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Using common viticultural practices to modulate the rotundone and 3-isobutyl-2-methoxypyrazine composition of *Vitis vinifera* L. cv. Fer red wines from a temperate climate wine region with very cool nights

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ABSTRACT

Aim: Rotundone and 3-isobutyl-2-methoxypyrazine (IBMP) are two potent aroma compounds responsible for pepper and bell pepper notes in red wines, respectively. The aim of the study was to modulate, through common viticultural practices, the volatile composition in these two molecules of Fer red wines from a temperate climate wine region with very cool nights, located in the southwest of France.
Methods and results: Three viticultural practices (leaf removal 10 days after berry set, removal of lateral shoots, and delayed harvest 7 days after the control) were investigated in 2015 and in 2016. Rotundone concentrations up to 69 ng/L were found in experimental wines. IBMP concentrations were below perception level in wines from 2016 and below detection level in wines from 2015, a vintage with particularly hot climatic conditions between berry set and bunch closure. Delayed harvest induced an increase in rotundone concentration while leaf removal and the removal of lateral shoots had no significant impact on rotundone concentration. Delayed harvest and the removal of lateral shoots were the most efficient practices to decrease IBMP in wines. The three techniques made it possible to increase the odour activity values (OAV) ratio of OAV rotundone to OAV IBMP, with the greatest impact observed for delayed harvest.
Conclusion: According to our results, delayed harvest appears to be the best practice to modulate the volatile composition of Fer wines toward an increase in the OAV rotundone to OAV IBMP ratio.
Significance and impact of the study: Our results may assist local grape growers to modulate the volatile composition of their wines.

KEYWORDS

IBMP, rotundone, leaf removal, removal of lateral shoots, delayed harvest

INTRODUCTION

Vitis vinifera L. cv. Fer is a dark-skinned grape variety which in 2015 covered 1548 ha exclusively in the southwest of France, according to FranceAgriMer (www.franceagrimer.fr). Together with Merlot, Cabernet Sauvignon, Cabernet Franc, Carmenère, Petit Verdot, Castets and other minor cultivars, this variety was considered part of the ecogeographic group named “carmenets” by Bisson (1995, following Levadoux, 1948), according to morphological traits and supposed geographical origin. The name Fer is derived from the latin *ferus,* meaning ‘wild, savage’ which is consistent with the common belief that Fer was domesticated from local wild grapevines (Lavignac and Audiot, 2001). It has several direct offspring belonging to the ancient southwest of France varietal heritage, proof of its seniority and its wide distribution in the vineyards of this part of France. For example, DNA parentage analysis indicated that Fer was related to Mauzac Noir du Lot-et-Garonne (which is not a colour mutation of Mauzac) and birthed Négret de Banhars and Gros Cabernet through spontaneous crossings with Manseng Noir and Txakoli, respectively (Lacombe *et al*., 2013). Thus, Fer is one of the grandparents of Carmenère, which is an offspring of Gros Cabernet and Cabernet Franc (Boursiquot *et al*., 2009). The earliest literary references to Fer appeared in 1783 (Rézeau, 1998) and this variety is presently known under several synonyms (i.e Braucol in Gaillac, Servadou in Lot-et-Garonne, Pinenc in Saint-Mont or Madiran, Mansois in Marcillac). More recently in 1966, Fer was used by INRA to breed Ferradou with Merlot (plantgrape.plantnet-project.org).

Fer gives medium bodied wines which often exhibit bell-pepper-like odours associated with substantial levels of 3-isobutyl-2-methoxypyrazine (IBMP) (Davaux, 2005). This attribute is particularly enhanced when grapes are not sufficiently ripe or are produced under cool and wet climate conditions (Roujou de Boubée, 2000). Notably with concentrations up to 110 ng/L (Davaux, 2005), IBMP has been identified as an aromatic marker of Fer wines from the Protected Designation of Origin (PDO) Marcillac in the French Aveyron department, which is locally considered as a cool climate vineyard. Fer wines from this wine region can also develop some distinctive peppery notes that are attributable to rotundone, a sesquiterpene discovered in 2008 (Wood *et al*., 2008), with concentrations up to 18 ng/L (Olivier Geffroy, unpubl. data, 2014). IBMP and rotundone are among the most potent aroma compounds identified in wine with detection thresholds in red wine of 15 ng/L and 16 ng/L, respectively (Roujou de Boubée, 2000; Wood *et al*., 2008). Both molecules have been described by Ferreira (2012) as two of the 16 most impactful aroma compounds in wine that can break its “buffer” without the support of additional odorants.

In addition to Fer, IBMP has been identified in cultivars mainly from the Carmenets group such as Sauvignon, Cabernet Sauvignon, Merlot and Cabernet Franc (Allen *et al*., 1995; Hashizume and Umeda 1996). Rotundone has been found in in a wide range of varieties, including Pinot Noir, Durif, Graciano and Riesling (Herderich *et al*., 2012), Duras (Geffroy *et al*., 2014), Gamay (Geffroy *et al*., 2016a), Malbec and Abouriou (Cullere *et al*., 2016) and Vespolina, Schioppettino and Grüner Veltliner (Caputi *et al*., 2011).

