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OENO One
VINE AND WINE OPEN ACCESS JOURNAL
By IVES International Viticulture & Enology Society

DOI: https://doi.org/10.20870/oeno-one.2022.56.3.5495

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

The sugarless grape trait characterised by single berry phenotyping

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Associate editor:
José Miguel Martínez Zapater

Received:
29 March 2022

Accepted:
2 June 2022

Published:
11 July 2022

ABSTRACT

In grape production, the selection of varieties well-adapted to climate fluctuations, especially warming, is based on achieving a balance between fruit sugars and acidity. In recent decades, temperature has been constantly rising during ripening causing excessive sugar concentrations and insufficient acidity in wine grapes in the warmest regions. There is thus an increasing interest in breeding new cultivars able to ripen at lower sugar concentration while preserving fruit acidity. However, the phenotyping of berry composition challenges both methodological and conceptual issues. Indeed, most authors predetermine either average harvest date, ripening duration, thermal time or even the hexoses concentration threshold itself to compare accessions at a hopefully similar ripe stage. In this study, we phenotyped the fruit development and composition of 6 genotypes, including 3 new disease-tolerant varieties known to produce wines with low alcoholic contents. The study was performed at single berry level from the end of the green growth stage to the end of phloem unloading, when water and solute contents reach a maximum per berry. The results confirm that sugarless genotypes achieve fruit ripening with 20-30 % less hexoses than the classical varieties, Grenache N and Merlot N, without impacting berry growth, total acidity or cation accumulation. The sugarless genotypes displayed a higher malic acid/tartaric acid balance than the other genotypes, but similar sucrose/H^+ exchanges at the onset of ripening. Data suggest that the sugarless phenotype results from a specific plasticity in the relationship between growth and the turgor imposed by organic acid accumulation and sugar loading. This opens interesting perspectives for the understanding of the mechanism of grapevine berry growth and for breeding varieties that will cope better with climate warming.

KEYWORDS: Fleshy fruit, fruit development, ripening, sugars, acidity, climate warming

INTRODUCTION

With a hexose concentration (glucose + fructose, [Hex]) of higher than 1.1 mol/l at the ripe stage, grape is one of the richest fleshy fruits in terms of sugars. [Hex] is known to depend on the GxExM interaction (Suter *et al*., 2021), and until recently the adaptation of cultivars to local conditions was thought to be related to the thermal time needed to reach specific vegetative and reproductive phenological stages, such as budburst, flowering or fruit veraison (Parker *et al*., 2020). The selection of grapevine varieties is mainly based on the balance between sugars and acidity (Torregrosa *et al*., 2017; Ollat *et al*., 2018; Duchêne *et al*., 2020). In cold climates, early ripening varieties are preferred in order to ensure the accumulation of sugars and secondary metabolites before autumn. Conversely, in warm regions, late ripening varieties which shift fruit ripening to cooler days preserve organic acids (Rienth *et al*., 2016), anthocyanidin (Zhang *et al*., 2015) and aroma compounds (Alessandrini *et al*., 2018; Asproudi *et al*., 2016; Gutiérrez-Gamboa *et al*., 2018). In practice, however, a range of oenological processes are implemented to correct the sugars in the must and/or its acidity, demonstrating that the supposed adaptation of the varieties based on thermal time phenology is oversimplified. Over the last decades, temperature during the period of grapevine fruit ripening has been constantly increasing, which can lead to excessive sugars and insufficient acidity in hot vine growing areas, such as Mediterranean regions (Santillán *et al*., 2019, Bécart *et al*., 2022). Given the strength and the rate of ongoing climate changes, it is critical to precisely define the development and the metabolism of fruits to accurately identify their adaptation potential (Bigard *et al*., 2018 and 2020). There is thus increasing interest in breeding new cultivars which can ripen at lower sugar concentration while preserving fruit acidity.

In fact, warming does not only accelerate the whole ripening process (which would be easily solved by harvesting earlier), but it also desynchronises different aspects of ripening, thus accelerating malic acid breakdown (Rienth *et al*., 2016, Sweetmann *et al*., 2014) and inhibiting the accumulation of secondary metabolites, such as anthocyanins; this leads to making the decision of shifting the harvest date to when grapes have a higher [Hex] (Arrizabalaga *et al*., 2018). Moreover, comparing cultivars at the same developmental stage (the so-called ripe stage) raises both methodological and conceptual issues. In most comparative studies, authors predetermine an average harvest date, ripening duration, thermal time or even [Hex] threshold to compare accessions at a hopefully similar ripe stage, which contradicts the recognised impact of GxE on these variables (Liu *et al*., 2007; Dai *et al*., 2011; Duchêne *et al*., 2012). To circumvent this inconsistency and lack of consensus, the moment at which berry phloem unloading stops was recently proposed as a relevant definition of ripe stage from both a physiological and transcriptomic point of view (Bigard *et al*., 2018; Bigard *et al*., 2020; Shahood *et al*., 2015; Shahood *et al*., 2020; Savoi *et al*., 2021). This key transition which marks the arrest of the most intensive fruit biochemical pathways is associated with the transcriptional extinction of many genes, encoding, amongst others, sugar transporters, aquaporins and cell wall-related enzymes (Savoi *et al*., 2021). Unfortunately, this developmental stage cannot be directly inferred from [Hex] kinetics, which continue to evolve after the completion of sugar storage (or accumulation) due to subsequent berry shrivelling. Thus, additional information on berry growth is required to address the net rates of sugar accumulation and malic acid breakdown in berries, together with their respective timings. Very recently, single berry phenotyping approaches improved our understanding of berry growth and metabolism during ripening, avoiding any biases resulting from berry asynchronicity (Shahood *et al*., 2020; Savoi *et al*., 2021). Based on this new paradigm, this study aimed to decipher the genetic differences existing for [Hex] and fruit acidity in a set of genotypes encompassing traditional varieties and new hybrids exhibiting low [Hex] at harvest (Escudier *et al*., 2017; Ojeda *et al*., 2017).

