	23.80	P<0.0001
	residuals	96		
total amino-acids in skin				
	bunch side	1	3.83	0.053
	berry side	1	5.83	0.018
	bunch side: berry side	1	0.37	0.546
	residuals	96		
total amino-acids in flesh (1 missing observation)				
	bunch side	1	0.00	0.992
	berry side	1	22.55	P<0.0001
	bunch side:berry side	1	0.08	0.780
	residuals	95		

Based on berry composition data, the analysis of variance results showed the strong influence of bunch side on the secondary metabolites investigated (Tables 1 and 2), suggesting an impact of incoming solar radiation or heating on the exposed side (*X*) of the bunch. The highly significant effect of berry side on secondary metabolism (Table 2) could be explained in the same way. However, apart from site effects on amino-acids, azimuth effects were very weak and actually non significant (Table 1); the weak apparent azimuthal effect on flavonols in *X* berries (Fig. 4) was probably mitigated by natural variability among bunches, whereas the effect on anthocyanins seemed likely to be caused by a more complex combination of factors (Fig. 5).

No obvious explanation for the berry side influence on amino-acid content, especially in pulp (Table 2), was supported by the present data or the literature (review in Rienth *et al.*, 2014). A putative mechanism would link the amino-acid gradient inside the berries to water fluxes, since transpiration is higher through the skin of the outside half (*ext*). Other speculative explanations include anatomical differences and net consumption by primary or secondary metabolism.

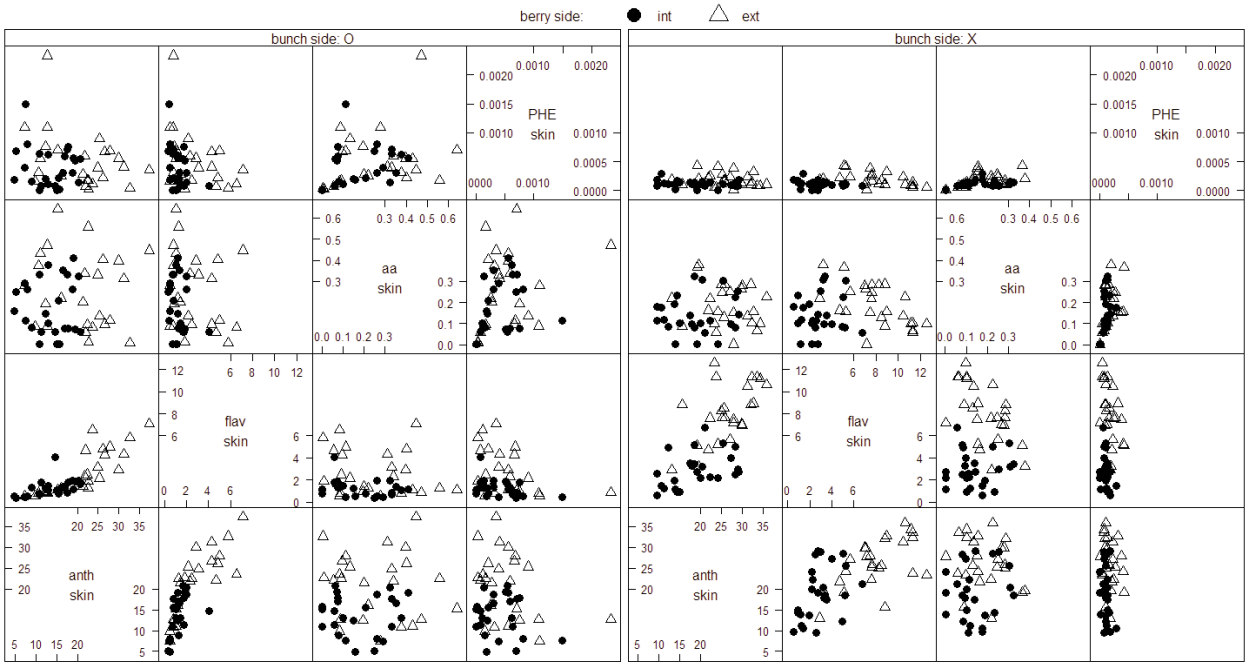

Figure 6 - Relationships of phenylalanine (PHE), total amino-acids (aa), total flavonols (flav) and total anthocyanins (anth) in skin of half berries from South-exposed bunches. Effects of bunch side (exposed "X" vs. opposite "O") and berry side (internal "int" vs. external "ext").

