Varietal differences in solute accumulation and grape development

Antoine Bigard

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VARIETAL DIFFERENCES IN SOLUTE ACCUMULATION AND GRAPE DEVELOPMENT

Présentée par Antoine BIGARD
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Sous la direction de Laurent TORREGROSA et Charles ROMIEU

Devant le jury composé de

Gregory GAMBETTA, Professeur, Bordeaux Sciences Agro	Rapporteur
Éric GOMES, Professeur, Université de Bordeaux	Rapporteur
René SIRET, Professeur, ESA d'Angers	Président du Jury
Markus RIENTH, Professeur, Changins, Suisse	Examinateur
Laurent TORREGROSA, Professeur, Montpellier SupAgro	Directeur de thèse
Charles ROMIEU, Chargé de recherche, INRA Montpellier	Co-directeur de thèse
Hernán OJEDA, Ingénieur de recherche, INRA UE Pech-Rouge (Gruissan)	Invité

UNIVERSITÉ DE MONTPELLIER

Montpellier SupAgro

Abstract

Until recently, varieties used for wine production (mainly *V. vinifera*) have been selected for high sugar accumulation and secondary metabolism compounds (aromas, tannins, anthocyanins). Some climate change parameters (temperatures, CO2) accentuate the trend towards higher sugar levels at harvest, resulting in more alcoholic wines. The increase in wine alcohol content is a global phenomenon, with annual increases of 0.16%. This alters the qualitative profile, in particular by impacting the alcohol/acidity balance and poses a problem for consumer health.

The grape is a non-climacteric fleshy fruit that develops in two phases. The first is the herbaceous phase during which malic and tartaric acids accumulate mainly accumulate. During this phase, the berry grows by mitosis and vacuolar expansion. The second phase of growth is associated with the massive import of hexoses, water and potassium. At the end of the second growth phase, the phloem stops unloading and the berry concentrate its main metabolites by evaporation. Some oenological practices make it possible to reduce the sugar content of must or alcohol wines content (CEE-606/2009 and CEE-53/2011), but are partial or costly and can have an impact on the wines quality. Cultivation practices do not sufficiently modify the development of the grape to be effective, except that they degrade the quality potential of the harvest. In the long term, the most promising approach is the variety selection.

The latter aspect was addressed in the thesis project. First, new phenotyping strategies/tools were developed to characterize grape development. Then, the diversity for the accumulation of primary metabolites in grapes (*V. vinifera*) or that can be generated by crossing with the microvine was analysed. In the last part, the physiological characterisation of genotypes resulting from a cross between *V. vinifera* and *M. rotundifolia* with a low sugar accumulation character during grape ripening was further developed.

The main results of this work indicate:

1) It is possible to assess the development of a berries population both in asynchrony (densimetric baths) and in heterogeneity (Dyostem). The colour of the fruit was not a good indicator of the beginning of ripening, appearing 1 to 5 days after the first signs of berry softening. In addition, monitoring at the berry population level has shown that for fine analyses, it is preferable to analyse the single fruit.

2) There is a great diversity in *V. vinifera* with regard to the composition of primary berry metabolites and their dilutions. The possibility of independently segregating the accumulation of water, sugars, acids and cations was revealed, opening up interesting prospects for varietal innovation.

3) Analysis of the low sugar concentration trait in descendants of *V. vinifera* and *M. rotundifolia* shows that this characteristic does not result from a limitation or delay in accumulation or from greater heterogeneity/asynchrony of the berries. The results suggest that there are mechanistic differences between growth level and osmotic pressure of the fruit during maturation between genotypes. This discovery raises many questions: are there differences in the cell wall structures or their associated enzymes? Are berry cells of low sugar genotypes larger or more numerous than traditional varieties?

Two of these descendants were crossed with the microvine to detect the associated QTLs to this trait in order to identify the functions controlling this agronomic interest trait.

Key words: growth, osmoticum, sugars, acidity, cations, physiological stage.

Résumé

Jusqu'à récemment, les variétés destinées à la production de vin (majoritairement *V. vinifera*) ont été sélectionnées pour forte accumulation en sucres et composés du métabolisme secondaire (arômes, tanins, anthocyanes). Certains paramètres du changement climatique (températures, CO_2) accentuent la tendance à l'augmentation des teneurs en sucres à la récolte donnant des vins plus alcooleux. L'augmentation de la teneur en alcool des vins est un phénomène planétaire, avec des augmentations annuelles de 0,16%. Cela altérant le profil qualitatif notamment en impactant l'équilibre alcool/acidité et pose un problème vis-à-vis de la santé des consommateurs.
Le raisin est un fruit pulpeux non-climactérique se développant en deux phases. La première est la phase herbacée durant laquelle s'accumulent majoritairement les acides maliques et tartriques. Durant cette phase, la baie grossit par mitose et expansion vacuolaire. La deuxième phase de croissance est associée à l'importation massive d'hexoses, d'eau et de potassium. A la fin de la deuxième phase de croissance, le phloème arrête son déchargement et la baie concentre ses principaux métabolites par évaporation. Certaines pratiques œnologiques permettent de réduire les teneurs en sucres des moûts ou en alcool des vins (CEE-606/2009 et CEE-53/2011), mais sont partielles ou coûteuses et peuvent impacter la qualité des vins. Les pratiques culturales ne modifient pas suffisamment le développement du raisin pour être efficaces, sauf à dégrader le potentiel qualitatif de la vendange. Sur le long terme, l'approche la plus prometteuse est la sélection variétale.
Ce dernier aspect fut abordé dans le projet de thèse. En premier lieu, de nouvelles stratégies/outils de phénotypage permettant la caractérisation du développement du raisin furent développés. Ensuite, la diversité pour l'accumulation des métabolites primaires dans le raisin (*V. vinifera*) ou pouvant être généré par croisement avec la microvigne fut analysée. Dans un dernier volet, la caractérisation physiologique de génotypes issus d'un croisement entre *V. vinifera* et *M. rotundifolia* présentant un caractère de faible accumulation en sucres durant la maturation des raisins fut approfondie.
Les résultats principaux de ce travail indiquent :
1) Il est possible d'apprécier le développement d'une population de baies tant en asynchronie (bains densimétriques) qu'en hétérogénéité (Dyostem). La couleur du fruit n'était pas un bon indicateur du début de la maturation, apparaissant 1 à 5 jours après les premiers signes de ramollissement des baies. Par ailleurs, les suivis réalisés à l'échelle de population de baies ont montré que pour des analyses fines, il était préférable d'analyser le fruit unique.
2) Il existe une grande diversité chez *V. vinifera* pour ce qui concerne la composition en métabolites primaires des baies et leurs dilutions. La possibilité de ségréger indépendamment l'accumulation d'eau, des sucres, des acides et des cations fut révélée, ouvrant d'intéressantes perspectives en termes d'innovation variétale.
3) L'analyse du caractère faible teneur en sucres chez des descendants de *V. vinifera* et *M. rotundifolia* montre que ce caractère ne résulte ni d'une limitation ou d'un décalage de l'accumulation, ni d'une plus grande hétérogénéité/asynchronie des baies. Les résultats suggèrent qu'il existe des différences mécanistiques entre niveau de croissance et pression osmotique du fruit lors de la maturation entre génotypes. Cette découverte soulève de nombreuses questions : existe-t-il des différences dans les structures des parois cellulaires ou de leurs enzymes associées ? Est-ce que les cellules des baies des génotypes à faible teneur en sucres sont plus grosses ou plus nombreuses que les variétés traditionnelles ?
Deux de ces descendants ont été croisés avec la microvigne pour détecter les QTLs associés à ce caractère dans l'objectif d'identifier les fonctions contrôlant ce trait d'intérêt agronomique.

Mots clés : croissance, osmoticum, sucres, acidité, cations, stades physiologiques.

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Acronyms and abbreviations

°C: Celsius degrees

Ψ_{PD}: Leaf water potential

ΔP_X: Xylemian gradient pressure

$Л_a$: Apoplasmic osmotic pressure

$Л_v$: Osmotic pressure

$[CO_2]$: Carbon Dioxide Concentration

CO_2 : Carbon Dioxide

DW: Dry Weight

E: Environment

EC: Enzyme Commission number

Eq or $eq.L^{-1}$: Equivalent per Litre

ESMs: Earth System Models

G: Genotype

g: Gravitational constant

GxE: Genotype x Environment (represents the interaction)

ha: Hectare

HCl: Hydrochloric Acid

HNO_3: Nitric Acid

M or $mol.L^{-1}$: Mole per Litre

meq: Millie-equivalent

mEq or $meq.L^{-1}$: Millie-equivalent per Litre

min: Minute

mM or $mmol.L^{-1}$: Millie-mole per Litre

mol: Mole

Mpa: Mega-Pascal (10^6 Pascal)

N: Equivalent concentration

N_2: Dinitrogen

O_2: Dioxygen

O_3: Ozone

pH : Potential of Hydrogen

ppm: Parts-per-million ($1/10^{-6}$)

RCP: Representative Concentration Pathway scenario

rpm: Revolutions per minute

SE: Standard Error

sec: Second

T°: Temperature

TA: Titrable Acidity

TPI: Total Polyphenol Index

TSS: Total Soluble Solid

µm: Micrometre

VPD: Vapour Pressure Deficit

Introduction

Introduction

In this section, several contextual aspects of the PhD will be exposed. First, the grapevine berry development will be presented for main solutes, i.e. primary and cations. Then, climate change will be described, with a focus on major impacts on vine phenology and wine production. Finally, an overview of the main techniques used to mitigate those consequences will be provided.

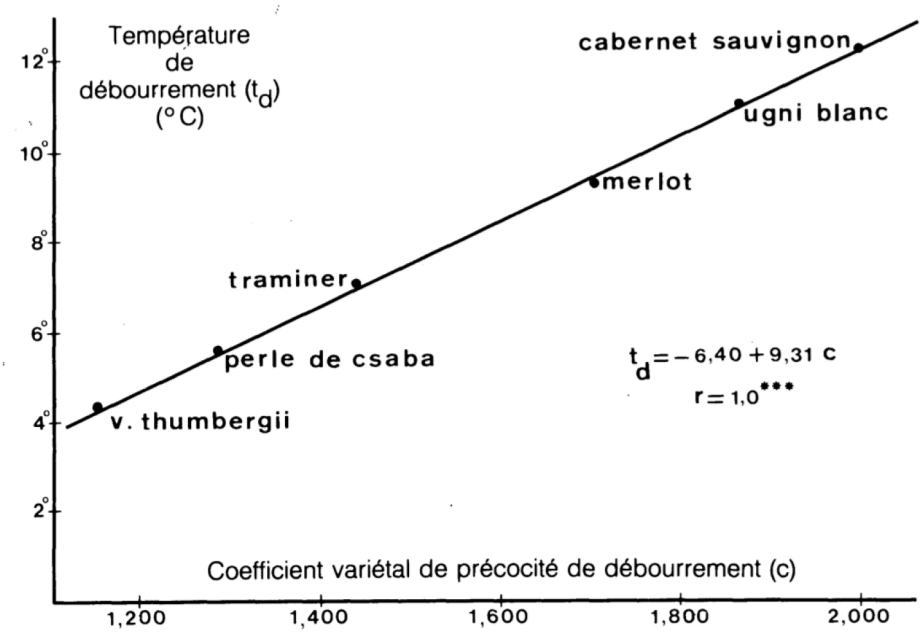
I. The grapevine fruit development

Grapevine which is one of the most worldwide and important cultivated plant with 7 535 917 ha planted in 2014, represents a yearly economic activity of about 11.4 billion Euros in France (www.franceagrimer.fr; www.oiv.int; Myles et al., 2011; Aleixandre et al., 2014). *Vitis vinifera* (family: Vitaceae – genus: *Euvitis*) (Winkler, 1962; This et al., 2006; Bacilieri et al., 2013; Adam-Blondon et al., 2016) is cultivated since long time with first signs of cultivation found in Georgia with the discovery of 8000 years old cultivated seeds (www.fao.org). Wine-grape is also one of the most if not the most sensitive crops to climate changes due to the determinant influence of meteorological factors on the organoleptic profile of the resulting wine (Webb et al., 2008; Mira de Orduña, 2010; Ollat et al., 2017).

I.1. The kinetic of berry development

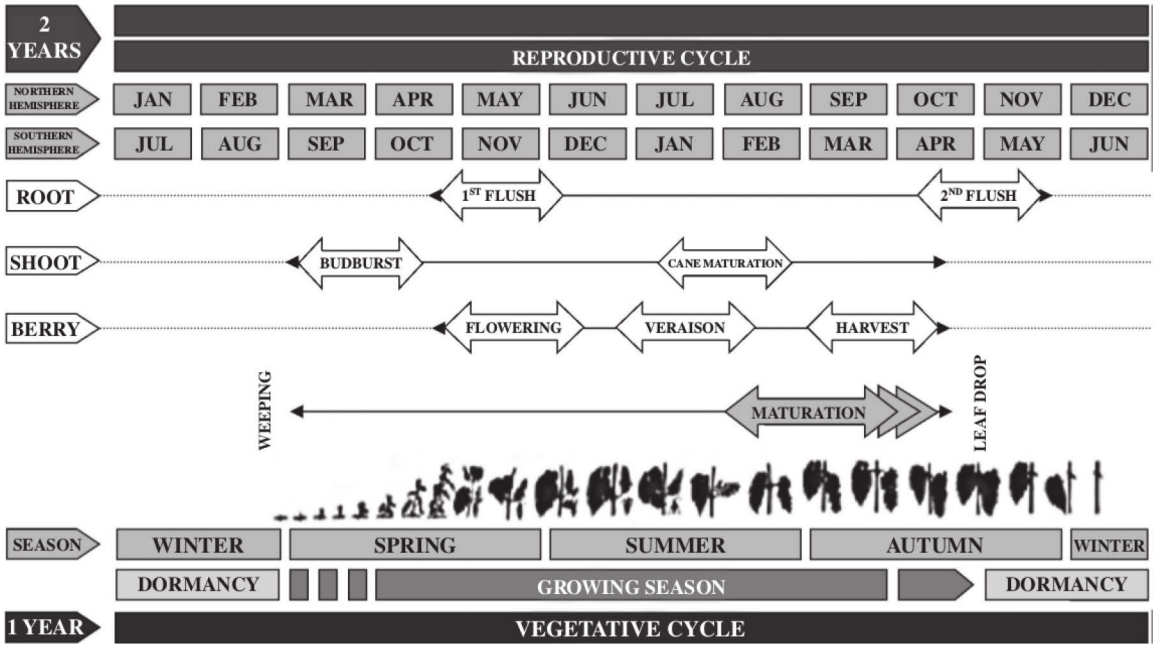
Grapevine is a perennial woody temperate-zone plant (Roubelakis-Angelakis, 2009). Vine development starts with bud-break and this require T° to break dormancy (Fraga et al., 2012, Carbonneau et al., 2015). Temperature needed is depending on varieties and can be correlated to precocity at harvest time (Figure 1) (Pouget, 1988).

Figure 1: Temperature needed for bud-break compared to varietal coefficient of precocity (higher it is, more early is the variety) (Pouget, 1988).



Then after bud-break for the reproductive organ, the following key stage is flowering (as represented in figure 2) (Carbonneau et al., 2015). The next stage is the veraison (fruit colour change) and the last one is ripe stage or harvest. At the end of August in average, bud dormancy is initiated and then when T° begins to slow-down, leaves are falling.

Figure 2: Vegetative and reproductive cycle for the vine associate to berry development (Fraga et al., 2012).



Grape is a non-climacteric fleshy fruit with a development characterised by a double sigmoid growth pattern including two growth phases (Coombe & Hale, 1973; Coombe, 1976; Mullins et al., 1992). Berry development starts with flowering and pollination which occurs between May and June in North hemisphere. Then, after the fruit-set, the first growth phase (green growth phase) can start. This first growth results from cell division and expansion (Ojeda et al., 1999). During this period, berry accumulates primary metabolites, in majority organic acids, mainly tartaric and malic acids (Kliewer, 1965). This phase is followed by a stage called veraison meaning colour change (Coombe 1992; Conde et al., 2007). After a second phase of growth (maturation or ripening period), mainly due to water uptake linked to sugars unloading, berry reaches it maximum volume as represented in figure 3 (also called physiological maturity in this document) (Dai et al., 2011; Keller, 2015; Bigard et al., 2018). After phloem unloading stops, the berry concentrates solutes by water loss (Coombe & McCarthy, 2000; Conde et al., 2007; Bondada et al., 2017).

The first growth phase starts with the development of the pericarp from ovary mesocarp through a short period of cell divisions triggered by fecundation (Coombe & Hale, 1973; Coombe, 1976). This phase, which is characterised by cells division and growth, lasts on average 60 days after anthesis at a population level (Vicens, 2007). Cell division occurs since the beginning of this phase

with a peak 5 days after anthesis and will end with this phase definitely determining the number of pericarp cells (Harris et al., 1968; Ojeda et al., 1999). During this developmental phase, the berry is green and hard, and accumulates tartaric and malic acids as the main contributors (69 to 92%) to berry osmotic potential (Terrier & Romieu, 2001; Conde et al., 2007; Keller et al., 2015), which reaches between 0.16 and 0.3 Mpa 50 days after anthesis (Thomas et al., 2008). Growth is then depending on seeds number, source-sink relations, phytohormonal control and environment (Ollat et al., 2002). Tartaric acid is predominantly accumulated at the very beginning of the green growth phase and can reach 300 meq.L^{-1} at the end of green growth phase in *V. vinifera* species (Champagnol, 1984; Bigard et al., 2018). Malic acid is latter accumulated until the end of the green growth phase and can reach 460 meq.L^{-1} at veraison in *V. vinifera* species (Champagnol, 1984; Bigard et al., 2018). During green growth phase, sugars are also accumulated (mainly glucose due to fructose higher metabolisation) to be directly used as energy to reach up to 150 mmol.L^{-1} (Davies et al., 2012; Houel et al. 2015; Bigard et al., 2018), with a minor contribution to fruit osmotic potential.

Calcium has also a maximum uptake rates during this phase and especially at the beginning when mitosis and respiration are fully activated (Mpelasoka et al., 2003; Bonomelli & Ruiz, 2010). This is probably related to it important structural role in the cell wall and membranes, or use as counter-ion for vacuole anions. Potassium will be accumulated slightly during this phase (Bashir & Kaur, 2018). Magnesium is accumulated during early stages of development as calcium (Duchène & Chardonnay, 1992). No information was found on ammonium accumulation during this stage except that it represents half of the nitrogen in the berry at this stage (Christensen, 2000).

After the first growth phase, berry will stop growing and starts softening (Robin et al., 1997; Castellarin et al., 2015), involving abscisic acid signalling (Kuhn et al., 2013; Pilati et al., 2017). This stage is called green stage (Thomas et al., 2008; Vicens, 2007). At this stage, berry pH is between 2.5 and 2.7 and fructose/glucose ratio between 0.5 to 0.1 (Champagnol, 1984; Varandas et al., 2004; Deloire, 2007), osmotic pressure is around 0.4 MPa (Thomas et al., 2006), and a lot of changes in genes transcripts are occurring creating cell wall modifications with metabolites synthesis, accumulation, degradation and accumulation pathway changes (Nunan et al., 2001; Marín-Rodríguez et al., 2002; Terrier et al., 2005). Softening, which is the first sign of ripening, occurs before colour change (Abbal et al., 1992; Robin et al., 1997; Castellarin et al., 2015). Using micro-arrays, Terrier et al. (2005) revealed that fifty-six transcripts putatively involved in cell-wall metabolism drastically changed in expression during berry development of Shiraz with four with an

expression related to cell-wall elongation in one of the clusters analysed (two beta-galactosidases (EC 3.2.1.23), a xyloglucan endotransglycosylase, XyloglucanEndoTransglucosidase, and a pectinesterase). Same observation was made in other fleshy fruits (Marín-Rodríguez et al., 2003; Vicens, 2007). Others enzymes such as alpha-galactosidase (EC 3.2.1.22), pectin methylesterase (EC 3.1.1.11), pectate lyase (EC 4.2.2.2), endopolygalacturonase (but activity not correlate with polygalacturonan content) and xyloglucan endotransglycosylase (EC 2.4.1.207) can be detected and play a role in cell wall modifications, polygalacturonase (EC 3.2.1.15) with mRNA detected may also play a role (Nunan et al., 2001). Cellulases are low in content and there is also trace of xyloglucanase which can be correlated to alkalisoluble and cellulosic polysaccharides loss during ripening but with no much impact on texture.

The pectate lyase catalyses the eliminative cleavage of de-esterified pectin inside the primary cell wall (Marín-Rodríguez et al., 2002). The presence of calcium is needed for depolymerizing cell-wall polygalacturonides and this action leads to the cell wall integrity loss. In strawberry, fully inhibition of pectate lyase gene expression permits to obtain firmer berries showing its important role for firmness in fleshy fruits (Marín-Rodríguez et al., 2003). Also in others fruits such as Banana, its activity was showed to be related to loss in firmness during maturity (Marín-Rodríguez et al., 2003). Its action on grapes during ripening, especially just after veraison, combined with polygalacturonase (principal enzyme responsible for pectin degradation in tomato) could explain the increased solubility of galacturonan in cell walls (Nunan et al., 2001; Marín-Rodríguez et al., 2003). The beta-galactosidase is an enzyme that could play a major role in the hydrolysis of cell wall type I arabinogalactan during berry development and softening due to its increasing activity just after veraison (Nunan et al., 2001). Alpha-galactosidase increases after veraison but its role stays unclear (Nunan et al., 2001). As describe by Nunan et al. (2001), there are two steps during softening, the enzymes first decrease the amount of galactose/galactan (mainly due to the beta-galactosidase). Then an increase of the water-soluble polysaccharides is noticeable (by possible action of pectin methylesterase, polygalacturonase and pectate lyases which mRNA is present particularly in the period immediately following veraison). This increase is combined with an increase in the amount of proline/hydroxyproline-rich proteins (reinforce the wall during berry expansion) in the cell walls.

All those cell wall modifying enzymes should lead to change cell-wall composition and structure together with berry texture (Nunan et al., 2001; Chen et al., 2015). However, Nunan et al. (2001) noticed few changes in non-cellulosic polysaccharides and cellulose inside grapevine berry cell

walls during softening. Showing that all those changes due to enzymes activities can't be directly correlated to higher or lower berry firmness. The cell wall structure itself as to be taken into account. For example, Ohanez variety is firmer than Gordo variety and it cells walls have significantly more cellulose, xyloglucans and hydroxyproline-rich proteins. Also, Gordo cells walls are enriched in polygalacturonans with more extensive pectic matrix phase. In that way both factors (enzyme activity and cell wall structure) must be taken into account to determine differences in berry texture added with the interaction of environmental components (Nunan et al., 2001; Marín-Rodríguez et al., 2003; Vicens, 2007; Zepeda et al., 2018). Even now what is determining berry softening is still unclear. For example Terrier et al. (2005) suggested that new enzymes and structural proteins candidate genes were not known yet, which shows that research must continue to understand the mechanisms of berry softening (Zepeda et al., 2018).

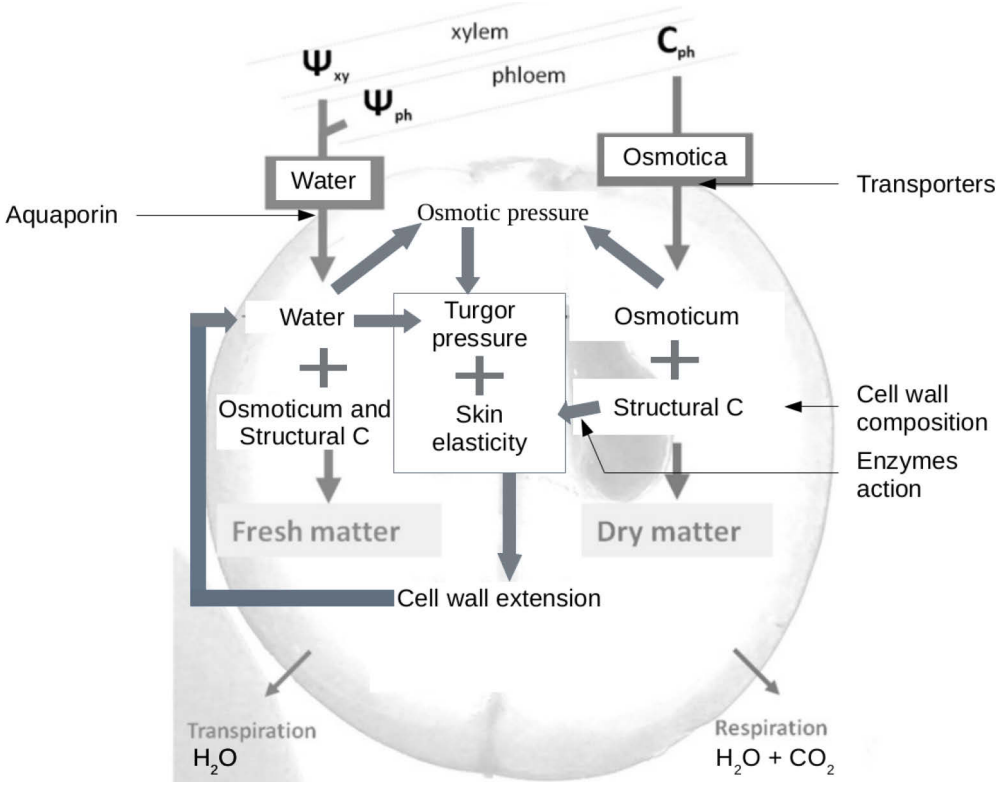
The maturation or ripening period which lasts between 40 and 50 days (McCarthy, 1999) starts immediately after softening at the population level. Since the beginning of this phase the berry has been accumulating sugars (glucose plus fructose in equal quantity) inside their vacuole cells and concentration can amount to 1.1 $mol.L^{-1}$ (Matthews et al., 1987; Varandas et al., 2004; Vicens, 2007; McCarthy, 1999; Xie et al., 2009; Duchêne et al., 2012; Bordenave et al., 2013; Bigard et al.., 2018). Malic acid is metabolised also really quickly and end up at maximum 100 mEq (Champagnol, 1998; Duchêne et al., 2013) for most varieties. Tartaric acid stays constant in quantity, but changes in concentration due to the dilution to 50 to 150 mEq at the end of this phase (Champagnol, 1998; Duchêne et al., 2013; Rösti et al., 2018; Bigard et al.,; 2018). During this phase, the fourth major osmoticum, Potassium, is accumulated faster than during first growth phase to 0.05-0.1$mol.L^{-1}$ (Storey, 1987; Rogiers et al., 2017) due to possible redistribution from leafs (Bashir & Kaur, 2018). Calcium is constantly accumulated (Bonomelli & Ruiz, 2010) but much lower level to arrive at 2.5 $mmol.L^{-1}$ (Mpelasoka et al., 2003) mainly concentrated in the skin (Duchène & Chardonnay, 1992). Magnesium is also present in higher amount in the skin (Conde, 2007), and rise up to 4.5 $mmol.L^{-1}$ at the harvest (Mpelasoka et al., 2003).

As seen above, berry development is complex and most studies characterized population of berries and by doing this, didn't take into account berry heterogeneity and asynchrony (Shahood, 2017).

Berry growth during this period is just due to cell enlargement (Matthews et al., 1987; Ojeda et al., 1999). Shahood (2017) suggested that a delay can be present between berry growth and sugars accumulation by comparing berry growth duration (15 to 20 days) to berry sugars accumulation

duration (about 30 days) at single berry level (Coombe, 1984; Ollat, 1997; Friend et al., 2009). This effect suggests an early accumulation without dilution, with consequence to increase osmotic pressure despite change in volume (Figure 3). This is might due to delay in skin extensibility as showed by Coombe (1984) Matthews et al. (1987), Huang & Huang (2001) and Shahood (2017). After this period, during ripening osmotic pressure due to sugars loading reach to -3 to -4 MPa keeping active the water gradient for water uptake to support cell enlargement (Diakou et al., 1997; Thomas et al., 2006; Xie et al., 2009).

Figure 3: Theoretical representation of the forces created with osmoticum accumulation in a berry. Adapted from Vivin et al. (2017).



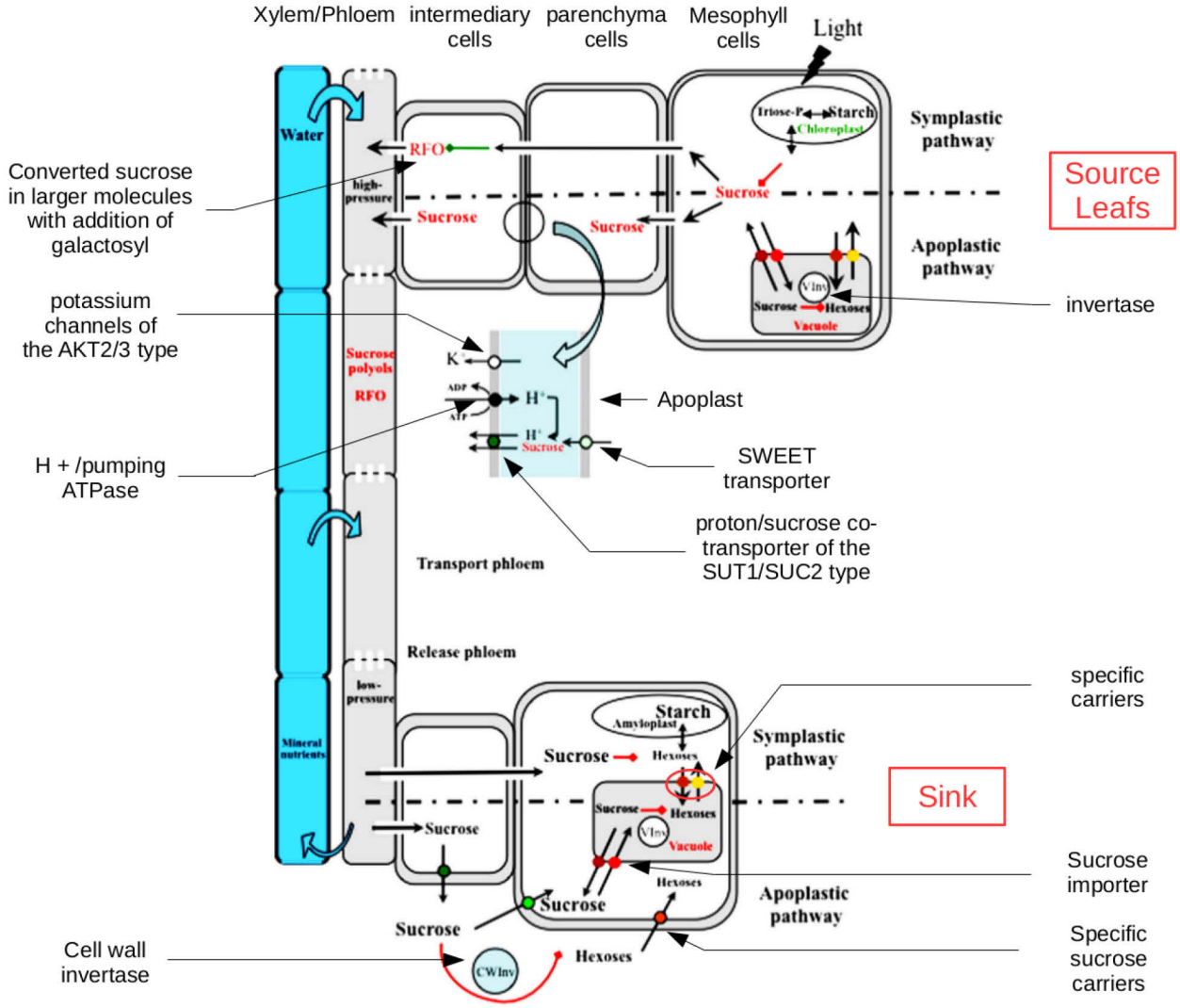
During development, two factors have to be taken into account to not misunderstand berry development and population development. The first factor is heterogeneity of the volume in a berry population. This factor is genotype dependant (Bigard et al., submitted) and may result from a large range of factors controlling inflorescence and fruit development (as seeds number), as fruit radiative and evaporative micro-environment (Pagay & Cheng, 2010; Böttcher et al., 2011; Dai et al., 2011; Houel et al., 2013; Kuhn et al., 2013; Doumouya, 2014; Reshef et al., 2017). It complicates the understanding of the interaction between the development of a single berry and a harvest (Nelson et

al., 1963; Lund et al., 2008; Böttcher et al., 2011; Rolle et al., 2013; Doumouya et al., 2014; Gouthu et al., 2014; Rienth et al., 2016). For classical grape development monitoring, at least 100 berries are commonly sampled to delete the effect of heterogeneity showing it important impact on results. The second factor, the asynchrony, is cumulated to the first one creating even more difficulties to understand fruit physiology such as systematic underestimation of water and solute flux (Coombe, 1984; Shahood, 2017). In this respect, research on gene expression for example or berry physiology are now performed on single berries (Lund et al., 2008, Shahood, 2017), or selected berries (density, volume, texture...) (Nelson et al., 1963; Singleton et al., 1966; Terrier et al., 2001; Carbonell-Bejerano et al., 2016; Friedel et al., 2016; Rienth et al., 2016) in order to minimize this effect. The delay can reach up to 14 days (Gouthu et al. 2014). For example, in previous studies sugars appears to be accumulated at 15-20 $\mu mol_{(hexoses)}.min^{-1}$ for a theoretical 1 kg harvest during maturation (McCarthy 1999) but this accumulation rate would be at least 2 times faster in single or synchronized berries (Coombe, 1984; Shahood et al., 2017). In the literature, kinetics of development systematically refer to the average composition of the fruit population, this protocol being inherited from grape monitoring as performed to determine the date of harvest. This approach is performed on batch of berries, not synchronized or unique berries as would be necessary for pertinent fruit physiology studies. It is implicitly assumed that all changes triggered on average population by GxE interaction reflect changes in berry metabolism, and changes in population structure are totally overlooked. Differences between individual berry and population of berries developmental patterns have to be clearly explicated in order to compare genotypes.

I.2. The accumulation of berry solutes

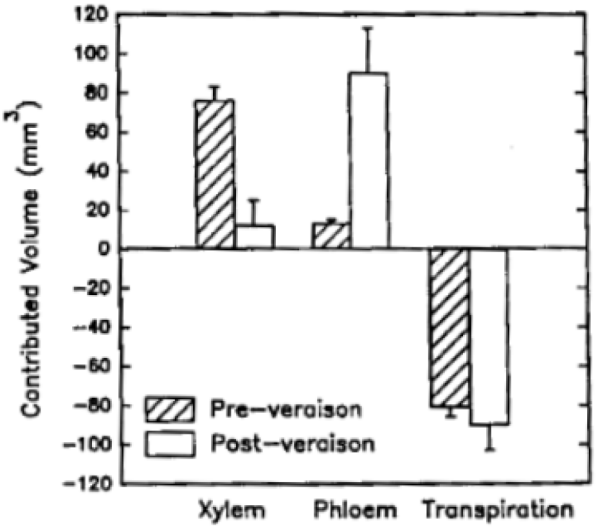
Since water transport is passive in plants, the berry needs first to accumulate solutes and develop an osmotic pressure in its vacuoles, in order to accumulate water, growth and development. Cell walls and peel has to be extensible to allow vacuolar enlargement. Phloem and xylem are the two main vascular tissues able to perform this continuous unload. Xylem is important for water conduction during green growth phase (Greenspan et al. 1994) and seems to be not functional in post-veraison (Bondada et al., 2005). Phloem is transporting photo-assimilates, mainly sucrose in grape-vine (Van Bel, 2003). Xylem and phloem are physically linked and exchange water, sugars, minerals and hormones (Van Bel, 1990; Metzner et al., 2010), each berry is linked to the plant by 5-6 peripheral and central vascular bundles. Exchanges between source and sink organs can proceed in two ways (cf. Figure 4). First one is the symplastic pathway that is characterised by a mass influx through plasmodesmata ensuring a cytoplasmic continuity between the phloem conductive bundles and the sink cells (Lalonde et al., 2003). The second road is the Apoplastic pathway, which is dominant after softening (Lalonde et al., 2003; Afoufa-Bastien et al., 2010). Assimilates are transported through plasma membranes and circulate in the apoplast. Sugars either follow their concentration gradient or are accumulated against it at energy cost, depending on different transporters.

Figure 4: Comparison of the symplastic and apoplastic phloem pathway for sugar transport in plants (adapted from Lemoine et al., 2013).



As the major component of fleshy fruits, water is primordial for berry growth and attributes concentration (Conde et al., 2007; Vicens, 2007; Keller et al., 2015). This water comes mainly from root (99 %) and circulates via both phloem and xylem (Greenspan et al., 1994, 1996; Ollat et al., 2002; Matthews & Shackel, 2005). Its quantity in fruits is highly dependent on climatic conditions (Jakab et al., 2013). During first growth period, water is mainly coming from xylem (88 %) (Greenspan et al., 1994, 1996; Choat et al. 2009) and then after softening, xylem flux decreases but remains functional (Keller et al., 2006; Rogiers et al., 2006; Tilbrook & Tyerman, 2009; Clearwater et al., 2012; Cuéllar et al., 2013; Keller et al., 2015; Zhang & Keller, 2017) so water import becomes mainly phloemian as represented in figure 5 (Chatelet et al. 2008 a, b; Choat et al. 2009; Dai et al. 2010; Keller et al. 2014). Keller et al. (2015) suggested that this decrease is due to the rapid increase of turgor, changing pressure gradient (ΔP_X) between extremities of the xylem.