While IBMP is usually considered by wine professionals as an undesirable aroma compound in red wine, rotundone is generally positively perceived by consumers (Geffroy *et al*., 2018). The modulation of the sensory profile of Marcillac red wines toward a spicier character is an issue frequently raised by local winemakers. This modulation, which may involve an increase in rotundone concentration coupled with a decrease in IBMP concentration, can be challenging. Indeed, rotundone and IBMP are produced *in situ* and are not sensitive to source-think variations (Geffroy *et al*., 2016; Koch *et al*., 2010; Zhang *et al*., 2016). In addition, both compounds are generally enhanced by cool and wet climatic conditions (Caputi *et al*., 2011; Mendez-Costabel *et al*., 2013).

However, some viticultural practices could be valuable to reach this objective. Indeed, the kinetic of accumulation during grape maturation differs between IBMP and rotundone. The bell pepper aroma compound is produced between berry set and bunch closure and its concentration in berries decreases during grape ripening (Roujou de Boubée *et al*., 2000; Ryona *et al*., 2010). Rotundone starts its accumulation from mid-veraison, and the highest rotundone concentrations are reached late in the berry development (Caputi *et al*., 2011; Geffroy *et al*., 2014).

In the same way, distinct responses to canopy management techniques and notably defoliation have been observed for the two aroma compounds. It has been shown that an early leaf removal, especially just after berry set (Roujou de Boubée, 2000; Davaux, 2005; Scheiner *et al*., 2010; Šuklje *et al*., 2012, Sivilotti *et al*., 2016), and the removal of lateral shoots (Roujou de Boubée, 2000) were effective to reduce IBMP in berries and wines. The effects of sunlight and temperature on fruit composition induced by defoliation are always difficult to separate, as many biochemical pathways are affected by these two abiotic factors. The most likely hypothesis is that bunch light exposure induces a photochemical degradation reaction of IBMP that could be affected secondarily by the increase in temperature (Šuklje *et al*., 2012). Conflicting results have been found concerning the impact of leaf removal on rotundone: leaf removal can either reduce, increase or have no impact at all on rotundone concentrations (Geffroy *et al*., 2014; Homich *et al*., 2017) depending on its timing, its intensity and the site. The impact of the removal of lateral shoots on rotundone concentration has not been yet investigated.

In 2015 and 2016, three viticultural practices (leaf removal 10 days after berry set, removal of lateral shoots and delayed harvest 7 days after the control) were investigated in a field trial to modulate the volatile composition of Fer wines from the Marcillac PDO toward an increase in the OAV rotundone to OAV IBMP ratio.

MATERIAL AND METHODS

1. Experimental design

The experiment was established on a 0.80-ha vineyard located within the Marcillac PDO (latitude 44° 27′ 5″′ N; longitude 02° 25′ 43″′ E) with a slope of 15%. This vineyard typical of the area with 2.20 m × 1 m vine spacing and vines was trained with vertical shoot positioning on a single Guyot pruning system. The soil was managed by chemical weed control under the vines and by grass cover in every inter-row. The date of establishment of the site and the rootstock were not determined because the vineyard was planted more than 50 years ago. The trial was conducted on four rows in a randomised complete block design with three replications per treatment. Each experimental unit of 26.4 m^2 contained 12 continuous vines. Orientation of the vine rows was north-west to south-east.

2. Viticultural practices

Three viticultural techniques were investigated and compared with a control treatment.

- 100% fruit zone leaves (leaf removal) were removed by hand on the east-facing side 10 days after berry set, on 17 June in 2015 and on 7 July in 2016. All leaves were removed from the basal node to the first node above the top bunch.

- Lateral shoots were removed manually twice (removal of lateral shoots). The first operation was undertaken at berry set on 7 June in 2015 and on 27 June in 2016 and the second one three weeks later, on 28 June in 2015 and on 3 July in 2016.

- For the delayed harvest treatment (delayed harvest), grapes were harvested exactly 7 days after the control and the two other studied treatments on 5 October in 2015 and on 26 October in 2016.

3. Vine and weather measurements

Harvest, except for the delayed harvest treatment, took place on 28 September in 2015 and on 19 October in 2016, which corresponds exactly to 54 days after mid-veraison. Yield (kg/vine) was monitored for each vine by weighing individually crop load with a Precia Molen C20 K balance (Precia SA, Privas, France). In 2015 and in 2016, nine temperature loggers (EBI 20-T1, Ebro Electronic, Germany) displayed in solar radiation shields were positioned in the bunch zone for each experimental unit except for the delayed harvest treatment. These data were used to study the impact of the techniques on bunch microclimate through calculation of mean air temperature (Tv-r), cool night index (FNv-r) and maximal air temperature (Txv-r) between veraison and harvest (Tonietto and Carbonneau, 2002). In 2015, bunch surface temperature was identified as one of the main determinants of rotundone, which could be predicted in grapes using the percentage of degree hours above 25°C (DH25) (Zhang *et al*., 2015). Consequently, DH25 was calculated using the data from the temperature loggers, and complementary measurements of bunch surface temperature were undertaken in 2016 using a non-contact infrared thermometer. These measurements were carried out for the control, leaf removal and removal of lateral shoots treatments between mid-veraison and harvest on 21 September 2016, a cloudless and windless day. Bunch surface temperature was determined halfway between the two end points of the bunch using a non-contact Raynger MX infrared thermometer (Raytek, USA). Measurements were made on 30 bunches for each experimental unit on each side of the vine at 12:00 h and 16:00 h. The measurements in the experimental plot were completed in less than 15 min for each time of measurement. Bunch surface temperature was first to be assessed for all the experimental units on the east-facing side and then on the west-facing side of the row. The same day, the opportunity was taken to determine stem water potentials (Ψ stem) and leaf nitrogen status. For each experimental unit, nitrogen status was characterised on 12 leaves (one leaf per vine) through measurements of Dualex® (Force A, Orsay, France), a fluorescence-based sensor that allows the calculation of a chlorophyll to flavonols ratio named NBI® (nitrogen balance index). Stem water potentials were measured according to the method proposed by Choné *et al.* (2001). Measurements were taken from three of the 12 vines and from two mature exposed leaves per vine. Leaf blades were initially enclosed in plastic bags between 11:00 h and 12:00 h. Measurements were made between 14:00 h and 15:00 h. Since 2005, rainfall and air temperature (minima, maxima and mean values) have been monitored by a CimAGRO weather station (Cimel Electronique, Paris, France) placed within 5 km of the experimental site. These data were used to characterise the climate of the area for the 2005–2014 period and the two vintages of study through calculation of Huglin index (Huglin, 1986) and cumulative rainfall. Cumulative rainfall between veraison and harvest were not determined for the 2005–2014 period as veraison dates were unknown. The time necessary to remove fruit zone leaves and lateral shoots was also assessed for each experimental unit.