MATERIALS AND METHOD

1. Plant Material and sampling method

Experiments were performed outdoors at the INRAE Pech Rouge experimental unit (Gruissan, France, 43.14'/3.14''W) under a semi-arid Mediterranean climate in 2017 (temperature, rainfall and evapotranspiration data described in Alem *et al*., 2021). Experimental plots were managed through drip irrigation keeping the predawn leaf water potential (ΨPD) higher than -0.5 MPa (Giorgi & Lionello, 2008) to correspond to a moderate water stress. The set of the genotypes comprised 3 traditional varieties, Grenache N, Merlot N and Morrastel N (https://plantgrape.plantnet- project.org/fr/), and 3 new disease-resistant varieties derived from 4 (3197-81B, 3197-373N) or 5 (3184-1-9N) backcrosses of *Muscadinia rotundifolia* with *V. vinifera* varieties (Bouqu*et al*., 1980). These three latter genotypes are known to display a reduced [Hex] at harvest, enabling the production of wines at low ethanol levels, called VDQA, "***Vins De Qualité à teneur modérée en Alcool***" (Ojeda *et al*. 2017). Hereafter, the names G5, G7 and G14 will be used for 3197-81B, 3197-373N and 3184-1-9N respectively. On a weekly and random basis, 60 berries were collected during a period of 2 weeks before to 2 weeks after the first signs of berry softening, and 30 berries were collected during the rest of the ripening period over a period of 1 week after fruit shriveling. Whole berries were sampled between 09:00 and 11:00 by cutting the fruit peduncle just below the calyx; they were kept in a plastic bag in a cool place and analysed on the same day.

2. Berry firmness and composition

Firmness was monitored with a digital penetrometer called Pénélaup™ (Abbal *et al*., 1992; Robin *et al*., 1997) as described in Shahood *et al*. (2020). Immediately after the firmness measurement, berries were immersed in 4 times their weight of 0.25N HCl. Seeds were immediately removed and samples incubated for 48h. Samples were vigorously shaken

and a first 100 L aliquot was diluted 11 times with 8.3 10^{-3} N acetic acid (internal control) + 16.4 10^{-3} N sulphuric acid and centrifuged for 5 min at 20°C at 18,500 g; supernatants were injected by HPLC to quantify glucose, fructose, malic and tartaric acids using a Biorad aminex-HPX87H column, which is also described in Shahood *et al*. (2020). A second 100 L aliquot was diluted 10 times in water and then centrifuged for 3 min at 12000 rpm (20 °C). Next, 10 µl of clear supernatant was injected into the HPLC using a Waters® IC-Pak Cation M/D 3.9x150 mm column with the same parameters used in Bigard *et al*. (2020), in order to obtain Potassium ($[K^+]$), Magnesium ($[Mg^{2+}]$) and Calcium ($[Ca^{2+}]$) concentrations. Titratable acidity (TA) was calculated as the sum of malic and tartaric acids minus K^+ in $mEq.L^{-1}$.

3. Data normalisation and presentations

R® software (version 4.1.2) was used to build graphical representations and to analyse the data (R Core Team, 2017). Tests as shapiro.test ("stats", for normality) and bartlett.test (for variance equality) were always performed before the selection of the appropriate statistical test. Software packages were "ggplot2" (Version 3.3.5), "car" (Version 3.0-12) and "rcompanion" (Version 2.4.6).

RESULTS AND DISCUSSION

The grapevine displays small fruits clustered in grape bunches showing a huge internal asynchrony (Gouthu *et al*., 2014; Doumouya *et al*., 2014; Bigard *et al*., 2019; Shahood *et al*., 2020), as illustrated in the following figures. In previous studies, extrapolating single fruit metabolic traits from the average values observed for the population of berries led to biased kinetic interpretations and chimeric metabolic concepts. In the grapevine, where the berry is the only truly relevant physiological unit, the most accurate method would be to non-destructively characterise each single fruit kinetics (Castellarin *et al*., 2015; Savoi *et al*., 2021), which is only possible for morphological attributes (fruit colour and volume) and firmness. Regarding solute accumulation, hypodermal sampling led to excessively elevated fluxes, possibly resulting from injury to the berries, thus the validity of this result was questioned (Coombe, 1992). In previous studies, destructive sampling of density sorted berries or large sets of individual fruits allowed to get accurate physiological insights on grapevine fruit development (Rolle *et al*., 2013; Bigard *et al*., 2019; Shahood *et al*., 2020). Here, we have phenotyped the ripening of 3 new sugarless genotypes (G5, G7 and G14) and 3 traditional varieties (Grenache N, Merlot N and Morrastel N) though the destructive

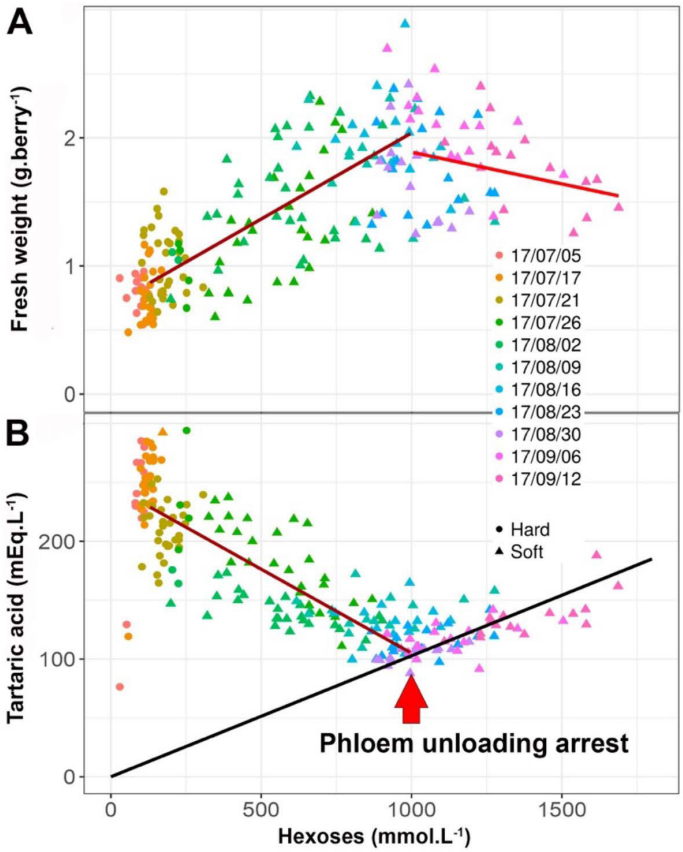

FIGURE 1. Evolution of fresh weight (A) and tartaric acid concentration (B) of single berries of Morrastel during ripening. Each dot corresponds to a single hard (solid circle) or soft (triangle) berry. Solid lines correspond to successive linear regressions for soft berries before and after phloem unloading arrest (indicated by the red arrow). In Figure 1B, a regression fit between the origin and all points beyond the arrow is displayed.

chemical analysis of thousands of single berries, as in Shahood *et al*. (2020). Since it was not possible to record each individual flowering or softening date, data are interpreted as a function of berry sugar concentration, a proxy for the internal time of fruit ripening (Rienth *et al*., 2016).