Figure 5: Water flow for each component of water budget in berries of Cabernet-Sauvignon without water limitation (Greenspan et al., 1994).



This xylemian gradient pressure (ΔP_X) between plant and berry constitutes the driving force for xylem hydraulic conductivity and depends on climatic conditions and plant water status (Keller et al., 2006; Tilbrook & Tyerman, 2008, 2009), on berry vacuolar osmotic pressure (Π_v) (sugars concentrations in phloem and berry) and phloemian water recycling via the xylem (Becker & Knoche, 2011; Zhang & Keller, 2017). ΔP_X affects berry growth during day/night cycle with a contraction during the day due to transpiration and water uptake by plant. As water stress tends to contract even more (Greenspan et al., 1996). During ripening, a considerable osmotic pressure develops in berry vacuoles, due to the accumulation of hexoses, which should make the berry the biggest sink for water in the plant (Greenspan et al., 1994). Keller et al. (2014) even showed that green berry shrivelled as a consequence of severe water stress start to grow again at the onset of ripening even before irrigation, showing the power of the sink for water uptake.

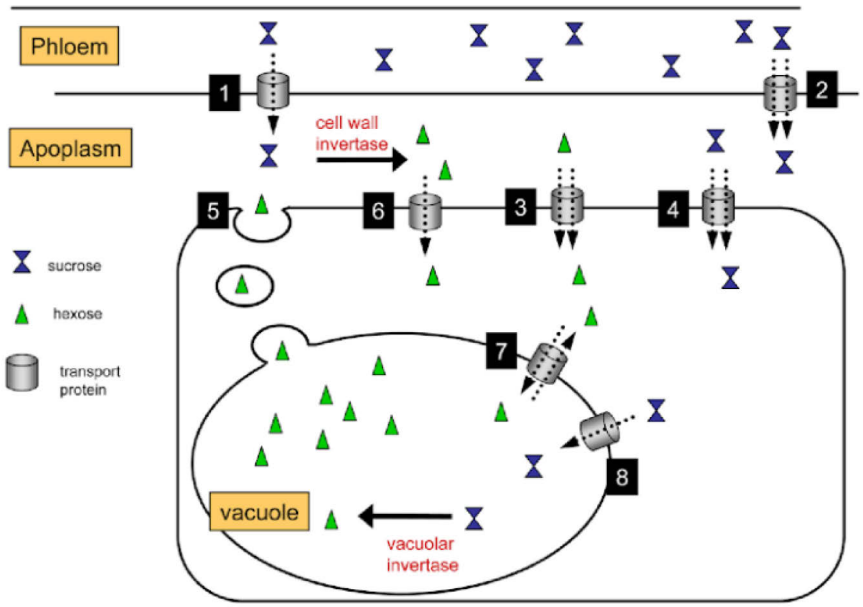
During ripening, when water flux becomes mainly phloemian, unloading in mesocarp cells go through from symplastic to apoplastic pathway (Zhang et al., 2006), resulting in an increase in apoplasmic osmotic pressure (Π_a) facilitating water and mass influx (Ruan & Patrick, 1995; Patrick, 1997; Keller & Shrestha, 2014; Keller et al., 2015). Keller et al. (2015) inferred from phloem sugar concentration measurements and berry water budget that only 20 % of the water from phloem would be used for berry growth and transpiration (which is climate dependant; Dreier et al., 2000) with the remaining water being recycled by xylem, and suggested that if berry still accumulate sugars after maximum volume, it means that water is going back to the xylem, but calculations

tends to invalid this hypothesis (Shahood, 2017). This back flux would be crucial for berry solutes unload to obtain normal berries and depends on the fruit solutes demand, leaf photosynthesis, plant water status and atmospheric vapour pressure deficit (VPD). Whatever, Shahood (2017) showed that berry growth and sugars accumulation stopped simultaneously, undermining the previous theory. After physiological maturity (maximum berry volume), berry shrivel due to transpiration and/or higher xylemian reflux than phloemian influx (Greer & Rogiers, 2009).

Sugars are important for vine plants and produced by leafs photosynthesis, and then transported inside the phloem mainly in the sucrose form (Swanson & Shishiny, 1958; Conde et al., 2007). The majority of sugars imported inside berry during green growth will be metabolised and sugars will never be higher than 150 mM during this phase, to rise up at 1 M after ripening (Wu et al., 2011; Davies et al., 2012; Houel et al. 2015). At veraison, ratio of main sugars (glucose/fructose) is between 2 and 10 at veraison stage to finish around 1 at maturity (Varandas et al., 2004). Sucrose is so representing maximum 2% of these sugars except in some table grape varieties or varieties derived from *Vitis labrusca* , but it seems that this trait, linked with a lower vacuolar invertase activity, is recessive (Shiraishi et al., 2012). These sugars are of primary importance for both wine and table grapes (Davies et al., 2012). During green growth, sucrose imported in the berry and cleaved by different invertase enzymes, creating a sucrose gradient that favours its entry in the vacuole (Fillion et al., 1999). Change in phloem inflow at veraison would result in an increase of sugars accumulation rate in berries (Zhang et al., 2006).

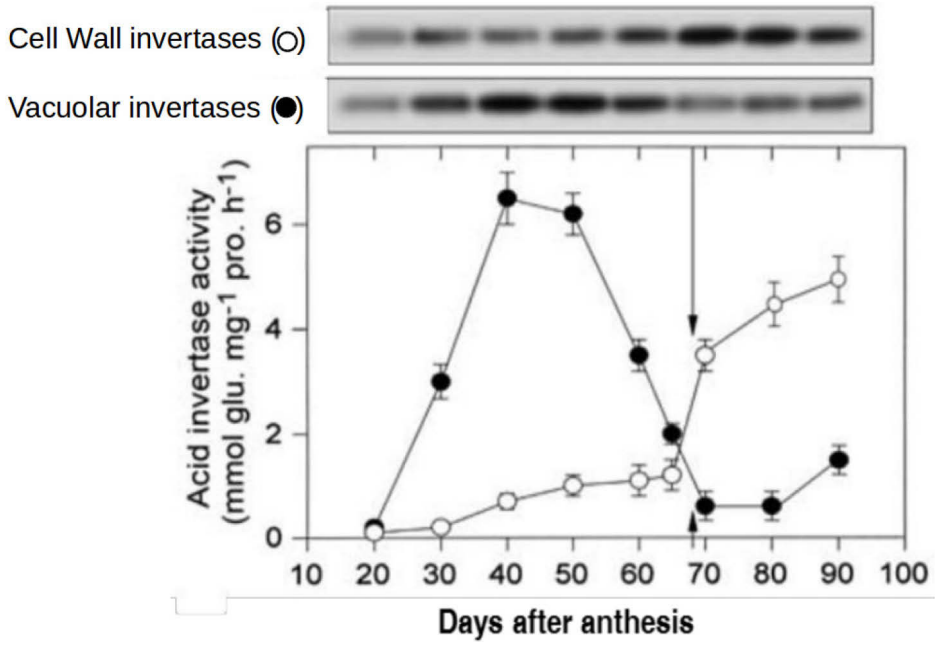
Sugar accumulation involves specific transporters (Hedrich et al., 2015). Transporters are needed for all assimilates and water transport through biological membranes. Sucrose, after a movement due to mass flux will be loaded inside the berry by both phloem pathways possible (apoplastic and mostly symplastic) (Zhang et al., 2006; Turgeon & Wolf, 2009). Then, changes in activity of the invertase and phloem pathway at veraison will lead to a higher capacity to import sucrose into berry but sucrose can’t pass alone through phospholipidic membranes. Sucrose will then requires transporters to pass or endocitosis (Figure 6) (Fontes et al., 2011).

Figure 6: Sugars transport into the berry (Davies et al, 2012), with **1**: Apoplastic pathway depending on pH; **2**: Apoplastic pathway with couple H+/sucrose; **3**: Monosaccharide transporters type *VvHT*; **4**: Sucrose transporters; **5**: Endocytisis; **6**: SWEETs; **7**: Monosaccharide transporters; **8**: Sucrose transporters.



Cell membranes have a hydrophobic double lipidic layer that permits to keep solutes gradient between cytoplasmic and extra-cellular environments. 3 groups of sucrose transporter may reside at the plasma membrane interface: Sucrose Carrier (SUC), Sucrose transporter (SUT) and Sucrose Facilitator (SUF) (SUC and SUT with *VvSUC2, 11, 12, 27* in grapevine) (Davies et al., 1999; Manning et al., 2001; Afoufa-Bastien et al., 2010), the expression of *VvSUC 27* gene being higher during green stage and that of *VvSUC11, 12* during ripening. Sucrose can also be cleaved in glucose and fructose by the activity of the three different vacuolar (Inv-V), cell wall (Inv-CW) et neutral (Inv-N) invertases. Zhang et al. (2006) showed that vacuolar invertase activity decreased while cell wall invertase increased at the onset of ripening, an argument in favour of the induction of the apoplastic pathway at this stage (Figure 7).

Figure 7: Vacuolar and cell wall invertase activities and immunoreactivity during berry growth (adapted from Zhang et al., 2006).



The vacuolar invertase activity is correlated to hexose and sucrose relative quantity inside berry vacuole, but not to the total sugars quantity accumulated (Takayanagi & Yokotsuka, 1997; Davies & Robinson, 1996). Neutral invertase would be used if sucrose comes directly to cytoplasm, but not much information are recorded on it (Davies et al., 2012). In grapevine, 9 neutral (*VvNIs; VvNI 1-5* being weak during ripening), 2 vacuolar (*GIN1* & *GIN2;* diminution of activity after the onset of ripening) and 1 cell wall (*VvcwINV;* increase slowly during green growth and decrease during ripening period) invertases were characterised (Davies & Robinson, 1996; Dreier et al., 1998; Nonis et al., 2008). *VvcwINV* being induced in parallel with *VvHT5* hexose transporter in some specific conditions (Lecourieux et al., 2014). After conversion, the transport of glucose and fructose formed in the apoplasm requires plasma membrane transporters too. It exists 7 classes of monosaccharide transporters: Sugar Transport Protein (STP), Vacuolar Glucose Transporter-like (VGT-like), Tonoplast Sugar Transporter (TST), Plastidic Glucose Transporter/Suppressor of G protein Beta1 (pGleT/SGB1), Early-Responsive to Dehydration-like (ERD6-like), Polyol Tranporter (PLT) and Inositol Transporter (INT) (Büttner, 2007). In grape vine, hexose transporters (*VvHT1, 2, 3, 4, 5* (STP on plasmic membranes), *6* (TST) *& 7*) and *VvGLT* were identified (Fillion et al., 1999; Vignault et al., 2005; Conde et al., 2006; Hayes et al., 2007; Davies et al., 2012; Lecourieux et al., 2014). *VvHT1* compared to *VvHT2* decrease in activity after the onset of ripening and is located in intermediary cells. Others transporters as SWEETs (Sugars Will Eventually be Exported Transporters) may play a primordial role in sugars unload by facilitating sucrose, glucose and

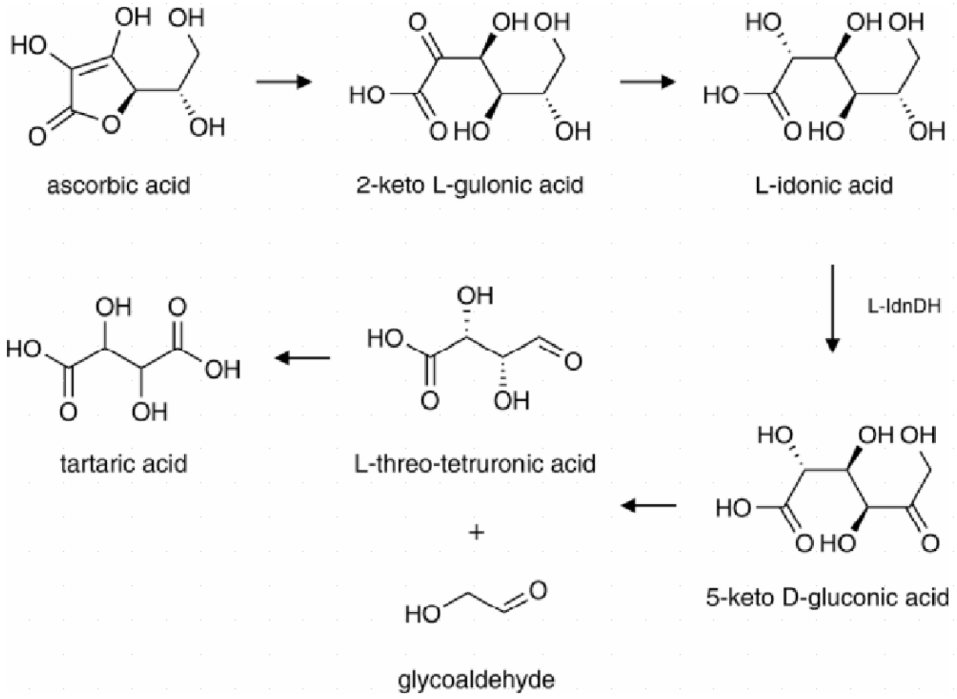
fructose loading through the tonoplasm (Chong et al., 2014). Seventeen SWEETs genes where found in grape-vine with different expressions in ripe berry (*VvSWEET4* (for glucose and located on plasmic membrane)*, 7, 10, 11, 15 and 17d*) and in flowers (*VvSWEET3, 4, 5a, 5b, 7, 10 and 11*). For a review of all transporters of the literature see Shahood (2017).

Proton pumps are also important to energize sugar transport by H+ symporters at the plasma membrane and H+ antiporters at the vacuolar one. H^+ pumps use the P~P link energy to transfer H^+ against its concentration gradient. To promote a concentration gradient force for sucrose and acids accumulation, three different vacuolar pumps (V-pyrophosphatase (V-PPase), V-ATPase, & type P3 $_{A/B}$ ATPase) are known to create a gradient of protons towards the vacuole (Faraco et al. 2014). All those pumps don't have similar H+/ATP coupling ratio (Lobit et al., 2006; Palmgren & Nissen, 2011; Etienne et al., 2013). Plasmic ATPase (PM H^+-ATPase) is necessary for having pH gradient for passive ions flux through specific channels and can be useful for salt tolerance, pH regulation and cell growth (Sussman, 1994).

Sucrose from phloem can be cleaved by both invertase (Inv) and sucrose synthase (SuSy), and re-synthesized by sucrose phosphate synthase (SPS). This makes possible to define 4 substrate cycles: degradation/synthesis of cytosolic sucrose, vacuolar degradation/cytosolic synthesis, apoplasmic degradation/cytosolic synthesis and synthesis/starch amyloplastic hydrolysis (Nguyen-Quoc & Foyer, 2001). Glycolysis (Hexose + 2 ADP + 2 Pi + 2 $NAD^+ \rightarrow$ 2 pyruvate +2 ATP+ 2 NADH, H^+) permit synthesis of primary and secondary metabolites, or permit storage in starch. Without oxygen, NADH as to be recycled mainly by ethanol production in plants (Shahood, 2017).

Metabolites are accumulated, diluted and metabolised during berry development (Conde et al., 2007). First, acids are mainly accumulated in the vacuole (Terrier & Romieu, 2001). Malic and tartaric acid are the two mains acids in grape-berry, and participate up to 90% of the juice acidity. The main difference between those two acids is that tartaric is more stable in quantity than malic acid (Lakso & Kliewer, 1975). L-(+)-tartaric acid is accumulated at the beginning of green growth phase (Champagnol, 1984). Its synthesis pathway begging with L-ascorbic acid and cleavage of one carbons pair (C2/C3 or C4/C5 depending on species) (DeBolt et al., 2006). The preferred path to synthesize tartaric acid in grapevine is using a glycolaldehyde as showed in figure 8 but the use of an oxalic acid (OxA) and a L-threonate can also be used. In 2006, the discovery of L-idonate dehydrogenase (L-IdnDH) argues in favour of the proposed path (DeBolt et al., 2006).

Figure 8: Possible path for tartaric acid formation in grapeberry (Conde et al., 2007).



L-(-)-malic acid is synthesized later in the green growing phase (Champagnol, 1984; Conde et al., 2007). This acid is the major one in many fruits (Etienne et al., 2013). This acid can be seen as a reserve of CO2 before photosynthesis, a support for respiration and neoglucogenesis, an efficient osmoticum in terms of carbon, it can also control physiological process as stoma opening (Kelly et al., 1976; Famiani et al., 2014; Sweetman et al., 2014). Malic acid is in majority synthesised inside berry from PEP (PhosphoEnolPyruvate) via the PEP-carboxylase (PEPC; inside the cytosol) and the malic dehydrogenase (MDH; inside the cytosol, glyoxysomes and mitochondria) (Taureilles-Saurel et al., 1995 a, b). PEPC and MDH activities are high during early stage of berry development and decrease just before the onset of ripening (Lakso & Kliewer, 1975; Terrier et al., 2005) and then during ripening MDH is re-increasing. Sweetman et al. (2014) showed that there is a positive linear correlation between malic content and PEPC activity. So PEPC and cytoplasmic MDH seem to be responsible for malic acid accumulation during green growth, mitochondrial MDH being mainly use for degradation by respiration. In any cases, the pathway hexose + $2CO_2$ <=> 2 $malate^{2-}$ + 4 H^+ can be active in both directions, never mind the flux. Malic acid can also be produced by photosynthesis during green growth (Ollat & Gaudillere, 2000), or inside the mitochondria via main enzymes (Fumarase (not limiting), mMDH (strong activity during green growth) (Ollat & Gaudillere, 2000; Fatland et al., 2005; Sweetman et al., 2014), or via the malate synthase in the glyoxylate cycle (Terrier et al., 2005).

During ripening, neoglucogenesis will use malic acid as substrate for PEP synthesis via MDH, malic enzyme (reversible activity increasing during development)plus pyruvate ortho-phosphate dikinase (PPDK; not detected in grape), or PEP carboxykinase (PEPK) (Ruffner & Hawker, 1977; Goodenough et al., 1985; Terrier et al. 2005). During green growth, low activity of PEPK and high activity of PEPC favour malic acid synthesizing, which changes after the onset of ripening. Also, malic acid can be degraded by the respiration, which is more important in berries during green growth than ripening phase (Ollat & Gaudillere, 2000). At the onset of ripening, vacuolar released malic acid is used to produce ATP via TCA cycle. Later, its use for this cycle seems to be correlated with mMDH activity (Taureilles-Saurel et al., 1995; Etienne et al., 2013). During ripening, NADP-ME (depend on $NADP^+$/NADPH, pH and regulators: Mn^{2+}, Mg^{2+}, ATP) and NAD-mtMDH seems to have a major role in malic acid degradation (Sweetman et al., 2009; Etienne et al., 2013). Pyruvate, formed by ME can be fermented in alcohol by the pyruvate decarboxylase (PDC) and the alcohol dehydrogenase (ADH) during low cytoplasmic pH and/or hypoxic environment, or in lactic acid via the lactate dehydrogenase (LDH) (Sweetman et al., 2009). Whatever, strong arguments suggest that the synthesis or degradation of malic acid is regulated by the capacity of the vacuole to accumulate it as the free acid. Terrier et al showed that tonoplast vesicles extracted from green berries were perfectly tight to H^+ (and accompanying anion), allowing V-ATPase to reach thermodynamical equilibrium (pHvac = 2.7). During ripening, futile H^+ recirculation cycles develops, preventing the vacuolar lumen to reach such an acidic pH (non equilibrium), so malic acid is necessarily released. Detailed investigations on the malic acid/sugar stoichiometry suggests that four hexoses are accumulated per malic acid consumed at the onset of berry ripening (Shahood, 2017), consistent with the induction of a sucrose/H^+ antiporter at the tonoplast membrane, as indicated by the induction of VvHT6 transcription at this stage (Terrier et al., 2005). However, Rienth et al., 2016 showed that in cold conditions, which reduce respiration of imported photoassimilates, a noticeable accumulation of hexose can occur before the global malic acid/sugar is induced. It must be also stated that sugar loading in berries continues after malic acid is consumed.

Berry development also needs micro-elements, at least 17, considered as essential (Bashir & Kaur, 2018). It's important to notice that inorganic cation osmotic potentials are higher than organic cations (Bonomelli & Ruiz, 2010). Potassium (K^+) is the most concentrated cation in berries and its concentration depends on many factors such as fertilisation, rootstock, etc. (Deloire, 2007). Potassium is mainly absorbed by the plant between bloom and veraison by the plant with *VvK1.1* gene activity and redistributed through the plant with *VvK1.2* gene activity via both phloem and

xylem (Mpelasoka et al., 2003; Bashir & Kaur, 2018). Two potassium channels genes, *VvKUP1* and *VvKUP2,* where found and they were highly active in the skin at the early stage of development showing it importance for berry (Cuellar et al., 2013). Expression will then decrease at veraison but stay active. At this time, other gene *VvK1.2 w*ill significantly increase in activity, promoting Shakers channels (*VvCIPK04–VvCBL01* and *VvCIPK03–VvCBL02)* sharply increasing potassium concentration (Mpelasoka et al., 2003). Potassium is mainly accumulated in the skin where it can be 1.7-6.9 times higher than in the flesh and has a role in sugars importation.

Calcium (Ca^{2+}) is also an important element of plant development as its involved in the cell wall and membranes structure, as counter-ion for acids and anions in the vacuole (Mpelasoka et al., 2003; Bonomelli & Ruiz, 2010). This cation which can't be transported via phloem have to traffic through xylem using an unidirectional stream (as in *Phaseolus vulgaris* (Steucek & Koontz, 1970)). It also can't be mobilized from older tissue showing it dependency to xylem flow (Mpelasoka et al., 2003). In fact calcium in fruit can be separated in three pools depending on activities (soluble Ca : nitrates, chlorides, organic acids; exchangeable Ca; Ca not physiologically active : oxalate, phosphates, and carbonates). Maximum uptake occurs at the early stage of berry development. Higher cell size, firmness and less dry matter were observed on berries from plants supplemented with Ca^{2+}.

Magnesium (Mg^{2+}) accumulation starts early during berry development (Duchène & Chardonnay, 1992). This cation is really mobile and can be redirected (Christensen, 2000), and Steucek & Koontz (1970) studies on *Phaseolus vulgaris* showed that magnesium was moving in the phloem. Mg^{2+}/H^{+} exchangers (AtMHX in *Arabidopsis*) permit it to enter inside the vacuole (Shaul, 2002). Others transporters were also found in *Arabidopsis* MGT6, MGT1, MGT7, MGT9, MGT2, and MGT3, which are expressed in roots, with (MGT1 and MGT6) also located in the plasma membrane and expressed in the epidermal cells (Mao et al., 2014). Studies shows higher amount in skin than flesh but a stable concentration at similar level to calcium (Conde, 2007).

Ammonium (NH_4^{+}) represents half of the nitrogen in the early stage of berry development (Christensen, 2000) and is assimilated via the glutamine synthetase (GS; EC 6.3.1.2) NADPH-glutamate synthetase (GOGAT; EC 1.4.1.14) pathway to form glutamine and glutamate in *Phaseolus vulgaris* L. (Hungria & Kaschuk, 2014). After being uptake by roots, long distance nitrogen is transported in the plant in nitrate, ammonium and amino acids forms (Schobert & Komor, 1992). All three forms are possibly navigating through the xylem (With really low amount

of NH_4^+ in *Phaseolus vulgaris* L. (Hungria & Kaschuk, 2014)), in contrast with phloem that contain only nitrogen in *Ricinus communis*. Then after veraison the production of amino acids will decline its concentration (Christensen, 2000). Researches on *Phaseolus vulgaris* L. suggested a diminution in ammonium uptake during warm conditions (Hungria & Kaschuk, 2014).

II. Climate change and its impact on berry development

Climate is dramatically changing since 1860 with the industrial revolution (Giorgi, 2005). Since then, Temperature (T°) is rising coupled with atmospheric CO_2 and precipitations are changing depending on areas (Anderson et al., 2016). Grapevine being one of the most sensible plants to climate change due to resulting organoleptic wine profiles and viticulture being spread worldwide, it is a major significance to qualify and quantify the impacts of climate change on grapevine (Jones & Davis, 2000; Bucur & Dejeu, 2014).

II.1. The "greenhouse" effect

Atmospheric CO_2 is constantly increasing in concentration mainly due to human activity (Michaelis, 1993; Szulejko et al., 2017) and today, it is one of the major challenges to reduce emission of this "greenhouse" gas.

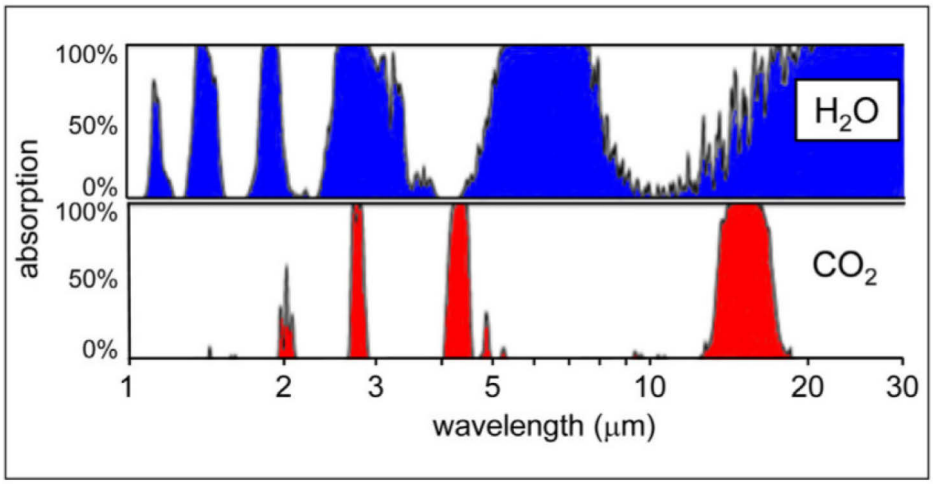
The atmosphere is formed with 2 main gases which are dinitrogen (N_2) and dioxygen (O_2) (Oktyabrskiy, 2016). Their part in the atmosphere volume is about 78% for N_2 and 21% for O_2. Both of those molecules belong to the $D_{\infty h}$ point symmetry group, meaning that they have a symmetry centre and no dipole moment. Consequently, they do not absorb or emit infrared compare to CO_2 and water which are major absorbent in the atmosphere. CO_2 belong also to the $D_{\infty h}$ point symmetry group and have a centre of symmetry but can have dipole moments under asymmetric and doubly degenerate deformation vibrations. It part in the atmosphere volume is only about 0.03%.

This gas is studied since 1890's with Svante Arrhenius and Arvid Högbom, creators of the first climate change model (Anderson et al., 2016). Few years later (1930's), Guy Stewart Callendar resumed the previous work and wanted to find a proof about the CO_2 and warming relationship. In the 1990's this relationship was still not clear, and CO_2 was considered to have just a possible impact on global warming (Michaelis, 1993).

With the impulse of Roger Revelle, CO_2 was studied and measured in a station based in Mauna Loa volcano in Hawaii (since 1956). He also showed that it absorbs approximately 12% of the integral power of the intrinsic long-wave emission of the Earth surface, compare to 68% for water (Abdussamatov, 2013; Anderson et al., 2016; Oktyabrskiy, 2016) (Figure 9) but compare to water it

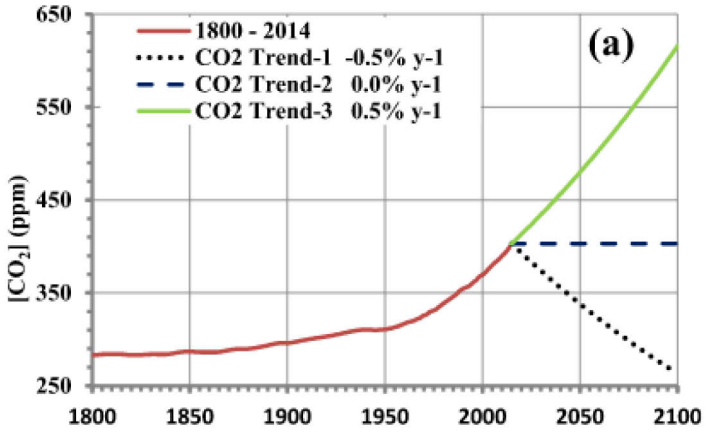
atmospheric concentration is not stable due to see slow uptake and emissions (Mistui & Abe, 1986; Meehl et al., 2006; Anderson et al., 2016; Oktyabrskiy, 2016). Water is belonging to C_{2v} point symmetry group and has no centre of symmetry so it reacts to all vibrations possible (Oktyabrskiy, 2016). It yearly variation is very small, about 0.0004% change in 2013 for example in USA.

Figure 9: Atmospheric absorption spectra for water vapour and CO_2, adapted from Wikimedia Commons (Anderson et al., 2016).



As we can see on figure 10, atmospheric CO2 started rising during 1860's due to industrial revolution (coal burning) and since then it increases exponentially (Szulejko et al., 2017). Values presented for 1800 to 2014 are measured values (based on Antarctic ice core data for values before 1958 and then on Mauna Loa Observatory data), CO_2 Trend-1 is a projection if the CO_2 concentration diminish by -0.5% [CO_2] yearly, CO_2 Trend-2 is a projection with a balance between sink and source to obtain a 0.0% CO_2 yearly augmentation. CO_2 Trend-3 is a projection based on a continuous augmentation +0.5% [CO_2] yearly. Looking at actuals data, CO_2 concentration was at 406.33 ppm in April 2017 and increase to 408.96 ppm in April 2018 (Team ESRL) showing that emissions are still higher than sink.

Figure 10: Historical and projected data of atmospheric CO_2 (Szulejko et al., 2017).



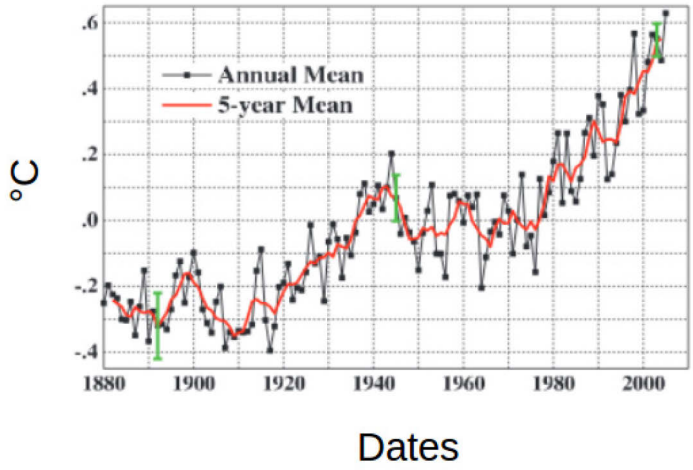
For projections, models are used as the Earth System Models or Representative Concentration Pathway scenarios which are very complex models using interactions (Anderson et al., 2016). But presently, researchers change their mind about the effects of those gases on climate change and about models because the Earth atmosphere does not really function as a greenhouse (Oktyabrskiy, 2016). The atmosphere is often defined as a glass but in reality it is a bit opaque mainly due to ozone (O_3), questioning the reliability and usefulness of the models (Anderson et al., 2006). Knowing this, the terms "greenhouse effect" and "greenhouse gases" remained symbolical (Oktyabrskiy, 2016).

II.2. The rising temperature

The human activity can have direct or indirect impacts on global warming (Michaelis, 1993) and today, the states aim to limit global warming (Kharin et al., 2018).

Temperature is the first indicator of global warming (Foster & Rahmstorf, 2011). Since the industrial revolution, emissions of "greenhouse" gases have impacts on T° and it rises is well-known since the 1970's (cf. Fig. 11) (Foster & Rahmstorf, 2011; Anderson et al., 2016). Unfortunately, the trend is not reversing as we burn fossil fuels. Callendar for example predicted that at in 2000 T° will have increase by 0.16°C, which was wrong because only 2 gases were selected in his model (CO_2 and water). In effect, during the last 30 years, global surface T° increased by approximately 0.2°C per decade (Hansen et al., 2006).

Figure 11: Average of extreme temperature trends (Hansen et al., 2006).

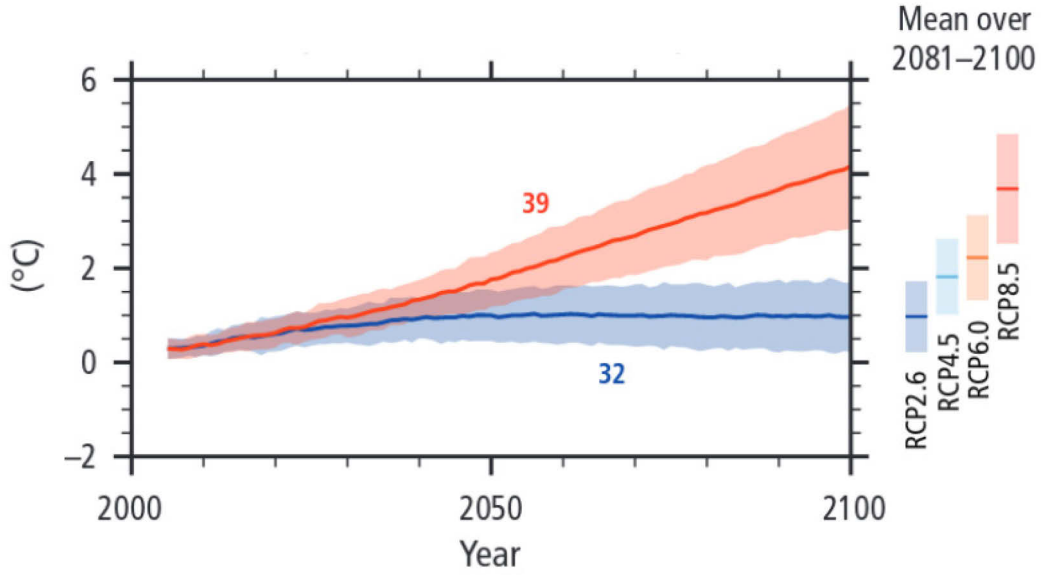


Global warming is at his highest since the 18th century and no indication of a slowdown or acceleration of this warming, even with natural factors evolution (Foster & Rahmstorf, 2011; Szulejko et al., 2017). That's why Parties to the United Nations Framework Convention on Climate Change found a compromise and tried to hold the increase in global average T° below 2°C above pre-industrial levels, which mean bellow 1.5°C compare to actual levels (IPCC, 2014; Kharin et al., 2018). To limit this increment as low as possible is today necessary because a global warming of 2°C would have more impacts than a warming of 1.5°C in extremes events formation probabilities (Kharin et al., 2018). For example, the probability of a extreme warm, which appear every 20 years on average, would increase 130% and 340% at the 1.5°C and 2.0°C warming levels relatively to pre-industrial level compare to cold extremes which would decrease from 28% to 83% respectively. Models also showed that with an increasing T° and the absence of moisture limitation, extremes precipitations intensity will increase exponentially all around the world (Clausius–Clapeyron relationship) even though some stations measurements suggest the opposite (Wang et al., 2017; Kharin et al., 2018).

Models are used for projections in order to predict the future climate conditions. Those projections, as ESMs (Earth System Models), showed that warming will continue in response to emissions of greenhouse gases to the atmosphere (Anderson et al., 2016). As represented in figure 12, whatever the scenario, T° will still increase. For RCP 2.6, which corresponds to a peak follow by a decline, T° will still increase and then stabilised at ≈ 1°C above pre-industrial level in 2100. In contrast, RCP 8.5 (representing a continual increase in CO_2 atmosphere concentration) shows that temperature will increase constantly to reach an increment of ≈ 4°C above pre-industrial level in

2100. RCP 4.5 and 6.0 (corresponding to stabilisation in CO_2 atmosphere concentration) show a small increase follow by a stabilisation at ≈ 1.9°C and ≈ 2.2°C respectively above pre-industrial level in 2100.

Figure 12: Global average surface temperature change projections (relative to 1986 – 2005) (IPCC, 2014)



But models tend to predicted higher high T° than observation and lower cold T° (Kharin et al., 2018). Also, each science team has his own method for correcting input data obtain from non-climatic influences (Foster & Rahmstorf, 2011). Added to probable uncertainties and complications of recorded data, surface or satellite, question the utility and reliability of models (Anderson et al., 2016; Szulejko et al., 2017) but have the advantage to give the global trend over the long term, which is increasing T° and extremes event (IPCC, 2014). Some simple indicators, as annual daily maximum/minimum temperatures and annual maximum 1-day precipitation give robust informations on climate extremes (Kharin et al., 2018). For example, maximum 1-day precipitation increases by about 7% each °C. In the end, T° is strongly impacted in the short-term by known factors as El Niño/southern oscillation (an internal quasi-oscillatory mode of the ocean–atmosphere system), volcanic aerosols or solar variations (Foster & Rahmstorf, 2011). Temperature increases as other impacts as sea rising level (Meehl et al., 2006). Finally, climate change will have different impacts on regions due to their own climate conditions (Kharin et al., 2018).

III. Climate change and viticulture

III.1. Effects on plant physiology

Climate change (increase of CO_2 in the atmosphere, rising T°, etc.) impacts on plant development and functioning. It impacts on grapevine (Sadras & Moran, 2012; Ollat et al., 2017; Arrizabalaga et al., 2018) is well study to challenge effects due to climate change on historical growing regions (Jones, 2004).