4. Berry sampling and analyses

At harvest for each experimental site, two samples consisting of 200 berries and 800 g of berries were collected on each experimental site. The 200-berry sample was weighted, crushed gently, and the musts obtained were centrifuged to enable traditional laboratory measurements. Sugar concentration (°Brix) was determined with a digital hand-held Pocket refractometer PAL (Atago, Japan). Titratable acidity was measured according to the Organisation Internationale de la Vigne et du Vin (OIV) method (OIV 2009) and pH was measured with a HI 3221 pH meter (Hana Instruments, France). A Konelab Arena 20 sequential analyzer (Thermo Electron Corporation, USA) associated with enzyme kits provided by several suppliers was used to determine amino acids, ammonium (Megazyme, Ireland) and malic acid (Thermo Fisher Scientific, USA). Potassium determination was carried out by flame photometry (Bio Arrow, France) according to OIV method (OIV 2009) and tartaric acid determination by colorimetric titration (Hill and Caputi, 1970). $\delta^{13}C$ based upon $^{13}C/^{12}C$ ratio was determined at harvest on grape sugars for each experimental unit according to a published protocol (Geffroy *et al.* 2014). In water stress conditions, sugars contain more ^{13}C compared to those produced when the water status is not limiting, and this isotopic ratio can be used as an integrative indicator of water deficit (van Leeuwen *et al.*, 2009) experienced by vine during grape ripening. The remaining berry skins were crushed for 2 minutes at low speed (600 rpm) using a food blender (Moulinex, Faciclic, France) to perform phenolic analyses. Anthocyanins and total phenolic index (TPI) were quantified in grapes according to the method described by Cayla *et al.* (2002), which can be summarised here briefly. 50 g of the crushed berries were macerated for 1 h in a medium containing 15 mL of ethanol (95%) and 85 mL of HCl (37%). Samples were stirred every 15 minutes and after centrifugation (14 000g for 6 min), anthocyanins and TPI were quantified the same way as in wine according to Ribéreau-Gayon and Stonestreet (1965) and Ribéreau-Gayon (1970), respectively, using an Evolution 100 spectrophotometer (Thermo Electron Corporation, USA).

The 800 g grape samples were used to perform microscale fermentations in 1 L Erlenmeyer flasks according to the protocol proposed by Geffroy *et al.* (2014). At the end of the 8-day maceration at 25°C, wines were pressed under controlled conditions (200 kPa for 2 min) using a Para Press laboratory press (Paul Arauner GmbH, Kitzingen, Germany). Wines were then centrifuged (14 000 × *g* for 6 min), received a sulfite addition of 80 mg/L before being bottled and stored at 4°C or below. After 1 month and as part of a contract service, rotundone and IBMP were determined in wines by the AWRI (Adelaide, Australia) with SPME-GC-MS using deuterated isotopes and parameters previously described (Geffroy *et al.*, 2014; King *et al.*, 2011). Reproducibility tests showed an average coefficient of variation of 5% across the microfermentors for the rotundone and IBMP concentrations. Limits of quantification were 2 ng/L and 5 ng/L for rotundone and IBMP, respectively. Odour activity values (OAVs) determined by dividing the concentrations of the two odorants by their perception thresholds in wine were used to calculate the OAV rotundone to OAV IBMP ratio.

5. Data treatment

Statistical analyses were conducted with Xlstat software (Addinsoft, France). All the analytical data measured both in 2015 and in 2016 that included rotundone were subjected to a three-way analysis of variance (ANOVA) treatment (vintage × treatment × block) with first-order interaction. Other variables which were just determined, detected or calculated during one year of study (i.e. IBMP, ratio of OAV rotundone to OAV IBMP) were treated by a two-way ANOVA (treatment × block) with or without first-order interaction depending if measurements were repeated for each experimental unit. Bunch surface temperature data were subjected to a four-way ANOVA (treatment × side of the row × time of measurement x block) with first-order interaction. Fisher's least significant difference (LSD) test was used as a post-hoc comparison of means at $P < 0.05$.

RESULTS AND DISCUSSION

1. Effect of the vintage

Results of the ANOVA treatments showed that among the studied factors the vintage had the greatest impact on the measured variables (Table 1). For most of the variables, no block effect or significant interactions between the factors were observed. However, for ammonium, potassium and anthocyanins, significant interactions were found, involving the vintage factor and/or the block factor and the treatment factor. This indicates that the impact of the studied techniques can differ notably according to the characteristics of the vintage.