1. Berry development and sugar accumulation

Figure 1 shows the evolution of berry weight and tartaric acid concentration during the ripening of Morrastel N, which is representative of the panel of varieties described in this study. Observed trends are typical of *V. vinifera* genotypes, with nearly double the berry weight and a twofold dilution of tartaric acid during ripening. As described in Rienth *et al*. (2016) and in Bigard *et al*. (2020), the dilution of tartaric acid appears to be a relevant indicator of berry relative growth, as its quantity does not evolve during or after ripening (Ruffner, 1982; Lang and Thorpe, 1989; Terrier and Romieu, 2001; Rienth *et al*., 2014; Rösti *et al*., 2018; Burbidge *et al*., 2021). When the uploading of sugars and water in the berries stops (Coombe & McCarthy, 2000; Conde *et al*., 2007; Savoi *et al*., 2021), hexoses and tartaric acids just continue to concentrate due to evaporation. In theory, evaporation necessarily results in a straight line passing through the origin when each solute concentration is plotted against each other, as can be observed in Figure 1b. Tartaric acid concentration was obviously less scattered than fresh berry weight, thus facilitating the identification of the max berry weight stage. With this method, it was possible to determine the berry [Hex] at the maximum fruit weight for each variety; i.e., 920 mmol/L for G5, 900 mmol/L for G7, 800 mmol/L for G14, 1125 mmol/L for Grenache N, 1125mmol/L for Merlot N and 1000 mmol/L for Morrastel N. We used 10 berries which were the closest to each other for each genotype and both stages (end of green growth and max berry weight) to perform statistics as recorded in Table 1. For the end of the green growth period, the ten berries showing the highest malic acid concentration were selected.

Despite genotypic differences in berry weight at the green stage (Figure 2A), the 6 genotypes displayed classical fruit expansion kinetics during ripening (Coombe, 1976). The relative weight increment (i.e., Vripe/Vveraison) was obtained using all possible combinations of green- and ripe-selected berries, ranging from $1.8^{+/-}0.5$ to $3.1^{+/-}0.7$ for Merlot N and G5 respectively.

Regardless of the genotype, we observed considerable heterogeneity in berry size at a similar [Hex], as previously reported (Gouthu *et al*. 2014; Vondras *et al*., 2016; Bigard *et al*. 2019; Shahood *et al*., 2020). Both the maximum weight of the berries and [Hex] at this stage varied depending on the variety (Figure 2A). [Hex] increased during ripening, from values of around 100 mmol/L at the end of the green growth phase to 0.8 - 1.1 mol/L at the physiological ripe stage (Table 1). During berry ripening, at around 0.5 M each, glucose and fructose became the major osmoticums, as has been previously reported for *V. vinifera* (Hawker *et al*., 1976; Liu *et al*., 2006; Shiraishi *et al*., 2010). The [Hex] observed in this study at phloem arrest for Merlot N and Grenache N is lower than the usual concentration threshold of 1.2 - 1.5 mol/L [Hex], at which the industry considers the berries as technologically ripe. Kliewer (1967) reported

TABLE 1. Berry weight, firmness, sugar concentration and acidity of the 6 studied genotypes at the end of the green growth phase and at the physiological ripe stage. FW (Fresh weight), Text. (Texture), Hex (Hexoses), TA (Tartaric acid), MA (Malic acid). Statistical significance: * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$), na ($p > 0.01$).

Genotype	Stage	FW ($g.berry^{-1}$)			Texture ($g.mm^{-1}$)			Hexoses ($mmol.L^{-1}$)			Acidity ($meq.L^{-1}$)		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
G14	Green	1.3	0.4	ab	1993	428	ab	66	11	a	576	23	ab
G5		0.9	0.1	cd	1911	331	ab	73	15	ab	712	54	c
G7		1.3	0.2	a	1683	605	ab	110	55	bcd	705	62	c
Grenache		1.1	0.3	abc	1861	446	ab	99	13	cd	562	38	a
Merlot		0.9	0.2	bcd	2088	431	b	78	17	abc	629	37	bd
Morrastel		0.7	0.2	d	1490	356	a	122	14	d	655	16	cd
Test & Post-Hoc Genotype effect		Kruskal-Wallis & Dunn $4\ 10^{-5}$ ***			Anova & Tukey $5\ 10^{-2}$ *			Kruskal-Wallis & Dunn $2\ 10^{-5}$ ***			Kruskal-Wallis & Dunn $1\ 10^{-8}$ ***		
G14	Ripe	2.5	0.3	b	163	79	na	809	33	a	80	25	a
G5		2.5	0.4	bc	152	41	na	918	13	b	90	36	a
G7		3.1	0.7	c	196	59	na	905	17	ab	128	39	a
Grenache		2.2	0.5	ab	184	33	na	1130	23	c	116	37	a
Merlot		1.6	0.3	a	217	89	na	1123	9	cd	104	36	a
Morrastel		1.8	0.4	a	154	43	na	997	9	bd	116	47	a
Test & Post-Hoc Genotype effect		Anova & Tukey $3\ 10^{-8}$ ***			Anova & Tukey $1\ 10^{-1}$			Kruskal-Wallis & Dunn $2\ 10^{-10}$ ***			Anova & Tukey $5\ 10^{-2}$ *		