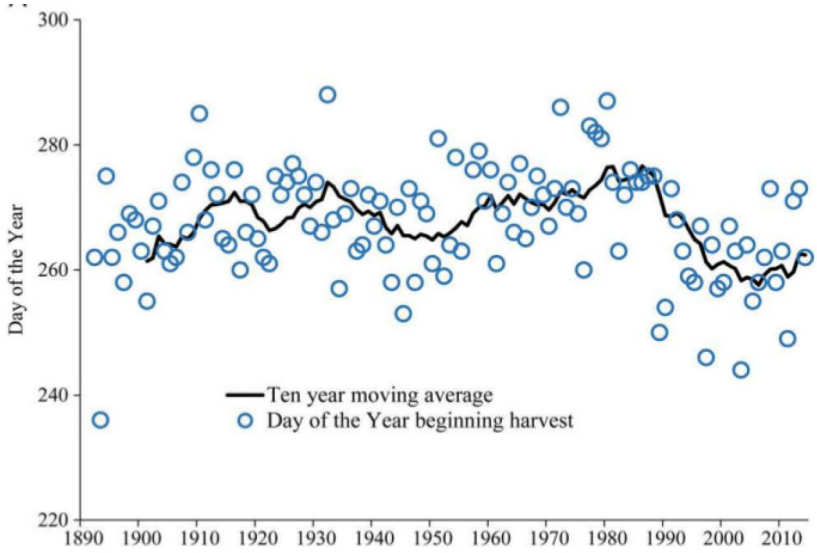
Many factors are influencing grapevine growth such as fertilisation, water availability, T°, light, CO_2 atmospheric concentration, etc. (Kriedmann, 1968; Lakso & Kliewer, 1975; Bindi et al., 2001b; Edwards et al., 2017; Arrizabalaga et al., 2018). Many of those factors are directly controlled by the viticulturist (Fraga et al., 2012; Etienne et al., 2013) and other are directly due to climatic conditions, which can be impacted by climate change (van Leeuwen & Darriet, 2016).

Temperature

Favourable temperature is one of the most important conditions to wine quality (Kriedmann, 1968; Fraga et al., 2012; Ollat et al., 2017; Wolkovich et al., 2018).

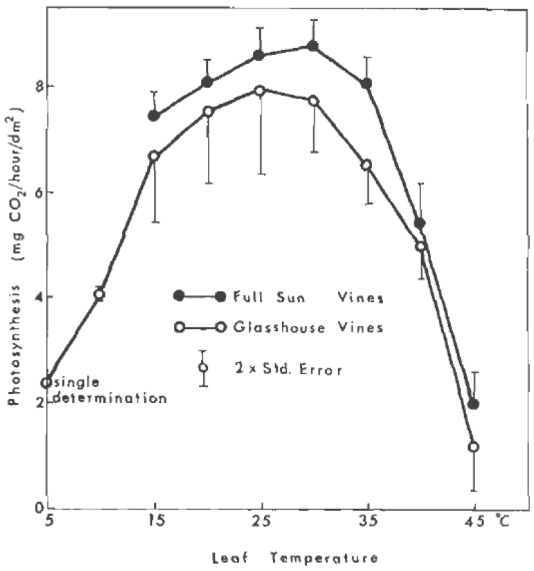
The phenology of the plant is impacted by high T° (Arrizabalaga et al., 2018). Elevated T° significantly shortened phenology of grapevine (van Leeuwen & Darriet, 2016; Edwards et al., 2017; Ollat et al., 2017). In fact, at least 10°C basal T° is needed break the vine dormancy and initiate its growing cycle (Pouget, 1988, Fraga et al., 2012). This T° is today reached earlier, advancing vine phenology (Carbonneau et al., 2015; Edwards et al., 2017). Budburst, flowering and then berry development was observed in advance with high T° in greenhouse experiments (Arrizabalaga et al., 2018), leading to earlier harvests (Jones, 2004; van Leeuwen & Darriet, 2016) (Figure 13). For example, Arrizabalaga et al. (2018) worked with different temperatures and found an average of 13 days between *V. vinifera.* cv. Tempranillo clone physiology.

Figure 13: Harvesting dates in Saint Emilion during 1892 to 2014 (van Leeuwen & Darriet, 2016).



Temperature level has direct impact on photosynthesis rate and respiration (Fraga et al., 2012; Etienne et al., 2013; Greer & Weedon, 2013). Kriedmann in 1968 studied this effect and saw that optimal T° for maximum photosynthesis was at 25°C inside glasshouse for Sultana clones. Figure 14 represents the average net photosynthesis depending on leaf T° for plants growing in greenhouse and plants fully exposed in field. This experiment also shows a substantial decline of this rate when leaf T° exceeded 35°C but even at 40° C the leaves are still photosynthetically active, which is in agreement with Greer (2018). Greer & Weedon (2013) estimated a 30-50% reduction in photosynthesis due high temperatures and radiation but this limitation may change according to genotypes (Greer, 2018). They also reported consistent effect on the stomatal conductances of the leaves in the shoot lower half, but after leaf position 10, stomatal conductance increased during the high temperatures to approximately 0.1 $mol.m^{-2}.s^{-1}$ (Greer & Weedon, 2013). In consequence, extreme heat or heat weaves can limit stomatal openings and mesophyll activity, reducing photosynthesis activity, declining C assimilation (Fraga et al., 2012; Greer, 2018).

Figure 14: The effect of temperatures on net photosynthesis for four plants in greenhouse and seven fully exposed in filed (Kriedmann, 1968). Measurement was performed with the same light intensity.

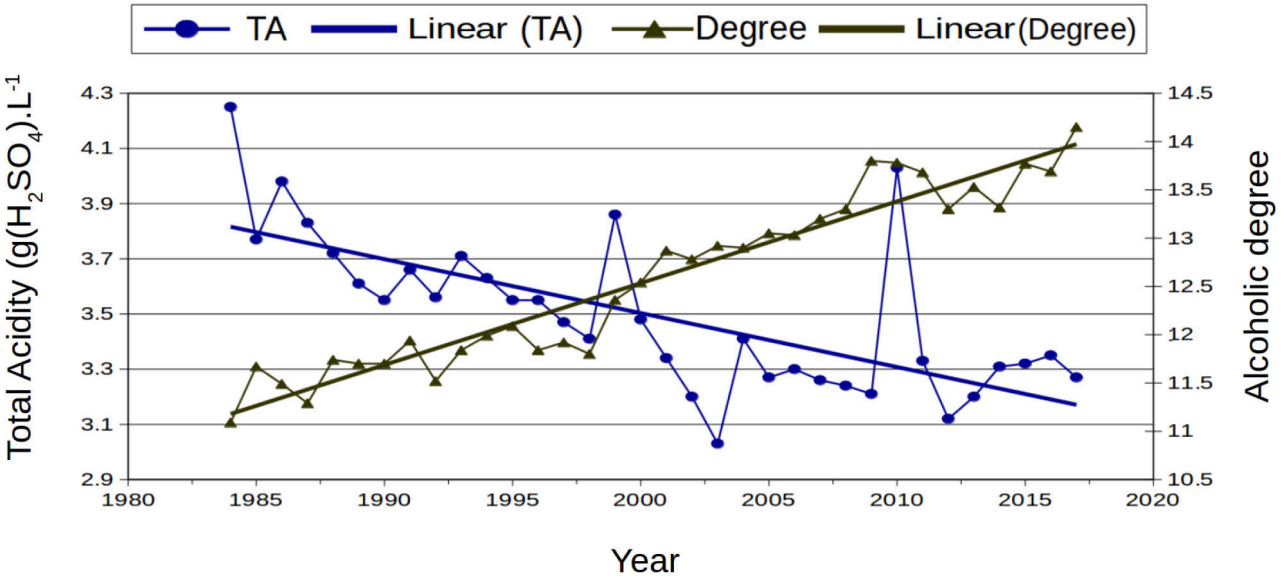


Temperature has also an impact on berry composition (Mori et al., 2007; Greer & Weedon, 2013). Sugar berry concentration is impacted by rising T° (Jones, 2004; Mira de Orduña, 2010) and heat waves (Arrizabalaga et al., 2018) even occurring before sugar accumulation period (Greer & Weedon, 2013). For samples at harvesting date in Bordeaux (France), sugars seemed more concentrated with higher T° (Pereira et al., 2006). Resulting wines tend to have more alcohol content (Sadras & Moran, 2012; van Leeuwen & Darriet, 2016). Even if the effect of T° on final berry sugar concentration is low (Petrie & Sadras, 2008), it impact is clear on resulting wine, with an increment of 0.14-0.17 % per year potential alcohol content (Sadras & Moran, 2012) (Figure 15). For Keller (2009) above 30°C the sugar accumulation can even stop.

The impact of warming on malic and citric acid concentrations is, compared to tartaric acid and sugars in grapevine, really important (Etienne et al., 2013; Arrizabalaga et al., 2018). In cool regions, juice tends to be more acidic than in other places, showing the importance of T° on acidity (Lakso & Kliewer, 1975; Barnuud et al., 2014). All grape berry acids have not the same response to T° elevation (Etienne et al., 2013). Rienth et al. (2016) showed that low temperatures can desynchronise malic acid accumulation/consumption and sugars accumulation leading to late accumulating malic acid berries during ripening. In contrast, heat has a negative impact on malic acid synthesis, storage and degradation (Etienne et al., 2013; Arrizabalaga et al., 2018). For malic acid accumulation optimum, T° as to be between 20 and 25°C (Lakso & Kliewer, 1975). After

38°C, there is a tendency to lose malic acid due to Phosphoenolpyruvate (PEP) carboxylase activity loss (Lakso & Kliewer, 1975). Increasing T° lead then to an up-regulation of VvPpdk (Pyruvate, phosphate dikinase) during night, an increase in activity and transcript level of NAD-dependent ME (NAD-ME), a decrease in PEPCK (Phosphoenolpyruvate carboxykinase) transcript and activity during day, a decrease in the PK (Pyruvate kinase) activity and PEPC (Phosphoenolpyruvate carboxylase) activity (Sweetman et al., 2014). NADP-ME, NADP-MDH, and NAD-MDH activities were unaffected by heat. With heat, both NAD-ME (for malic acid degradation) and PEPC activities (for malic acid accumulation) are impacted. Lakso & Kliewer (1975) showed that in in-vitro NAD-ME activity increase constantly with T° and after a heat shock, the recovery of the activity being fast. PEPC activity increases with T° up to 38°C and then decreases strongly. This enzyme recovers also activity after a heat shock but slower and less than NAD-ME. They also showed that there was no synthesizing of heat-stable PEP carboxylase and malic enzyme isozymes in immature grape berries in response to high T°. In addition, high T° limits proton pumps (thermodynamic equations) slowing down the diffusion of organic anions through the tonoplast due to modified lipid properties affecting membrane fluidity (Etienne et al., 2013). During ripening, the increase in proton pump transport activity can partially compensate the leakage of solutes, creating an efflux of malic and citric acid to the cytosol where they will be degrading. Finally, all those parameters (tonoplast membrane properties, NAD-ME activity up-regulation, PEPC activity down-regulation) influenced by T° lead to a diminution in ripe berries malic acid concentration (Lakso & Kliewer, 1975; Etienne et al., 2013; Sweetman et al., 2014; Rienth et al., 2014). Tartaric acid showed no major differences in concentration mainly due to the fact that during ripening it content doesn't evolve (Etienne et al., 2013; Rienth et al., 2014; Arrizabalaga et al., 2018; Rösti et al., 2018), Even if high T° may have an impact on it final concentration (Kizildeniz et al., 2015). This loss of fruit malic acid cut down the titratable acidity and results in an unbalance of the sugar-acid balance (Barnuud et al., 2014; Sweetman et al., 2014) (Figure 15). This loss is well correlated with an increase of the juice pH, even considering Potassium, which evolves with T° too (Mira de Orduña, 2010).

Figure 15: Potential alcohol and total acidity of wines in Languedoc-Roussillon from 1984 to 2016 (Source: Dubernet Lab, Narbonne, France).



Anthocyanins are also impacted by T°, conducting to a decline in their final concentration and a change in their total composition (Mori et al., 2007; Mira de Orduña, 2010; Fraga et al., 2012; Sadras & Moran, 2012; Arrizabalaga et al., 2018). In apple, Arabidopsis, grape, maize, petunia, red orange, and rose, the expression of the anthocyanin biosynthetic genes is up-regulated by low temperature and down-regulated by high temperature (Mori et al., 2007; Rienth et al., 2014). Mori et al. (2007) showed that T° can reduce the total anthocyanin content to less than half between two T° treatments (first: 25°C day and 20°C night; second: 35°C day and 20°C night) but most of the genes were just slightly repressed by high temperature (Rienth et al., 2014). mRNA levels analyses revealed that most anthocyanin biosynthetic genes are increased at 2 weeks after "*veraison*" and then decreased 2 weeks later but the difference observed in mRNA levels is smaller than difference in content and no inhibition was reported. One enzyme, the UFGT (UDP-glucose: Flavonol 3-O-glucosyltransferase (for anthocyanins accumulation)), had a better activity with higher T°. Changes in composition are more difficult to estimate due to solar radiation effect (Mira de Orduña, 2010) but the ^{13}C-labelled anthocyanins seem to decrease after a heat treatment (Mori et al., 2007). Finally, many factor others than biosynthesis inhibition, such as chemical and/or enzymatic degradation, could also reduce the anthocyanins in grape skins under high T°. Consequently, with higher T°, there is less colour in wines and in order to have more colour, harvesting is delay, increasing sugars in musts and so increasing the effect on fermentation (Sadras & Moran, 2012) (Figure 15).

High temperatures have others direct and indirect disadvantages on plant and berries as increasing volatilization of aroma compounds (lower MP (Methoxypyrazines; which gives egetal, herbaceous, or bell-pepper like aromas) levels) (Mira de Orduña, 2010; Fraga et al., 2012). The decrease in malic acid affects the malolactic fermentation (high pH values). Temperatures may also affect positively the potassium level (Mira de Orduña, 2010). Temperature can also promote diseases or/and diseases vectors as for *Xylella fastidiosa,* the vector for Pierce's disease, or the black rot (*Guignardia bidwellii*), the flatid planthopper (*Metcalfa pruinosa)* (Sadras & Moran, 2012; Sunitha, 2017). It also may have a possible impact on roots mycorrhiza (Mira de Orduña, 2010).

CO_2

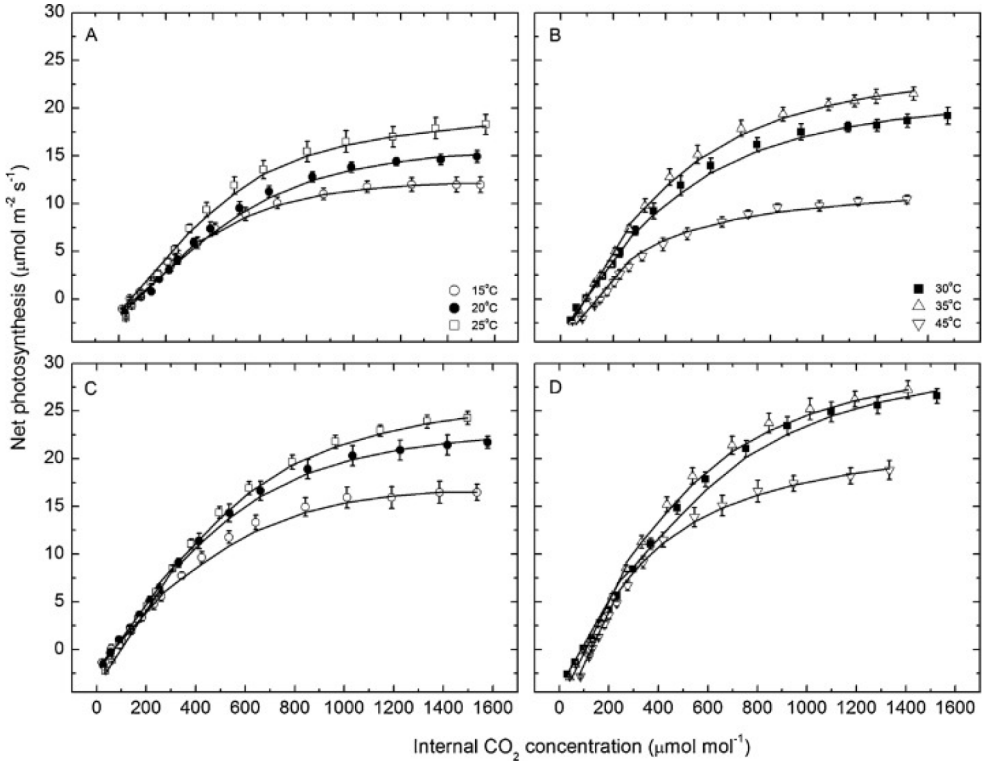
Atmospheric CO_2 concentration also impacts the vine development (Bindi et al., 2001b; Martínez-Lüscher et al., 2015; Edwards et al., 2017). Bindi et al. (2001a) for example developed a system call FACE (Free Air CO_2 Enrichment). This tool permits an enrichment in field conditions with good repeatability (on 1 min average measurement, less than 20% deviation for over 80% of the time). This tool is also adequate studying both long and short time periods treatment.

As well as increasing T°, increasing CO_2 atmospheric concentration have other impacts on grapevine development and resulting wine (Bindi et al., 2001b; Mira de Orduña, 2010). Increasing CO_2 speeds up the vine phenology (Bindi et al., 2001b; Kizildeniz et al., 2015). CO_2 also improve leaf area, leaf DW (dry weight), stem DW and significantly improve root DW (Bindi et al., 2001b; Kizildeniz et al., 2015).

It impacts on photosynthesis is also studied since CO_2 is directly used to produce sugar in leafs (Stitt, 1991; Bindi et al., 2001b; Kizildeniz et al., 2015; Martínez-Lüscher et al., 2015). Martínez-Lüscher et al. (2015) noticed a significant increase of the carbon fixation rate on Tempranillo plants under climate change conditions (700 ppm CO_2; 28°C day/18°C night). This result has to be mitigated because T° alone as an impact on photosynthesis rate and when they are both applied they are in interaction (Mira de Orduña, 2010; Fraga et al., 2012; Etienne et al., 2013; Greer & Weedon, 2013). This interaction is important and has to be studied (Ollat et al., 2017). Bindi et al. (2001b) showed that with higher CO_2 concentration, accumulation of fruit and vegetative biomass is at a higher rate. Figure 16 represents the net photosynthesis for 2 genotypes and 6 different temperatures (Greer, 2018). In these graphics, patterns are closely following the C_3 model developed by Farquhar et al. (1980). Only 45°C treatment impairs photosynthesis in a significant way (Figure 16). In

consequence, if the plant has all the nutriments needed, photosynthesis rate will increase with CO_2 concentration (+30/40 % per 250 ppm augmentation) and lead to permanent plant changes, with differences between genotypes (Figure 16) (Stitt, 1991; Martínez-Lüscher et al., 2015; Edwards et al., 2017; Greer, 2018).

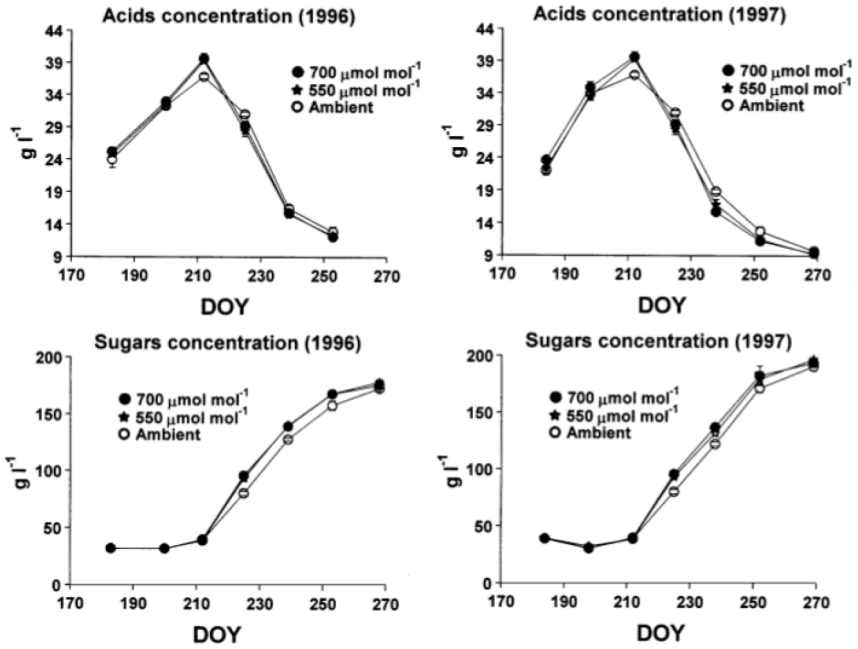
Figure 16: Photosynthesis in ($\mu mol.m^2.s^{-1}$) relation to CO_2 concentration ($\mu mol.mol^{-1}$) and 6 different leaf temperatures (15, 20, 25°C on A,C and 30, 35, 45°C on B,D) using 2 genotypes (Chardonnay A, B and Merlot C, D) with no light limitation (Greer D.H., 2018).



The impact of increasing atmospheric CO_2 on berry composition has to be taken into account (Kizildeniz et al., 2015; Edwards et al., 2017). Bindi et al. (2001b) showed that TSS accumulation (mainly sugars) during ripening increase with high CO_2 atmospheric concentration compare to ambient one but there is no difference between 550 and 700 $\mu mol.mol^{-1}$ enrichment (Figure 17). The duration of the treatment seems to play also a role in the increment in TSS (Edwards et al., 2017). Those observations are different from Kizildeniz et al. (2015) observations on Trampranillo. In this case, CO_2 elevation alone was inefficient to improve berry Brix (sugars content). Bindi et al. (2001b) saw that CO_2 enriched atmosphere created higher accumulating rate fruit biomass than ambient concentration, but without differences between treatments (550 and 700 $mmol.mol^{-1}$ CO_2 atmospheric concentration). CO_2 enriched atmosphere improve at maturity fruit DW between 40 and 45 % and total DW between 45 and 50 %. Same observation was made on berry and bunch DW

in red Tempranillo (Kizildeniz et al., 2015). For the acids, no real significant impact of CO_2 was found (Bindi et al., 2001b; Kizildeniz et al., 2015). Kizildeniz et al. (2015) revealed that elevated CO_2, regardless of T°, increased the total anthocyanins concentration in red Tempranillo under full irrigation conditions and it effect on TPI (Total Polyphenols Index) is negligible.

Figure 17: Concentrations ($g.L^{-1}$) of acids and sugars depending on CO_2 treatment (ambient, 550 $\mu mol.mol^{-1}$, 700 $\mu mol.mol^{-1}$) for 1996 and 1997 (Bindi et al., 2001b). Vertical bars indicate SE. DOY = Day of year.



CO_2 treatments effect the fermentation and can create unwanted ethyl acetate and diacetyl (co-products from fermentation) (Mira de Orduña, 2010). CO_2 concentrations may act on disease changing plant/insect or disease interaction (Sunitha, 2017). But increasing CO_2 can have advantages as improving yield (Edwards et al., 2017). In the long-term, a stabilization of CO_2 concentrations may reduce damage to yield and quality (Fraga et al., 2012).

Water

Water availability is a major factor for the vine development (Etienne et al., 2013; Ollat et al., 2017). The impacts of drought are largely depending on grapevine developmental stage (small shoot growth, poor flower cluster and small berry size) (Fraga et al., 2012). Water stress can have direct impacts on the vine physiology, reducing shoot growth, berry size, leaf area, dry weight, flower abortion and cluster abscission (Fraga et al., 2012; Kizildeniz et al., 2015; van Leeuwen & Darriet, 2016).

Water deficit has an impact on vine and decreases net photosynthesis (Fraga et al., 2012; van Leeuwen & Darriet, 2016) mainly due to stomatal closure (Stitt, 1991).

It can have also an impact on berry composition. For example, data suggest that, under water stress conditions, organic acid content and TA tend to increase in ripe fruits due to concentration effects (Etienne et al., 2013). Also, coupled with high CO_2 concentrations at ambiant T°, cyclic drought (until plants show visual signs of water deficit) can increase Brix (Kizildeniz et al., 2015). Water deficit has the advantage to favour higher anthocyanin/sugar ratios of Shiraz and also increase tannin concentration (Sadras & Moran, 2012; van Leeuwen & Darriet, 2016). Same observation was reported for Cabernet Sauvignon in the Riverland of Australia and the Columbia Valley of USA (Sadras & Moran, 2012). So, during berry development, a stress can be applied on the plant to partially restore anthocyanin/sugar ratios disrupted by high temperature (Zsófia et al., 2011; Ojeda et al., 2002). On contrary, excessive humidity during ripening promote sugars dilution, which is unfavourable for quality and can lead to damage on leaves (mainly due to diseases) and stuck grape ripening (Fraga et al., 2012; van Leeuwen & Darriet, 2016). Elevated CO2 can compensate the decrease in bunch weight induced by elevated T° and drought, maybe due to C balance compensation (Mira de Orduña, 2010; Kizildeniz et al., 2015).

III.2. An overview of putative means to mitigate grape/wine composition

As seen above, climate change has a lot of effects on wine composition (Ollat et al., 2017; Kharin et al., 2018). Cultural practices as corrections on must or wine potentially can mitigate some of the modifications in vine and berry development due to climate factors (Varela et al., 2005).

Cultural practices

The minimal pruning reduces berry sugars concentration (Novello & Palma, 2013; Martínez De Toda et al., 2015). This change in architecture leads to a metabolic change, lengthening the different growth cycles. Canopy management (Varela et al., 2005), as leaf thinning provide interesting results in reducing sugar concentration in grapes but its efficiency depends on the timing and intensity of the defoliation (Stoll et al., 2013). In fact, only severe leaf thinning allows a significant reduction sugar accumulation rate but with negative impacts on aromatic quality. Antitranspirant can also be sprayed on the leaves to reduce sugar concentration by altering photosynthetic capacities (Novello & Palma, 2013). Leaf exposition having a benefit for photosynthesis (Kriedmann, 1968; Fraga et al., 2012), shade management can reduce photosynthesis and also reduce canopy T° (Greer & Weedon, 2013). Also, throughout the plant cycle, irrigation management with severe stress as impact on sugars concentration in berries (Zsófia et al., 2011; Ojeda et al., 2002). Indeed, a light water deficit (-0.5MPa) decreases the volume of the berries without reducing the sugar concentration. Only high stress can reduce carbon uptake before veraison followed by moderate stress after veraison (Ojeda et al., 2002). Shading can also reduce sugars content when performed at the right time (Greer & Weedon, 2013). Early harvesting or partial green harvesting would make possible to reduce sugar concentrations, but these methods provide herbaceous compounds that are not in demand (Pickering, 2000; Torregrosa et al., 2014).

Oenological corrections

Sugars/Alcohol - Current oenological corrections consist of reducing the alcohol content of fermented or fermenting wines or reducing the amount of sugars in must (Table 1) (Pickering, 2000; Varela et al., 2005; Novello & De Palma, 2013; Aguera et al., 2010) and are legally regulated with a system excluding everything not authorized (Cottereau, 2005). There are several methods to extract alcohol from a wine such as thermal methods, the use of membranes, of yeasts that can produce less alcohol or even using a tracer gas (Ciani et al., 2016). De-alcoholization do not have many

influences on the organoleptic quality of the wine (Aguera et al., 2010; Bordenave et al., 2013), but it is difficult to implement both technically and in terms of regulations (European regulations: 20% maximum ethanol removal from the wine is authorized) (Meillon et al., 2010). It should also be noticed that 2 alcoholic degrees de-alcoholised wines from different methods (removal of sugars from the must, alcohol removal during fermentation (distillation) and from wine (membranes)) had similar profiles (Aguera et al., 2010). Another simple method is to add water either to the fermented or fermenting wine or to the must (Pickering, 2000). This process is called wetting (or galvanising) and consists of a simple dilution with water, to which flavours can be added to correct their dilutions (Pickering, 2000). This process is prohibited in Europe.

Acidity - This parameter can be modified in both must and wine using acid addition or cations (potassium) removal (Escudier et al., 2012; Sweetman et al., 2014). Both techniques which can't be used in the same time are strictly regulated (CEE-606/2009: addition of malic or tartaric acid up to 20 meq.L^{-1}; CEE 53/2011: removal potassium to increase acidity up to 54 meq.L^{-1}). But those solutions are just sustainable on the short term (Ollat et al., 2017).

Table 1: Methods possible to reduce fermentable sugar concentration or to remove alcohol from wine (Pickering, 2000).

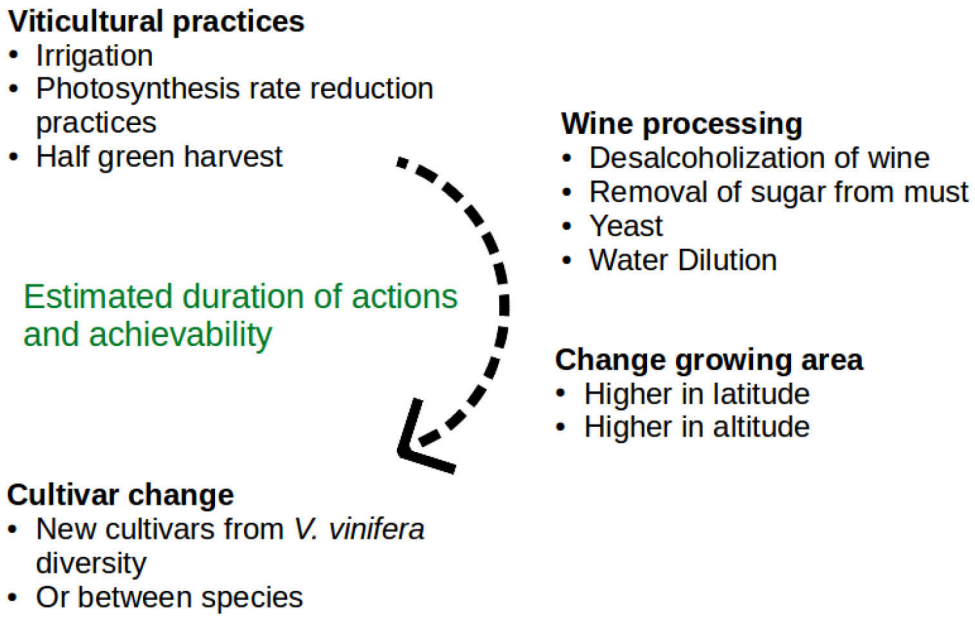
Principle	Method
Reduction of fermentable sugar concentration in grape or juice	Use of unripe fruit
	Juice dilution
	Freeze concentration and fractionation
	Enzymes (e.g. glucose oxidase)
Removal of alcohol from wine	Thermal: distillation under vacuum or atmospheric pressure; evaporation; freeze concentration
	Membrane: dialysis; reverse osmosis
	Adsorption: resins; silica gel
	Extraction: organic solvents; supercritical carbon dioxide
Other	Dilution of wine
	Arresting fermentation early
	Low-alcohol-producing yeast
	Combinations of above methods

Location and varieties change

The region can strongly influence berry development (Sadras & Moran, 2012; Ollat et al., 2017; Arrizabalaga et al., 2018). Planting in cold areas or at high altitudes reduces sugar concentrations (Torregrosa et al., 2014). The choice of rootstock is very important for berry solute accumulation (Jakab et al., 2013; Bordenave et al., 2013, Koundouras et al., 2009). For example, new CSIRO rootstocks can provide 20% more berry anthocyanins or a weakly vigorous rootstock can reduce berry sugar concentrations (Torregrosa et al., 2014; Novello & Palma, 2013). Another option can be provided by the use of varieties that limit sugar accumulation during ripening (Hannah et al., 2013; Torregrosa et al., 2014). In addition, researching on new varieties based on diversity rather than cultural practices would be cheaper on the long term (Arrizabalaga et al., 2018; Bigard et al., 2018).

Ollat et al. (2017) made a report on different research programs in France. Conclusions are represented in the figure 18, varietal innovation and changing production location should be sustainable (Fraga et al., 2012; Novello & Palma, 2013; Wolkovich et al., 2018), oppositely to viticultural, winemaking practices (Torregrosa et al., 2014; Ollat et al., 2017).

Figure 18: The different levers to mitigate the negative impacts of climate change (adapted from Torregrosa et al., 2014)



Issues

G5, G7 and G14 are part of the new varieties derived from *Muscadinia rotundifolia* experimented in a previous research program (Escudier et al., 2017; Ojeda et al., 2017). During the experimentation, traditional maturity controls revealed that new varieties presented a low sugar concentration trait with a "normal" polyphenolic maturity at harvest. This character is potentially interesting for breeding as a solution against increasing wine alcohol content but no studies have been conducted to understand this character.

In the order to characterise this trait, a precise look at the berry population development were needed. To characterise berry population asynchrony and heterogeneity, density sorting, Dyostem® (individual volume and colour) and individual berry firmness monitoring were first tested. The chapter one is describing those simple methods in order to determine as precisely as possible key periods in the ripening of grapevine fruits. This chapter will be presented in an article submitted in OenoOne.

New varieties are the descendants of the fourth and fifth generation derived from *Muscadinia rotundifolia G2* with a *Vitis vinifera* Malaga seedling. Genetic of descendants being mostly coming from *Vitis vinifera,* it was interesting to characterise the diversity for berry composition in *V. vinifera* for traits that potentially could help to mitigate some adverse effects of climate warming (sugars, acids, cations, and water). A subset of 12 *V. vinifera* varieties was selected based on expert's advice and preliminary experiments. Berry softening and volume kinetics were monitored in order to characterise fruit composition at the onset of ripening and maximum berry volume, i.e. at the physiological ripe stage.

In the aim of further breeding perspectives, we wanted to check if a cross could segregate main berry characters. For this purpose, a microvine population derived from a cross between a Picovine00C001V0008 (Vvgai1/Vvgai1) and the Ugni Blanc fleshless berry mutant was also phenotyped as above. Results are presented as an article already published in Frontiers in Plant Sciences for the diversity in sugars, acids and water, and as a paper draft for cations and titrable acidity.

Low sugar concentration genotypes, G7 and G14, were first tested using methods describe above in order to characterise berry population asynchrony and heterogeneity during development compared

to *V. vinifera* controls (Merlot, Morrastel and Grenache). G5, G7, G14, Merlot, Morrastel and Grenache analysis were then down-scaled to single berry analysis during berry growth in order to be more accurate in the description of the berry development in comparison with classical varieties. Firmness was also analysed to characterise as precisely as possible the onset of ripening. Sugar concentration then being used as scale for ripening, methods were tested in order to select berries at their maximum volume stage. After berry selections, genotypes were compared to characterise low sugar concentration trait and berry growth. Results form chapter three. This chapter will be presented in two parts with their own material and methods and results and discussion.

Finally, two microvine populations derived from a cross between G5 and V3 microvine and a cross between G14 and V3 microvines were created. After being phenotyped at the onset of ripening and physiological maturity, and being genotype by GBS (Genotyping By Sequencing; *ApeK1* enzyme; Illumina Hiseq), QTLs (Quantitative Trait Loci) analysis were performed for each population. The data are still being analysed.

Chapter I - How to monitor grape development using simple tools?

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How to monitor grape development using simple tools?

Antoine Bigard[1,2], Yannick Sire[2], Dargie T. Berhe[3], Mélanie Veyret[2], Hernán Ojeda[2], Charles Romieu[1]*, Laurent Torregrosa[1,2]

[1]*AGAP, University of Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France*

[2]*UE INRA de Pech Rouge, University of Montpellier, Gruissan, France*

[3]*Dilla University, SNNPRS, Dilla, Ethiopia*

Abstract

Aim: The purpose of this work is to select the most simple and efficient method to determine key periods in the ripening of grapevine fruits.

Methods and results: Experiments were performed in 2014 and 2015 in an irrigated vineyard located at the INRA Centre of Pech-Rouge, Gruissan (France). Berries from GX and Merlot varieties were sampled weekly and analysed to describe the advancement of ripening. The traits collected were: apparent firmness, density, fresh weight, volume assessed through Archimedes' method, Dyostem® or Tartaric acid relative dilution, primary (sugars and organic acids) and secondary (anthocyanidins) metabolites. Results showed that softening occurs before coloration with a time gap depending on the genotype x year interaction. Berry density sorting allowed the grouping of single fruits with similar Brix and provides interesting information about the extent of berry heterogeneity in sugar concentration. Density sorting also revealed that malic acid breakdown and anthocyanin accumulation were promoted when compared to sugar concentration, in late or slow berries. Berries at similar sugar concentration still exhibited considerable size heterogeneity. All fruit volume measurement methods displayed similar kinetics during ripening for both genotypes, allowing the accurate determination of the period when the berry population reached its maximum statistical volume.

Conclusion: Berry softening is a more reliable indicator of the onset of ripening than colour change and the delay between both traits is a pertinent variable. During ripening, berry density sorting gives useful information about the heterogeneity of sugar concentration and its interaction with acids and anthocyanin. Ripe stage may be objectified through periodic measurements of average berry volume or weight in order to detect the stop of phloem unloading and the onset of

berry shrivelling. Time resolution is considerably impaired by asynchronic berry development and berry weight heterogeneity.

Significance and impact of the study: Results presented here can help for the definition of suitable processes of grapevine fruit sampling.