Climatic data presented in Table 2 and Table 3 allow us to characterise the climate of the region and the two vintages of the study.

According to Tonietto and Carbonneau (2002), the climate of the Marcillac PDO can be considered as temperate with very cool nights.

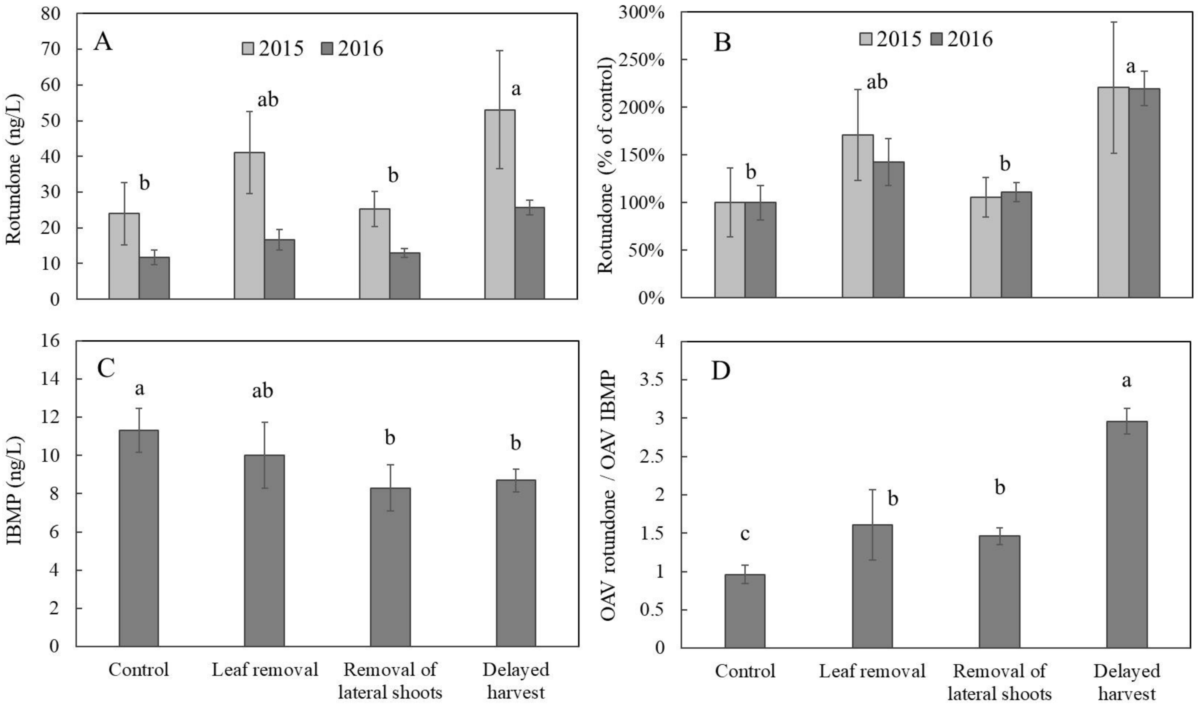
TABLE 1. Results of the ANOVA performed on the variables measured during the trial.

Variable	*P*-value					
	Vintage (V)	Treatment (T)	Block (B)	V × T	V × B	T × B
Mass of 200 berries	0.102	0.341	0.900	0.410	0.559	0.137
Sugar concentration	< 0.001	0.542	0.718	0.903	0.412	0.849
Titratable acidity	< 0.001	0.168	0.204	0.218	0.626	0.479
pH	< 0.001	0.169	0.319	0.283	0.349	0.136
Malic acid	< 0.001	0.183	0.100	0.084	0.486	0.077
Tartaric acid	0.672	0.290	0.761	0.152	0.802	0.492
Amino acids	0.005	0.005	0.320	0.548	0.510	0.219
Ammonium	0.004	0.004	0.009	0.020	0.233	0.004
Potassium	0.001	0.009	0.356	0.040	0.165	0.964
Anthocyanins	< 0.001	0.001	0.724	0.072	0.029	0.011
Total phenolic index (TPI)	< 0.001	0.076	0.834	0.647	0.358	0.551
Yield at harvest	< 0.001	0.313	0.090	0.878	0.207	0.477
Mean air temperature (Tv-r)	0.227	0.798	0.815	0.999	0.785	0.985
Cool night index (FNv-r)	0.011	0.521	0.521	0.396	0.769	0.644
Maximal air temperature (Txv-r)	0.038	0.981	0.877	0.678	0.939	0.199
DH_{25}^{a}	0.003	0.713	0.433	0.402	0.876	0.692
$\delta_{13}C$	0.776	0.027	0.393	0.500	0.858	0.306
Stem water potential (Ψ_{stem})	n.d.	0.009	0.003	n.d.	n.d.	0.064
Nitrogen balance index (NBI)	n.d.	0.205	0.522	n.d.	n.d.	0.069
Rotundone	<0.001	0.002	0.403	0.310	0.498	0.455
2-methoxy-3-isobutylpyrazine	n.d.	0.060	0.736	n.d.	n.d.	n.d.
OAV of rotundone to OAV of IBMP ratio	n.d.	<0.001	0.086	n.d.	n.d.	n.d.