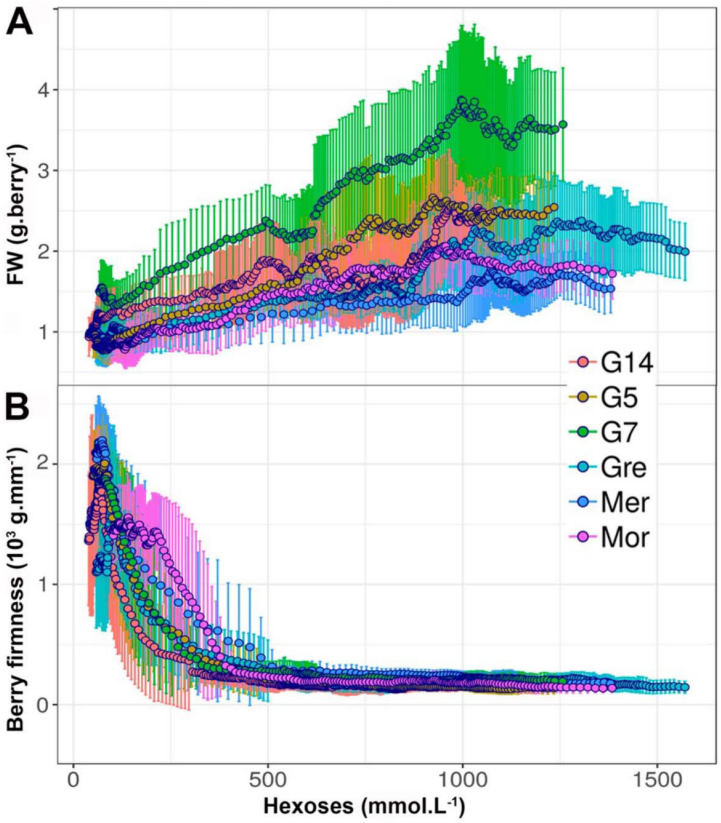

FIGURE 2. Evolution of berry fresh mass (A) and firmness (B) during the fruit ripening of 6 grapevine varieties. Each dot represents the average of sets of 10 berries ranked according to their hexose concentration (bars on each dot represent the standard deviation).

the range of 1 mol/L- 1.5 mol/L [Hex] as being the technical ripeness threshold for usual *V. vinifera* varieties. This apparent discrepancy is due to the very common practice of pushing grapes towards over-ripeness to obtain redder, fuller and more aromatic and concentrated wines (Antalick *et al*., 2021). In the absence of supplementary physiological landmarks, the use of [Hex] for comparative studies is very hazardous, as this parameter steadily increases after phloem unloading arrest because of fruit shriveling (Friend *et al*., 2009; Shahood *et al*., 2020; Figure 2A).

Sucrose unloading in berries of all genotypes dramatically increased at softening, or at relaxation of turgor pressure (Figure S1). All genotypes displayed a glucose/fructose higher than 2.2 before fruit softening, then the ratio progressively converged to 1, as reported for other *V. vinifera* varieties (Varandas *et al*., 2004). No specific metabolic trends were observed for the G genotypes. It is known that the berry glucose/fructose balance, which depends on grapevine organs and the developmental stage, can be used as a metabolic indicator of fruit ripening (Kliewer *et al*., 1966). During green growth, the preferential use of fructose is obvious, leading to elevated G/F ratio. At softening, the import of sucrose dramatically accelerates, exceeding metabolic needs, insofar as malic acid replaces sugar as a respiratory substrate; consequently, the G/F ratio rapidly becomes 1 (Amerine and Thoukis, 1958; Liang *et al*., 2011; Houel *et al*., 2015).

For a range of extreme varieties and offsprings, Bigard *et al*. (2018) showed that [Hex] can vary from 750 to 1350 mmol/L when solute unloading stops just before berry shrivelling. In the present study, when phloem arrest was considered as the physiological ripe stage, the G genotypes displayed [Hex] levels of between 0.8 and 0.9 mol/L, while Morrastel N showed levels of 1 mol/L, and Merlot N and Grenache N levels over 1.1 mol/L (the highest). These data agree with previous results obtained at the whole berry population levels with similar genotypes (Ojeda *et al*., 2017; Bigard *etal*., 2019). Interestingly, the final quantity of sugar per fruit unit in sugarless genotypes is of the same magnitude as classical varieties; i.e/, 2.8 +/- 0.3 mmol (G7), 2.5 +/ 0.6 mmol (Gre), 2.3 +/- 0.4 mmol (G5), 2.0 +/- 0.3 mmol (G14), 1.8 +/- 0.3 mmol (Merlot) and 1.8 +/- 0.4 mmol (Morrastel) per berry.

Before softening, some differences were found in terms of berry firmness at the end of the green growth phase, with Morrastel N and Merlot N showing the least and the most firm fruits respectively (Table 1). From the green growth phase onwards, the mechanical properties of the berries evolved in the same way for all three genotypes (Figure 2B): the berries softened rapidly at the beginning of ripening to reach a low level of firmness until 500 mmol.L^{-1} of [Hex];

i.e., at more or less mid-ripening. This was followed by a slow and continuous decrease in firmness up to the physiological ripe stage and over-ripening, during which no differences in firmness were observed between the sugarless genotypes and traditional cultivars (Table 1, Figure S1). Therefore, although firmness is widely accepted as a sensitive and early indicator of the onset of ripening (Coombe *et al*. 1992; Abbal *et al*., 1992; Castellarin *et al*., 2015; Shahood *et al*., 2020; Bigard *et al*., 2020), this parameter cannot be used to tag the transition at the arrest of phloem unloading. Consequently, the only way to non-destructively determine the shift from fruit expansion to shrivelling remains the monitoring of berry growth. As discussed below, this can be done indirectly using tartrate concentration.

2. Evolution of the main determinants of fruit acidity

2.1 Tartaric acid

During ripening, tartaric acid concentration depended on the variety and the stage of berry development (Table 2). At the onset of ripening, tartaric acid concentration was 25 % lower (150 vs 200 mEq/L) in all G genotypes. Tartaric acid dilution (Figure S2) proceeded at a negligible rate until 220 mmol/L (G5, G14) to 300 mmol/L [Hex] (Grenache N) and then accelerated, confirming the delay between berry softening and growth resumption (Castellarin *et al*., 2015; Shahood *et al*., 2020 and other literature cited in this paper).

The change in tartaric acid from the start to the end of ripening (Figure 3) is consistent with an initial two- to three-fold dilution followed by an increase in concentration due to shrivelling, leading to a linear increase in sugar and tartaric acid passing the origin. At the arrest of phloem unloading, the concentration of tartaric acid reached a minimum, ranging from 75 $^{+}/_{-}$ 10 mEq/L (G5) to 126 $^{+}/_{-}$ 18 mEq/L (Merlot N). Grenache N displayed a 104 $^{+}/_{-}$ 12 mEq/L concentration of tartaric acid at the ripe stage.