Introduction

For many countries, viticulture plays a considerable socio-economic role. Vine growing area represents more than 8 million hectares worldwide, making it one of the most important horticulture crops (Myles et al., 2011; Aleixandre et al., 2014). In France, for example, the industry represents a yearly economic activity of about 11.4 billion Euros (Franceagrimer, *www.franceagrimer.fr*). Wine-grape is one of the most sensitive crops to climate changes due to the determinant influence of meteorological factors on wine composition (Webb et al., 2008). Most widely-used grapevine cultivars belong to the *Vitis vinifera* that originated from South Caucasus towards Mediterranean regions (This et al., 2006; Bacilieri et al., 2013). Grape is a non-climacteric fleshy fruit characterised by a double sigmoid growth pattern. Pericarp develops from ovary mesocarp through a short period of cell divisions triggered by fecundation and followed by two cycles of vacuolar expansion (Coombe & Hale, 1973; Coombe, 1976; Ojeda et al., 1999). During berry development and ripening, metabolite concentrations evolve depending on net biosynthesis or metabolization, and growth dilution, both mechanisms being genotype-dependant (Dai et al., 2011; Keller, 2015; Bigard et al., 2018).

The first growth phase results from both mitosis, which activity peaks 5 days after anthesis, and a first period of cell enlargement (Ojeda et al., 1999). During this developmental phase, the berry is green and hard, and accumulates tartaric and malic acids as the main contributors to berry osmotic potential (Terrier and Romieu, 2001; Keller et al., 2015). Tartaric acid is predominantly accumulated at the beginning of the green growth phase while malic acid is accumulated later until the onset of ripening. The final number of cells of the pericarp is definitively determined at the end of green growth phase (Ojeda et al., 1999). Between phase one and phase two of growth, there is a non-growing period of the fruit called lag phase or green plateau (Thomas et al., 2008; Vicens, 2007). The second growth phase, known as ripening, starts with a sudden berry softening (Coombe, 1984, Robin et al., 1997) involving abscisic acid (ABA) signalling (Kuhn et al., 2013; Castellarin et

al., 2015, Pilati et al., 2017). Considerable changes in gene expression occur simultaneously with berry softening (Terrier et al., 2005; Deluc et al., 2007; Rienth et al., 2016; Balic et al., 2018).

Most grapevine phenology scaling systems consider the change in berry skin pigmentation as the starting point of ripening (Baggiolini, 1952; Eichhorn and Lorenz, 1977; Symons et al., 2006; Toffali et al., 2011). The most widely used reference to qualify the onset of grape ripening is the so-called "mid-véraison" stage that corresponds to the presence of 50% pigmented berries (Grotte et al., 2001). During ripening, the increase of sugar and water contents is associated with a decrease in organic acids concentration (Davies & Robinson, 1996, Terrier et al., 2005; Vicens, 2007). This period is characterised by an increase in phloem unloading and a decrease in xylem water influx (Greenspan et al., 1994, Keller et al., 2005). Sucrose import occurs through the apoplastic phloem unloading pathway (Zhang et al., 2006), before being cleaved in glucose and fructose by invertases (Hawker, 1969; Takayanagi & Yokotsuka, 1997). The considerable osmotic potential resulting from sugar accumulation during berry ripening (Matthews et al., 1987) associated with cell wall modifications promote the second wave of cell enlargement by water influx (Xie et al., 2009). Potassium appears as the fourth more abundant solute in berry (0.05-0.1mol.L^{-1}) (Storey, 1987) with a discrete contribution to grape osmotic potential (Rogiers et al., 2017). During ripening period, berry sugar concentration is proportional to berry volume (Matthews & Nuzzo, 2007). Theoretical models of sugar and water accumulation in the grapevine berry were elaborated (Dupin et al., 2010; Dai et al., 2011). Secondary metabolites such as anthocyanins (red grapes) or flavonols are also accumulated in ripening berries (Toffali et al., 2011). After the end of sugar phloem unloading, sugars and other metabolite concentration continues due to water loss (Coombe & McCarthy, 2000; Conde et al., 2007; Bondada et al., 2017).

Phenological shifts must be clearly distinguished from intrinsic physiological changes in the interpretations of the GenotypexEnvironment effects on fruit traits (Carbonell-Bejerano et al., 2013, 2016). In this respect, it is of major importance that key transition stages, like the onset of sugar accumulation or the arrest of phloem unloading could be experimentally objectivised (Rienth et al., 2016; Bigard et al., 2018). Unfortunately, only few methods are available to monitor grape development in the field and most of them are destructive or lack of precision. In most studies, the determination of the onset of ripening is still performed by monitoring berry colour change (Symons et al., 2006; Parker et al., 2011; Toffali et al., 2011; Arrizabalaga et al., 2018) which was shown to occur after sugar accumulation starts (Robin et al., 1997; Castellarin et al., 2015). No precise non-destructive handy tools except following berry growth exist for detecting the maximal

volume (Coombe, 1984; Lang & Thorpe, 1989; Rienth et al., 2016; Friedel et al., 2016). Whatever, berry heterogeneity complicates the relation between fruit physiology and wine quality (Nelson et al., 1963; Lund et al., 2008; Böttcher et al., 2011; Rolle et al., 2013; Doumouya et al., 2014; Rienth et al., 2016; Shahood et al., 2017).

Berry heterogeneity may result from a large range of factors controlling inflorescence and fruit development, in particular fruit radiative and evaporative microenvironment (Kuhn et al., 2013; Doumouya, 2014; Reshef et al., 2017). Grape development is generally described in terms of berry growth, sugars, acids and anthocyanins concentrations, on pools of a significant number of randomly sampled berries, so that the composition of the average population in the considered parcel should be accurately described (De Montmollin et al., 2004; Geraudie, 2009; Parker et al., 2011; Arrizabalaga et al., 2018). This approach allows an optimization of harvesting date and important oenological features predictions, as influenced by the year. It is widely accepted that berry heterogeneity is so strong that hundreds of berries must be sampled, unfortunately the GxE plasticity of this heterogeneity and its impact on wine quality are unknown. Classically, fruit size is analysed by photo imaging, size separation using a sieve or downscaling at single berry phenotyping (Rienth et al., 2016; Friedel et al., 2016). There are also new technologies to follow berry ripening like Vis-NIR spectroscopy (Geraudie, 2009) or texture analysis (Coombe, 1984; Doumouya, 2014; Castellarin et al., 2015). To gain some precision and standardize samples, berries are sorted according to their apparent density or internal sugar concentration in more and more studies to select berries at (Nelson et al., 1963; Singleton et al., 1966; Lanier & Morris, 1978; Terrier et al., 2001 & 2005; Fournand et al., 2006; Kontoudakis et al., 2011; Rio Segade et al., 2013; Carbonell-Bejerano et al., 2016; Friedel et al., 2016). However, with this approach other factors such as berry size or other primary metabolites are not considered (Friedel et al., 2016).

With the objective to select the more efficient or handy method to determine the beginning and the end of ripening, we experimented with a set of approaches to analyse the rheological and biochemical fruit changes during ripening: apparent firmness and density, fresh weight and volume, primary (sugars and organic acids) and secondary metabolite (anthocyanidin) contents.

Materials and methods

Plant material

Grape berries were sampled from outdoor vines at the INRA of Pech-Rouge, Gruissan, France (43.14' N latitude and 3.14'' W longitude, elevation 6m above sea level). The experimental vineyard is located in semi-arid Mediterranean climate (Giorgi & Lionello, 2008) and managed though drip irrigation to keep the predawn leaf water potential (Ψ_{PD}) higher than 0.5MPa. In this study, three different genotypes were used (Table 1): Merlot and Grenache, two widely-grown varieties and a new powdery and downy mildew resistant hybrid, 3184-1-9N (Escudier et al., 2017; Ojeda et al., 2017), named GX in the rest of the manuscript.

Table 1 - Experimental design

Method/Parameter	2014	2015
Density sorting	x	x
Dyostem®	x	x
Archimed		x
Fresh weight	x	x
Glucose and Fructose	x	x
Malic and tartaric acids	x	x
Anthocyanidins	x	x
Osmolality	x	
Dry matter	x	

Sampling methods

During the vintages 2014 to 2015, samplings were performed once or twice a week starting 1-2 weeks before the first signs of berry softening and up to berry shrivelling. In 2014, 600 berries were randomly sampled from the entire field for each genotype and date. In 2015, the experimental plot was divided in three distinct blocks for each genotypes, with 200 berries were sampled and analysed separately from each block. For all experiments, berries were separated from bunches by cutting the pedicel the nearest possible from the berry, in order to minimise the impact of this organ for the volume measurement and to limit juice leaking.

Density sorting

As soon as possible after sampling, i.e. before 1 hour, a sorting of the berries was performed through their apparent density as described in Nelson et al. (1963) and Singleton et al. (1966) with slight modifications, i.e. using NaCl instead of sucrose (Rolle et al., 2011; Carbonell-Bejerano et al., 2013) to prevent any microbiological issues. Twelve solutions were prepared from 80g to 190g $NaCl.l^{-1}$ with same increments as Carbonell-Bejerano et al. (2013) (Table 2).

Table 2 - NaCl concentration (g/L) and correspondence between apparent density and sugars concentration in g/L and mmol/L.

Bath number	NaCl (g/L)	Density	Sugars (g/L)	Sugars (mmol/L)
1	190	1.12	279	1549
2	180	1.11	264	1465
3	170	1.11	248	1377
4	160	1.10	233	1293
5	150	1.09	218	1210
6	140	1.09	202	1121
7	130	1.08	187	1038
8	120	1.08	172	955
9	110	1.07	156	866
10	100	1.06	141	783
11	90	1.06	126	699
12	70	1.05	110	611
13	< 70	< 1.05	< 110	< 610

Volume measurement

The average berry volume of each apparent density class was measured independently by image analysis and immersion. The Dyostem® (Vivelys company, *www.vivelys.com*) device takes a picture of 100 berries immobilized on regular wells on a blue plate, and calculates each berry volume following contour adjustment with a perfect circle, before reporting berry size distribution in the sample. Alternatively, net-bagged berries were hanged in a beaker containing pure water on a balance, in order to measure displaced water volume according to the Archimedes law ($P_{Moved\ Liquid} = w_{Moved\ Liquid} \times g = \rho_{Liquid} \times V \times g$) (Lang & Thorpe, 1989). Change in berry volume during ripening was also estimated from the decrease in tartaric acid concentration from the onset of ripening, assuming a constant amount per berry during ripening as generally accepted.

Osmolality and Dry Matter

The osmolality was measured using a Single-Sample Freezing Point Osmometer (Gonotec, *www.gonotec.com*). After a three-point calibration (distilled water as zero-point calibration, 300mOsmol.kg^{-1} NaCl/H_2O and 850mOsmol.kg^{-1}), 3 replicates of 50µL juice sample were directly analysed by the device expressing average measurement in mOsmol per kilograms. For dry matter, samples were vortexed and centrifugation 5min (8000g) at room temperature. Two hundred fifty µL of clear juice were weighted before and after 24 hours 105°C dry followed by an hour desiccation.

Primary and secondary metabolites

In 2014, every density class was analysed as a separate sample (except when the number of berries per class was lower than 10, then they were pooled with the nearest class). Immediately after berry sorting, samples were crushed with a domestic crusher during approximately 15sec at room temperature. Then three crude juice samples were taken with one immediately frozen at -30°C. Anthocyanins quantification was done with one fresh sample (10-20mL) which was weighted and three times diluted in a hydro-alcoholic solution (2.631mol.L^{-1} ethanol + 0.01mol.L^{-1} HCl). After one hour orbital stirring, 10mL of solution were centrifuged 5min at 12000g (20°C), then the supernatant was 20-50-time diluted depending on colour, before 520nm absorbance measurement in a 1cm optical path Evolution 300 UV-VIS spectrometer (Thermo Scientific, *www.thermoscientific.fr*). Anthocyanins (mg.L^{-1}) were calculated as total dilutionxOD520nmx22.76 (Ribéreau-Gayon et al., 1998). The second fresh sample was centrifuged same as above, before titration to pH 7 of 20mL supernatant with 5mol.L^{-1} NaOH using a TitroMatic KF 2S 2B (Crison, *www.crisoninstruments.com*). Total acidity was expressed as meq(H_2SO_4).L^{-1}. The frozen sample was used to quantify primary metabolites. Samples were thawed in a 60°C water bath during 30min, vigorously shacked with an orbital shaker for 15sec, and centrifuged as above. Supernatants were ten times diluted with 0,2N HCl and filtered on cellulose acetate 0.2µm membranes, before injection on HPLC (Biorad aminex-HPX87H column) according to Bories et al. (2011) with same conditions as described in Bigard et al. (2018).

Data analysis

R-software version 3.4.3 was used for statistical analysis (R Core Team, 2017).

Results and Discussion

1. Determination of the onset of ripening

This developmental stage corresponds to the beginning of sugars uploading and to the maximum of organic acid concentration and content per berry. At this stage cell division is completed (Ojeda et al., 1999; Fernandez et al., 2006) and the final berry size can already be anticipated (Coombe, 1984; Houel et al., 2013; Bigard et al., 2018).

Figure 1 - Merlot and GX berry softening and colour change (Dyostem®) in 2015.

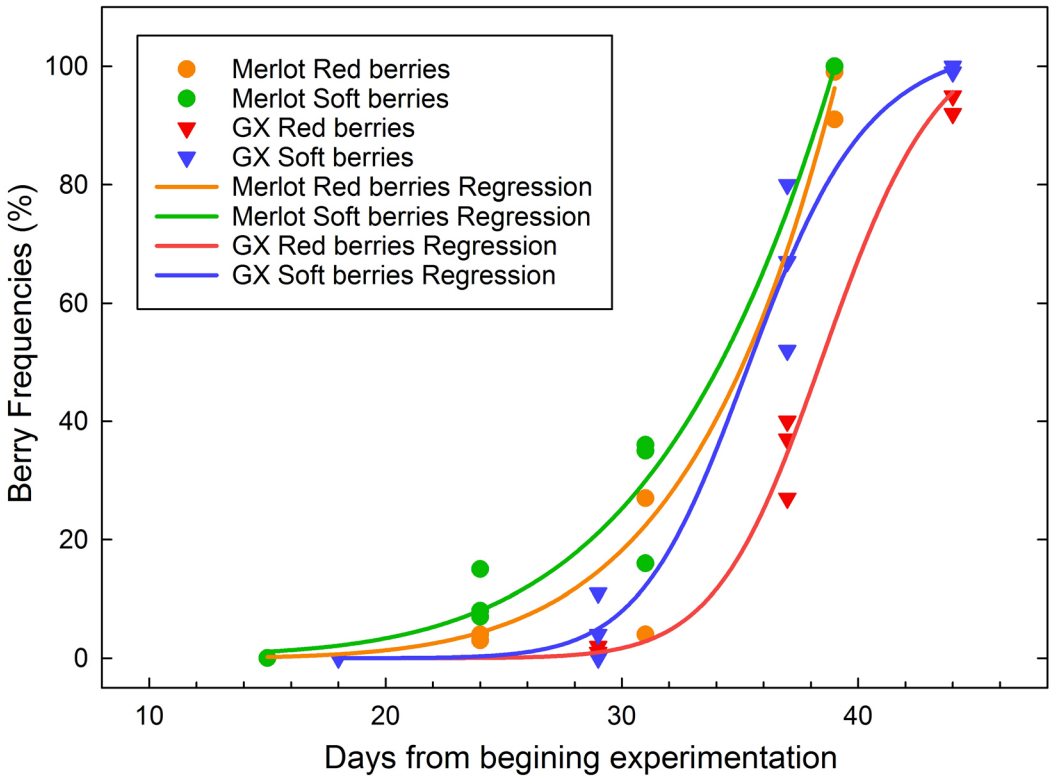


Figure 1 represents how the respective frequencies of soft or coloured berries increase with time inside the Merlot and GX populations in 2015. The difference in firmness between hard and soft berries is so high that it can even be detected by hand (Terrier et al., 2005; Bigard et al., 2018). Obviously, the onset of ripening does not occur simultaneously inside berry population but spreads over a ten to fifteen days period. Moreover, skin coloration was delayed from softening by several days, which is in agreement with previous reports using more accurate methods (Robin et al., 1997; Castellarin et al., 2015). Consequently, at "mid-véraison" stage, which is defined as “half of pigmented berries”, a bunch actually contains more ripening than unripe berries. This figure shows

that the duration between colour change and softening depends on varieties. In 2015, the average delay from mid-softening to mid-colour change was about 3.6 days in GX, compared to 1 day for Merlot. In addition, we observed that this parameter also strongly depended on the year (for Merlot the delay was 1 day in 2014 and 4.5 days in 2015) and even on samples location in the field (GX 2015, repetition 1 = 4.5 days, repetition 2 = 1.7 days, repetition 3 = 4.6 days). Mixing ripening berries that are diluting and metabolizing organic acids with hard green berries that are still accumulating them doesn't appear as the best way to estimate the maximum of acidity potential at the onset of ripening.

To get more accurate insight on maximum acidity and berry size at the completion of green growth stage just before ripening starts, it is convenient to eliminate the first soft berries as soon as they appear and characterize the remaining ones, however such a precaution does not preclude that the contribution of late berries will not led to an underestimation of these parameters. This method was successfully used in Bigard et al. (2018) to get more pertinent insights on the genetic diversity of berry size and primary metabolites *Vitis vinifera* at the end of green growth. The determination of berry firmness by hand is as quick as monitoring berry colour changes and provides much more accurate samples and information. For increased precision, single berry can also be analysed with tools as Penelaup® (Abbal et al., 1992) or portable devices allowing non-destructive measurements (Coombe, 1992; Castellarin et al., 2015).

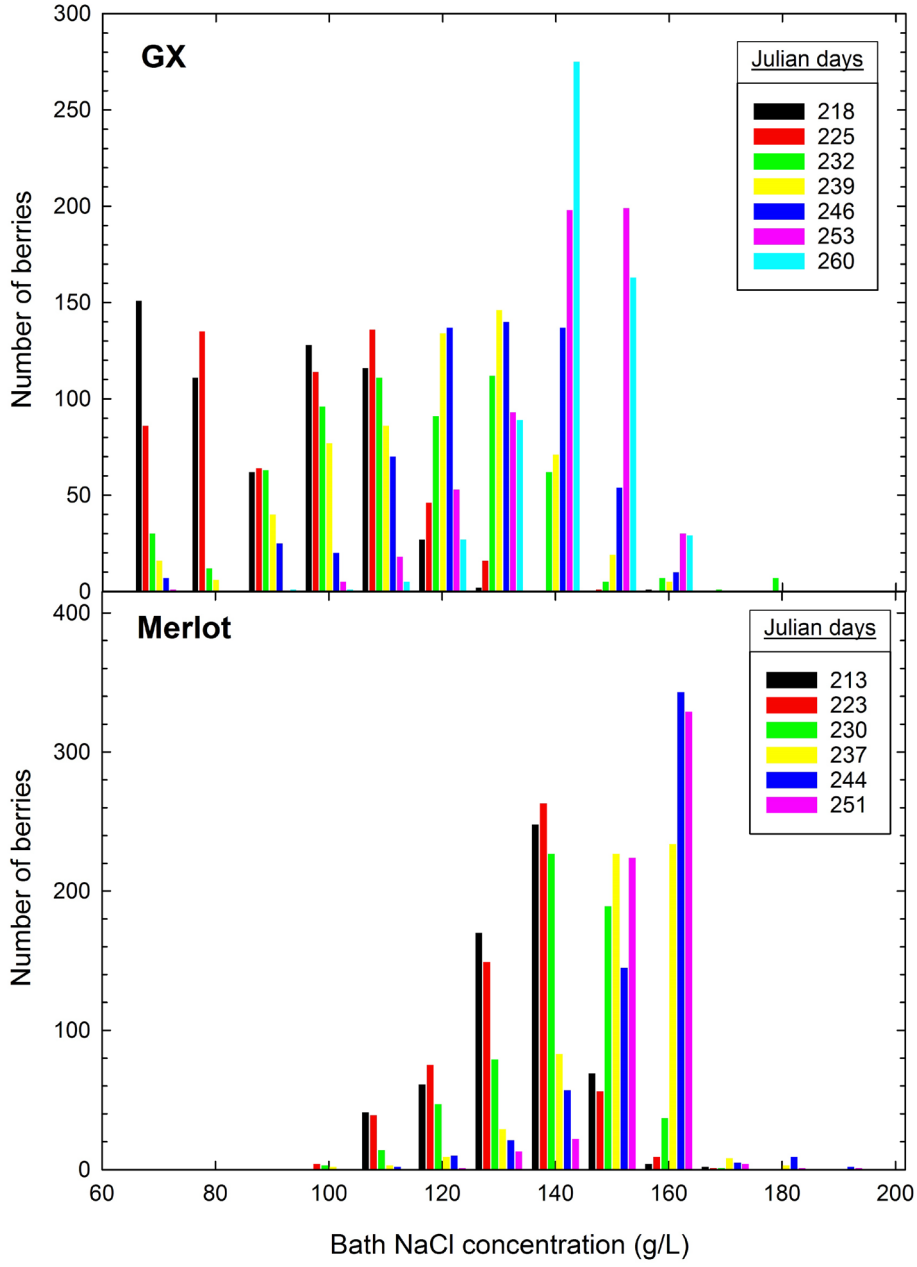
2. Heterogeneity of berry development during ripening

As well-known since Coombe (1984) and represented in figure 1, berries don't start ripening simultaneously. Berries with different amount of sugars, organic acids and secondary metabolites can thus be mixed in samples (Kontoudakis et al., 2011; Friedel et al., 2016). If controlled by the viticulturist, this asynchrony may permit to produce different wine types with the same cultivar but can also have a negative impact causing uneven maturity or inter-seasonal fluctuations (Dupin et al., 2010). However, the heterogeneity within bunches potentially disturb the accurate analysis of fruit development and metabolism in most genetic and physiological studies.

Figure 2 represents the frequencies of fruits at different densities and its evolution during the 2014 season for GX and Merlot. Lower the NaCl concentration is, lower are the berry density and its internal sugars concentration i(Table 1). This histogram shows that it is possible to separate berries at different stages of sugars concentration as described in Nelson et al. (1963), Singleton et al.

(1966), Böttcher et al. (2010) and Friedel et al. (2016). In the conditions of present study, early samplings showed a huge heterogeneity in densities spreading the berries on 7 baths. Then, during ripening, heterogeneity tended to decrease, in agreement with Kontoudakis et al. (2011), Gouthu et al. (2014) and Belviso et al. (2017). All together, these results show that berry heterogeneity is present in all stages of ripening.

Figure 2 - Number of berries per bath during the ripening of GX and Merlot in 2014.



The figure 3 represents the total acidity for each class of apparent density depending on sampling dates for GX and Merlot in 2014. This result confirmed that the acidity level tends to decrease

during the season in all density batches, as initially mentioned by Singleton et al. (1966) and recently described by Friedel et al. (2016). This suggests that early ripening berries accumulate sugars at lower malic acid consumption when compared to the late ones. Different mechanisms could explain this shift in primary metabolism: e.g. sugar availability may be higher for the first ripening berries, associated with delayed or reduced malate breakdown in fruits with a more comfortable glucidic status (Rienth et al., 2016). Alternatively, the relative evolution of malic acid can be modulated by the progressive evolution of environmental factors during the season (Davies & Robinson, 1996; Vicens, 2007; Rienth et al., 2016).

Figure 3 - Evolution of the juice total acidity ($gH_2SO_4.l^{-1}$) per bath during the ripening of GX and Merlot in 2014.

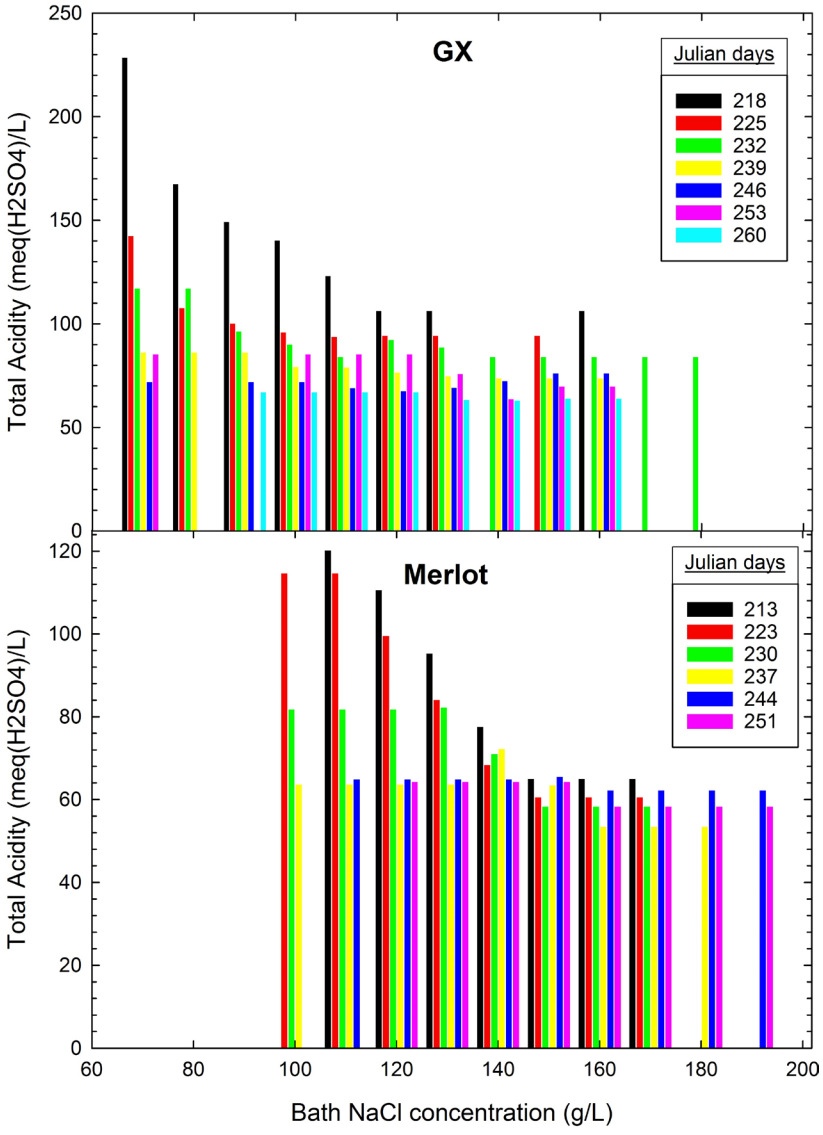


Figure 4 represents the GX and Merlot berry volume heterogeneity assessed by the Dyostem® for bath number 8 (ca 170g.L^{-1} sugars, cf. Table 2). The frequencies of small berries below 1.5g decreased from ca 67% to less than 8% while those heavier than 2.5g increased from less than 4% to 22% in GX during the season, indicating that the net rate of sugar unloading may become a limiting factor in the bigger berries, that would therefore ripen slower or later. This tendency was not confirmed in Merlot lacking such large berries. Whatever, fruit size diversity at the same sugar concentration appeared considerable, in agreement with Coombe (1984) and Friedel et al., 2016 , but in contradiction with Matthews & Nuzzo (2007).

Figure 4 - Evolution of the berry size in the bath n°8 (120g.l^{-1} NaCl) during the ripening of GX and Merlot in 2014.

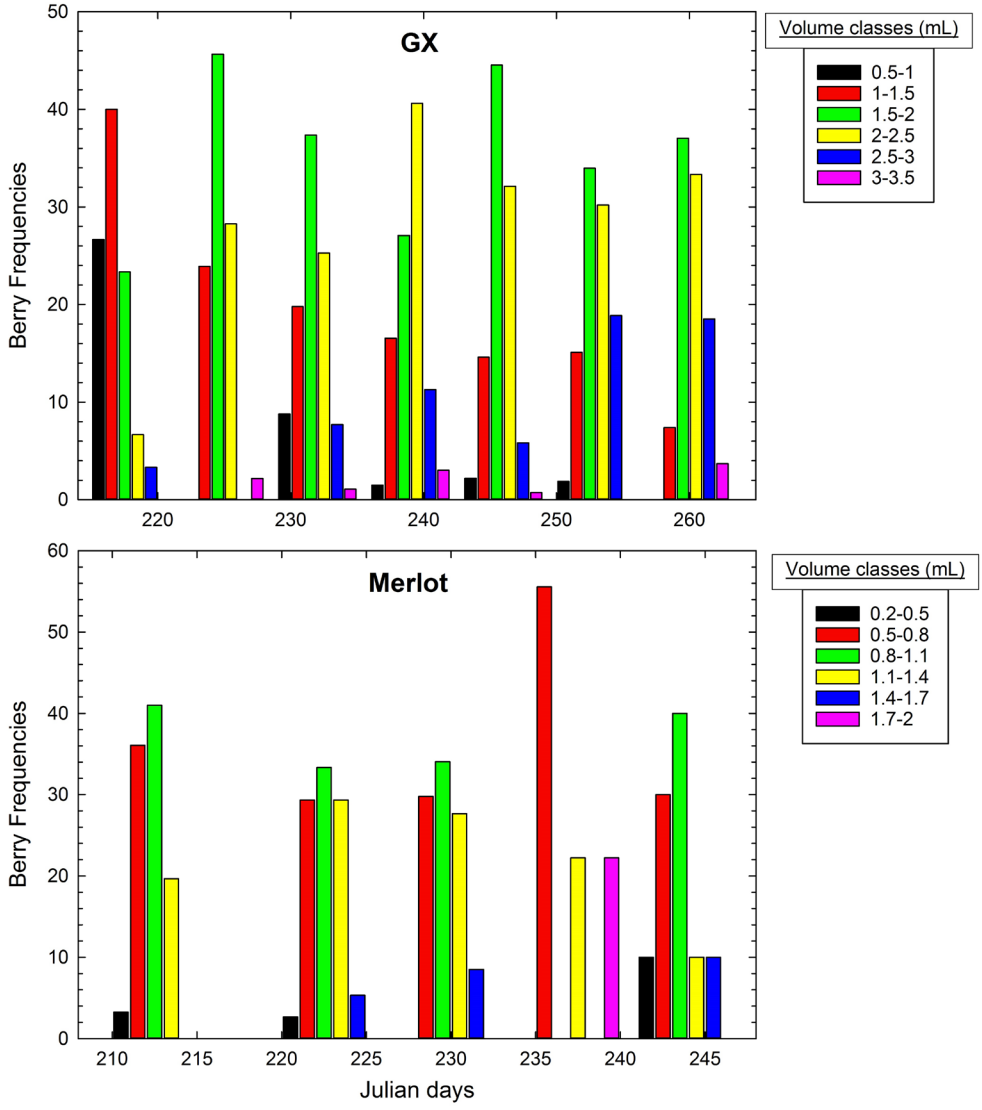
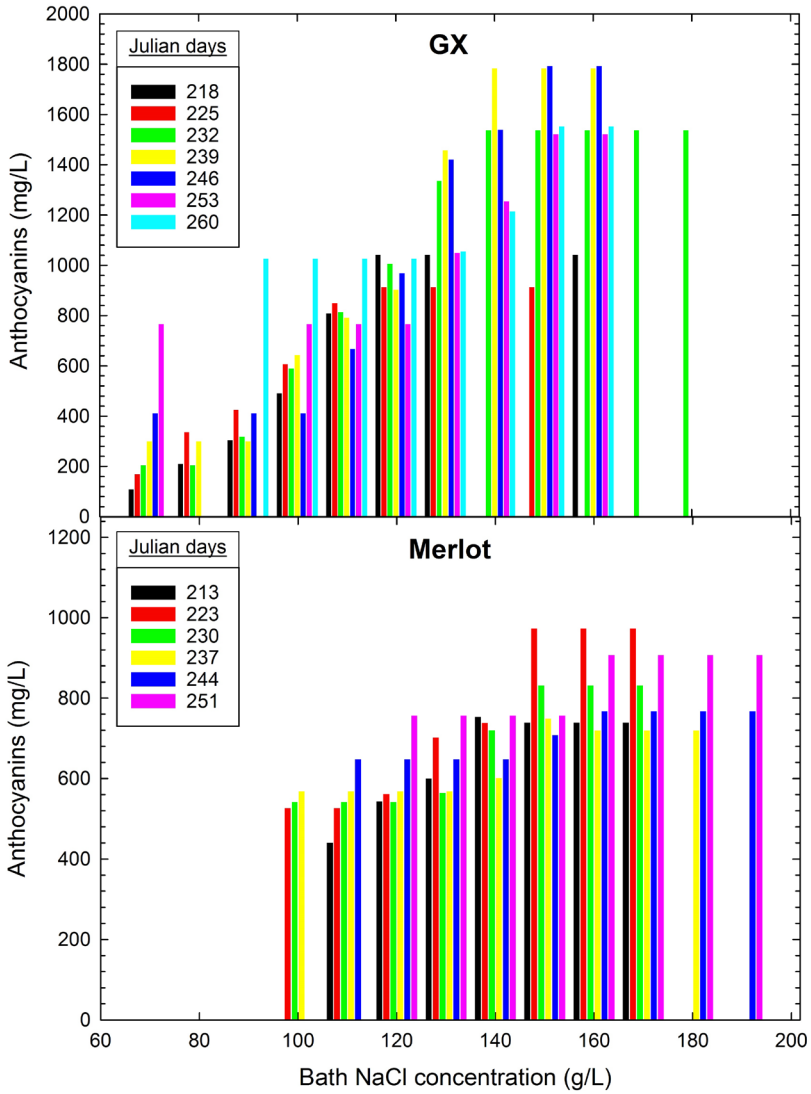


Figure 5 represents the evolution of anthocyanin concentration per batch and sampling date. As compared with figure 3, for the same level of sugars, late ripening berries tend to produce and accumulate more anthocyanins than early ones, suggesting that first ripening berries would finally accumulate sugars quicker relatively to organic acid and colour changes when compared to the late ones (cf. First baths for GX in 2014). This observation agrees with Belviso et al. (2017), who showed that the abundance of protocatechic acid in berry skin did not follow the same pattern of development in early and late berries. The interpretation becomes more difficult for sugars concentration greater than 202g.L^{-1} maybe due to berry shrivelling or due to environment conditions as discuss in Bigard et al. (2018).

Figure 5 - Evolution of the anthocynaidins (mg.L^{-1}) per bath during the ripening of GX and Merlot in 2014.



Osmolality and dry matter content were analysed in all 2014 samples. Osmolality values were strongly correlated with Brix for both genotypes with correlation coefficients higher than 0.98 (p-Value < $2.2e^{-16}$) as observed by Matthews et al. (1987). Same observation was made for dry matter content which correlated with Brix (Data not shown). Despite osmolality or dry matter measurements are accurate methods to appreciate the osmotic potential and total solutes per fruit, but they do not appear very convenient to monitor ripening and figure out berry heterogeneity as compared to Brix.

During ripening, density sorting appears to be a pertinent method to prepare lots of berries with homogeneous sugar concentration (Nelson et al., 1963; Singleton et al., 1966; Lanier & Morris, 1978; Terrier et al., 2001 & 2005; Fournand et al., 2006; Kontoudakis et al., 2011; Rio Segade et al., 2013; Carbonell-Bejerano et al., 2016; Friedel et al., 2016). However one must be particularly aware that other ripening–related metabolites display a marked plasticity with respect to sugars (Belviso et al., 2017), and berry size as reported by Singleton et al. (1996) and Friedel et al. (2016).

During experimentation, a small shift (5-10%) of the berry sugars concentration value corresponding to each bath was observed as also described by Singleton et al. (1996). Many factors could explain the observed shift. Firstly, as observed in figure 3, there is a drift in the average of total acidity by bath when acids are the major soluble solids. This could result from the impacts of developmental changes in organic acids and/or other solutes or respective contribution of flesh, seeds and skin to average fruit density.

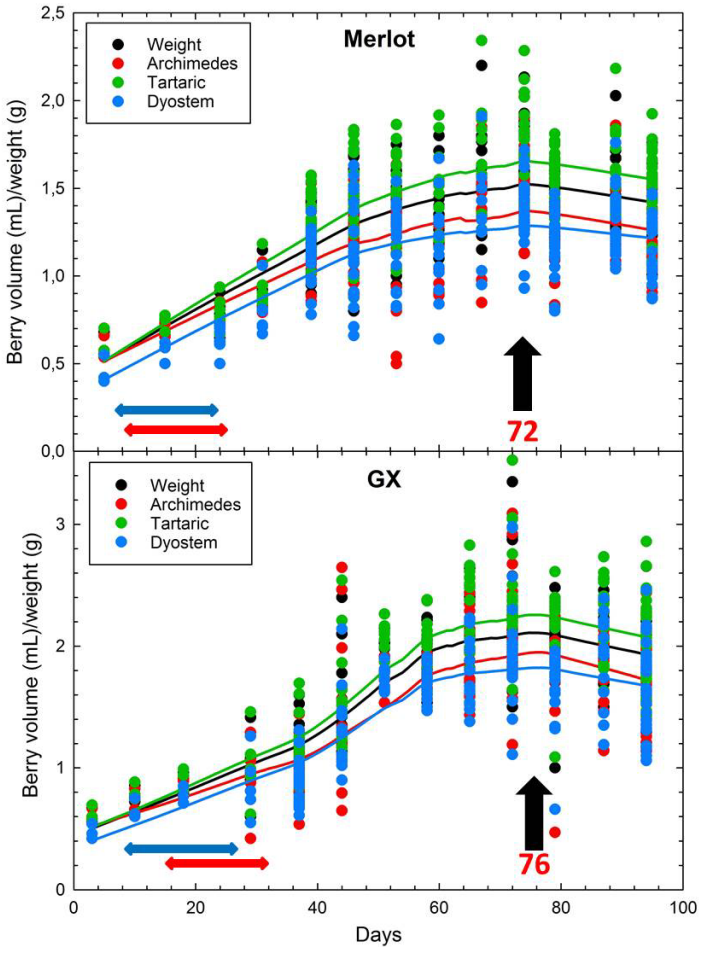
Apparent density sorting can provide an overview of the heterogeneity in soluble contents within a berry population (Friedel et al., 2016). However, density separation is a tedious process (e.g. 1.5h to sort 600 berries through 12 baths) that can't be performed on a large number of environmental conditions or genotypes.