If the main assumption about identical primary metabolism was met, as strongly suggested by sugar and organic acid results, the berry composition differences may only have been caused by the variations in microclimate exposure. The highly significant effect of berry side on secondary metabolism (Table 2) and the clear differences between *int* and *ext* half berries, especially in exposed berries (Figs. 4 and 5), suggest that solar radiation plays a major role in this microclimate effect : whereas the temperature gradient across an individual berry is naturally damped by heat conduction in a semi-liquid aqueous medium, the solar radiation is absorbed by a thin layer in the skin and therefore the *int* berry halves remain in the shade (Smart and Sinclair, 1976 ; Cola *et al.*, 2009). Therefore, the strong influence of berry side was more likely due to the impact of solar radiation. This view is, however, somehow simplistic since the structure of the bunch itself, its compactness and the stage of berry growth and bunch closure all influence the actual solar radiation gradient across a single berry. On the other hand, in defoliated bunches, the external half of *O* berries in South-exposed bunches may receive direct solar radiation during certain parts of the day – morning and afternoon, when the sun direction is nearly parallel to the row axis – a fact that could explain the intermediate position of *O ext* berry halves with respect to the first principal component axis in PCA (Fig. 3).

Total flavonols actually exhibited a strong response to microclimate, where solar radiation was dominant, as observed in other studies (Price *et al.*, 1995 ; Spayd *et al.*, 2002 ; Downey *et al.*, 2004 ; Cortell and Kennedy, 2006 ; Downey *et al.*, 2006 ; Matus *et al.*, 2009 ; Azuma *et al.*, 2012 ; Diago *et al.*, 2012 ; Koyama *et al.*, 2012). Here however, the results suggested a strong involvement of solar radiation direct impact on the berry skin at the local infra-berry fine scale. This positive effect of solar radiation on total flavonol concentration in the berry skin was very significant and intense, and could consistently explain the variations observed at berry scale as well as at bunch scale (Fig. 4). The significant bunch side-berry side interaction (Table 2, Fig. 4B) could also be driven by incoming solar radiation, although radiation load at "*int*" berry sides was unknown.

Anthocyanins also responded to microclimate at both scales ; like in other studies (Bergqvist *et al.*, 2001 ; Spayd *et al.*, 2002 ; Cortell and Kennedy, 2006 ;; Downey *et al.*, 2006 ; Tarara *et al.*, 2008 ; Matus *et al.*, 2009 ; Azuma *et al.*, 2012 ; Diago *et al.*, 2012), part of the effect was likely due to solar radiation. For instance, intercepted solar radiation probably explained the higher levels of skin anthocyanin concentrations in *ext* berry halves (Fig. 5B). On the other hand, lower levels in *O* berries of South-exposed bunches, compared with *O* berries of North-exposed bunches (Fig. 5A), hinted at a decay of total

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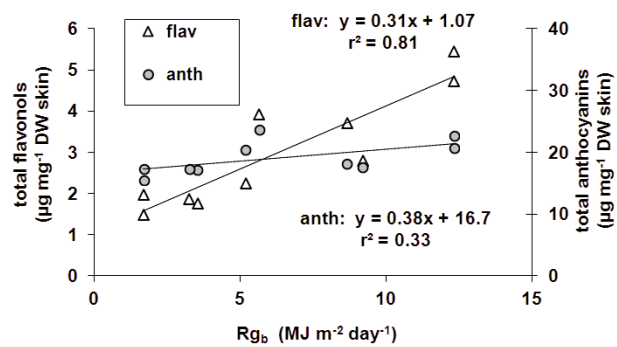
anthocyanin concentrations in the skin in hotter conditions, confirming other results (Haselgrove *et al.*, 2000; Bergqvist *et al.*, 2001; Spayd *et al.*, 2002; Mori *et al.*, 2005; Downey *et al.*, 2006; Mori *et al.*, 2007; Cohen *et al.*, 2008; Tarara *et al.*, 2008; He *et al.*, 2010; Azuma *et al.*, 2012; Sadras and Moran, 2012; Rienth *et al.*, 2014). These apparently mixed effects of solar radiation and temperature might result from a threshold response function of radiation combined with an optimum response function with respect to berry temperature (Cohen *et al.*, 2008; Tarara *et al.*, 2008). They might also be due to different sensitivities of synthesis and catabolism to radiation and/or temperature, respectively (He *et al.*, 2010; Sadras and Moran, 2012). Finally, these microclimate effects combine with the developmental programming of anthocyanin production that seems linked to genotype (Downey *et al.*, 2004).

As in every outdoor experiment, solar radiation and berry temperature variations were coupled since intercepted solar radiation was the main energy input, while post-veraison berries were characterized by a reduced capacity of passive cooling by transpiration and by a non negligible thermal inertia (Smart and Sinclair, 1976; Rebucci *et al.*, 1997; Spayd *et al.*, 2002; Tarara *et al.*, 2008; Cola *et al.*, 2009). This coupling was therefore a direct consequence of energy balance at berry or bunch scale. Simulated values of incoming solar radiation (Rg) and berry temperature (T) representative of the different categories of bunches in the experiment (Fig. 1) were indeed correlated. Most of this correlation was due to exposed *X* berries, where most radiation contrast was found, as a consequence of exposure to the outside of the row combined with differing azimuth. Additionally, temperature contrast in *X* berries between East- and West-exposed bunches, which received the same radiation level, was not enough to achieve a decoupling at bunch scale.