TABLE 2. Characterisation of the area's climate and the two vintages by Huglin index and cumulative rainfall.

		Cumulative rainfall (mm)		
Vintage	Huglin index (HI)	1 Jan – 31 Dec	1 Apr – 30 Sep	Veraison – Harvest
2005–2014	1838	804	425	n.d.
2015	2031	736	367	164
2016	1871	1107	525	123

FIGURE 1. Effect of the techniques on A) rotundone expressed in ng/L in 2015 and in 2016; B) rotundone expressed in percentage of control in 2015 and in 2016; C) IBMP in 2016; and D) the ratio of OAV rotundone to OAV IBMP in 2016 (mean of three observations). a–d indicate statistically significant differences at $P<0.05$ except for IBMP ($P=0.06$).



The 2015 vintage was characterised by limited rainfall events and hot weather conditions over the whole vine vegetative cycle. The beginning of the 2015 summer was one of the warmest ever recorded in the area, with two heat waves occurring in June and in July. During these two extreme climatic events, maximum temperature exceeded 40°C for several days. Frequent thunderstorms and rain showers were observed during the month of August, which was marked by cooler and wetter climatic conditions. In comparison with 2015, the 2016 vintage (which remains a hot vintage) was cooler and rainier, as reflected by Huglin index and cumulative rainfall. Despite rainy and cool weather conditions from spring to the beginning of summer, which induced a late-flowering, an outstanding flowering and berry-set rate were observed that lead to high yield at harvest for the area. Grapevine yield formation extends over two consecutive seasons. The formation of inflorescence primordia that occurs in the first season, typically in June under our experimental conditions, is enhanced by temperature and light (Srinivasan and Mullins, 1981). We can assume that the particularly hot and sunny conditions observed in June 2015 might explain the large crop level obtained at harvest in 2016, the second season. Conditions were hot and dry in August and throughout the maturation in 2016. The delay in phenological development observed at flowering was maintained until harvest, which occurred 3 weeks later than in 2015.

TABLE 3. Effect of the studied techniques on the variables for which no significant interaction involving the treatment factor was observed (mean and standard deviation of three observations)

Variable	Control			Leaf removal			Removal of lateral shoots			Delayed harvest		
	2015	2016		2015	2016		2015	2016		2015	2016	
Mass of 200 berries (g)	283±11	302±24	a	267±19	292±16	a	287±13	284±18	a	292±22	297±13	a
Sugar concentration (°Brix)	22.2±0.8	19.4±1.0	a	22.6±0.4	19.7±1.2	a	20.8±0.4	19.2±0.8	a	22.8±0.8	19.4±0.4	a
Titratable acidity (g/L of tartaric acid)	7.08±0.11	8.20±0.19	a	7.38±0.19	8.86±0.42	a	7.12±0.19	8.83±0.30	a	7.36±0.24	8.17±0.25	a
pH	3.14±0.02	3.05±0.03	a	3.14±0.03	3.02±0.04	a	3.17±0.01	3.02±0.03	a	3.17±0.03	3.06±0.05	a
Malic acid (g/L)	1.35±0.08	2.26±0.14	b	1.40±0.13	2.44±0.13	a	1.37±0.08	2.41±0.26	ab	1.48±0.08	2.25±0.11	ab
Tartaric acid (g/L)	5.05±0.30	4.57±0.17	a	4.89±0.05	5.49±0.51	a	5.35±0.21	4.92±0.07	a	4.34±0.02	5.01±0.75	a
Amino acids (mg/L)	46.1±2.1	61.1±6.8	b	46.1±8.2	54.7±9.7	b	51.2±5.2	57.4±2.4	b	63.9±3.4	71.9±6.8	a
Total phenolic index (TPI)	59±1	96±3	a	68±5	103±10	a	62±7	102±3	a	59±6	91±4	a
Yield at harvest (kg/vine)	2.24±1.00	2.84±1.11	a	1.95±1.06	2.71±1.74	a	2.26±1.17	2.64±0.82	a	2.46±1.02	3.16±1.72	a
Mean air temperature (Tv-r)	18.09±0.54	18.43±0.25	a	18.22±0.08	18.58±0.16	a	18.06±0.15	18.40±0.15	a	n.d.	n.d.	
Cool night index (FNv-r)	11.02±0.24	9.24±0.26	a	10.24±0.95	9.29±0.16	a	10.90±0.16	9.12±0.06	a	n.d.	n.d.	
Maximal air temperature (Txv-r)	29.17±2.94	27.02±0.89	a	30.10±0.78	26.22±1.00	a	29.49±2.77	27.33±1.18	a	n.d.	n.d.	
DH_{25}[a]	3.31±0.38	2.42±0.14	a	3.64±0.08	2.32±0.24	a	3.45±0.03	2.42±0.10	a	n.d.	n.d.	
$\delta_{13}C$	-26.69±0.16	-26.41±0.34	a	-26.99±0.16	-26.89±0.40	ab	-26.85±0.07	-26.76±0.61	a	-27.19±0.26	-27.49±0.37	b
Stem water potential (MPa)	n.d.	-0.57±0.10	b	n.d.	-0.50±0.09	a	n.d.	-0.47±0.06	a	n.d.	n.d.	
Nitrogen balance index (NBI)	n.d.	5.43±0.98	a	n.d.	5.24±1.19	a	n.d.	5.69±0.80	a	n.d.	n.d.	