3. Detection of the end of the growing phase

Maximum of berry volume may be taken as an objective criterion for the timing of phloem arrest as definition for physiological maturity, while sugar concentration continuously increases when berry shrivelling replaces phloem mass flow as a concentration mechanism. The only way today to determine accurately the point of water maximum content per fruit is to monitor growth kinetics. Figure 6 represents berry growth pattern assessed by Dyostem®, Archimedes' method, fresh weight

or tartaric acid dilution, assuming tartaric content is stable during ripening (Lang & Thorpe, 1989; Terrier and Romieu, 2001) but using this acid for berries volume estimation is tricky due to risk of precipitations in presence of potassium (Rösti et al., 2018). As shown in figure 6, each method provides different absolute volumes, but maximum volume is simultaneously reached at 76 days after beginning of sampling for GX in 2015, which corresponds to 50 days after veraison, as widely documented in the literature. Considering the ease of performing Archimedes' method and the possibility to implement it in a non-destructive way (Lang & Thorpe, 1989; Bigard et al., 2018), this approach appeared the most suitable to determine, at population level, the point when a balance occurs within the population of berry between the fruit growing and shrivelling.

Figure 6 - Comparison of the berry volume kinetics measured by fresh weight (g) and volumes (mL) (Archimedes' method, estimated by Dyostem® or tartaric concentration) during the ripening of Merlot and GX. Curves represent the loess for each method with a span equal to 0.6. Blue and red arrows represent respectively the softening and the colour change periods, black arrow represents the maximum volume stage.



Conclusion

In order to better understand the effects of environmental factors on grape quality or to properly phenotype genetic resources, critical transitions in berry development needs to be objectified by additional variables than average sugar concentration in berry population. However, there is an inherent paradox in looking for precise stages of development in a non-synchronous population, and the dynamic structure of this population must necessarily be accounted for. We have shown that berry softening is a much pertinent indicator than colour change to detect the onset of ripening. Changes in berry firmness can be determined either manually or by electronic devices, in a destructive or non-destructive way. During ripening, the heterogeneity of berry development can be characterized by a combination of NaCl density sorting and Dyostem® or other image analysis systems, these approaches allow the characterization of several hundred of individual berries. One must remain however particularly aware that organic acid and anthocyanins display significant plasticity with respect to sugar concentration. Then, accurate determination of berry population structure requires statistical evaluation of these metabolites in hundreds berries. To properly identify berry at specific stages during ripening, the only accurate method remains to downscale the analyse at single berry level as described by Rienth et al. (2016) or Shahood (2017) but this method, which is time consuming, can only be performed in limited dimension experiments. These aspects will be examined in a following paper.

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Author contributions: LT, CR and AB designed the experiments. AB, MV and YS performed the experiments. AB, LT and CR drafted the manuscript. AB, LT, CR and HO edited the manuscript. All authors reviewed the final version of the manuscript.

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Chapter II - Vitis vinifera L. Fruit Diversity

II.1 - *Vitis vinifera* L. Fruit Diversity to Breed Varieties Anticipating Climate Changes

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Vitis vinifera L. Fruit Diversity to Breed Varieties Anticipating Climate Changes

Antoine Bigard[1,2], Dargie T. Berhe[2,3], Eleonora Maoddi[1], Yannick Sire[2], Jean-Michel Boursiquot[1], Hernan Ojeda[2,4], Jean-Pierre Péros[1], Agnès Doligez[1], Charles Romieu[1] and Laurent Torregrosa[1,2]*

[1]AGAP, University of Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France

[2]UE INRA de Pech-Rouge, University of Montpellier, INRA, Montpellier, France

[3]SNNPRS, Dilla University, Dilla, Ethiopia

[4]UE INRA de Vassal, Grapevine Biological Resource Centre, University of Montpellier, INRA, Montpellier, France

Abstract

The wine industry is facing critical issues due to climate changes since production is established on very tight Genotype × Environment interaction bases. While, some cultivation practices may reduce adverse effects of abiotic stresses on the vines, e.g., the use of irrigation to mitigate drought, the deleterious impacts of warming on fruit development are difficult to manage. Elevated temperature alters grapevine fruit growth and composition, with a critical increase of the sugars/organic acids ratio. Select grapes with improved metabolite balances to offset high temperature effects is a valuable option to sustain viticulture. Unfortunately, the lack of knowledge about the genetic diversity for fruit traits impacted by temperature impairs the design of breeding programs. This study aimed to assess the variation in berry volume, main sugars and organic acids amounts in genetic resources. Fruit phenotyping focused on two critical stages of development: the end of green lag phase when organic acidity reaches its maximum, and the ripe stage when sugar unloading and water uptake stop. For that purpose, we studied a panel of 33 genotypes, including 12 grapevine varieties and 21 microvine offspring. To determine the date of sampling for each critical stage, fruit texture and growth were carefully monitored. Analyses at both stages revealed large phenotypic variation for malic and tartaric acids, as well as for sugars and berry size. At ripe stage, fruit fresh weight ranged from 1.04 to 5.25 g and sugar concentration from 751 to 1353 mmol.L^{-1}. The content in organic acids varied both in quantity (from 80 to 361 meq.L^{-1}) and in composition, with malic to tartaric acid ratio ranging from 0.13 to 3.62. At the inter-genotypic level, data showed no link between berry growth and osmoticum accumulation per fruit unit, suggesting that berry water uptake is not dependent only on fruit osmotic potential. Diversity among varieties for berry size, sugar accumulation and malic to tartaric acid ratio could be exploited through cross-breeding.

This provides interesting prospects for improving grapevine to mitigate some adverse effects of climate warming on grapevine fruit volume and quality.

Introduction

With 75–85 million tons of grapes produced yearly in the world, the grapevine is the main fruit crop. Grapevine fleshy berry, classified as a non-climacteric fruit (Coombe, 1976), undergoes a complex development process including two growth phases (Mullins et al., 1992). The first growth phase results from cell division and expansion coupled with the accumulation of organic acids, mainly tartrate and malate (Kliewer, 1965). After a lag phase called green plateau, fruit softens and massive uptake of sugars triggers a second phase of flesh cell enlargement (Matthews et al., 1987). Considering their sequential accumulation, organic acids (up to 250 $mmol.L^{-1}$) and sugars (up to 1 M) appear as the main drivers of berry osmotic potential during green and ripening growth phases, respectively. Other solutes, such as potassium, which only peaks at 30 $mmol.L^{-1}$ at ripe stage, would be minor players in fruit osmotic potential (Rogiers et al., 2017). The final concentrations of sugars and organic acids at ripe stage determine the ethanol to acidity ratio after yeast fermentation, which is a primary factor of wine quality (Champagnol, 1984; Ribéreau-Gayon et al., 2006).

Domesticated *Vitis vinifera* L., the major grapevine species cultivated for wine production, is supposed to have been diffused from the South Caucasus toward Mediterranean regions (This et al., 2006; Bacillieri et al., 2013), using a little fraction of the genetic diversity present in this species (Myles et al., 2011; Zhou et al., 2017). Modern wine, juice and table grape industries only use a limited number of *V. vinifera* cultivars (Wolkovich et al., 2018) which are established in very tight interactions with climatic conditions and cultivation practices (Carbonneau et al., 2015). In 2016, the first 30 *V. vinifera* cultivars represented 85% of the plant material released by French nurseries, with the top 10 genotypes accounting for more than 65% of the production3. In traditional European vine growing regions, as well as in more recently developed areas (United States, Australia, China), only a few elite cultivars are planted that represents a small fraction of the grapevine germplasm (Galet, 2000; Goldammer, 2015; Wolkovich et al., 2018).

Climate change has already induced noticeable changes in the grapevine development cycle and wine composition (Ganichet, 2002; Seguin et al., 2004; Duchêne and Schneider, 2005; Drappier et al., 2017; Ojeda et al., 2017a). Current models anticipate a further increase from +2°C to +5°C within a few decades (Bock et al., 2013; Fraga et al., 2013; Hannah et al., 2013), which represents a

serious threat for wine production in several regions. The impact of environmental factors has been studied on grapevine vegetative or reproductive organs (Butrose, 1969a,b; Webb et al., 2007; Greer, 2012; Coupel-Ledru et al., 2014; Xu et al., 2014; Luchaire et al., 2017) and fruit composition (for a review, see Dai et al., 2011). Butrose et al. (1971) reported that the increase in temperature decreased berry size while increasing sugar concentration. Elevated temperature has been shown to reduce malic acid (Butrose et al., 1971; Lakso and Kliewer, 1978; Sweetman et al., 2014) and anthocyanidin contents in berries (Kliewer and Torres, 1972; Mori et al., 2007). In the last 15 years, the molecular regulation of the synthesis and transport of main primary and secondary metabolites in the grapevine has received considerable attention (Terrier et al., 2001; DeBolt et al., 2006; Hichri et al., 2011; Rienth et al., 2016b). The first process-based models of metabolite accumulation in grapevine fruit have only recently been established (Dai et al., 2013; Vivin et al., 2017).

Changing cultural practices is the first option to reduce adverse climatic effects (Van Leeuwen et al., 2013). For instance, watering is a very efficient measure to mitigate drought (Ojeda et al., 2002). However, the effects of heat stress on berry development and composition are more difficult to control. Several attempts were made to decrease the rate of sugar accumulation into the berry, e.g., using anti-transpirant sprays or leaf removal to reduce carbon assimilation (Gatti et al., 2016a), shading nets to decrease photosynthetic capacity (Greer et al., 2011), minimal pruning to change vine canopy structure (Martínez De Toda et al., 2015). Some of these practices were found effective to reduce sugar accumulation, but with deleterious effects on vegetative growth and secondary metabolite accumulation into fruits (Greer et al., 2011; Bobeica et al., 2015). Delaying winter pruning to shift berry development toward cooler periods in the autumn (Ravaz, 1912; Gatti et al., 2016b) was found irrelevant. Since none of these adaptations proved efficient enough to offset the expected changes in temperature, a promising alternative could be to take advantage of the grapevine genetic diversity to select grapes with improved developmental and metabolic properties (Ollat et al., 2014; Torregrosa et al., 2017a).

Phenotypic variability, which is an intrinsic property of all species, results from genetic (G), environment (E) or GxE interactions (Conde et al., 2007). Wolkovich et al. (2018) recently claimed that enough genetic diversity exists in *V. vinifera* phenology to mitigate the adverse effects of climate warming on grapes quality. However, Ollat et al. (2015) showed that late ripening cultivars from southern European regions are inefficient to compensate the ripening time shifts that are expected in Bordeaux region. Indeed, Xinomavro from Greece, or Carignan from Spain would even ripe earlier than Petit Verdot, which is already used in Bordeaux wines. While climate models

anticipate an phenology advance of several weeks, the latest varieties experimented by Ollat et al. (2015) only ripen a few days later than Cabernet-Sauvignon, the emblematic variety of Bordeaux. Moreover, the effects of global warming on the composition of the grape at harvest can not only be analyzed on the acceleration of reproductive development since water, metabolites and inorganic compounds into the fruit are differentially impacted by temperature (Kliewer and Lider, 1970; Kliewer and Torres, 1972; Barnuud et al., 2014).

Therefore, there is an urgent need to evaluate the grapevine diversity for berry development and composition (Gascuel et al., 2017), focusing on attributes that are impacted by temperature, i.e., the berry volume and the accumulation of sugars, organic acids and secondary metabolites. Few studies exist on the diversity of grape composition in *V. vinifera* germplasm (Shiraishi et al., 2010; Houel et al., 2013; Preiner et al., 2013; Teixeira et al., 2013; Yinshan et al., 2017) or in breeding populations (Doligez et al., 2006, 2013; Liu et al., 2006, 2007; Mejia et al., 2007; Duchêne et al., 2012, 2013; Chen et al., 2015; Costantini et al., 2015; Houel et al., 2015). Unfortunately, in most of these studies, fruit developmental stages were ambiguously defined and berry parameters were characterized independently one from each other, resulting in some confusion between water and metabolites accumulation or concentration. In this study, we have measured at the same time the main berry traits that could vary with temperature increase in 33 *V. vinifera* genotypes. The whole genotype set consisted in a first subset of wine grape cultivars and a second subset of microvine offspring, this latter model being very promising for both physiological and genetic studies (Chaib et al., 2010; Rienth et al., 2016b; Luchaire et al., 2017; Sanchez-Gomez et al., 2017). The phenotypic diversity for growth and solutes accumulation was characterized at two critical stages of grapevine fruit development: (i) the end of green growth phase, when the berry stops loading organic acids and (ii) the end of ripening, when the contents of water and sugars reach their maximum.

Materials and methods

Plant material and growing conditions

Based on expert's advice and preliminary experiments, all genotypes included in this study displayed contrasted features for berry size and soluble solid contents at ripening. The first subset of genotypes consisted in 12 *V. vinifera* varieties (Supplementary Table S1). In 2016, the 12 *V.*

vinifera varieties were phenotyped at the Grapevine Biological Resources Centre (GBRC) of Vassal (Marseillan, France), where the vines were grown in sandy soils as ungrafted and non-irrigated plants (Experiment 1). In 2017, the phenotyping was repeated for six of the varieties that were present on the grapevine collection of Montpellier SupAgro Campus (Montpellier, France). In this collection, which was established from the GBRC 15 years ago, the vines were grown in gravelly soils as grafted and fertirrigated plants (Experiment 2). In both experiments, each variety was established as 5–20 replicated plants managed by spur pruning with vertical shoot positioning (VSP). To avoid the effects of source/sink unbalance, the number of clusters was reduced to 4–8 per vine after berry set. The second subset included 21 offspring of microvines from a cross between the Picovine00C001V0008 (*Vvgai1*/*Vvgai1*), which confers to the progeny Dwarf and Rapid Cycling and Flowering (DRCF) traits (Chaib et al., 2010), and the Ugni Blanc fleshless berry mutant (*flb*; Fernandez et al., 2006b). Microvine phenotypes were recorded in two experiments performed in two different greenhouses. In 2016 (Experiment 3), two replicates of 4-years-old own-rooted potted plants for each of the 21 microvine offspring (Supplementary Table S1) were established at the INRA experimental unit of Pech-Rouge (Gruissan, France). In 2017 (Experiment 4), 2–4 replicates of 3–5 years-old own-rooted potted plants for six microvines offspring were established at the Montpellier SupAgro Campus (Montpellier, France). In both experiments, night/day temperatures were maintained at 15/25 ± 5°C and the microvines were watered at full PET (potential evapotranspiration). To standardize vegetative and reproductive development of the microvines, lateral branches were systematically removed as described by Luchaire et al. (2017), to keep a single proleptic shoot per plant (Figure 1). The experiments for varieties and microvines were performed in different environmental contexts to appreciate the stability of the phenotypes. For varieties, main changes between Experiments 1 and 2, corresponded to grafting, watering, soil type, exposition and temperatures (Supplementary Table S2). For microvines, main changes between Experiments 3 and 4, corresponded to the plant age and air temperature (Supplementary Table S2). Thus, in the rest of the manuscript, the terms experiments, environment or year are indifferently used.

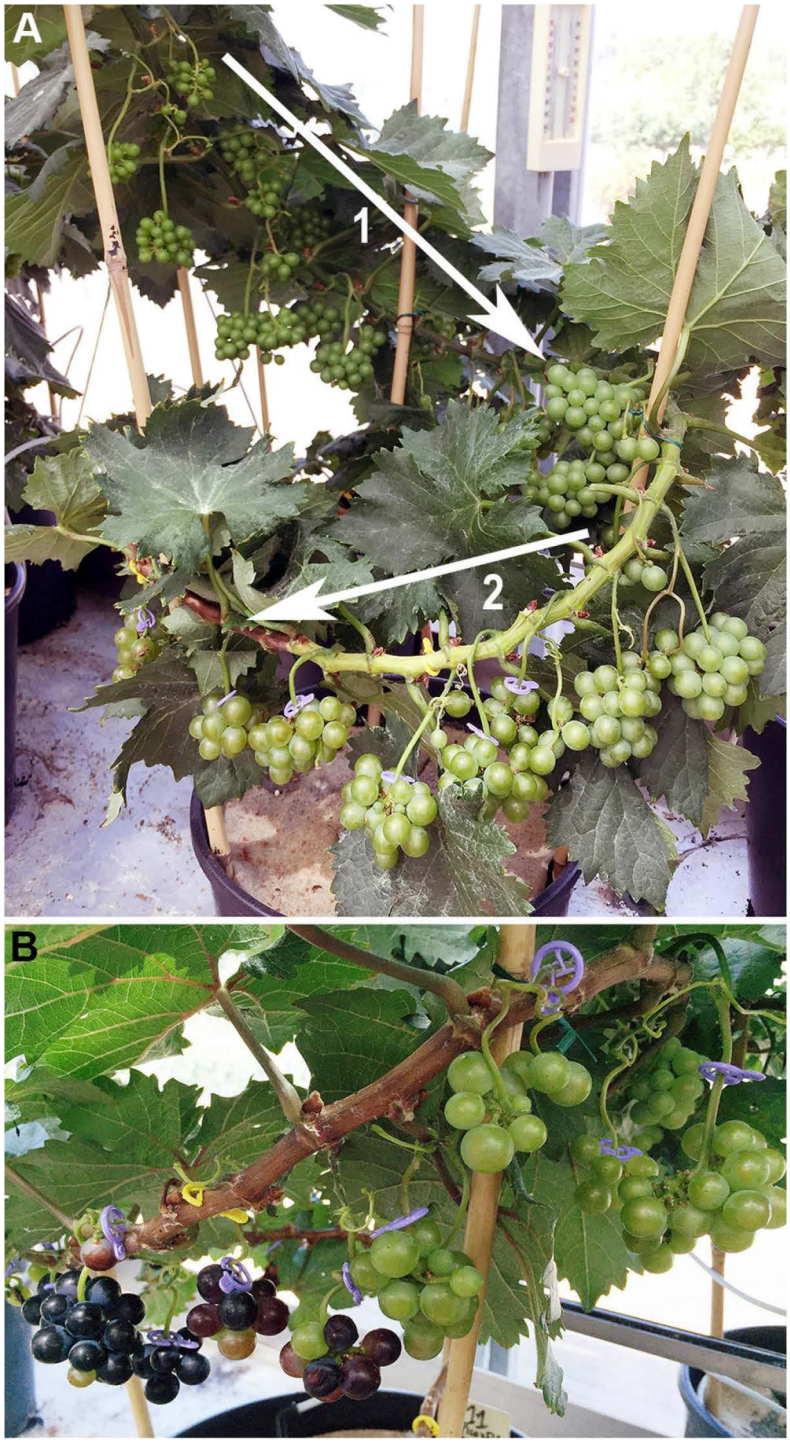

FIGURE 1. The typical continuous fruit development along a microvine proleptic shoot. **(A)** The offspring n°98 displaying non-pigmented fruits during green (arrow 1) and ripening (arrow 2) growth stages. **(B)** The onset of ripening (*véraison*) as it is observable on the offspring n°11 which develops anthocyanidin-pigmented berries.

Fruit sampling methods

In Experiment 1, starting before fruit softening, nine clusters per varieties were monitored weekly for single berry firmness. When the first soft berries was detected, 4–30 hard green berries were sampled to represent the stage with the highest contents in organic acids. For each of the nine clusters, 2–54 berries were sampled 3, 4, and 5 weeks later. In Experiment 2, first sampling date was determined as 2016 with a higher precision and only clusters presenting both hard and soft berries were maintained in plants to address synchronized bunches. To gain in the accuracy of the determination of ripe stage, two clusters per variety were immersed 3 times a week to non-destructively monitor the evolution of berry volume as described in Torregrosa et al. (2008). Several samples were collected at 3-day intervals when berry growth started to slow down. All samplings were performed in triplicate (3 × 30 berries). In Experiment 3, 2–11 microvine hard berries were sampled from individual clusters with the same procedure as described above. Berry firmness was manually assessed twice a week to identify which cluster displayed the first signs of berry softening, and 2–13 berries were then sampled on each of the two clusters above. Thanks to the continuous production of clusters in microvines, at least three replicates were collected at 1- to 2-weeks intervals from each plant for each developmental stage. In Experiment 4, microvine plants were grown up to simultaneously display all reproductive stages from flowering to berry shriveling. For each plant replicate, 5–8 berries were systematically sampled on clusters present between 3 and 5 levels above the first bunch showing berry softening and 3-5 levels below the onset of berry shriveling. Berries of the same clusters were pooled for biochemical analyses, except for clusters at the onset of ripening (i.e., presenting both hard and soft berries) for which 5–8 single berries were separately analyzed. This allowed a precise selection of samples corresponding to the last stages of green berry development and maximum berry volume. For all genotypes, when the berry volume from successive clusters was very close or irregular, we selected the cluster displaying the maximum of sugar contents per berry and the lowest concentration in tartaric acid, assuming that it corresponded to the arrest of sugar unloading and water uptake.

Berry growth and composition determination

For Experiments 1 and 3, fresh berries were ground with a mortar and pestle at room temperature and stored at -30°C. To complete extraction and dissolve organic salts, samples were heated at 60°C for 30 min, vortexed during 30 s and then centrifuged at 18,500 *g* during 5 min at 20°C. Clear juice

was diluted 10 times in 0.2 N HCl, and then filtered with sterile, non-pyrogenic, hydrophilic cellulose acetate 0.2 µm membranes before HPLC injection. In Experiments 2 and 4, we performed a new protocol that was validated in preliminary experiments to simplify primary metabolite extraction (data not shown). Single or pooled berries were added with 5X fresh weight of 0.25 N HCl. After 48 h incubation at room temperature, samples were diluted 10 times with 8.3 10^{-3} N acetic acid (internal control) + 16.4 10^{-3} N sulphuric acid. After centrifuging as above, supernatants were directly injected for HPLC to separate glucose, fructose, malic and tartaric acids through a Biorad aminex-HPX87H column according to Bories et al. (2011) with slight modifications (60°C and 0.6 $ml.min^{-1}$ rate flow).

Data presentation and statistical analyses

Except for Figure 2, presented data corresponded to targeted fruit developmental stages: the last stages of green berry development and the maximum volume of the berries. Statistical analyses for G, E and GxE interactions, were performed with R-software version 3.4.3 (R Core Team, 2017) on the six varieties and six microvine genotypes experimented in two environments. Pearson correlations were calculated between variables with interception to 0 (type of regression expected). The slope of the regressions was used to compare environmental effects. For mean comparisons, several tests were used depending on homoscedasticity pre-tests. Parametric Student's *t*-test (one parameter) or ANOVA I and II (G, E, GxE interaction) were performed to data displaying a normal distribution and equal variance between treatments. Otherwise, non-parametric Wilcoxon (one parameter) and two-way ordinal regression (G, E, GxE) were performed. For classification tests, a comparison of least-square means at a 0.05 significance level and a Tukey adjustment was performed (Supplementary Tables S3, S4). Raw data and R codes will be provided upon request.

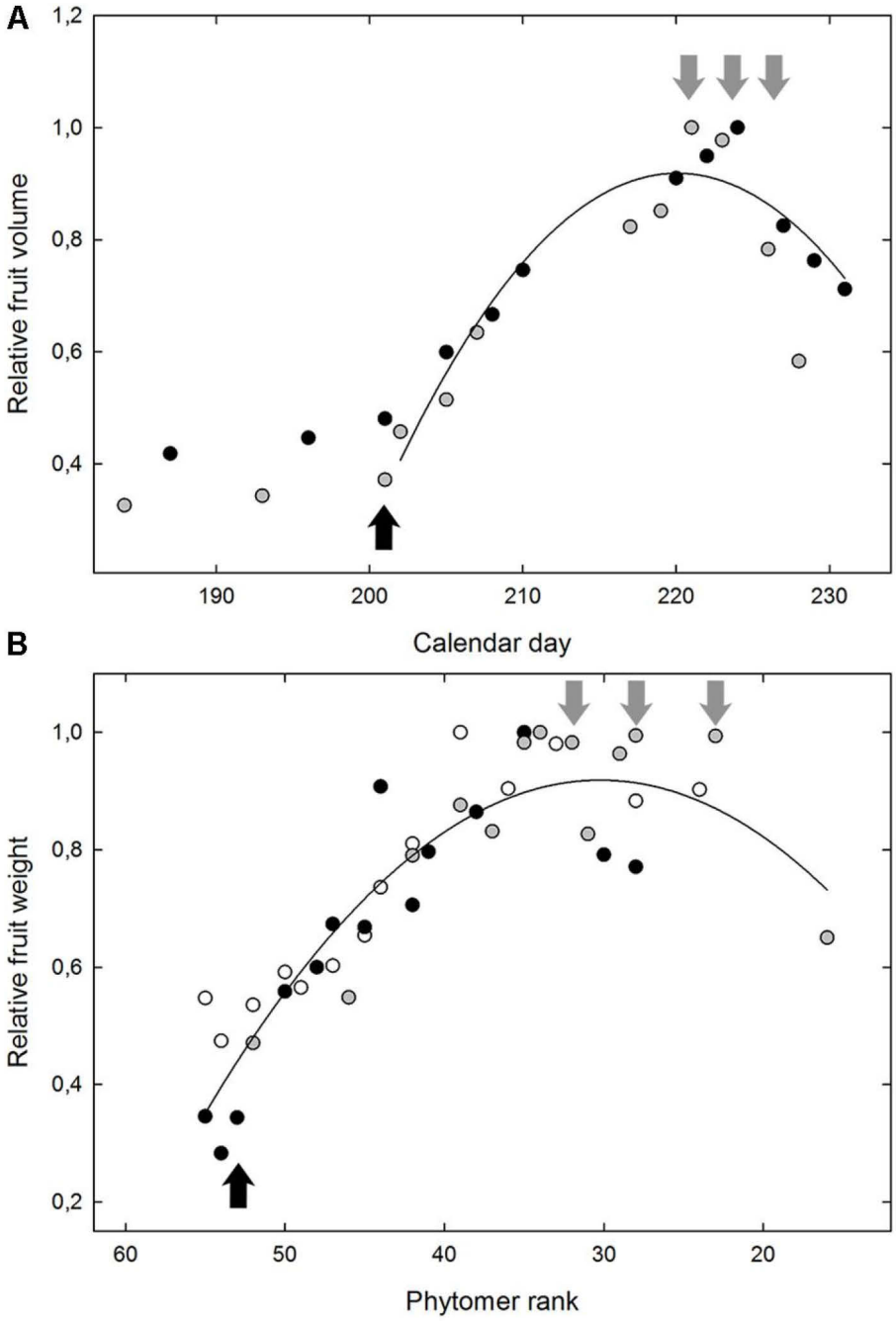

FIGURE 2. The typical fruit growth from onset of ripening for the Grenache variety **(A)** in Experiment 2 and for the microvine offspring n°114 **(B)** in Experiment 4. In **(A)**, the data correspond to the evolution of the relative fruit volume, as a function of the calendar day, with the maximum berry volume as 1. The average berry volume was non-destructively monitored by the immersion of 2 reference clusters (gray and black dots). In **(B)**, the relative berry weight is represented for 3 replicate plants of the microvine n°114 (gray, black, and white dots) as a function of the phytomer position from the base of the main shoot, with the maximum average berry weight as 1. Black arrows indicate the date/position of the samples for green berry. Gray arrows indicate the 3 dates/positions of the samples analyzed for ripe berries.

Results

Berry growth during ripening

All varieties displayed similar kinetics of fruit growth, regardless of the large variation observed for berry volume at both green and ripe stages. Likewise, microvine fruits followed the same developmental trends as a function of the position along the primary shoot (Figure 2). The quantity of sugar accumulated per berry did not increase any longer in the two samples following maximum fruit volume (data not shown). Following maximum fruit volume, sugar concentration (or °Brix) increased through water loss, i.e., decrease in fruit volume, which may be marked for some genotypes. The contents in main metabolites considerably varied within genotypic subsets and samples, with a clear distinction between the two targeted stages of fruit development (Figure 3).

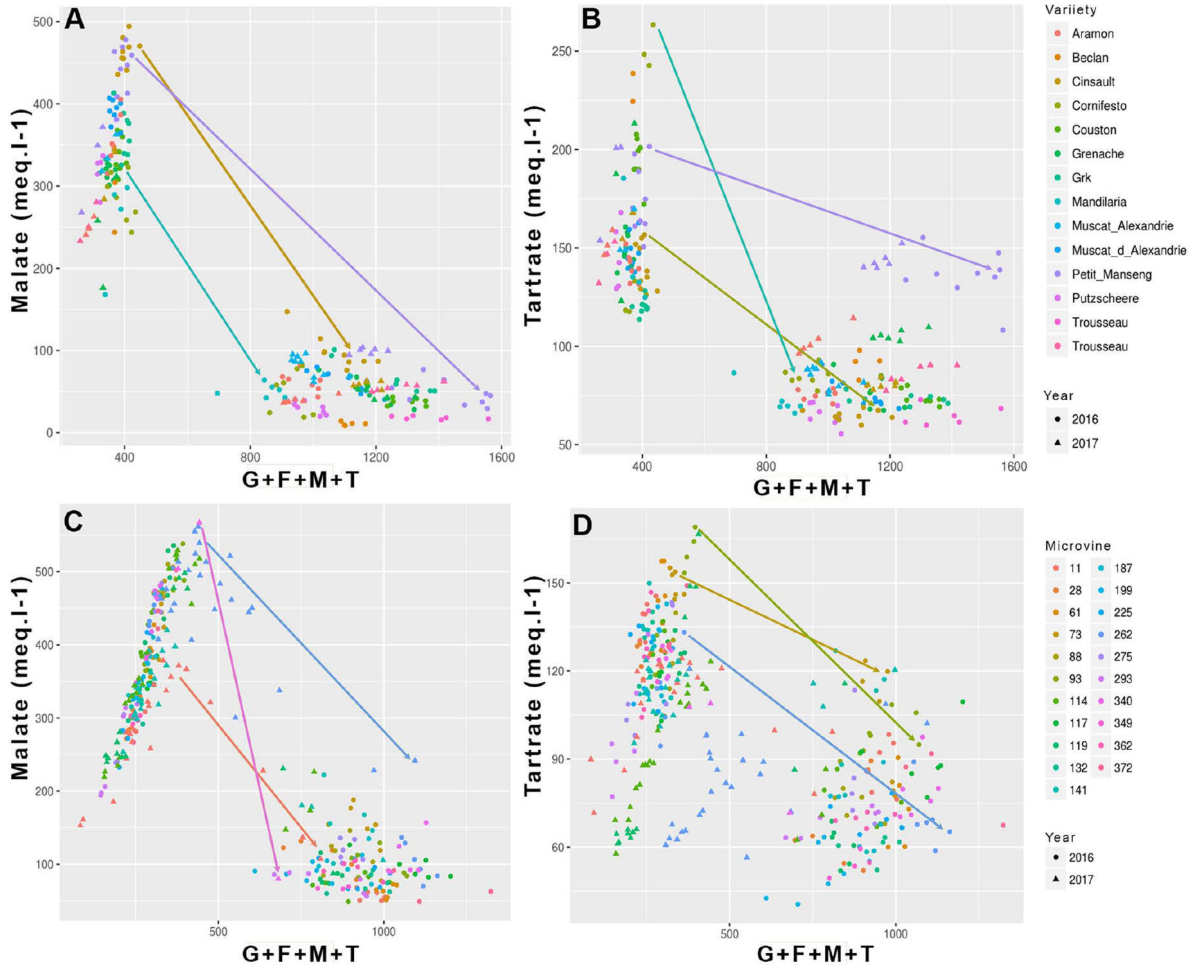

FIGURE 3. Evolution of the main acids (malic and tartaric) concentration as a function of sum of major osmoticum (glucose + fructose + malate + tartrate) concentration during ripening for variety **(A,B)** and microvine **(C,D)** subsets. Arrows show several contrasted trends for the evolution of malate and tartrate concentrations from the onset of ripening to ripe stage.

Berry size

For varieties, in Experiment 1 (2016), berry weight ranged 1.04–5.25 g/berry at maximum berry volume (Figure 4 and Supplementary Tables S3, S4), increasing on average by 2.10 ± 0.36 between green lag phase and ripe stage, with a coefficient of correlation of 0.97 (p-value = 6.53 10^{-7}). In 2017 (Experiment 2), the increment in weight between green and ripe stage was similar (2.10 ± 0.53), with a coefficient of correlation of 0.92 between stages (p-value = 9.81 10^{-3}). The increase of berry weight during ripening ranged from 1.4 for Petit Manseng to 2.9 for Cinsaut. In the microvine progeny, 2016 berry weight (Experiment 3) ranged from 1.15 to 2.56 g/berry at maximum berry volume, increasing by 1.39 ± 0.13 between the two stages, with a coefficient of correlation of 0.89 (p-value = 8.37 10^{-8}). In 2017 (Experiment 4), the increase of berry weight during ripening was 1.84 ± 0.47 with a coefficient of correlation of 0.81 (p-value = 5.25 10^{-2}). This increment ranged from 1.15 to 2.4, and was not correlated to maximum berry volume. The plots inserted in Figure 4 show the year-to-year relationships for the six varieties and six microvines reproduced in 2017 (Supplementary Tables S3, S4). Statistical analyses showed a significant effect of genotype, environment and GxE interaction on both green and ripe berry weights for varieties and only on ripe berry weight for microvines. For microvine green fruit weight, only the effect of genotype was found statistically significant.

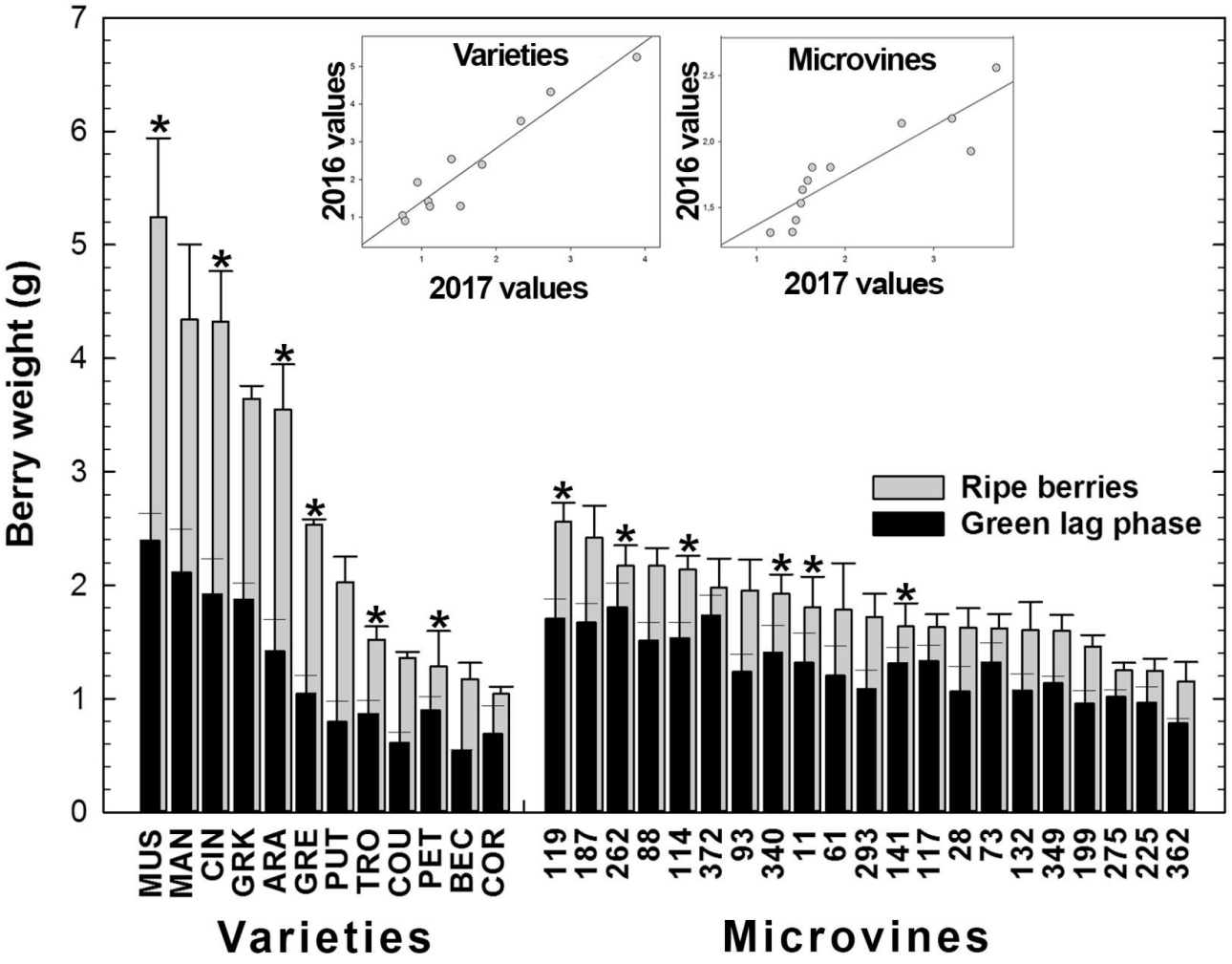

FIGURE 4. Diversity for the berry weight at the end of green growth and at ripe stage in varieties and microvine subsets. Bar chart represent 2016 mean values with the corresponding SE. Contrasted genotypes experimented in 2016 and 2017 are indicated by an asterisk. Inserted plots show the relationships between the mean values of both years (Supplementary Tables S3, S4 for detailed numeric values and statistics).