In order to link berry composition to microclimatic factors, the microenvironmental variables T and Rg were considered separately as possible variables explaining changes in berry total anthocyanins and total flavonols. A clear relationship of total flavonols with radiation was observed, confirming that more flavonols were produced in berry skin in response to increasing radiation load (Fig. 7). This result was consistent with the well-known sensitivity to solar radiation of the flavonol net synthesis and flavonol synthase gene expression (Haselgrove *et al.*, 2000; Spayd *et al.*, 2002; Downey *et al.*, 2004; Downey *et al.*, 2006; Matus *et al.*, 2009; Azuma *et al.*, 2012, Koyama *et al.*, 2012). However, the present data strongly supported a linear relationship (Fig. 7) and the efficiency of conversion was quantified by its slope: about 0.31 µg total flavonols (g DW skin)$^{-1}$ (MJ m^{-2} day^{-1})$^{-1}$. A weaker positive and linear relationship of berry total anthocyanins with radiation was observed (Fig. 7). While residuals of the total flavonols vs. radiation linear relationship were insensitive to temperature, residuals of the total anthocyanins vs. radiation linear relationship exhibited a nearly linear negative trend, confirming the repressive effect of higher temperature on total anthocyanin content. When merged into a bilinear relationship of total anthocyanins with radiation and temperature, the quality of fit was improved (r^2 = 0.67).

All these results validate the hypothesis of a local metabolic response to microclimatic factors and therefore confirm the existence of an acclimation mechanism that modulates secondary metabolism according to radiation and temperature. This study in field conditions and at two nested scales therefore confirms previous work (Spayd *et al.*, 2002; Downey *et al.*, 2006; Tarara *et al.*, 2008; Dai *et al.*, 2011; Azuma *et al.*, 2012; Rienth *et al.*, 2014), but gives more insight on the accurate location of flavonoid gradients. Furthermore, it provides quantified responses to environmental factors, especially with regard to the flavonol synthesis response to solar radiation load. Recent studies of gene expression and transcription factors considering the flavonoid pathway in the grape berry skin or other plant tissues (Matus *et al.*, 2009; He *et al.*, 2010; Dai *et al.*, 2011; Azuma *et al.*, 2012; Cohen *et al.*, 2012; Gouthu *et al.*, 2014; Rienth *et al.*, 2014) will certainly help elucidate this acclimation mechanism, although the actual sensors of solar radiation and temperature in the berry skin still remain elusive.



Figue 7 - Relationship of measured total anthocyanin (anth) and flavonol (flav) contents in berry skins with mean simulated incoming solar radiation at berry scale (Rgb).

CONCLUSIONS

The main results demonstrated a strong and significant microclimate effect. This effect was prominent and intense at berry scale and consistent with the better known effect observed at bunch scale. Even if the respective influences of solar radiation and temperature could not be fully distinguished, results at berry scale hinted at a major influence of solar radiation on both anthocyanins and flavonols. These results were therefore a clear evidence of a strictly local mechanism of plant response to microclimate at skin tissue level. The complex response of anthocyanins to microclimate was likely the outcome of two contradictory mechanisms: a triggering of synthesis by solar radiation and a reduced net synthesis (or enhanced net degradation) due to higher temperature. However, natural variations led to a microclimate contrast (for instance East- vs. West-exposed bunches) that was insufficient to confidently distinguish the respective influences of solar radiation and temperature on anthocyanin content. Nevertheless, the use of a simplified model of radiation interception and berry temperature showed that total flavonol content in the berry skin was clearly linearly linked to solar radiation, which led to a first estimation of radiation use efficiency for flavonol production. Total anthocyanin content in the berry skin was linearly linked to both solar radiation and temperature, positively with solar radiation and negatively with berry temperature.

These results contribute to an improvement of global knowledge about grape maturation and have interesting implications for the control and management of grape quality. Vine training system, bunch position and shading, and bunch structure and compactness are all microclimate-related factors that could be involved in a sensible adaptation of the grapevine production system to the marketing strategy or the climate change.

Acknowledgements: The authors gratefully acknowledge Château Cheval Blanc and Château Figeac for allowing the field experiment, the Région Aquitaine council and INRA for funding, J.-P. Gaudillère and C. Van Leuween for helpful discussions and S. Delrot for helpful criticism of the manuscript.

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