Tartaric acid is the first organic acid to accumulate during young berry development and remains one of the main acids in ripe fruits of *Vitis vinifera* (Amerine *et al*., 1965; Kliewer, 1966). In the single berry analysis of this study, tartaric acid ranged from 170 to 250 mEq/L at the beginning of ripening to decrease to 70-120 mEq/L at phloem stop, as previously observed (Bigard *et al*., 2019). The statistical analyses confirmed that at the onset of ripening sugarless genotypes already displayed a lower tartaric acid concentration than traditional cultivars. This trend is amplified at the phloem loading arrest (or maximum weight) stage due to the higher expansion and resulting dilution during the ripening of these genotypes (Table 1). Morrastel N, also called Graciano in Spain, is a traditional cultivar that produces wines rich in polyphenols with moderate ethanol levels (Ramos and Martínez de Toda, 2021). Our study performed at single berry level confirms that Morrastel N can produce ripe fruit at lower [Hex] (i.e., below 1 mol/L) in comparison to other traditional varieties.

2.2 Malic acid

Malic acid concentration peaked at 370-550 mEq/L at the very end of the green growth period and then decreased to less than 90 mEq/L at maximum berry volume regardless of

TABLE 2. Berry composition in major organic anions and inorganic cations of the 6 studied genotypes at the end of the green growth phase and at the physiological ripe stage. TA (Tartaric acid), MA (Malic acid), K (Potassium), Mg (Magnesium), Ca (Calcium). Statistical significance: * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$), na ($p > 0.01$).

Genotype	Stage	TA (meq.L^{-1})			MA (meq.L^{-1})			K (mmol.L^{-1})			Mg (mmol.L^{-1})			Ca (mmol.L^{-1})		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
G14	Green	172	16	a	447	10	a	43	6	b	1.9	0.6	a	4.9	2.7	na
G5	Green	209	33	abc	547	25	b	44	8	b	1.6	1.0	a	4.5	2.2	na
G7	Green	194	38	ab	552	30	b	42	5	b	2.8	1.1	ab	5.3	1.8	na
Grenache	Green	219	32	bc	375	27	c	31	5	a	2.7	0.9	ab	4.6	0.7	na
Merlot	Green	254	34	c	430	17	ac	55	5	c	3.7	1.0	b	5.4	1.6	na
Morrastel	Green	248	17	c	451	11	a	45	6	b	2.9	0.6	b	4.4	1.0	na
Test & Post-Hoc Genotype effect		Kruskal-Wallis & Dunn $2\ 10^{-5}$ ***			Anova & Tukey $9\ 10^{-10}$ ***			Kruskal-Wallis & Dunn $1\ 10^{-9}$ ***			Anova & Tukey $7\ 10^{-5}$ ***			Kruskal-Wallis & Dunn $6\ 10^{-1}$		
G14	Ripe	80	8	ab	57	26	ab	56	6	b	2.0	0.4	a	1.9	0.5	a
G5	Ripe	75	10	a	72	30	ab	57	4	b	1.8	0.4	a	2.6	0.7	ab
G7	Ripe	98	19	bc	87	27	b	57	4	b	2.9	0.3	b	2.2	0.6	a
Grenache	Ripe	105	12	cd	59	31	ab	47	8	a	2.7	0.5	b	2.8	0.8	ab
Merlot	Ripe	126	18	e	42	19	a	64	6	b	3.7	0.6	c	3.7	0.8	b
Morrastel	Ripe	121	22	de	59	27	ab	64	8	b	2.9	0.7	b	3.0	1.9	ab
Test & Post-Hoc Genotype effect		Anova & Tukey $4\ 10^{-10}$ ***			Anova & Tukey $1\ 10^{-2}$ *			Kruskal-Wallis & Dunn $5\ 10^{-7}$ ***			Anova & Tukey $2\ 10^{-11}$ ***			Kruskal-Wallis & Dunn $5\ 10^{-4}$ ***		

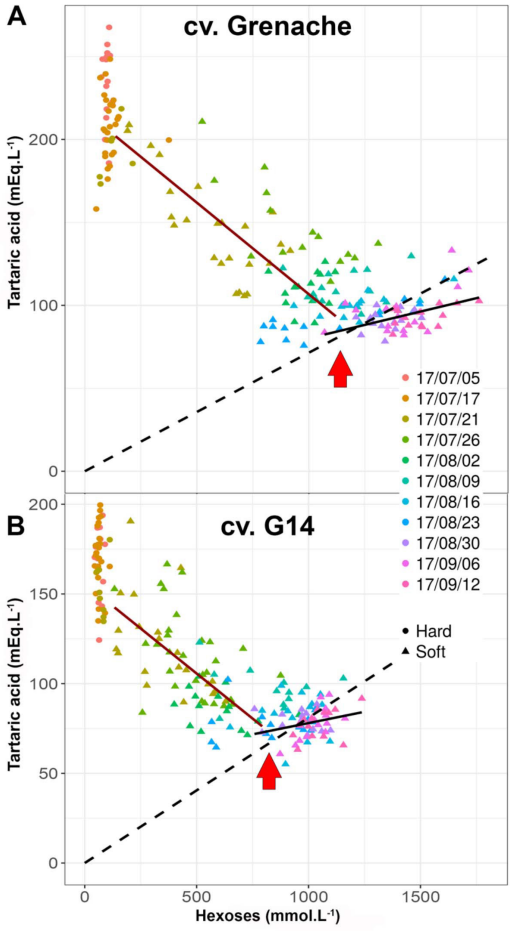

FIGURE 3. Evolution of tartaric acid concentration during the berry ripening of Grenache (A) and G14 (B). Lines correspond to linear fitting during and after phloem unloading. Each dot corresponds to a single hard (solid circle) or soft (triangle) berry. Solid lines correspond to successive linear regressions for soft berries before and after phloem loading arrest (3 latest dates). Hatched lines show the linear regression forced through the origin, as expected from evaporation alone, on all points after phloem arrest (red arrow).

the genotype (Table 1). At the onset of ripening, in contrast to tartaric acid, malic acid concentration was higher in sugarless genotypes than in traditional varieties. At the arrest of phloem unloading, the concentrations in malic acid ranged from 42 $^{+}/_{-}$ 20 mEq/L (Grenache N) to 87 $^{+}/_{-}$ 26 mEq/L (G7), with no obvious genotypic effects. After phloem arrest, in contrast to tartaric acid, malic acid concentration stayed stable or even slightly decreased (Figure 4), as observed in previous studies performed at berry population levels (Ojeda *et al*., 2017; Bigard *et al*., 2019).