Organic acids

Among all genotypes, in both years, the total concentration of malic and tartaric acids ranged from 401 to 644 meq.L^{-1} at the end of green growth phase and from 75 to 362 meq.L^{-1} at maximum berry volume (Figure 5 and Supplementary Tables S3, S4). At ripe stage, the malate concentrations varied from 12 to 99 meq.L^{-1} among varieties and from 57 to 276 meq.L^{-1} among microvines (Figures 3A,C). The tartrate concentration varied from 60 to 146 meq.L^{-1} among varieties and from 51 to 114 meq.L^{-1} among microvines (Figures 3B,D) and such concentrations at ripe stage were higher in 2017 (Supplementary Table S4). For varieties, tartaric acid concentration between green lag phase

and maximum berry volume decreased by 2.09 ± 0.43 in Experiment 1 and 1.67 ± 0.26 in Experiment 2. A significant correlation between this decrease and berry growth was observed in Experiment 2 (0.83, *p*-value 3.70 10^{-2}). The malate/tartrate ratio ranged from 1.42 to 6.05 at the end of green growth stage and 0.14 to 3.62 at ripe stage (Figure 6 and Supplementary Tables S3, S4). For varieties, this ratio was correlated with berry size at green stage with a correlation coefficient of 0.75 (*p*-value = 3.49 10^{-4}) in 2016 (Experiment 1) and 0.68 (*p*-value = 4.68 10^{-7}) in 2017 (Experiment 2).

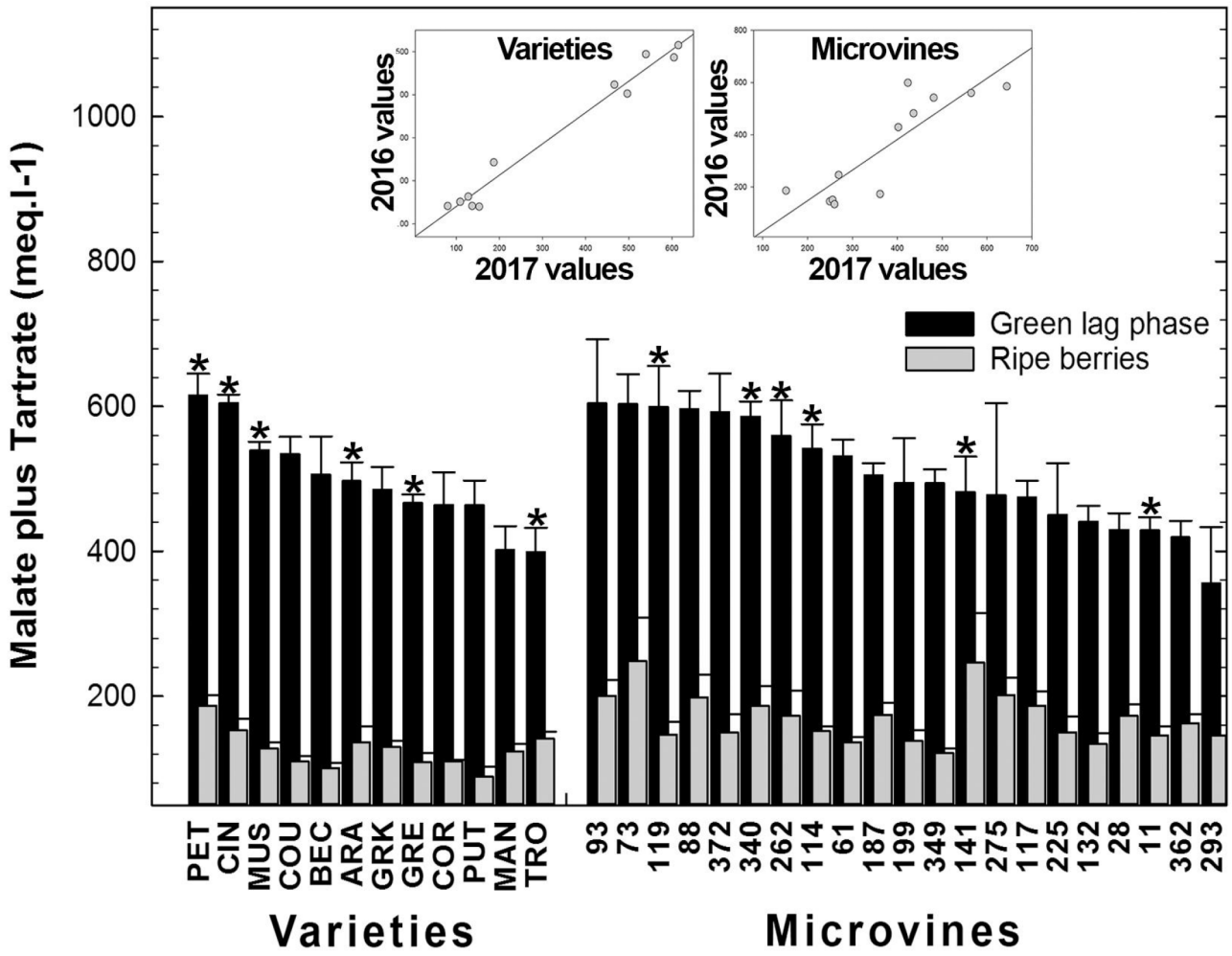

FIGURE 5. Diversity for the sum of malic and tartaric acid fruit concentrations at the end of green growth and at ripe stage in varieties and microvine subsets. Bar chart represent 2016 mean values with the corresponding SE. Contrasted genotypes experimented in 2016 and 2017 are indicated by an asterisk. Inserted plots show the relationships between the mean values of both years (Supplementary Tables S3, S4 for detailed numeric values and statistics).

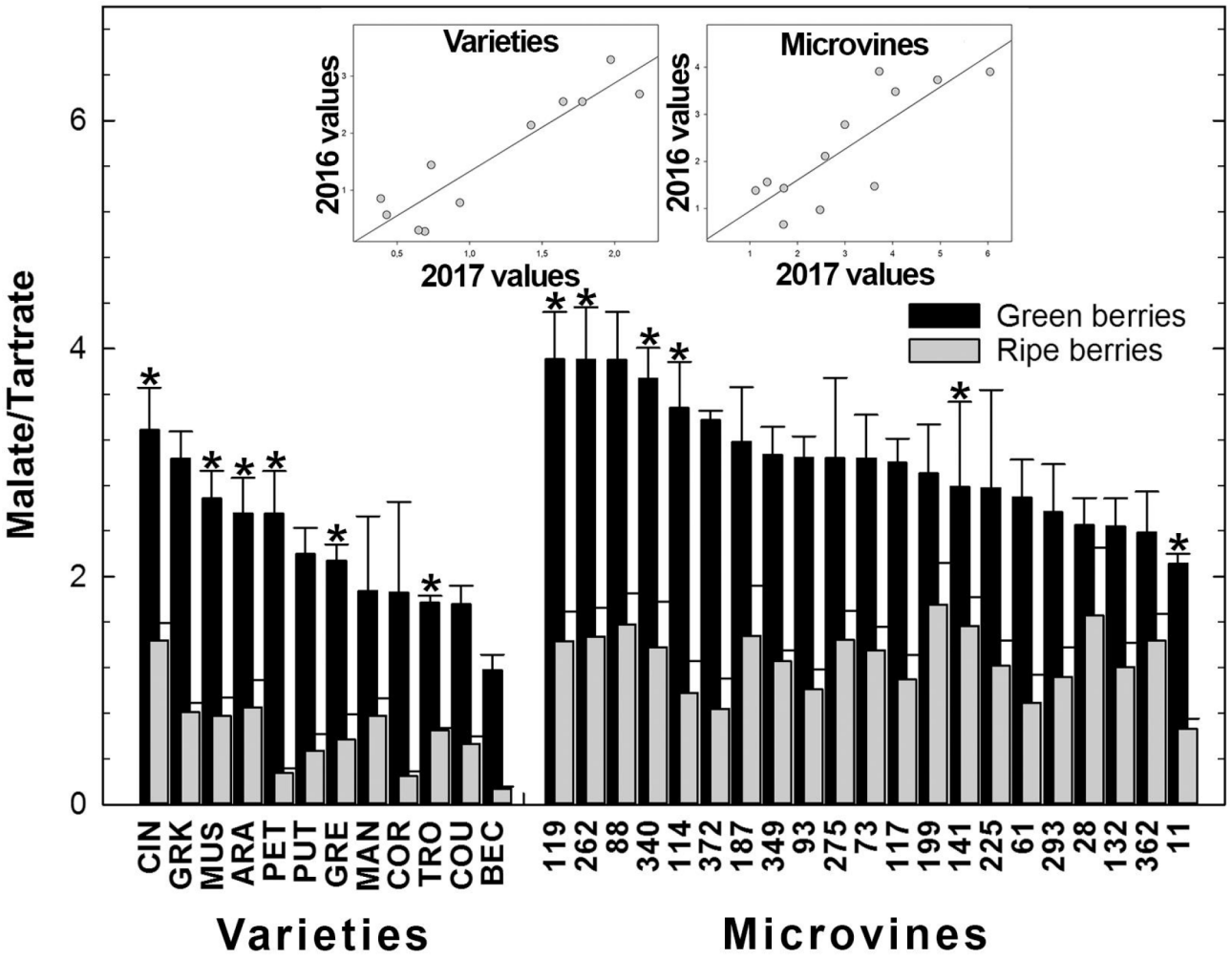

FIGURE 6. Diversity in the ratio malate/tartrate of the fruit at the end of green growth and at ripe stage in varieties and microvine subsets. Bar chart represent 2016 mean values with the corresponding SE. Contrasted genotypes experimented in 2016 and 2017 are indicated by an asterisk. Inserted plots show the relationships between the mean values of both years (Supplementary Tables S3, S4 for detailed numeric values and statistics).

The plots inserted in Figures 5, 6 show the year-to-year relationships for the six varieties and six microvines reproduced in 2017 (Supplementary Tables S3, S4). Statistical analyses showed a significant effect of genotype, environment and GxE interaction in both genotype subsets for the total acids content at ripe stage, but no environmental effect for microvine green fruits. For the malate/tartrate ratio at ripe stage, we found a significant effect of genotype, environment and GxE interaction in both genotype subsets for green fruits but no environmental effect in ripe fruits for varieties.

Sugars

Among all genotypes, in both years, the Glucose + Fructose concentration varied from 12 to 153 mmol.L^{-1} at green lag phase, to 752–1353 mmol.L^{-1} at ripe stage (Figure 7 and Supplementary Tables S3, S4) with higher average concentrations in varieties but no correlation was found between developmental stages. Correlations between sugar concentration at ripe stage and maximum berry volume was observed for varieties (-0.75 in 2016 and -0.54 in 2017). The rate of sugar accumulation during ripening ranged from 25 to 52 mmol.L^{-1}.day^{-1}. The plots inserted in Figure 7 show the year-to-year relationships for the six varieties and six microvines reproduced in 2017 (Supplementary Tables S3, S4). Statistical analyses showed a significant effect of genotype, environment and GxE interaction in both genotype subsets for sugars contents at ripe stage, but no environmental effect in green fruits for varieties.

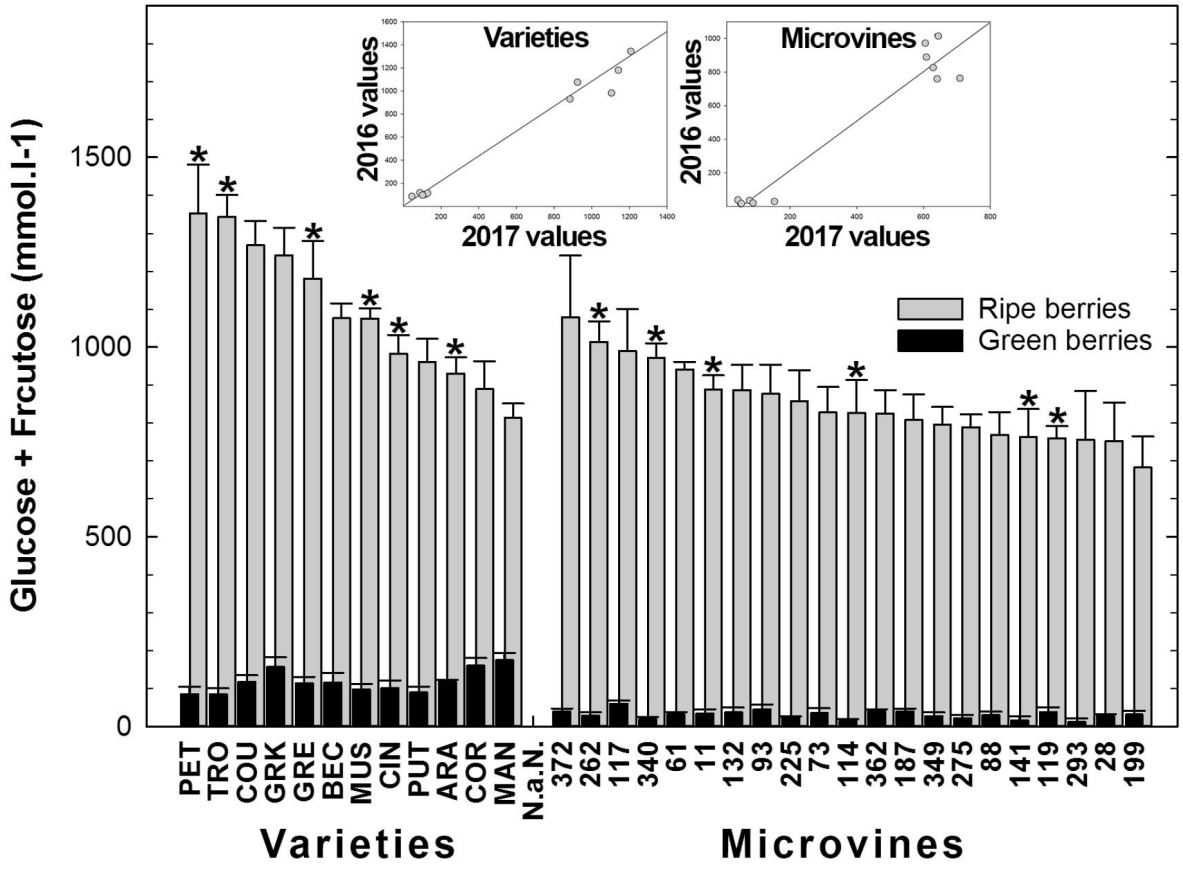

FIGURE 7. Diversity in the sum of glucose and fructose concentrations in fruit at the end of green growth and at ripe stage in varieties and microvine subsets. Bar chart represent 2016 mean values with the corresponding SE. Contrasted genotypes experimented in 2016 and 2017 are indicated by an asterisk. Inserted plots show the relationships between the mean values of both years (Supplementary Tables S3, S4 for detailed numeric values and statistics).

Osmoticum accumulation

Among all genotypes, in both years, the total of main osmotica (Glucose + Fructose + Malate + Tartrate) varied from 190 to 436 mmol.L^{-1} at green lag phase to 605–1446 mmol.L^{-1} at maximum berry volume (Figure 8 and Supplementary Tables S3, S4). Maxima for malic and tartaric acid concentrations were observed in green berries (Figure 3). At this stage, organic acids accounted for the main osmotica while, during ripening, sugars became predominant. The plots inserted in Figure 8 show the year-to-year relationships for the six varieties and six microvines reproduced in 2017 (Supplementary Tables S3, S4). Statistical analyses showed a significant effect of genotype, environment and GxE interaction in both genotype subsets for the total content in major osmotica at the ripe stage, but no environmental effect in green fruit for microvines.

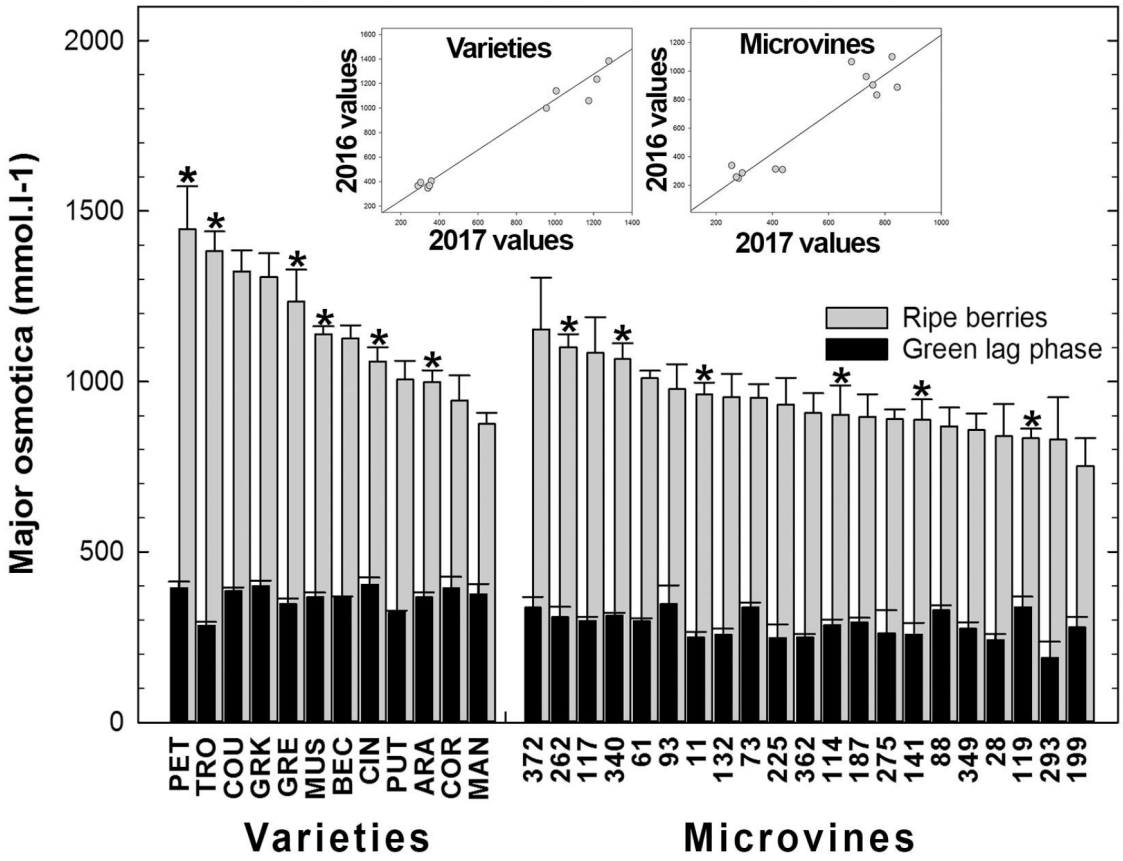

FIGURE 8. Diversity in the sum of major fruit osmotica (glucose, fructose, malic and tartaric acids) concentrations at the end of green growth and at ripe stage in varieties and microvine subsets. Bar chart represent 2016 mean values with the corresponding SE. Contrasted genotypes experimented in 2016 and 2017 are indicated by an asterisk. Inserted plots show the relationships between the mean values of both years (Supplementary Tables S3, S4 for detailed numeric values and statistics).

Correlations between traits

On average, microvines produced smaller berries than varieties. In both subsets, there was no link between fruit volume increase and osmotica content increase (Figure 9). In varieties, two significant correlations emerged between fruit traits in varieties at green lag phase: glucose and fructose concentrations (0.60, *p*-value = $1.54\ 10^{-10}$), as well as tartaric concentration and fruit volume (-0.56, *p*-value = $3.03\ 10^{-9}$). At ripe stage, the only significant correlation was between glucose and fructose concentrations (0.92, *p*-value < $2.2\ 10^{-16}$). In microvines, only one significant correlation was found between glucose and fructose concentrations at green lag phase (0.93, *p*-value < $2.2\ 10^{-16}$). At ripe stage, glucose and fructose concentrations were correlated (0.98, *p*-value < $2.2\ 10^{-16}$) as well as malate concentrations with either glucose (-0.50, *p*-value = $8.49\ 10^{-10}$) or fructose (-0.54, *p*-value = $3.03\ 10^{-9}$).

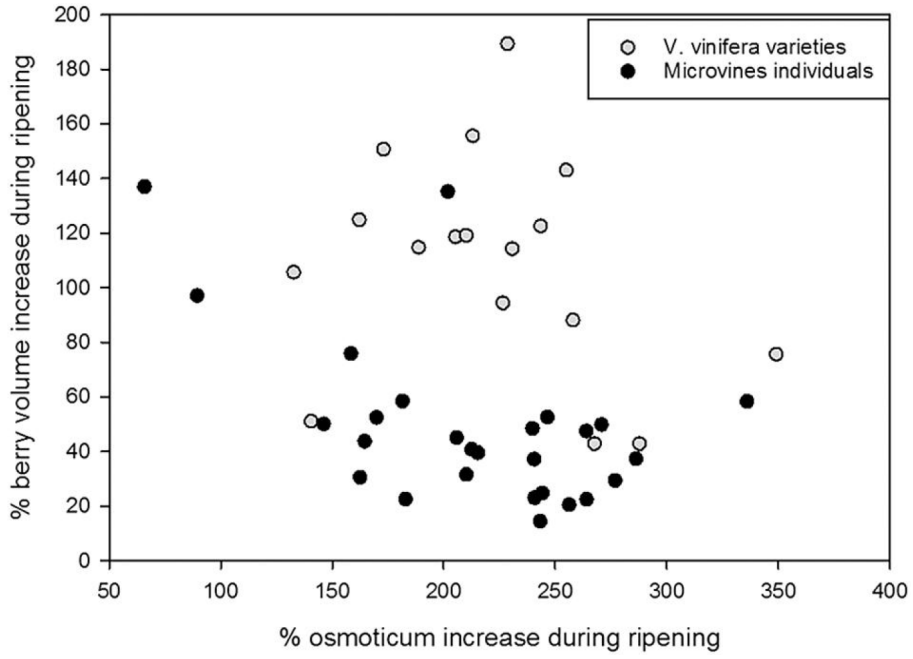

FIGURE 9. Major osmoticum concentration and fruit volume changes during ripening in varieties (grey dots) and microvine (black dots) subsets. % of osmoticum increase is calculated as 100*(osmoticum contents at max berry growth stage – osmoticum contents in green hard berry)/osmoticum contents in green hard berry. % of berry volume increase is calculated as 100*(berry volume at max berry growth stage – berry volume of green hard berry)/berry volume of green hard berry.

In the PCA analyses (Figure 10), the first two principal components explained more than 70% of the phenotypic variability for both subsets and stages. Correlations shown above were represented on PCA plots, which highlighted the low dependence of berry weight on major osmotica. In varieties, Petit Manseng showed a unique localisation with large tartaric acid and sugar concentrations at ripe stage, with a good reproducibility between experiments. For microvines, except for microvines n°141 at ripe stage, PCA suggested a strong environmental effect in relation to the higher size and acidity of the fruits and the lower sugars contents in 2017 as compared to 2016.

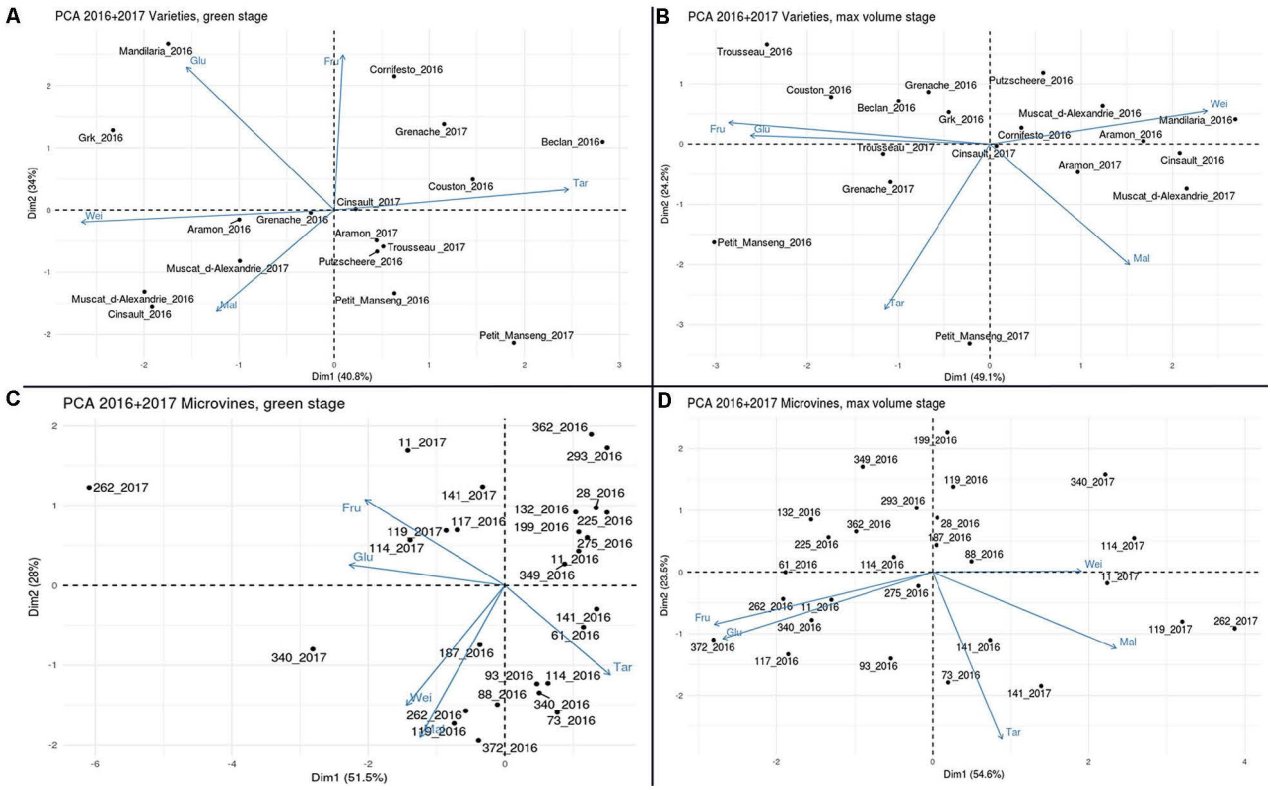

FIGURE 10. Principal component analyses the all variables collected with varieties **(A,B)** and microvine **(C,D)** subsets, for hard green (left) and ripe (right) berries. Glu (glucose), Fru (Fructose), Tar (tartaric acid), Mal (malic acid) concentrations. Wei (berry weight).

Discussion

Major descriptors of grapevine fruit development and composition

The first critical fruit developmental stage is the green lag phase, which corresponds to the end of the first growing phase when the concentration in organic acids is maximum (Kliewer, 1965; Kliewer et al., 1967). At this stage, berry weight showed a respective 230 and 440% variation among the microvine progeny and varieties. The range observed in the subset of varieties is equivalent to the one reported by Houel et al. (2013) for wine grape cultivars, resulting from the high polymorphism present within the *V. vinifera* variety germplasm (Boursiquot et al., 1995; Fernandez et al., 2006a; Houel et al., 2013). The smaller extend of the berry size diversity observed among microvines can be explained by the intrinsic segregation limitation present in a bi-parental progeny.

The size of the fruit depended on the genotype, the environment and their interaction for varieties, but only on the genotype for microvines, may be because of the little environmental differences between Experiments 3 and 4. In this study, all the genotypes displayed seeded fruit, excluding seedlessness as a potential source for fruit size diversity. In this respect, Houel et al. (2013) demonstrated that seed number or weight did not explain berry growth variation among genotypes. Tartaric and malic acids are major organic acids in *V. vinifera* fruit (Kliewer, 1965; Terrier and Romieu, 2001; Conde et al., 2007; Yinshan et al., 2017). During ripening, tartaric acid concentration decreases by dilution due to fruit enlargement while malic acid concentration decreases through both dilution and respiration (Lakso and Kliewer, 1978; Dai et al., 2011; Famiani et al., 2014). It was previously reported that organic acid concentration and the relative proportions of malate and tartrate varied according to the genotype at ripe stage (Kliewer, 1967a; Liu et al., 2006; Shiraishi et al., 2010). As tartaric acid is not metabolized during ripening (Terrier and Romieu, 2001), its level at the end of green stage is a determinant factor in the final concentration at ripe stage. In this study, the maximum tartaric acid content observed in green berries was 260 meq.L^{-1} for varieties and 180 meq.L^{-1} for microvines, which are higher values than in previous reports (Kliewer et al., 1967; Preiner et al., 2013).

At ripe stage, when the phloem unloading stops, the final quantity of solutes and water per berry determines fruit quality (Matthews et al., 1987; Coombe, 1992; Keller and Shrestha, 2014). Houel

et al. (2013) showed that most of the wine varieties displays 1–4 g berries at ripening, which is equivalent to the values observed in this study. Our data confirmed that berry weight at ripe stage varies according to genotype, environment and GxE interactions. For varieties, we observed similar increases in weight between green lag phase and ripe stage in both environments. This suggests that the final fruit size is determined very early during green growth phase. As reported in Houel et al. (2013), fruit weight doubled on average between the herbaceous plateau and ripe stage, but with some extreme behaviors. Indeed, fruit size increment during ripening ranged from x1.4 for Petit Manseng to x2.9 for Cinsaut, suggesting some variability in the control of fruit expansion. For microvines, we found a similar average fruit weight ratio between green and ripe stages in 2017, based on detailed spatial patterns of berry growth. In 2016, we observed smaller fruits at ripe stage for all microvines, but with little impact on the genotype ranking, suggesting a systematic underestimation of the maximum berry volume in Experiment 3.

Regarding the contents in organic acids into *V. vinifera* ripe fruit, Kliewer et al. (1967) reported concentrations ranging from 20 to 100 meq.L^{-1} for malate and from 50 to 100 meq.L^{-1} for tartrate. Using a set of *Vitis* genotypes including interspecific hybrids, Liu et al. (2006) reported a range of 5 meq.L^{-1} to 100 meq.L^{-1} for malate and 20 to 120 meq.L^{-1} for tartrate. Here, we have also identified a huge diversity in the relative abundance of both major organic acids in ripe berries, with a malate to tartrate ratio ranging from 0.13 to 3.62. The sum of concentrations of the two major organic acids in ripe berries ranged from 80 to 361 meq.L^{-1} with respective variations for malate and tartrate from 12 to 276 meq.L^{-1} and 51 to 146 meq.L^{-1}, which is larger than previously reported. On average, microvines displayed a higher malic acid concentration at ripe stage than varieties. This can be explained either by genetic or environment effects as microvines were grown in greenhouses, protecting them from pronounced increases in temperature that strongly activate the respiration of malic acid (Kliewer and Lider, 1970; Famiani et al., 2014; Keller, 2015; Rienth et al., 2016b).

During berry ripening sugars progressively become the major osmoticum (Keller, 2015). Among the different sugars accumulated in *V. vinifera* fruits, glucose and fructose are largely dominant (Hawker et al., 1976; Liu et al., 2006; Shiraishi et al., 2010). Famiani et al. (2014) and Keller et al. (2015) confirmed the low quantity of sucrose (<100 mmol.L^{-1}; i.e., less than 10% total sugars) in ripe berries with a ratio Glucose/Fructose tending to 1 at ripe stage. Sugar concentration was reported to vary according to environment, cultivation practices and variety (Liu et al., 2007; Dai et al., 2011; Duchêne et al., 2012). Studying 78 genotypes, including table and wine grape cultivars, Kliewer (1967b) reported sugar concentrations ranging from 18.7 (1 mol.L^{-1}) to 27 (1.5 mol.L^{-1})

°Brix at ripe stage. Recently, Yinshan et al. (2017), reported a huge diversity for sugar concentration in a panel of 45 genotypes, including wine grape varieties from North–East of China. However, these data should be considered with caution due to the imprecision about the stage of sampling. Here, we have observed sugar concentrations ranging from 813 to 1353 $mmol.L^{-1}$ among varieties. This represents a larger range of variation than in most previous studies and corresponds to a slightly lower average value. These differences may be of genetic origin or result from the method used to determine ripe stage. Indeed, when sampling is performed after the maximum berry volume, the concentration of sugar increases by fruit shriveling, even though the quantity of sugar per fruit remains stable. In the microvine progeny, the concentrations of sugars at ripe stage were found lower than in varieties with values ranging from 752 to 1078 $mmol.L^{-1}$.

Low PAR (Photosynthetic Active Radiations) or VPD (Vapor Pressure Deficit) in greenhouses could have reduced leaf carbon assimilation in the greenhouse limiting sugar accumulation flow directed to the fruit despite the source/sink balance was improved by cluster thinning. Moreover, a lower VPD could also be involved in limiting phloem transport of sugars from source organs to the fruit (Keller et al., 2015). Finally, it is also possible that parents of the microvine population carried alleles limiting berry sugar accumulation. Lastly, in previous studies with microvines from various genetic backgrounds, the level of sugar accumulated in berry during ripening was often found to be rather moderate, i.e., 1 $mol.L^{-1}$ or less (Houel et al., 2015; Rienth et al., 2016a; Luchaire et al., 2017). This could indicate that dwarf mutation itself (Boss and Thomas, 2002) or some biological process associated with the dwarf phenotype (Chaib et al., 2010; Torregrosa et al., 2016) are limiting for the accumulation of sugar into the berry.

Phenotyping at key stages of grapevine berry development

The study of the genotypic performances for berry growth and metabolites accumulation needs an accurate protocol to identify key stages of fruit development for each genotype. At the onset of ripening, a cluster is composed of berries with ripening related pathways only activated in a fraction of them (Coombe, 1992; Lund et al., 2008; Gouthu et al., 2014; Rienth et al., 2016b). Similarly, at the end of ripening, the bunch is a mix of berries concentrating primary metabolites by shriveling while other are still importing sugars and water (McCarthy and Coombe, 1999; Shahood, 2017). Because the phenology sequence and berry development asynchronism are both genotype and environment dependent (Costantini et al., 2008; Parker et al., 2011; Doumouya et al., 2014; Rolle et al., 2015; Torchio et al., 2016) it is not possible to predetermine the date of sampling. Duchêne et al.

(2012) proposed to compare the genotypic performances in sugar accumulation at defined thermal time points. However, several studies questioned the accuracy of thermal time scaling to study grapevine berry ripening (McIntyre et al., 1987; Rienth et al., 2016b; Romieu et al., 2016). In genetic studies, it is generally not possible to perform a comprehensive fruit sampling sequence for all genotypes since either the time is lacking or the number of fruits is limited. As a consequence of these limitations, almost all genetic studies just described the genotypic diversity at a single stage of berry development, with no precision regarding the real physiological stage of the berry (Preiner et al., 2013; Yinshan et al., 2017).

To offset these limitations and get relevant phenotypic data, we propose to perform the phenotyping at the two stopping phases of berry growth. At the end of the first growth phase, the detection of the first signs of berry softening allows a precise determination of the onset of sugar accumulation (Robin et al., 1997; Abbal et al., 1999; Terrier et al., 2005; Lund et al., 2008; Castellarin et al., 2016). The determination of the end of sugar unloading is more intricate (Doumouya et al., 2014; Shahood et al., 2015; Shahood, 2017). At a berry population level, it has been widely accepted that ripening takes about 40 days after colour change (Mullins et al., 1992), but Costantini et al. (2008) reported ripening periods varying from 10 to 80 days in a *V. vinifera* segregating population. For varieties, which only produce one to three clusters per shoot and reproductive cycle, the monitoring of berry softening during green growth phase allows the selection of clusters with both hard and soft berries. Hard berries can be sampled at that time to represent the very last stages of green berry development. Two or three of these clusters could be further used to non-destructively monitor berry growth by immersion. When the growth of these clusters begins to slow down, a regular sampling of berries on the other synchronized clusters allows the selection of berries at the max fruit volume. For microvines, the best option is to establish controlled conditions of growth to support a continuous and stable reproductive development (Luchaire, 2016). In that case, it is possible to phenotype several stages of development from each plant and use the same plant to harvest successive biological replicates.

Breeding prospects

The climate change models (Hannah et al., 2013; Wolkovich et al., 2018) and previous studies on the effect of temperature elevation on grapevine fruit development, provide clues to determine phenotypic targets of future breeding programs. A pre-requisite is to appreciate the magnitude and stability of the fruit trait diversity (Ollat et al., 2014; Gascuel et al., 2017). In this respect, fruit

quality at ripe stage result from multi-faced regulatory mechanisms, i.e., metabolite synthesis and degradation together with water accumulation, each one potentially genotype-dependant.