TABLE 4. Effect of the studied techniques on the variables for which at least one significant interaction involving the treatment factor was observed.

Variable	Control			Leaf removal			Removal of lateral shoots			Delayed harvest		
	Block			Block			Block			Block		
	1	2	3	1	2	3	1	2	3	1	2	3
2015												
Ammonium (mg/L)	44.6	41.8	45.6	22.1	25.4	60.1	53.1	55.6	50.0	49.3	58.0	63.1
Potassium (g/L)	0.85	0.76	0.85	0.91	0.85	0.90	0.91	0.83	0.82	0.60	0.60	0.68
Anthocyanins (mg/kg)	1090	1066	1160	1304	1347	1224	1241	1003	1065	1091	1007	949
2016												
Ammonium (mg/L)	43.1	48.4	45.1	29.5	21.5	51.5	37.0	40.3	33.8	35.2	52.0	40.7
Potassium (g/L)	0.87	0.94	0.82	1.02	1.01	1.01	0.99	0.95	0.93	1.01	0.96	0.81
Anthocyanins (mg/kg)	1474	1528	1633	1451	1730	1588	1549	1483	1581	1490	1394	1394

The effect of the studied factors on classical enological parameters, yield at harvest, leaf nitrogen status, water status and bunch microclimate, is summarised in Tables 3 and 4. IBMP and rotundone concentrations as well as the ratio of OAV rotundone to OAV IBMP, are shown in Figure 1 (A–D).

No differences in mean air temperature between veraison and harvest were observed between the two vintages, but 2015 was characterised by a greater cool night index, maximal air temperature and percentage of degree hours above 25 °C between veraison and harvest. These climatic features lead to lower concentration in malic acid and titratable acidity, and a higher pH at harvest in 2015 in accordance with previous findings (Coombe, 1987). In 2016, the larger yields observed and the late harvest date lead to a lower sugar concentration at harvest (Kliewer and Dokoozlian, 2005). While higher crop load should have negatively impacted the accumulation of phenolic compounds in grapes (Kok, 2016), anthocyanins and TPI were surprisingly greater for this vintage. We can suppose that the elevated day temperature conditions experienced in 2015 contributed to decrease anthocyanins content, whose degradation is promoted by temperatures above 30°C (Mori *et al*., 2007). Cooler nights might have also enhanced anthocyanins synthesis in berries in 2016 (Kliewer and Torres, 1972). The reasons why TPI was superior in 2016 remain unclear as the total levels of proanthocyanidins, which can be considered as the main contributor to TPI (Blouin *et al*., 2000), are weakly impacted by temperature (Cohen *et al*., 2012). Distinct pre-veraison water status notably at pea size stage which was not assessed during our study, might help explain the TPI differences between vintages (Zarrouk *et al*., 2012).

Greater concentrations in amino acids were observed in 2016 during the rainier vintage. In 2015, we can assume that the dry soil conditions experienced in spring and in early summer did not promote the mineralisation of soil organic matter and the assimilation of ions by the root system. Moreover, the cooler nights experienced during maturation in 2016 might also play a role. It has been recently highlighted that cool night index was inversely correlated to several amino acids, suggesting that the synthesis or accumulation of some amino acids might be enhanced by cool temperatures (Gutiérrez-Gamboa *et al*., 2018). This observation was not confirmed for ammonium and potassium, which are more difficult to interpret due to a significant vintage × treatment interaction.

Concentrations in rotundone up to 69 ng/L were found in wines, which indicates that the molecule is likely to have a large contribution to the sensory profile of Fer wines from the Marcillac area. Rotundone was affected by the vintage, with greater concentrations found in wines from 2015. Higher concentrations would have been expected in 2016, the coolest vintage with the smaller DH25 (Caputi *et al*., 2011; Zhang *et al*., 2015). However, Geffroy *et al. (*2014) emphasised that the amount of rainfall in the late berry development stages and the plant water status during maturation were key variables to explain the differences in rotundone between vintages in the southwest of France. Indeed, if 2015 was dryer than 2016 between the 1 January and

31 December period and $\delta^{13}C$ did not allow to discriminate the two vintages, 2015 was wetter over the maturation period. In addition, greater yields and the phenological delay observed in 2016 might have contributed to lowering the sugar concentration in berries at harvest. Even if rotundone appears to be produced in berries (Geffroy *et al*., 2016; Zhang *et al*.; 2016) and is not impacted by source-sink ratio in conditions favourable to a satisfactory level of maturity (Geffroy *et al*., 2014), the low level of sugar concentration in 2016 might have negatively impacted the accumulation of rotundone. Actually, as other secondary metabolites, rotundone synthesis must be an energy demanding process that requires carbohydrates. As rotundone is hydrophobic and was measured indirectly in wine, we can also suppose that the difference in ethanol content between the two vintages might have impacted its extraction from the berry pericarp, which is enhanced by fortification and elevated alcohol concentration (Zhang *et al*., 2017).

IBMP concentrations were below detection level in wines from 2015 which could be a result of the heat wave experienced between berry set and bunch closure in July. Indeed, it has been highlighted that the concentration of IBMP in grapes strongly decreases with temperature (Falcão *et al*., 2007). In 2016, IBMP concentrations remained below the odour threshold of 15 ng/L established by Roujou de Boubée (2000). With 2 years of IBMP data below the perception threshold of the molecule, it is legitimate to wonder about the sensory impact of IBMP in Fer wines from this region. Research by Poitou *et al*. (2017) after our study was conducted showed that 1,8-cineole, a monoterpene responsible for eucalyptus and mint aroma, could have an additive effect with IBMP and may decrease its perception threshold. As the same authors also highlighted that 1,8-cineole was found at high level in Fer wines from the Marcillac wine region, we can assume that IBMP might play a sensory role in 2016, notably in the control wine whose level of concentration is just below the 15ng/L threshold.