Malic acid respiration provides a major fraction of the energic supply of the berry during early ripening (Famiani *et al*., 2014; Shahood, 2017), hence the decrease in concentration is faster than when only dependent on dilution due to berry growth. Here, despite different starting points (Table 2, Figure S3), the decrease in malic acid during the first phase of ripening (i.e., from 250 to 800 µmol/berry [Hex]) was characterised by an initial slope of -1 mEq per 2 hexoses; the slopes were noticeably similar for all 6 genotypes (Figure S3). During early ripening, the initial changes in the respective amounts of malic acid and sugar per fruit (concentration x weight; Figure 5) are fully consistent with the activation of a sucrose/H^+ exchanger on the tonoplast of all *V. vinifera* cultivars investigated, including sugarless genotypes, agreeing with previous observations for Syrah and Pinot (Shahood *et al*., 2020). In single berries, genes linked to sucrose transport are strongly expressed until phloem arrest (Savoi *et al*., 2021). At the beginning of ripening, the sucrose/H^+ exchange is electro-neutralised by the release of vacuolar malate, as detected here, while more and more H^+ must be redirected to the vacuole as malic acid vanishes, as illustrated by the progressive activation of vacuolar ATPase and PPiase (Terrier *et al*., 2001).

When the cumulative evolution of tartaric + malic acids was monitored during early ripening (Figure S4A), no specific behaviours were observed in the sugarless genotypes in comparison to the other varieties. Considering that, depending on their concentrations, sugars and organic acids are the main contributors to the osmotic potential of the berry (Matthews *et al*., 1987), the present results totally exclude

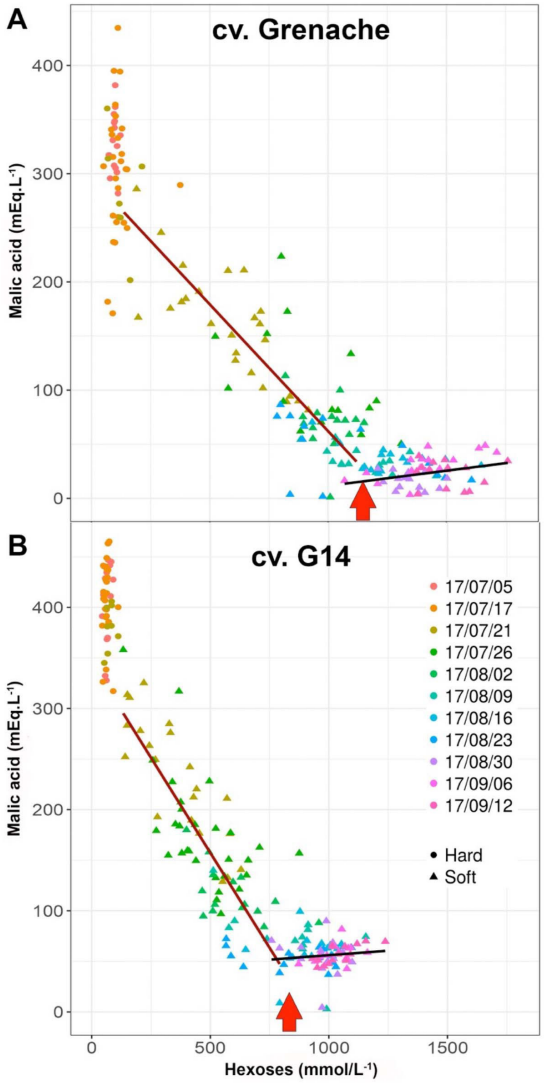

FIGURE 4. Evolution of malic acid concentration during the berry ripening of Grenache (A) and G14 (B). Lines correspond to linear fitting during and after phloem unloading. Each dot corresponds to a single (solid circle) hard or soft (triangle) berry. Solid lines correspond to successive linear regressions for soft berries before and after phloem loading arrest (the 3 last dates). Red arrows indicate the stage of phloem loading arrest.

the possibility of the reduction in sugar concentration being compensated for by a greater accumulation of organic acids in the sugarless genotypes. As already mentioned, sugarless genotypes displayed lower tartaric and higher malic acid than traditional controls and consequently a higher malic acid/tartaric acid ratio (Figure S4B).

2.3 Potassium

K^+, the main cation in the grapevine fruit, accumulates during both phases of growth (Cuellar *et al*., 2013). Here, concentrations before ripening ranged from 31 $^{+}/_{-}$ 5 mEq/L for Grenache N to 55 $^{+}/_{-}$ 5 mEq/L for Merlot N (Table 2). During ripening, [K^+] increased moderately (Figure S5) with increments ranging from 16 % (Merlot N) to 50 % (Grenache N) and both genotypes displaying the lowest and the highest levels of [K^+] at the ripe stage: 47 $^{+}/_{-}$ 8 mEq/L for Grenache N and 64 $^{+}/_{-}$ 6 mEq/L for Merlot N respectively. After the period of phloem loading arrest, [K^+] steadily increased as a result of water loss associated with shrivelling, as was the case for tartaric acid (Figure 6).

After the main organic compounds, K^+ is the fourth contributor to berry osmotic potential, which also neutralises a fraction of organic acids (Storey, 1987; Rogiers *et al*., 2017). During the early stages of development, low sugar accumulators (Morrastel N included) had a higher [K^+] than high accumulators (Table 2). The fact that this element mainly accumulates in the skin may play a role in the difference in elasticity of the G genotypes. At the end of phloem unloading the average [K^+] was around 50 mmol/L in the 6 genotypes, indicating strong homeostasis for this element. As observed at population level with other genotypes (Bigard *et al*., 2020), K^+ concentration increased 20-40 times less than hexoses during the phloem unloading period from veraison to max