Berry size, that determines fruit yield and quality (Boursiquot et al., 1995; Yamada and Sato, 2016), could be reduced by 30% upon temperature elevation (Kliewer and Lider, 1970; Butrose et al., 1971; Luchaire et al., 2017). Present study underlines how huge the diversity for fruit size is in *V. vinifera* varieties and the possibility to generate new phenotypes by hybridization. This constitutes a favorable context for breeders, even if GxE interactions may disturb the ranking of genotypes.

According to Kliewer and Lider (1970), Butrose et al. (1971), Seguin et al. (2004), and Rienth et al. (2016b), climate warming decrease the acidity of the wines up to 50%, with a marked reduction on malic acid, with already noticeable consequences on wine quality (Escudier et al., 2017). Fruit malic and tartaric acid concentration is depending on genotypic, environmental and GxE interaction effects. Their poor stability and heritability has already impaired the identification of QTLs in grapevine (Chen et al., 2015; Houel et al., 2015). In this study, we observed a huge variability in the contents of malic and tartaric acids and some correlation with berry growth. Thus, among the genotypes characterized here, the larger the berry at ripe stage, the higher the malic acid concentration and the lower the tartaric acid concentration. This explains the high correlation observed between berry size and malic/tartaric acids ratio. The diversity in berry acidity illustrated here represents a smart alternative to present physical or chemical corrections of juice and wine acidity (Escudier et al., 2012; Sweetman et al., 2014). Indeed, European regulations (CEE-606/2009) restrict the supplement of organic acid in grape juices at 20 meq.L^{-1} of either tartaric or malic acid. Moreover, the use of ion exchange resins or bipolar membranes to remove the cations neutralizing organic acids is limited to 54 meq.L^{-1} (CEE 53/2011) for conventional wines and remains prohibited for organic wines is some countries.

Other critical factors for the selection of grapevine fruits better coping with climate warming are the concentration in sugars and the sugars to organic acids ratio (Ojeda et al., 2017a). Kliewer and Lider (1970), Butrose et al. (1971), Rienth et al. (2016a), and Luchaire et al. (2017) showed that temperature elevation could increase sugars concentration up to 3°Brix (0.28 M sugar). Today, European regulations only authorize 20% ethanol removal from the wine (Meillon et al., 2010), which roughly corresponds to 0.12 mol.L^{-1} fruit sugar. Consequently, the genetic diversity for sugar contents observed here and the negative correlation between malic acid and sugar observed here, appear suitable to mitigate the negative impacts of heat on sugar/malic acid ratio (Ojeda et al.,

2017b; Torregrosa et al., 2017b). On this respect, the identification of the genetic bases of the extreme phenotypes exhibited in particular by Petit Manseng or Cinsault would provide useful markers for breeding.

Conclusion

Vitis vinifera belongs to an inter-fertile group of species adapted to a diverse range of climates, from hot desert areas to humid tropical regions, which potentially carry valuable reproductive and vegetative traits (Chen et al., 2015; Brillouet et al., 2016; Yamada and Sato, 2016; Koyama et al., 2017). This study highlighted that, despite the high genetic pressure performed on this clonally propagated perennial crop (Zhou et al., 2017; Wolkovich et al., 2018), consistent fruit trait diversity still exists in this taxon. Due to some independence in the segregation of main factors controlling berry growth or primary metabolite accumulation, we also showed that phenotypes with new trait value combinations can be generated by cross-breeding. To be suitable for genetic improvement, phenotypic plasticity must be assessed in a large range of fluctuating environments, a process that remains long and tedious when addressing fruit composition. Fortunately, new genetic resources such as the microvine can boost the identification of fruit traits and their physiological response to abiotic factors (Rienth et al., 2014a,b; 2016b; Luchaire et al., 2017), as well as the discovery of associated QTLs (Chaib et al., 2010; Dunlevy et al., 2013; Houel et al., 2015; Torregrosa et al., 2016, 2017a). Altogether, these observations open prospects for the breeding of varieties with fruit improved in size and composition, to challenge the consequences of climate warming.

Author contributions: CR, AB, and LT designed the experiments and drafted the manuscript. AB, DB, EM, and YS performed the experiments. J-MB provided the list of the *V. vinifera* variety subset. LT, AB, CR, J-PP, HO, and AD edited the manuscript. All authors reviewed the final version of the manuscript.

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Supplementary material

S1 - Table 1 - List of the genotypes included in the study and their phenotypes for berry size and sugar contents, as proposed by experts or from preliminary experiments.

Genotype name		Variety references Microvines progenitors	Expected berry Size*	Expected sugar concentration**
Varieties	Muscat d'Alexandrie	Vass7R08s336-340	Big	High
	Grenache	Vass8R07s001-005	Big	High
	Cinsaut	Vass2R05s551-555	Big	High
	Mandilaria	Vass4R13s596-600	Big	Low
	Grk	Vass4R03s306-310	Big	Low
	Aramon	Vass2R02s046-065	Big	Low
	Plant de Couston	Vass7R12s406-410	Small	High
	Trousseau	Vass2R14s356-360	Small	High
	Petit Manseng	Vass2R12s071-075	Small	High
	Putzscheere	Vass8R02s126-130	Small	Low
	Cornifesto	Vass6R05s001-005	Small	Low
	Béclan	Vass7R11s406-410	Small	Low
Microvines	P88	Picovine00C001V0008 x UB*flb*	Big	High
	P114	Picovine00C001V0008 x UB*flb*	Big	High
	P117	Picovine00C001V0008 x UB*flb*	Big	High
	P187	Picovine00C001V0008 x UB*flb*	Big	High
	P262	Picovine00C001V0008 x UB*flb*	Big	High
	P28	Picovine00C001V0008 x UB*flb*	Big	Low
	P61	Picovine00C001V0008 x UB*flb*	Big	Low
	P119	Picovine00C001V0008 x UB*flb*	Big	Low
	P132	Picovine00C001V0008 x UB*flb*	Big	Low
	P293	Picovine00C001V0008 x UB*flb*	Big	Low
	P340	Picovine00C001V0008 x UB*flb*	Big	Low
	P349	Picovine00C001V0008 x UB*flb*	Big	Low
	P11	Picovine00C001V0008 x UB*flb*	Small	High
	P73	Picovine00C001V0008 x UB*flb*	Small	High
	P93	Picovine00C001V0008 x UB*flb*	Small	High
	P141	Picovine00C001V0008 x UB*flb*	Small	Low
	P199	Picovine00C001V0008 x UB*flb*	Small	Low
	P225	Picovine00C001V0008 x UB*flb*	Small	Low
	P275	Picovine00C001V0008 x UB*flb*	Small	Low
	P362	Picovine00C001V0008 x UB*flb*	Small	Low
	P372	Picovine00C001V0008 x UB*flb*	Small	Low

* Big berries (> 1.5 g), ** High sugar contents (> 1 mol/L-1)

S2 - Table 2 - Sum of the GDD (growing degree days) in base 10 and means of the maximum temperatures measured during the 4 months of sampling in field (Exp 1 and 2) and greenhouse (Exp 3 and 4).

Subset	**Varieties (Field)**				**Microvines (Greenhouse)**			
Experiment	Exp 1 (2016)		Exp 2 (2017)		Exp 3 (2016)		Exp 4 (2017)	
Month	GDD10	Avr Tmax	GDD10	Tmax	GDD10	Tmax	GDD10	Tmax
June	337	25.4	419	29.9	467	32.7	374	28.4
July	428	29,0	464	30.8	474	29,0	394	29.5
August	407	28.2	455	30.6	448	27.1	390	29.1
September	347	26.7	265	25,0	408	27.9	332	28.3
Sum*/Avr**	**1520**	**27.3**	**1603**	**29.1**	**1796**	**29.2**	**1490**	**28.8**

* Sum of the GDD10 for the 4 months of fruit ripening. ** Average of the maximum air T°

S3 - Table 3 - Mean values of the fruit parameters measured for the six *V. vinifera* varieties experimented in years 2016 (Exp 1) and 2017 (Exp 2).

Stage of berry development	**Variety**	**Experiment**	**FW*** Mean *SE*	**M+T**** Mean *SE*	**Tartrate**** Mean *SE*	**M/T**** Mean *SE*	**G+F***** Mean *SE*	**Osmotica***** Mean *SE*
Green plateau	Aramon	N°1 (2016)	1.4 *0.3* def	497 *26* bc	140 *7* a	2.6 *0.3* de	118 *6* d	366 *16* cd
		N°2 (2017)	1.1 *0.0* cde	403 *18* a	152 *6* abc	1.6 *0.0* ab	87 *4* abc	289 *13* a
	Cinsaut	N°1 (2016)	1.9 *0.3* f	605 *12* de	142 *11* a	3.3 *0.4* f	102 *20* bc	404 *20* e
		N°2 (2017)	0.9 *0.1* abc	487 *42* bc	164 *8* abc	2.0 *0.1* bc	114 *2* cd	358 *19* cd
	Grenache	N°1 (2016)	1.0 *0.2* bcd	467 *12* ab	149 *8* ab	2.1 *0.1* cd	115 *16* cd	348 *15* c
		N°2 (2017)	0.7 *0.1* a	424 *115* ab	175 *46* abc	1.4 *0.0* a	128 *47* bcd	340 *31* abc
	Muscat d'Alexandrie	N°1 (2016)	2.4 *0.2* g	539,8 *11* c	147 *9* ab	2.7 *0.2* e	98 *15* bc	368 *13* cd
		N°2 (2017)	1.8 *0.0* efg	494 *34* bc	156 *12* abc	2.2 *0.1* cde	101 *7* bcd	348 *23* bc
	Petit Manseng	N°1 (2016)	0.9 *0.1* ab	614,8 *30* e	175 *18* c	2.6 *0.4* e	86 *19* ab	394 *19* de
		N°2 (2017)	0.8 *0.0* a	515 *81* cd	185 *27* bc	1.8 *0.1* abc	45 *6* a	303 *35* ab
	Effect *p*-values	Genotype	$< 2.2\ 10^{-16}$	$4.22\ 10^{-16}$	$5.04\ 10^{-4}$	$1.89\ 10^{-11}$	$1.12\ 10^{-4}$	$5.48\ 10^{-7}$
		Environment	$1.12\ 10^{-9}$	$1.22\ 10^{-10}$	$9.41\ 10^{-6}$	$< 2.2\ 10^{-16}$	0.157	$5.03\ 10^{-11}$
		G x E	$1.12\ 10^{-3}$	$2.42\ 10^{-2}$	$3.78\ 10^{-1}$	$4.77\ 10^{-3}$	$9.48\ 10^{-5}$	$5.30\ 10^{-5}$
Ripe fruit	Aramon	N°1 (2016)	3.6 *0.4* g	140 *21* bcde	74 *3* bc	0.9 *0.2* de	931 *43* ab	999 *35* ab
		N°2 (2017)	2.3 *0.1* a	141,4 *10* bcd	102 *7* ef	0.4 *0.0* ab	885 *61* a	955 *66* a
	Cinsaut	N°1 (2016)	4.3 *0.4* fg	153,3 *15* cde	63 *3* a	1.4 *0.2* f	983 *49* abc	1059 *42* abc
		N°2 (2017)	2.7 *0.2* e	139,7 *5* cd	81 *2* cd	0.7 *0.1* d	1105 *37* cde	1175 *36* cd
	Grenache	N°1 (2016)	2.5 *0.0* e	109,1 *12* ab	70 *2* ab	0.6 *0.2* bcd	1180 *100* def	1235 *94* cde
		N°2 (2017)	1.4 *0.1* cd	151 *6* cde	106 *3* f	0.4 *0.1* bc	1142 *64* def	1217 *63* cde
	Muscat d'Alexandrie	N°1 (2016)	5.2 *0.7* f	127,8 *9* bc	72 *2* ab	0.8 *0.2* de	1076 *26* bcde	1140 *22* bcd
		N°2 (2017)	3.9 *0.2* g	165,9 *19* de	84 *13* d	1.0 *0.1* e	925 *164* a	1006 *170* ab
	Petit Manseng	N°1 (2016)	1.3 *0.3* bcd	187 *14* ef	147 *9* g	0.3 *0.0* a	1353 *128* f	1447 *126* de
		N°2 (2017)	1.1 *0.0* b	243 *6* f	144 *5* g	0.7 *0.0* d	1052 *40* bcd	1174 *43* cd
	Trousseau	N°1 (2016)	1.3 *0.1* d	80 *0* a	62 *2* a	0.3 *0.1* a	1343 *57* f	1383 *57* e
		N°2 (2017)	1.5 *0.1* c	141 *10* cd	86 *5* de	0.6 *0.0* cd	1208 *82* ef	1279 *87* e
	Effect *p*-values	Genotype	$< 2.2\ 10^{-16}$	$2.92\ 10^{-12}$	$3.93\ 10^{-15}$	$8{,}05\ 10^{-10}$	$1.73\ 10^{-10}$	$7.71\ 10^{-12}$
		Environment	$1.52\ 10^{-15}$	$4.48\ 10^{-5}$	$1.58\ 10^{-13}$	0.33	$3.66\ 10^{-3}$	$8.97\ 10^{-3}$
		G x E	$1.73\ 10^{-4}$	$3.12\ 10^{-4}$	$4.42\ 10^{-8}$	$2.43\ 10^{-15}$	$9.75\ 10^{-6}$	$4.08\ 10^{-4}$

* FW, Fresh weight in g, ** M+T (Malate+Tartrate), M/T (Malate/tartrate) and Tartrate in meq.L-1, *** G+T (Glucose + Fructose) and Sum of major osmotica (M+T+G+F) in mmol.L-1

S4 - Table 4 - Mean values of the fruit parameters measured for the six microvine offspring experimented in years 2016 (Exp 1) and 2017 (Exp 2).

Stage of berry development	Genotype	Experiment	FW* Mean	FW* SE		M+T** Mean	M+T** SE		Tartrate** Mean	Tartrate** SE		M/T** Mean	M/T** SE		G+F*** Mean	G+F*** SE		Osmotica*** Mean	Osmotica*** SE	
Green plateau	011	N°3 (2016)	1.3	*0.3*	abc	429	*18*	ab	137.8	*6*	d	2.1	*0.1*	a	34.7	*11*	abcd	249	*16*	ab
		N°4 (2017)	1.4	*0.3*	abc	402	*85*	a	111.6	*19*	bc	2.6	*0.4*	b	78.3	*69*	d	279	*104*	ab
	114	N°3 (2016)	1.5	*0.1*	bc	542	*34*	bcde	121.9	*13*	cd	3.5	*0.4*	cd	15.2	*5*	a	286	*15*	ab
		N°4 (2017)	1.5	*0.2*	bc	481	*131*	abcd	93.9	*19*	ab	4.1	*0.5*	de	52.3	*34*	cd	293	*92*	ab
	119	N°3 (2016)	1.7	*0.2*	bc	599	*59*	e	122.3	*10*	cd	3.9	*0.4*	de	38.6	*12*	abcd	338	*31*	bc
		N°4 (2017)	1.6	*0.4*	bc	424	*158*	a	92.9	*43*	a	3.7	*0.4*	de	43.6	*26*	bcd	255	*100*	a
	141	N°3 (2016)	1.3	*0.1*	ab	482	*49*	abcde	129.4	*15*	cd	2.8	*0.8*	ab	16.8	*10*	a	258	*34*	ab
		N°4 (2017)	1.2	*0.1*	a	436	*101*	abc	109.2	*24*	bc	3.0	*0.4*	bc	53.8	*27*	cd	272	*68*	ab
	262	N°3 (2016)	1.8	*0.2*	c	560	*19*	cde	115.0	*14*	bc	3.9	*0.5*	de	29.1	*9*	abc	309	*30*	ab
		N°4 (2017)	1.6	*0.5*	bc	565	*55*	de	81.7	*15*	a	6.1	*0.9*	f	153.3	*84*	e	436	*86*	c
	340	N°3 (2016)	1.4	*0.2*	abc	586	*21*	de	123.9	*5*	cd	3.7	*0.3*	de	19.9	*5*	ab	313	*9*	ab
		N°4 (2017)	1.4	*0.1*	abc	644	*45*	bcde	108.3	*1*	abc	4.9	*0.4*	ef	89.1	*22*	cde	411	*45*	bc
	Effect p-values	Genotype	3.38 10^{-9}			5.26 10^{-8}			1.77 10^{-8}			< 2.2 10^{-16}			1.18 10^{-7}		5.4 10^{-8}		
		Environment	0.09			0.039			8.46 10^{-14}			8.52 10^{-9}			7.18 10^{-13}		0.13		
		G x E	0.46			8.76 10-3			6.14 10^{-1}			1.72 10^{-4}			2.24 10^{-3}		1.66 10^{-4}		
Ripe fruit	011	N°3 (2016)	1.8	*0.3*	ab	146	*13*	a	87.8	*9*	bc	0.7	*0.1*	a	889	*36*	cd	962	*35*	bc
		N°4 (2017)	1.8	*0.2*	abc	250	*68*	c	92.3	*12*	bc	1.7	*0.6*	bcd	609	*122*	ab	733	*89*	a
	114	N°3 (2016)	2.1	*0.1*	bc	152	*7*	ab	78.0	*10*	ab	1.0	*0.3*	ab	827	*87*	bc	903	*86*	ab
		N°4 (2017)	2.6	*0.1*	de	256	*52*	c	73.3	*12*	ab	2.5	*0.1*	cd	629	*28*	a	757	*46*	a
	119	N°3 (2016)	2.6	*0.2*	de	147	*18*	a	60.7	*6*	a	1.4	*0.3*	b	760	*32*	abc	833	*28*	ab
		N°4 (2017)	3.7	*1.0*	e	260	*81*	bcd	101.0	*21*	bc	1.7	*1.4*	abcd	641	*147*	abc	771	*107*	ab
	141	N°3 (2016)	1.6	*0.2*	a	247	*68*	c	96.9	*26*	bc	1.6	*0.3*	bc	763	*74*	abc	887	*61*	ab
		N°4 (2017)	1.5	*0.2*	a	269	*38*	cd	114.7	*6*	c	1.4	*0.4*	bc	709	*153*	abc	844	*134*	ab
	262	N°3 (2016)	2.2	*0.2*	cd	173	*34*	abc	69.7	*10*	ab	1.5	*0.3*	b	1014	*55*	e	1101	*39*	d
		N°4 (2017)	3.2	*0.6*	de	362	*33*	d	84.8	*25*	abc	3.6	*1.6*	d	644	*260*	abcd	825	*250*	abcd
	340	N°3 (2016)	1.9	*0.2*	abc	187	*28*	abc	79.0	*10*	b	1.4	*0.4*	b	972	*38*	de	1066	*46*	cd
		N°4 (2017)	3.4	-	e	152	-	abc	71.7	-	abc	1.1	-	ab	605	-	ab	681	-	a
	Effect p-values	Genotype	4.81 10^{-13}			3.16 10^{-4}			2.71 10^{-4}			3.20 10^{-4}			5.79 10^{-6}		1.19 10^{-5}		
		Environment	3.80 10^{-5}			5.62 10^{-10}			3.25 10^{-2}			9.36 10^{-6}			6.17 10^{-10}		6.37 10^{-8}		
		G x E	1.60 10^{-4}			3.35 10^{-4}			2.70 10^{-2}			2.95 10^{-5}			1.33 10^{-2}		1.61 10^{-2}		

* FW, Fresh weight in g, ** M+T (Malate+Tartrate), M/T (Malate/tartrate) and Tartrate in meq.L-1, *** G+T (Glucose + Fructose) and Sum of major osmotica (M+T+G+F) in mmol.L-1

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Chapter II: *Vitis vinifera* L. Fruit Diversity

II.2 - The existing diversity in cations in *Vitis vinifera* and it segregation in a progeny of microvines

Draft paper for Vitis

The existing diversity in cations in *Vitis vinifera* and it segregation in a progeny of microvines

A. Bigard[1,2], C. Romieu[1], Y. Sire[2], J.P. Péros[1], L. Torregrosa[1,2]*

[1]AGAP, University of Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France

[2]UE INRA de Pech Rouge, University of Montpellier, Gruissan, France

*Corresponding author: laurent.torregrosa@supagro.fr

Abstract

This paper is providing new cations data on samples used for Bigard et al. (2018). Samples being used in this experiment are the exact same as our previous work. In consequence primary attributes will not be study in this work. This paper will be entirely dedicated to study the cations diversity and segregation. Statistical analysis provided also information about the Environment (E), the Genotype (G) and the Environment x Genotype interaction (GxE) effects for those attributes. Bibliography is not well documented on cations diversity and this study aim to give an overview of this diversity. Analysing berries cations contents showed that diversity was as high as previously shown and sometimes even higher. Interesting correlations were also founded as between potassium, calcium and magnesium concentrations at ripe stage between two years of *Vitis vinifera* samples showing the need of having a ratio quite equilibrate of cations. Finally this paper gives a good overview of the existing diversity in *Vitis vinifera* and it possibilities as segregated characters using a microvine progeny.

Key Words: Grapeberry, Diversity, Cations, Ammonium, Calcium, Potassium, Magnesium, Acidity.

Introduction

Please refer to the introduction of this document.

Materials and methods

Plant material and growing conditions

In this experiment, plants and growing conditions are the *exact same as* describe in Bigard et al. (2018). Two subsets of genotypes were then analysed:

i) 12 *Vitis vinifera* varieties in 2016 (Grapevine Biological Resources Centre of Vassal, Marseillan, France; sandy soils, ungrafted and non-irrigated plants) and 6 repeated in 2017 (SupAgro campus, Montpellier, France; gravelly soils, grafted and ferti-irrigated plants) (Table 1). In this subset and for both years, 5-20 replicated plants were available and managed by spur pruning with vertical shoot positioning (VSP). To avoid the source/sink unbalance effects, the number of clusters was reduced to 4-8 per vine.

ii) a progeny of 21 microvines (3-6 years old) in 2016 (cross between the Picovine00C001V0008 (*Vvgai1/Vvgai1*) to obtains the Dwarf and Rapid Cycling and Flowering (DRCF) trait (Chaib et al., 2010) in the pregeny and the Ugni Blanc fleshless berry mutant (Fernandez et al., 2006) in Pech-Rouge greenhouse, Gruissan, France) and 6 repeated in 2017 (in SupAgro campus greenhouse) (Table 1). For both years, night/day temperatures were maintained at 15/25 +/- 3°C and the microvines were watered at full PET (potential evapotranspiration). To standardize vegetative and reproductive development of the microvines, lateral branches were systematically removed as described by Luchaire et al. (2017), to keep a single proleptic shoot per plant.

Growing conditions were selected in different growing regions to display heterogeneity and find genetics stability of the phenotypes. For the rest of the manuscript, the terms experiments, environment or year are indifferently used.

Table 1 – Genetic material used in experiments.

	Genotype Name Microvine Number	N° Vassal Accesssion N° microvines Line
Varieties	Aramon	Vass2R02s046-065
	Béclan	Vass7R11s406-410
	Cinsaut	Vass2R05s551-555
	Cornifesto	Vass6R05s001-005
	Grenache	Vass8R07s001-005
	Grk	Vass4R03s306-310
	Mandilaria	Vass4R13s596-600
	Muscat d'Alexandrie	Vass7R08s336-340
	Petit Manseng	Vass2R12s071-075
	Plant de Couston	Vass7R12s406-410
	Putzscheere	Vass8R02s126-130
	Trousseau	Vass2R14s356-360
Microvignes	P11	Picovine00C001V0008 x UB *flb*
	P28	Picovine00C001V0008 x UB *flb*
	P61	Picovine00C001V0008 x UB *flb*
	P73	Picovine00C001V0008 x UB *flb*
	P88	Picovine00C001V0008 x UB *flb*
	P93	Picovine00C001V0008 x UB *flb*
	P114	Picovine00C001V0008 x UB *flb*
	P117	Picovine00C001V0008 x UB *flb*
	P119	Picovine00C001V0008 x UB *flb*
	P132	Picovine00C001V0008 x UB *flb*
	P141	Picovine00C001V0008 x UB *flb*
	P187	Picovine00C001V0008 x UB *flb*
	P199	Picovine00C001V0008 x UB *flb*
	P225	Picovine00C001V0008 x UB *flb*
	P262	Picovine00C001V0008 x UB *flb*
	P275	Picovine00C001V0008 x UB *flb*
	P293	Picovine00C001V0008 x UB *flb*
	P340	Picovine00C001V0008 x UB *flb*
	P349	Picovine00C001V0008 x UB *flb*
	P362	Picovine00C001V0008 x UB *flb*
	P372	Picovine00C001V0008 x UB *flb*

Sampling methods

For the *Vitis vinifera* subset and the microvine one, in both years maximum berry volume was key point to determine maturity called ripe stage as defined in Bigard et al. (2018) and berry firmness was manually monitored to determine the onset of ripening called green stage.

For varieties in 2016, when possible 9 bunches were selected and 4-30 hard berries were sampled as green stage when first sign of softening appear and then 2-54 berries were sampled three, four and five weeks later. Unfortunately, Petit Manseng was already soft when experimentation started. In 2017, when first sign of softening appear, hard berries were sampled as green stage samples. Also,

to gain accuracy for the determination of ripe stage, two clusters per variety were immersed 3 times a week to monitor the evolution of berry volume in a non-destructively way as described in Torregrosa et al. (2008). All samplings here were performed in triplicate (3 x 30 berries).

For microvines, in 2016, 2-11 berries were sampled per cluster for green stage as describe above. Then, 2-13 berries were collected from 3 successive bunches when the oldest one presented first signs of shrivelling to determined ripe stage. In 2017, plants were grown up to simultaneously display all reproductive stages from flowering to berry shrivelling. Then 5-8 berries were analysed in pool per cluster (except clusters at the onset of ripening when hard and soft berries were analysed separately). With this the precision of the selection for samples corresponding to the green stage and ripe stage.

Berries were then selected for green stage as hard berries just before softening and for ripe stage as berries at maximum volume using different approach as describe in Bigard et al. (2018). After sampling, berries were carried on plastic bags and analysed.

Weight measurement and sample preparation

After sampling, in 2016 for both varieties and microvines, berries were weighted and ground with a mortar and pestle (room temperature) and then frizzed at -30°C for storing (Bigard et al., 2018). To finalise preparation, samples were heated at 60°C in a water bath for 30 min, vortexed during 30 seconds and then centrifuged at 18,500 g during 5 min at 20°C. Clear juice was diluted 10 times in 0.2 N HCl, and then filtered with sterile, non-pyrogenic, hydrophilic cellulose acetate 0.2 µm. Filtered clear juice obtain was then refrizzed at -30°C. In 2017, berries were weighted as describe in Bigard et al. (2018) and right after, lots were immersed in 4 times their weight using hydrochloric acid (0.25 N). Seed were handy removed in the solution and weighted. After 2 days incubation at room temperature, liquid from samples was diluted 10 times using water (for cations) and frizzed at -30°C. Unfortunately 2017 varieties green stage sample were lost, no data could be saved on cations.

Cations analysis

Samples were firstly defrozen at room temperature and then they were shacked by turning and turning back samples. After 3 min centrifugation at 12000 rpm (20°C), 10 µl clear supernatant juice

was injected via a Waters® 717 (Waters, *www.waters.com*) injector in the HPLC through a Waters® IC-Pak Cation M/D 3.9x150 mm column (20°C) using HNO3 as mobile phase (0.004 N). Waters® 600 pump was set as 1ml/min rate flow. Data are then obtain using a Shimadzu® CDD-10A conductimeter (Shimadzu, *www.shimadzu.fr*) and analysed using Waters® EMPOWER-3 software. Potassium, Magnesium, Ammonium and Calcium concentrations are then provided as data. Samples were also prepared as describe in Bigard et al. (2018) for separation of glucose, fructose, malic and tartaric acids and quantifications.

Data analysis and graphic representations

Data obtained were statistically analysed using R-software version 3.4.3 (R Core Team, 2017). For statistical analysis, only repeated samples in 2016 and 2017 will be used for microvines green and ripe stage and for varieties ripe stage. Histograms of 2016 samples (except Petit Manseng at green stage) were made using SigmaPlot® version 14 (Systat Software, *www.systatsoftware.com*). Excel® was also used as table to collect and classify data.

Results

In this chapter, only cations results will be described. Bigard et al. (2018) provide results for sugars, acids and weight of those samples.

Figure 1 represents the concentration of Potassium (mmol.L^{-1}) per variety and microvine for green and ripe stage in 2016. In varieties, Potassium accumulation at green stage ranged from 21 to 43 mmol.L^{-1} for Muscat d'Alexandrie and Béclan compared to 35 to 54 mmol.L^{-1} at ripe stage for Couston and Petit Manseng respectively. In microvines, Potassium accumulation at green stage ranged from 25 to 47 mmol.L^{-1} for microvines 73 and 199 compared to 28 to 57 mmol.L^{-1} at ripe stage for microvines 117 and 349 respectively. Statistical analysis showed that at green stage for the microvine progeny, there was an effect of E, G and GxE on Potassium accumulation. Despite, microvines 119 and 340 tended to have a high content compared to microvine 141 which had a low one. At ripe stage, there was an effect of E and G without interaction. 2016 values were significantly higher than 2017 and microvines 119, 114 and 262 were top 3 in accumulations, others fighting for last place. In varieties at ripe stage, there was a small interaction GxE with a big effect of G. Muscat d'Alexandrie and Grenache varieties was then always high, and in the opposite Petit Manseng was always poor.

Figure 1 – Potassium concentrations (mmol.L^{-1}) in varieties and microvines at green stage and ripe stage. Bar charts represent 2016 mean values with the corresponding SE.

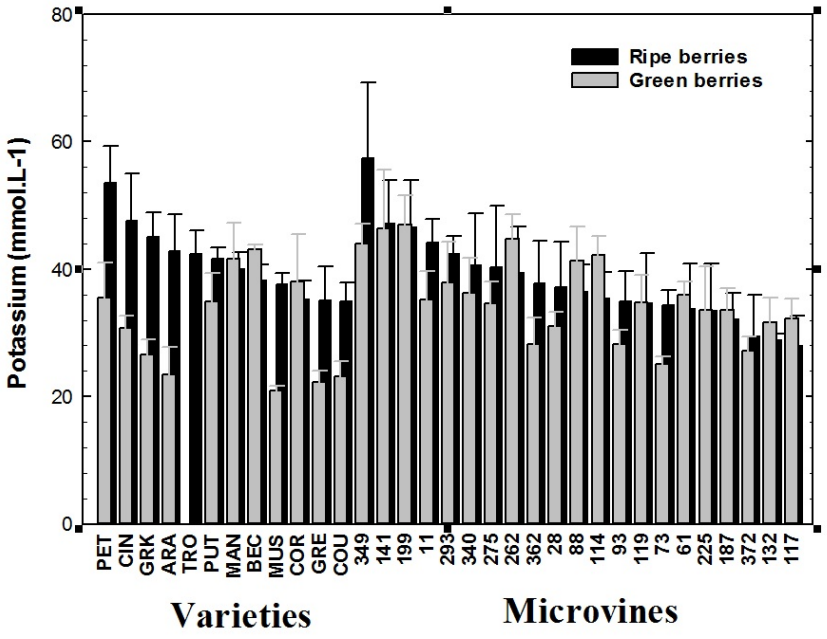


Figure 2 represents the concentration of Ammonium (mmol.L^{-1}) per variety and microvine for green and ripe stage in 2016. For varieties, Ammonium accumulation at green stage ranged from 0.9 to 19.6 mmol.L^{-1} for Béclan and Grenache compared to 0 to 5.5 mmol.L^{-1} at ripe stage for Mandilaria and Cornifesto respectively. In microvines, Ammonium accumulation at green stage ranged from 9.5 to 33.7 mmol.L^{-1} for microvines 114 and 372 compared to 3 to 15 mmol.L^{-1} at ripe stage for microvines 349 and 141 respectively. Statistical analysis showed that at green stage for the microvine progeny, there was an effect of G and GxE on Ammonium accumulation. Only microvine 114 stayed high in content in both years. At ripe stage, there was an effect of E and G with GxE interaction given no results. In varieties at ripe stage, there was an effect of E, G and GxE. Cinsault and Grenache varieties were thus always poor.

Figure 2 – Magnesium concentrations (mmol.L^{-1}) in varieties and microvines at green stage and ripe stage. Bar charts represent 2016 mean values with the corresponding SE.

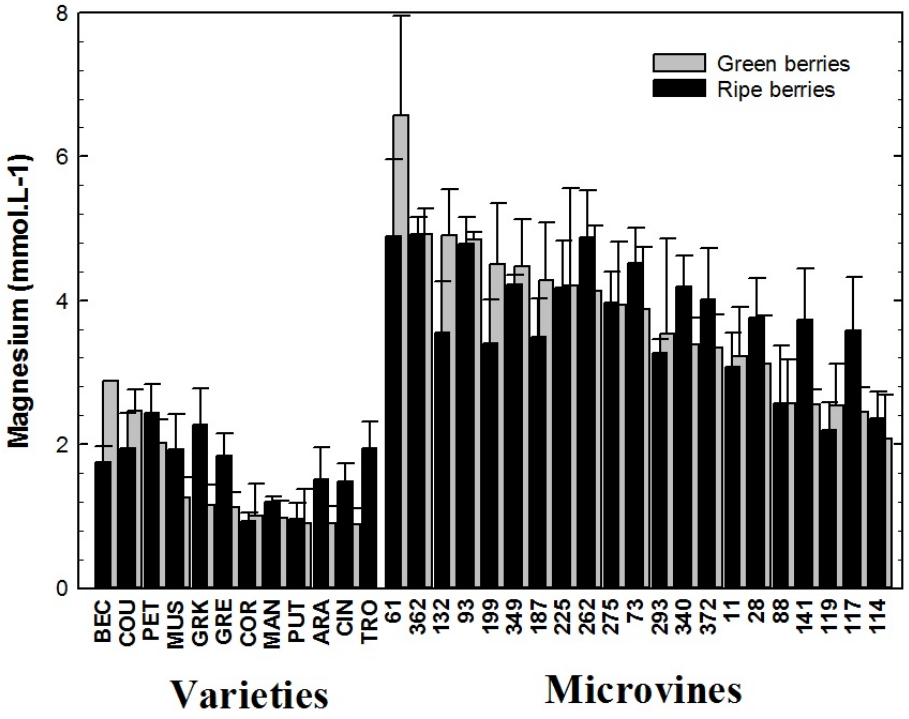


Figure 3 represents the concentration of Calcium (mmol.L^{-1}) per variety and microvine for green and ripe stage in 2016. Concentrations at green stage ranged from 1.1 to 10.5 mmol.L^{-1} for Cinsaut and Béclan varieties and from 0.8 to 3.2 mmol.L^{-1} for 114 and 362 microvines. At ripe stage values ranged from 0.1 to 3.2 mmol.L^{-1} for Mandilaria and Béclan varieties and from 0.4 to 2 mmol.L^{-1} for 119 and 372 microvines. Statistical analysis showed that at green stage for the microvine progeny, there was an effect of G, E and GxE but 2016 values were clearly higher and more homogeneous than 2017. At ripe stage, there was an effect of E and G. Microvine 119 and 114 were the highest and microvine 141 and 262 the lowest. Also, 2016 values were significantly higher than 2017. In varieties at ripe stage, there was an effect of E, G and GxE. Results showed that 2016 values were slightly higher than 2017 and that Cinsaut had a high concentration of Calcium, Petit Manseng a low one.

Figure 3 – Calcium concentrations (mmol.L^{-1}) in varieties and microvines at green stage and ripe stage. Bar charts represent 2016 mean values with the corresponding SE.

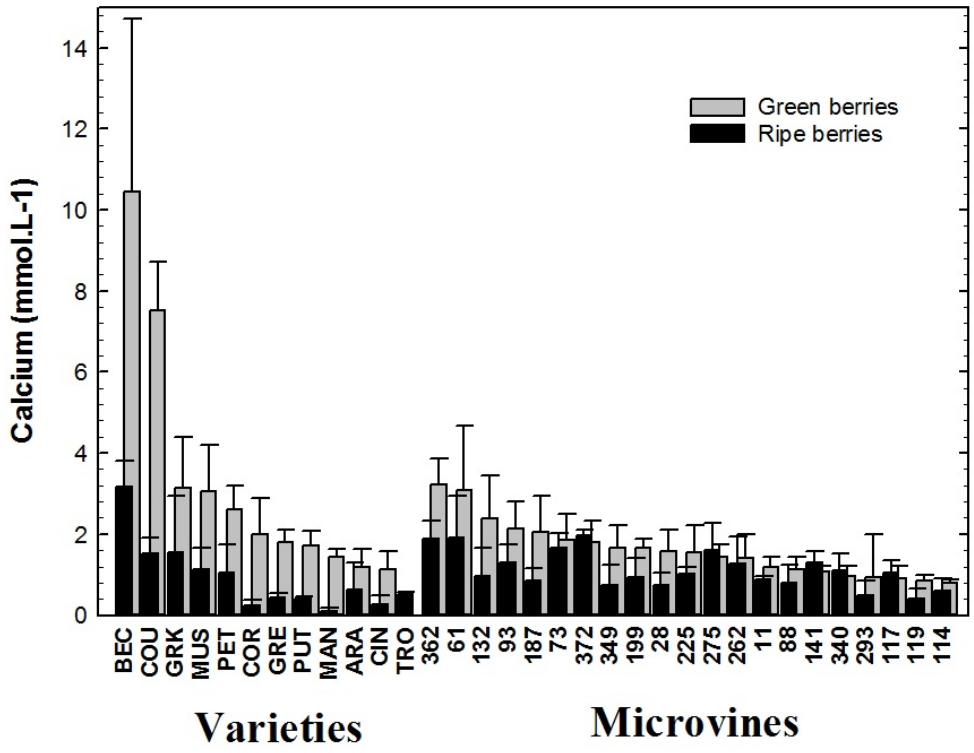


Figure 4 represents the concentration of Magnesium (mmol.L^{-1}) per variety and microvine for green and ripe stage in 2016. Concentrations at green stage ranged from 0.9 to 2.9 mmol.L^{-1} for Cinsaut and Béclan varieties and from 2.1 to 6.6 mmol.L^{-1} for 114 and 61 microvines. At ripe stage values ranged from 0.9 to 2.4 mmol.L^{-1} for Cornifesto and Petit Manseng varieties and from 2.2 to 4.9 mmol.L^{-1} for 119 and 362 microvines. Statistical analysis showed that at green stage for the microvine progeny, there was an effect of G and GxE on Magnesium accumulation. At ripe stage, there was an effect of E and G with a small GxE interaction. Microvine 114 remained with a high content. In varieties at ripe stage, there was an effect of E, G and GxE. Cinsault variety was with high content compared to Petit Manseng which was poor. Year 2016 was with slightly higher content than 2017.