2. Effect of the techniques

The time necessary to remove bunch zone leaves on one side of the row and lateral shoots was estimated at 43 ± 2 hours per hectare and 93 ± 6 hours per hectare, respectively.

Yields at harvest and mass of 200 berries were not impacted by the techniques. Carbohydrate supply at flowering is a key driver of berry set and berry development (Coombe, 1962). For the removal of lateral shoot treatment, this observation was expected because shoots were removed early before bearing two adults leaves and becoming source organs (Kliewer, 1970). For defoliation, this absence of effect is somewhat unexpected, as Kliewer (1970) showed that defoliation after berry set affected berry size. According to this author, the decrease in berry size is more intense when defoliation is performed early just after berry set. Therefore, it appears that the timing of defoliation applied in our experiment 10 days after berry set is appropriate to avoid any crop loss through berry size reduction.

A decrease in yield would have been expected for delayed harvest, a practice which was likely to induce a crop weight loss through water evaporation. Even if the xylem flow is decreased after veraison and the berry volume is less sensitive to soil moisture (Bondada *et al*., 2005), the transpiration must have been compensated by the large amount of rainfall recorded on the site between the two harvest dates (26 mm in 2015 and 24 mm in 2016). This water accumulation into the berry could explain the decrease of sugar concentration, TPI and anthocyanins, the changes in acidity (i.e. titratable acidity, malic acid and pH) and in potassium (in 2015 only) for this treatment. In addition, the decrease in anthocyanins could be related to the drop of temperature and to the lower level of water deficits experienced by the vine after the first harvest (Roggero *et al*., 1986; Somers, 1976). The reasons why a decrease in potassium was not observed for the delayed harvest treatment in 2016 remain unclear.

Leaf removal and the removal of lateral shoots had no significant impact on sugar levels which suggests that the vine can compensate the loss in foliar surface induced by these practices. Indeed, most of the researches indicates that compensation can take place through i) a stronger development of lateral shoots for defoliated vines (Kliewer and Fuller, 1973), ii) an increased photosynthesis efficiency of the main leaves when lateral shoots are removed (Candolfi-Vasconcelos *et al*., 1994), and iii) the translocation of sugars from starch mobilisation in roots and woody fractions to the berries (Zufferey *et al*., 2012).

TABLE 5. Effect of leaf removal and removal of lateral shoots on bunch surface temperature (mean and standard deviation of 30 observations) and results of the ANOVA.

Time of measurement (TM)	Side of the row (S)	Block (B)	Bunch surface temperature (°C) Control	Leaf removal	Removal of lateral shoots	*P*-value TM	S	B	T	TM × S	TM × B	TM × T	S × B	S × T	B × T
1200 h	East-facing	1	19.46±1.89	20.54±1.80	19.69±1.47	<0.001	<0.001	<0.001	0.120	<0.001	<0.001	0.150	<0.001	0.587	0.002
		2	18.38±1.16	19.85±1.24	20.35±1.63										
		3	16.09±1.18	16.41±1.39	15.73±1.40										
	West-facing	1	20.31±1.29	20.62±0.91	21.06±1.29										
		2	20.09±0.86	20.36±0.94	20.70±1.22										
		3	18.30±0.79	17.45±0.73	18.25±1.87										
1600 h	East-facing	1	26.15±0.96	25.89±0.69	25.23±0.64										
		2	26.16±0.77	25.79±0.87	25.97±0.92										
		3	25.66±0.64	25.19±0.63	25.69±0.85										
	West-facing	1	30.02±3.09	29.43±3.61	28.19±2.70										
		2	29.83±3.02	31.01±2.59	29.62±2.42										
		3	28.88±2.80	30.18±3.27	31.76±2.77										

Surprisingly, malic acid and in most cases potassium were increased for the defoliated treatment. The increase in berry temperature and the lower foliar density should have enhanced malate degradation and led to a lower potassium content, respectively (Kliewer and Bledsoe, 1987; Smart, 1985; Wolf *et al.*, 1986). The gains observed in our experimental conditions could be attributable to a higher lateral shoot growth. For malic acid, the absence of impact of leaf removal on bunch surface temperature (Table 5) and bunch air temperature between veraison and harvest (i.e. mean and maximal values, cool night index) might also have played a role. This could be explained by the specific features of the experimental site (presence of a hedge of trees on the southern border, steep slope and row orientation) and the development of newly emerged leaves, which must have minimized the penetration of solar radiation within the bunch zone. This likely low impact of solar radiation on berries is also supported by the anthocyanins concentrations that should have increased in both vintages (Tardáguila *et al.*, 2008). However, a gain in anthocyanins was only observed in 2015. During the late 2016 vintage, we can assume that the high solar azimuth angle during the late maturation period limited berry illumination and prevented the pigment synthesis. Trivially, our results also indicated that bunch surface temperature increased between 12:00 and 16:00 h and was higher (in the afternoon only) and more variable as reflected by standard deviation values in the side of the row exposed to direct sun exposure (Table 5) which is relevant with previous researches (Moffat *et al.*, 2013). The reasons why bunch temperature was lower on the east-facing side of the row at 12:00 remain unclear. In most cases, bunch temperature was greater on blocks 1 and 2 that are distant from the hedge of trees.