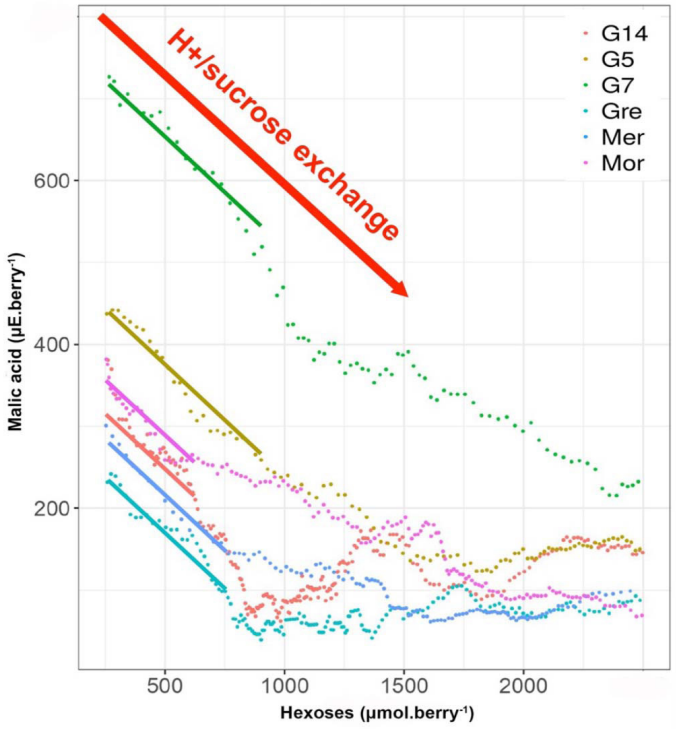

FIGURE 5. Repeatability of the malic acid/sugar exchange expressed in quantity per fruit during berry ripening of 6 grapevine varieties. Each dot represents the average of sets of 10 berries ranked according to their hexose concentration (error bars are not displayed to improve readability).

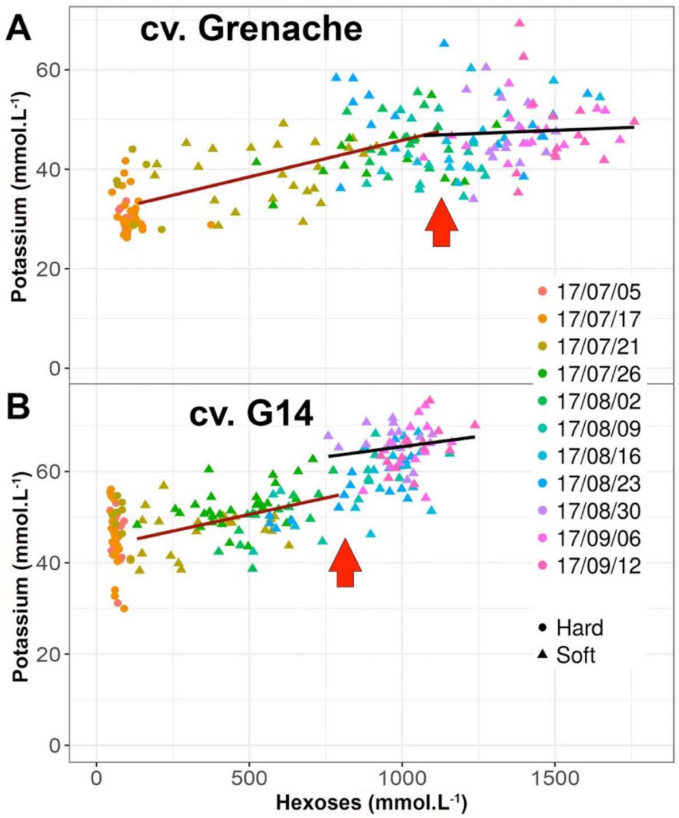

FIGURE 6. Evolution of potassium concentration during the berry ripening of Grenache (A) and G14 (B). Lines correspond to linear fitting during and after phloem unloading. Each dot corresponds to a single (solid circle) hard or soft (triangle) berry. Solid lines correspond to successive linear regressions for soft berries before and after phloem loading arrest (the 3 last dates). Red arrows indicate the stage of phloem loading arrest.

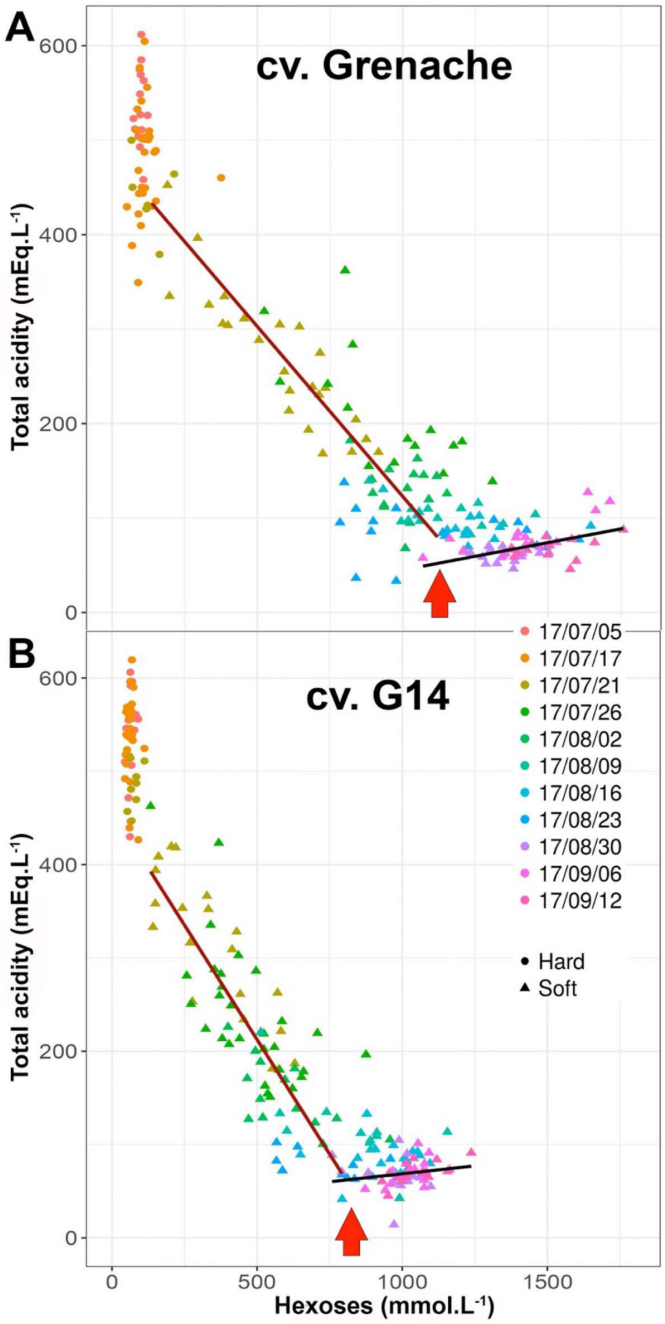

FIGURE 7. Evolution of juice total acidity during the berry ripening of Grenache (A) and G14 (B). Lines correspond to linear fitting during and after phloem unloading. Each dot corresponds to a single (solid circle) hard or soft (triangle) berry. Solid lines correspond to successive linear regressions for soft berries before and after phloem loading arrest (the 3 last dates). Red arrows indicate the stage of phloem loading arrest.

berry weight, as observed at population level with other genotypes (Figures 5 and S5, Table 2).

This obviously contradicts the so-called "massive" K^+ import into the ripening berry (Villette *et al*., 2020). As recently discussed by Savoi *et al*. (2021), the belief that K^+ transport will compensate for an intrinsic deficiency in the energisation of sugar imports is not supported by experimental data. In this respect, the simultaneous and parallel increases in [Hex] and [K^+] observed after the arrest of phloem unloading is not indicative of a co-transport mechanism, but only a result of net water loss and berry shrivelling. Despite significant progress in the understanding of the import of potassium into grapevine berries (Rogiers *et al*., 2017; Villette *et al*., 2020; Savoi *et al*., 2021), the putative mechanistic links between potassium and sugar imports remain speculative.

2.4 Evolution of fruit acidity

Green berries displayed high acidity (Figure 7, Table 1) ranging from 560 $^{+}/_{-}$ 40 mEq/L (Grenache N) to 710 $^{+}/_{-}$ 50 mEq/L (G5). Similar to tartaric acid dilution,

malic acid respiration and dilution, and K^+ accumulation, acidity was reduced to between 80 +/- 25 mEq/L (G14) and 130 +/- 40 mEq/L (G7) at the ripe stage, with no statistical differences between genotypes. Noticeably, total acidity tended to increase quite late during the ripening process for all genotypes: around 1250 mmol/L [Hex] in Grenache N, and 1000 mmol/L [Hex] in G14 (Figure 7).

Obtaining optimal grape acidity for the production of quality wines is a major challenge (Champagnol, 1984; Sweetman *et al*., 2014; Ollat *et al*., 2018). The effect of temperature on grape acidity is well documented (Kliewer and Lider, 1970; Butrose *et al*., 1971; Seguin *et al*., 2004; Rienth *et al*., 2016). By virtue of the electroneutrality principle, titratable (or total) acidity represents the difference between acids (mainly tartaric and malic in grapevine) and cations (mainly K^+ in plants). Bigard *et al*. (2018; 2020) and Duchène *et al*. (2020) have detailed genetic diversity for anions (i.e., organic acids) and cations and its consequence on grape acidity in a set of extreme *V. vinifera* varieties and offsprings. In the present study, we analysed the determinants of the acidity of 6 varieties, including 3 sugarless genotypes. As shown in previous sections, sugarless genotypes tended to display a higher malic acid/tartaric acid ratio than the 3 traditional cultivars, but they had similar contents in malic + tartaric acids and K^+. As a result, the sugarless genotypes showed similar levels of acidity at the same physiological ripe stage as other varieties.

3. Other cations (Mg^{2+}, Ca^{2+})

Far less Magnesium (Mg^{2+}) than K^+ accumulated in all the genotypes. [Mg^{2+}] displayed very few changes during ripening (Table 2, Figures S6 and S7). At the arrest of phloem unloading, [Mg^{2+}] ranged from 1.6 +/- 1.0 (G5) to 3.7 +/- 1.0 (Merlot N) mEq/L. Ranging from 4.5 +/- 2.2 (G5) to 5.4 +/- 1.6 (Merlot N) mE/L, a higher accumulation of Calcium (Ca^{2+}) was found in the green berries than of Mg^{2+} (Table 2); during ripening, [Ca^{2+}] tended to decrease (Figures S8 and S9), until it reached concentrations of 1.9 +/- 0.5 (G14) to 3.7 +/- 0.8 (Merlot N) mEq/L, which is a relative decrease quite comparable to that of tartaric acid. Therefore, its total amount per berry remains constant during ripening, which is widely accepted in grapevine, and consistent with its almost exclusive transport by xylem (Glad *et al*., 1992; Creasy *et al*., 1993). Neither cation had a major impact on wine quality or varied much within the range of varieties.

CONCLUSION

Obtaining fruits with reduced [Hex] while preserving their acidity is an option for mitigating the effect of climate warming on grapevine fruit quality (Torregrosa *et al*., 2017). This objective cannot be fully achieved by applying viticultural practices (e.g., harvesting before complete sugar unloading or removing a fraction of the leaves to reduce C photoassimilation) without impacting the quality of the wines (Bobeica *et al*., 2015; Antalick *et al*., 2021). Some diversity in water, sugars and the determinants of acidity of the grape can be found in *V. vinifera* varieties, or it can be obtained by crossbreeding (Bigard *et al*., 2018, 2020). In this study, using advanced methods of berry phenotyping we characterised the fruit development and ripening of a set of new disease-tolerant varieties producing low alcoholic wines (Escudier *et al*., 2017). In previous studies, we have shown that combining single fruit phenotyping and precise physiological landmarks can significantly improve the understanding of berry development (Shahood *et al*., 2020; Savoi *et al*., 2021). Indeed, in these conditions, studying the relationships between the main solutes are no longer biased by averaging unsynchronised, hence developmentally and metabolically chimeric, samples. In the present study, to circumvent the imprecision of berry growth curves resulting from the heterogeneity of berry size, tartaric acid dilution was used to detect the timing of phloem unloading arrest. The sugarless genotypes were found to display a [Hex] reduced by 20-30 % at the ripe stage without impacting berry growth, organic acid or cation accumulation levels. No major differences were found in terms of fruit growth rates and quantity of sugars per berry in comparison to the control varieties, suggesting that the sugarless phenotypes undergo a greater cellular expansion at similar osmotic or turgor pressure. This property is not specific to genotypes derived from *Muscadinia rotundifolia* and table grape varieties, because Morrastel N also displayed a limited [Hex] in the ripe fruit (< 1 mol/L). Moreover, similar behaviours can be found in other traditional varieties, such as Aramon, Cornifesto and Mandilaria (Bigard *et al*., 2018) and Glera, a variety used for Prosecco wine production (https://plantgrape.plantnet-project.org/fr/cepage/Glera). Taken together, our results show that adaptive traits to climate changes can be pyramidised with QTLs of tolerance to diseases. By crossing G5 and G14, we generated microvine segregating progenies (Torregrosa *et al*., 2019) to further characterise the physiology of this trait and to investigate the genetic determinism of water, sugar and organic accumulations (Savoi *et al*., 2021).

ACKNOWLEDGEMENTS

We would like to thank Eleoneora Maoddi, Yannick Sire and Philippe Abbal for their excellent contribution to the experiments.

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