The removal of lateral shoots had no effect on acidity components and anthocyanins, which also suggests that the technique had a weak impact on bunch microclimate and notably berry temperature as reflected by bunch microclimate measurements. As this technique has been little studied, it remains difficult to generalise these findings due to the specific features of our experimental site (see discussion above).

Leaf nitrogen status (NBI) determined between veraison and harvest did not make it possible to discriminate the treatments. In grapes, the greatest amino acids content was noted for the delayed harvest treatment, which is in accordance with previous research highlighting an increase in amino acids over the maturation period (Kliewer, 1968) and particularly in cool night climatic conditions (Gutiérrez-Gamboa *et al*., 2018).

In most cases, ammonium content in berries was lowered for leaf removal. More than half of the berries' nitrogen is imported after veraison through the phloem (Keller, 2015). We can assume that the technique induced a decrease in the source-sink ratio which has contributed to lower ammonium in berries. There remains no explanation as to why a loss in amino acids, and losses in amino acids and ammonium were not observed for leaf removal and the removal of lateral shoots treatment, respectively.

Leaf removal as well as the removal of lateral shoots induced an increase in stem water potentials, which could be the consequence of a lesser loss of water through transpiration. These differences in water status were not confirmed through measurements of $\delta^{13}C$.

In 2016, IBMP concentrations were not impacted by the treatment factor at $P<0.05$ but differences were observed at $P=0.06$. The removal of lateral shoots and the delayed harvest treatments showed the lowest IBMP concentrations (-24% and -26% in comparison with the control treatment, respectively). IBMP in wines from the leaf removal treatment did not differ significantly from the control, however a trend of lower levels was found (-12%). These conclusions are consistent with previous findings for delayed harvest (Roujou de Boubée *et al*., 2000, Ryona *et al*., 2010) but deserve further discussions for leaf removal and removal of lateral shoots. As in our experimental conditions, berry temperature did not allow to discriminate these two practices, the quantity of light received by the berries is expected to be the main determinant of IBMP in wines. As discussed previously, we can suppose that the development of newly emerged leaves and lateral shoots limited the penetration of light through the canopy and IBMP photodegradation for the defoliated treatment in comparison with the removal of lateral shoots treatment.

For rotundone, the largest gain was observed for the delayed harvest treatment (+121% in 2015 and +120% in 2016) while levels in wines from the leaf removal treatment showed intermediate results in trend (+71% in 2015 and +43% in 2016). Rotundone accumulation during grape maturation is known to increase steadily before plateauing (Geffroy *et al*., 2014; Zhang *et al*., 2015). In the southwest of France, this plateau was reached for Duras cultivar 44 days after mid-veraison (Geffroy *et al*., 2014). In our experimental conditions, the significant increase observed between the two harvest dates indicates that maximum concentrations were not yet reached 54 days after mid-veraison. According to Zhang *et al. (*2015), the rotundone plateau is higher and reached earlier during cooler and wetter growing seasons. We can suppose that the hot climatic conditions observed during maturation in 2015 and to a lesser extent in 2016 might have contributed to delayed rotundone accumulation. Therefore, we can not completely exclude that no gain in rotundone would be observed for the delayed harvest treatment under less hot weather conditions.

The observations on leaf removal somewhat contradict previous research highlighting a decrease in the grape rotundone concentration for defoliated vines (Geffroy *et al*., 2014) but are in agreement with other findings emphasising an increase (Homich *et al*., 2017). This non-significant increase cannot be imputable to a stress experienced by physical wounding (Zhang *et al*., 2016), or to a lesser level of water deficit as the removal of lateral shoots, which induced the same effects, had no impact on rotundone. Recently, Geffroy *et al*. (2019) suggested that light received by the berries could stimulate the production of rotundone. However, if this were to be the mechanism involved a larger gain would have been observed for the removal of lateral shoots treatment, which seems more favourable to light penetration (see discussion above). However, the removal of lateral shoots had no impact on rotundone. Further studies will be necessary to understand the mechanisms involved in rotundone production after defoliation.

When focusing on the ratio of OAV rotundone to OAV IBMP, the three studied techniques made it possible to significantly increase the ratio, with the largest gain observed for delayed harvest.

With only one year of data for IBMP and the ratio of OAV rotundone to OAV IBMP, we cannot exclude the fact that our results should be considered preliminary and that one extra year would be necessary to fully validate our observations. However, our findings appear particularly consistent from one vintage to the other for rotundone (expressed in % of control), which tends to confirm the strong repeatability and robustness of our measurements.

CONCLUSION

Our results indicate that common viticultural practices can be used to modify the composition in rotundone and IBMP of Fer wines. Delayed harvest appears to be the best practice to increase rotundone while decreasing IBMP. In our experimental conditions, this practice had no impact on yields and did not induce any extra costs. Its only drawback was to induce a decrease in anthocyanins. Defoliation, which in 2015 induced a gain in anthocyanins, is also a recommended practice to increase the ratio of OAV of rotundone to OAV of IBMP. In our conditions, 43 hours per hectare were required to perform defoliation, an operation that can be mechanised except for terraces. Despite its relative efficiency, the removal of lateral shoots is not an advisable technique to modify the aroma composition of the wines, as it required more than 90 hours per hectare of manual work.

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