Figure 4 – Ammonium concentrations (mmol.L^{-1}) in varieties and microvines at green stage and ripe stage. Bar charts represent 2016 mean values with the corresponding SE.

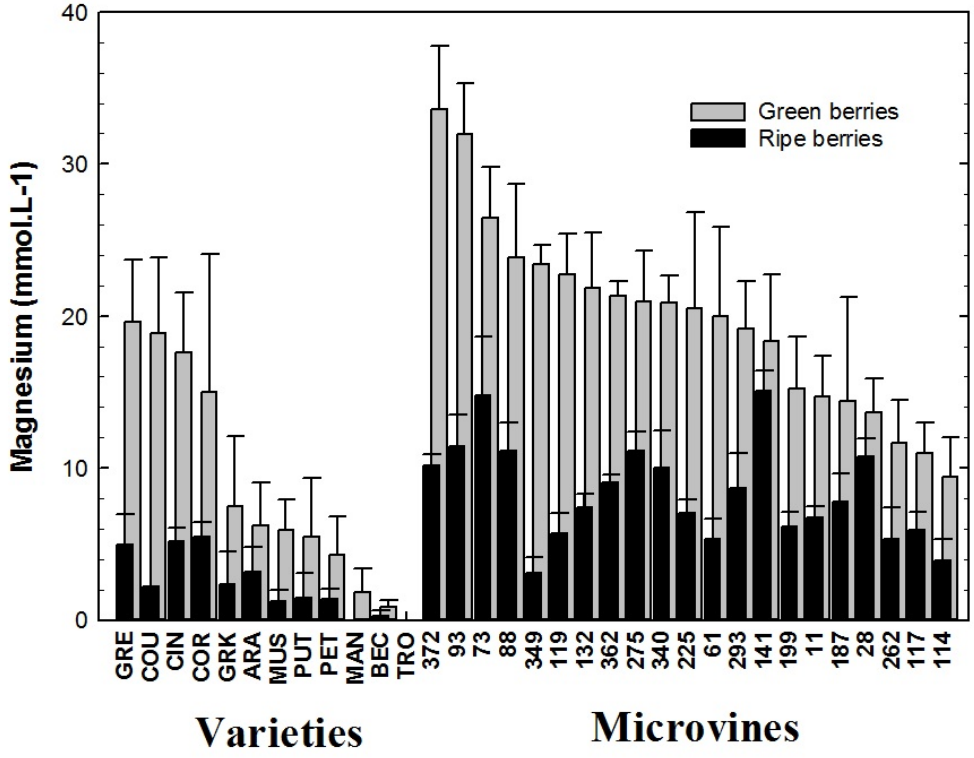
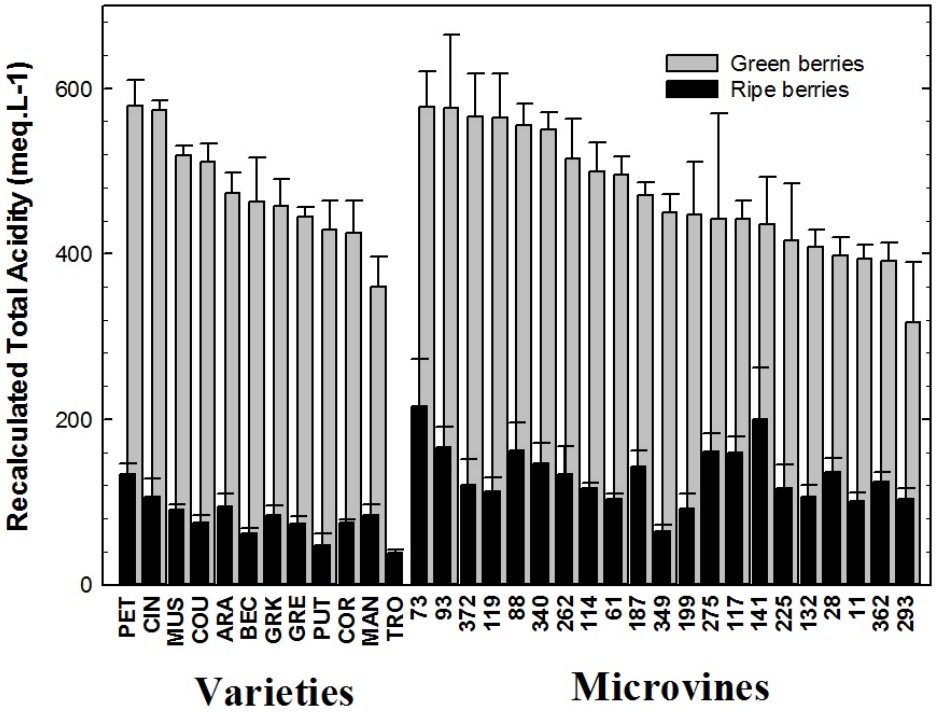


Figure 5 represents the concentration of Recalculated Total Acidity (meq.L^{-1}) per variety and microvine for green and ripe stage in 2016. Recalculated Total Acidity at green stage ranged from 360 to 580 meq.L^{-1} for Mandilaria and Petit Manseng varieties and from 318 to 578 meq.L^{-1} for 293 and 73 microvines. At ripe stage values ranged from 38 to 134 meq.L^{-1} for Trousseau and Petit Manseng varieties and from 64 to 215 meq.L^{-1} for 349 and 73 microvines. Statistical analysis showed that at green stage for the microvine progeny, there was just an effect of G on Magnesium accumulation. Microvine 11, 141 and 262 had the highest accumulation potential at green stage, 340 and 119 were poor in that element. At ripe stage, there was an effect of E, G and GxE with no trend noticeable. In varieties at ripe stage, there was an effect of E, G and GxE. Despite, Petit Manseng was always the poorest.

Figure 5 – Recalculated Total Acidity (meq.L^{-1}) in varieties and microvines at green stage and ripe stage. Bar charts represent 2016 mean values with the corresponding SE.



Correlations were also study. Significant correlations were found on microvine progeny at ripe stage with both years data: 0.77 between calcium concentration and potassium (p-Value < 0.05), 0.73 between total acidity and ammonium concentration (p-Value < 0.05), 0.62 between total acidity and potassium concentration (p-Value < 0.05). No correlations where found on green stage. Significant correlations were found on varieties at ripe stage with both years data : 0.96 between calcium and magnesium concentrations (p-Value < 0.05), 0.86 between potassium and magnesium

concentrations (p-Value < 0.05), 0.81 between potassium and calcium concentrations (p-Value < 0.05) and all cations except ammonium, had a correlation slightly higher than 0.60 with total acidity (p-Value < 0.05).

Discussion

Primary metabolites (acids and sugars), berry weight and sampling method were well described in Bigard et al. (2018). Samples used in this experiment being the same, this paper will focus on cations diversity only considered as "independant".

First observation was that diversity in cations in both stages exists even if years shown differences (place change). Potassium values observed were higher than some previous observations (Mpelasoka et al., 2003) and lower than some others (Storey, 1987; Rogiers et al., 2017). Temperatures maybe had an impact on potassium accumulation (Mira de Orduña, 2010) but this study showed that diversity exists for low potassium cultivars creation, in order to increase acidity (Sweetman et al., 2014). The European low permit to remove only 54 mmol.l-1 potassium to rise up acidity (CEE 53/2011) (Bigard et al., 2018). Microvines progeny showed that segregation can help by half removal necessities. Microvine for this trait also showed higher diversity at maturity than varieties which is an interesting information for breeders.

Ammonium values were really low at ripe stage in some cultivars witch can have consequences on fermentations. There is also no such much studies on this cation. Data shown that it concentration has a strong climate / genotype interaction making difficulties analysis and breeding.

Calcium was higher in green berry as showed in previous reports (Bonomelli & Ruiz, 2010; Bashir & Kaur, 2018), possibly due to his structural role in the cell wall and membranes, or use as counter-ion for vacuole anions in the early stages. Diversity was 3 times higher in cultivars than microvine progeny, showing that there is a small chance to achieve a high segregation of this trait. To understand better segregation of this trait, parents should be also study. However, varieties presented higher concentrations than bibliography (Mpelasoka et al., 2003). This cation is also localised manly on skin and our protocol were not the best for this extraction, underestimating probably real values (Duchène & Chardonnay, 1992).

Magnesium was higher in the microvine progeny than varieties. This phenomenon was probably due to higher fertilisation used for microvines. In field, varieties presented lower diversity than the bibliography (Mpelasoka et al., 2003). Microvines progeny maximum capacities were at same level as bibliography. Also for this cation not so many studies talks about diversity.

Total acidity is of a major challenge in grape juice production (Ollat et al., 2017). Impacts by climate change is known and well documented in the literature (Butrose et al., 1971; Kliewer & Lider, 1970; Seguin et al., 2004; Rienth et al., 2016). This work permits to have another overview of the diversity in total acidity. Acidity was higher for microvine progeny in green house probably due to lower temperatures compared to field. Microvines progeny showed that acidity is high to improve by cross maybe due to low heritability already found as describe in literature (Houel et al., 2015).

Highest correlation found was between magnesium and calcium in varieties ripe stage samples, both cations being known at having quite similar pattern of accumulation (Conde, 2007). But it was interesting to see that they were also both correlated with potassium. This suggests a common control of the berry accumulation for those cations at least for the end of sugars accumulation in field condition. On microvine progeny only potassium and calcium were correlated showing that the possible control for those cations is even higher and difficult to change during a cross. Total acidity, mainly due to acids and potassium, seems to also dictate cations concentrations in ripe stage varieties except ammonium. Oppositely to microvine progeny observations with a total acidity correlated with ammonium and potassium only.

Conclusion

In this experiment, the strong impact of the environment was clearly visible as 2016 was a better year than 2017 for cations accumulation, but does not much interfere on correlations as data showed. Also, a difference can be induced between greenhouse and field due to temperature, water controlled and also nutriments. Data showed interesting diversity in all cations experimented (potassium, calcium, magnesium and ammonium) in both diversity in *Vitis vinifera* and microvine progeny. Data showed that it was maybe possible to use diversity for further perspectives of breeding.

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Author contributions: LT, CR and AB designed the experiments. AB, YS, EM, SM, KG and CR performed the experiments. AB, LT and CR drafted the manuscript. AB, LT, CR, YS and JPP edited the manuscript. All authors reviewed the final version of the manuscript.

Literature cited

Please refer to the global Literature of this work

Chapter III: The low sugars content trait

The previous chapters aimed to compare several tools to monitor berry growth and metabolite accumulation (Chapter 1) and to study the diversity for these traits exiting in *V. vinifera* (Chapter 2). Chapter 3 will present the studies we have performed with some of low sugar accumulator genotypes to identify the developmental mechanisms associated with this trait.

This chapter is presented as successions of ideas/hypothesis tests on the physiological origin VDQA trait, in which normal sugars accumulating and low sugars accumulating genotypes are compared.

First, we checked if the low sugars content trait was not just the consequence of delayed ripening. Then, heterogeneity and asynchrony were analysed using density bath and texture/colour changes in order to check if the low sugar trait affected all berries in the population, or if it was just the consequence of the existence of a strongly delayed sub-population.

After having eliminated the two first possibilities, it was concluded that the VDQA trait was not an artefact, and we checked which could be its possible developmental origin. It was thus primordial to find proper, objective definitions of veraison and maturity stages to compare efficiently the two genotypes. For the determination of the stages, different tools were tested to find the most relevant one, especially for maturity. Data showed that variability in berry weights was too high to determine precisely maximum volume stage. Dilution of Tartaric acid was found more efficient. Then, once maximum volume stage was determined, the concentration in sugars was connected to growth. The first idea was to look if another osmoticum participated to the turgor pressure, permitting the growth with fewer sugars.

In the end, new hypothesis on low sugars content genotypes will be described and tested when possible.

III.1. Average berries population characterisation

In this part, the kinetics of metabolites accumulation and dilution/degradation in the two genotype types will be described. At the beginning of the experiment, in 2014, some Bouquet's new genotypes were qualified as low sugars content by specialists but growth kinetics were rather empirical. In this regard, the first aim of this study was to get more precise insights on these developmental patterns din order to determine if the low sugars content trait is really existed.

Materials and Methods

Plant material

Grape berries were sampled from outdoor vines at the INRA of Pech-Rouge, Gruissan, France in 2014 and 2015 (43.14' N latitude and 3.14'' W longitude, elevation 6m above sea level). This place is characterised by a semi-arid Mediterranean climate (Giorgi & Lionello, 2008). The vineyard was also managed though drip irrigation to keep the predawn leaf water potential (Ψ_{PD}) higher than 0.5MPa. In this part, 2 new powdery and downy mildew resistant hybrids also characterised by specialists as low sugars content (G7 and G14, 3197-373N and 3184-1-9N respectively coming from a cross between *Muscadinia rotundifolia* G2 with a *Vitis vinifera* Malaga seedling (Escudier et al., 2017; Ojeda et al., 2017)) will be compared to 3 *V. vinifera* controls (Grenache, Merlot, Morrastel).

Sampling methods

During the vintages 2014 to 2015, samplings were performed once or twice a week starting 1 to 2 weeks before the first signs of berry softening and up to berry shrivelling. In 2014, 600 berries were randomly sampled in pool for each date from the 3 experimental blocks per genotype. In 2015, 200 berries were randomly sampled and analysed separately from each block x genotype treatment. For all experiments, berries were separated from bunches by cutting the pedicel the closest possible from the berry, in order to minimise the impact of this organ for the volume measurement and to limit juice leaking.

Density sorting

As soon as possible after sampling, i.e. after max 1 hour, the berries were sorted through their apparent density as described in Nelson et al. (1963) and Singleton et al. (1966) with slight modifications, i.e. using NaCl instead of sucrose (Rolle et al., 2011; Carbonell-Bejerano et al., 2013) . Twelve solutions were prepared from 80g to 190g NaCl.l^{-1} with 10 g.l-1 increments as Carbonell-Bejerano et al. (2013) (cf. Table 1).

Table 1: Baths concentration in NaCl, correlated to sugars content and apparent density.

Bath number	NaCl (g/L)	Density	Sugars (g/L)	Sugars (mmol/L)
1	190	1.12	279	1549
2	180	1.11	264	1465
3	170	1.11	248	1377
4	160	1.10	233	1293
5	150	1.09	218	1210
6	140	1.09	202	1121
7	130	1.08	187	1038
8	120	1.08	172	955
9	110	1.07	156	866
10	100	1.06	141	783
11	90	1.06	126	699
12	70	1.05	110	611
13	< 70	< 1.05	< 110	< 610

Volume and weight measurement

The average berry volume of each sample was measured by image analysis and immersion. The Dyostem® (Vivelys, *www.vivelys.com*) is a device that takes a picture of 100 berries immobilized on regular wells on a blue plate, and calculates each berry volume following contour adjustment with a perfect circle, before reporting the distribution of berry volume by sample. Alternatively, net-bagged berries were hanged in a beaker containing pure water on a balance, in order to measure displaced water volume according to the Archimedes law ($P_{\text{Moved Liquid}} = w_{\text{Moved Liquid}} \times g = \rho_{\text{Liquid}} \times V \times g$) (Lang & Thorpe, 1989). Average berry weight were measured at the same time as volume using a Ohaus® scale precise at +/-0.01g (OHAUS, *www.us.ohaus.com*).

Primary and secondary metabolites

Immediately after measuring volume, samples were crushed with a domestic crusher during approximately 15 sec at room temperature. Then three crude juice samples were taken and immediately frozen at -30°C. Anthocyanins quantification was done with one fresh sample (10-20 mL) which was weighted and three times diluted in a hydro-alcoholic solution (2.631 $mol.L^{-1}$ ethanol + 0.01$mol.L^{-1}$ HCl). After one hour orbital stirring, 10 mL of solution were centrifuged 5min at 12000 g (20°C), then the supernatant was 20-50-time diluted depending on colour, before 520nm absorbance measurement in a 1cm optical path Evolution 300 UV-VIS spectrometer (Thermo Scientific, *www.thermoscientific.fr*). Anthocyanins ($mg.L^{-1}$) were calculated as total dilutionxOD520nmx22.76 (Ribéreau-Gayon et al., 1998). The second fresh sample was centrifuged as above. Refractive index was measured with a digital refractometer and then total acidity was obtained by titration to pH 7 of 20 mL supernatant with 5 $mol.L^{-1}$ NaOH on a TitroMatic KF 2S 2B (Crison, *www.crisoninstruments.com*). The machine gives also the juice pH as first data. Total acidity was expressed as $meq.L^{-1}$. The frozen sample was used to quantify primary metabolites. Samples were thawed in a 60°C water bath during 30 min, vigorously shacked with an orbital shaker for 15sec, and centrifuged as above. Supernatant were ten times diluted with 0,2N HCl and filtered on cellulose acetate 0.2µm membranes, before injection on HPLC (Biorad aminex-HPX87H column) according to Bories et al. (2011) with same conditions as described in Bigard et al. (2018).

Data analysis

R-software version 3.4.3 was used for statistical analysis (R Core Team, 2017). Data asymmetry and flattening were calculated with skewness and kurtosis functions on R from package "e1071". Parametric and non-parametric tests were used to find differencies and then a Two-way Ordinal Regression was performed as PostHoc test.

Results and discussion

Growth Kinetics of average berries population

Figure 1 represents the evolution of the concentration in soluble solids during ripening. In this graphic several informations can be extracted, first, soluble solids accumulation starts at mid-softening, showing no delay in the sugars accumulation (cf. Table 2). Also, this graphic shows two

different patterns, one for Merlot and Grenache and the other for G7, G14 and Morrastel. At day 45 for example, Merlot and Grenache had a sugars concentration around 1.35 mol.L^{-1} while G7, G14 and Morrastel were around 1 mol.L^{-1}. Considering that massive sugars accumulation beginning occurs between day 26 and 34 (cf. Table 2), day 45 should be at berry maturity (considering the bibliography (McCarthy, 1999)). Considering those two facts and considering a normal berry growth for all genotypes, G7, G14 and Morrastel appears to have the capacity to accumulate less sugars, or to dilute them more, when compared to other varieties as Merlot and Grenache.

Figure 1: Evolution of the sugars concentration (mol.L^{-1}) of 2014 and 2015 samples depending on days (0 represents mid-softening).

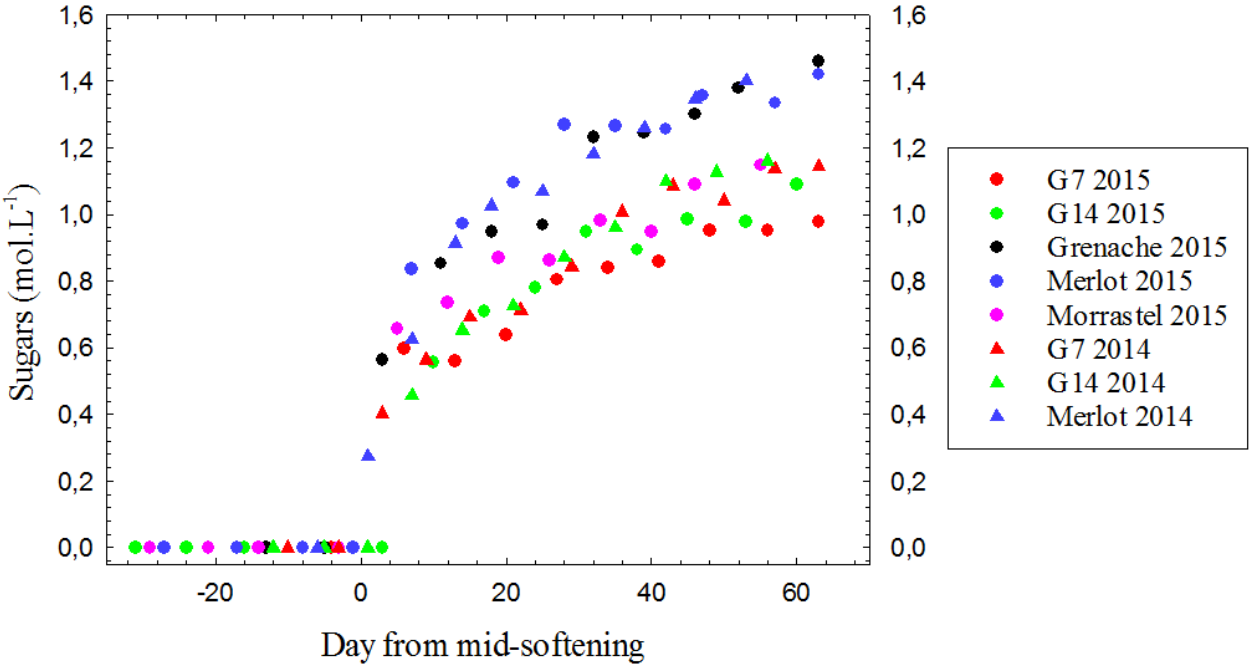
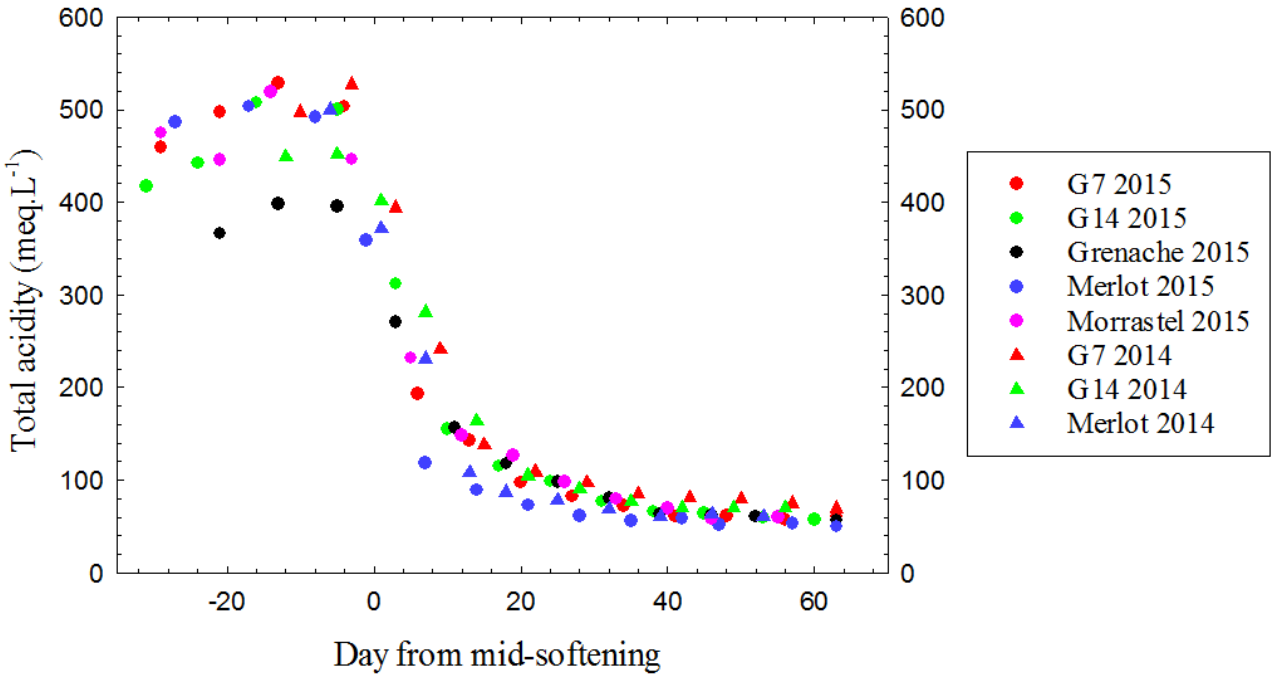


Table 2: Date of half veraison stage (50% soft berries with 50% hard berries) for all genotypes and both years.

Genotype	Year	Date
Merlot	2014	18/07/2014
Merlot	2015	24/07/2014
G7	2014	22/07/2014
G7	2015	22/07/2015
G14	2014	24/07/2014
G14	2015	26/07/2015
Morrastel	2015	25/07/2015
Grenache	2015	21/07/2015

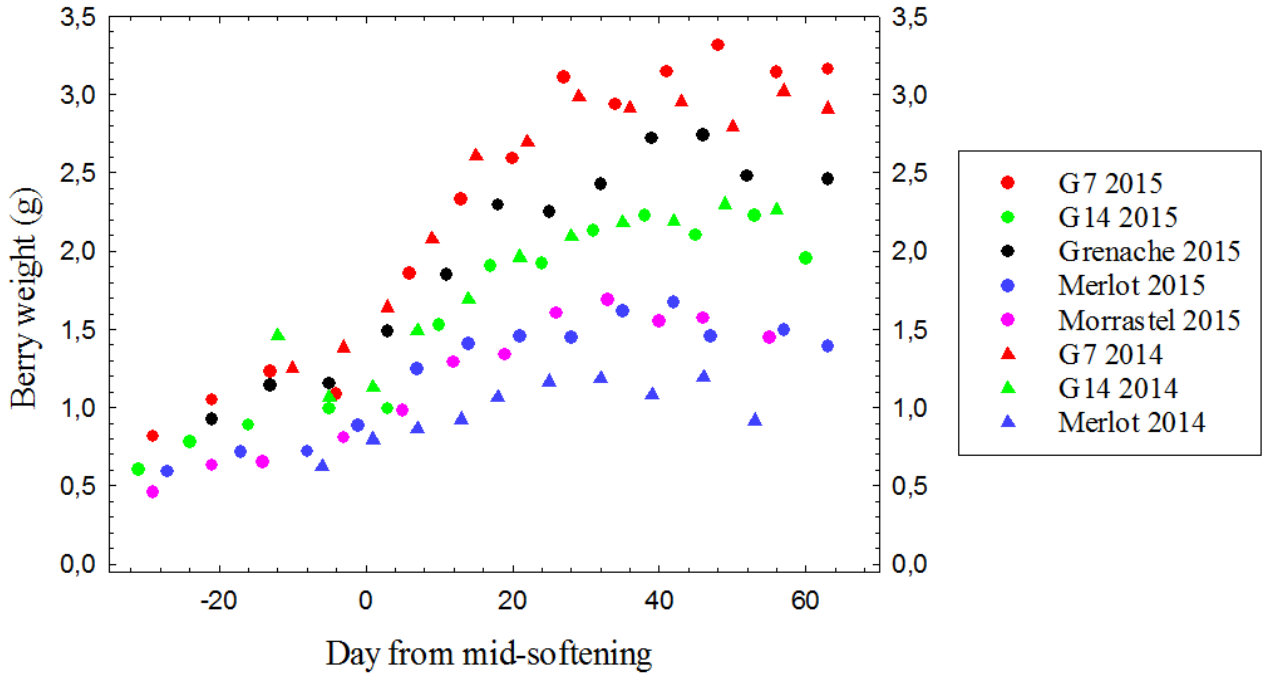
Figure 2 shows the evolution of titration acidity during ripening. Same as Figure 1, Acidity starts to decrease at mid-softening. This observation concurs the bibliography, reporting that the acidity is decreasing right after veraison (Coombe, 1984; Vicens, 2007; Xie et al., 2009; Bordenave et al., 2013). This figure shows that they are no delay between sugars accumulation and acidity breakdown, representing a normal growth pattern under standard climate conditions (Rienth et al., 2016). The decrease in acidity was extremely fast, 80% less than 20 days.

Figure 2: Evolution of the total acidity (meq.L^{-1}) of 2014 and 2015 samples depending on days (0 represents mid-softening).



Proof was made that genotypes with low sugars content trait displayed a normal pattern of sugars concentration and acidity loss (Champagnol, 1984; Rienth et al., 2016). Figure 3, which represents the average berry volume for each sample during time, shows that at day 50, all samples (except maybe G7 in 2015) had already reach their maximum volume. However, this figure also show erratic variations of the berry volume not synchronized between cultivars, suggesting that they may result from sampling difficulties, rather than by environmental changes as a common source of variation. Maximum berry volume stage is the stage when phloem unloading stops and berry starts shrivelling (Coombe & McCarthy, 2000; Conde et al., 2007; Bondada et al., 2017). At this stage, berry has reached a maximum in sugars content (not in concentration) and in water content, after which concentrations still increases because of the evaporation. Using figure 1 and 3 together, it is possible to claim that G7, G14 and Morrastel are displaying the low sugars content trait.

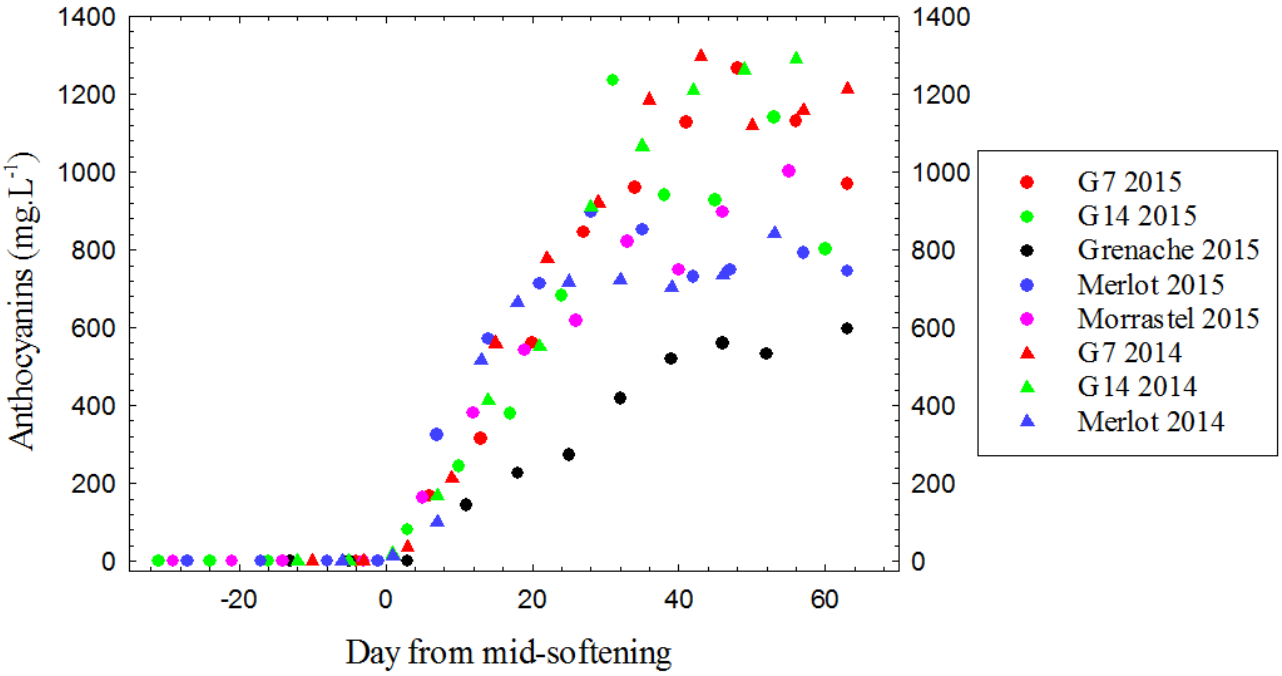
Figure 3: Evolution of the average berry weight (g) for 2014 and 2015 samples (0 represents mid-softening).



Interestingly, especially for wine production, G5, G7 and G14 displayed a really high concentration in anthocyanins. There final concentrations are around 1200 $mg.L^{-1}$ and compare to Jeandet et al. (1995), values are low but in this paper Morrastel were at 2000 $mg.L^{-1}$, which is higher than this experiment. Other papers showed lower concentration (Ribéreau-Gayon, 1982). G7 and G14 presented high anthocyanins accumulation potential compared to Merlot, Morrastel and Grenache at lower sugars concentrations, which is interesting agronomically speaking. Figure 4 represents this

concentration depending on days for all genotypes and years. For example, G7 and G14 maximum anthocyanins concentrations are twice higher than in Grenache and they are also higher than Merlot and Morrastel.

Figure 4: Time evolution of the average berry anthocyanins concentration ($mg.L^{-1}$) for 2014 and 2015 (0 represents mid-softening).



First conclusion

These results demonstrated that the limitation in sugar concentration in Morrasted, G5, G7 and G14 genotypes is not caused by an artefact of berry sampling or a phenological shift. This trait which is not year dependant could result from a limitation of sugar accumulation or an increase of dilution by water uptake. Although when sugar concentration in the flesh is comparatively low, anthocyanins are accumulated to a higher level in the skin. But all those results have to be mitigated by the fact that data are provided by sample of berry population, where fruit heterogeneity and asynchrony development wasn't taking into account.

So are the heterogeneity and the asynchrony among berries in the population harvested different between Morrasted, G5, G7 and G14 genotypes and Merlot, Grenache? Is it possible that the difference in observed average concentrations could result a higher fruit development asynchrony?

Characterisation of the fruit heterogeneity and asynchronous development

Berry heterogeneity is known to complicate the interpretation of the relation between fruit physiology and wine quality (Nelson et al., 1963; Lund et al., 2008; Böttcher et al., 2011; Rolle et al., 2013; Doumouya et al., 2014; Rienth et al., 2016; Shahood et al., 2017). Single fruit heterogeneity results from various factors controlling inflorescence and fruit development (radiative and evaporative micro-environment) (Kuhn et al., 2013; Doumouya, 2014; Reshef et al., 2017). The protocol used above on chapter III section 1 was based on pooling a significant number of randomly sampled berries, to estimate the developmental pattern of the average population, not the one of the berry (De Montmollin et al., 2004; Geraudie, 2009; Parker et al., 2011; Arrizabalaga et al., 2018). With this in mind, it is important to find a way to analyse this asynchrony and the impact of it to determine the real potential for each genotype.

To calculate indicators of asynchrony, 2015 data were used to look at the repetition effect; we first look at the shape of the distribution of the number of berries per baths and date, which correspond to different levels of berry maturity (Nelson et al., 1963; Singleton et al., 1966). The distribution was really similar to a Gaussian curve (cf. Fig 5). In this respect, asymmetry and flattening parameters can be calculated and analysed (cf. Fig. 6 & 7). Then, statistical analysis can be performed to compare genotypes and biological replicates (cf. Table 3).

Figure 5: Distribution of berries per bath and sampling date for G14 in 2014.

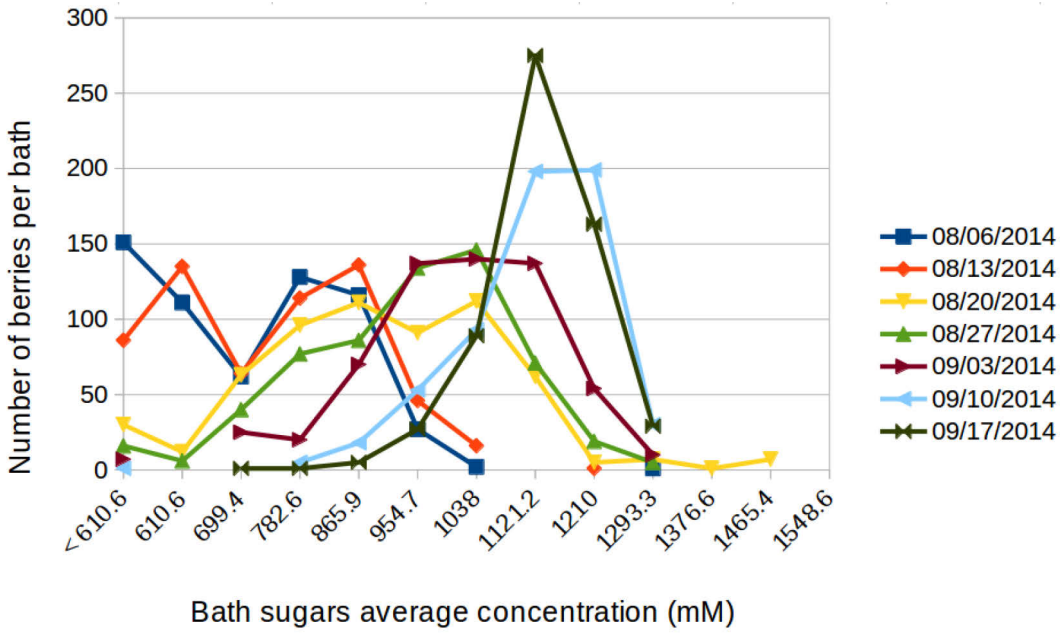


Figure 6: Calculated asymmetry of berry number distribution depending on sampling date for all genotypes and repetitions.

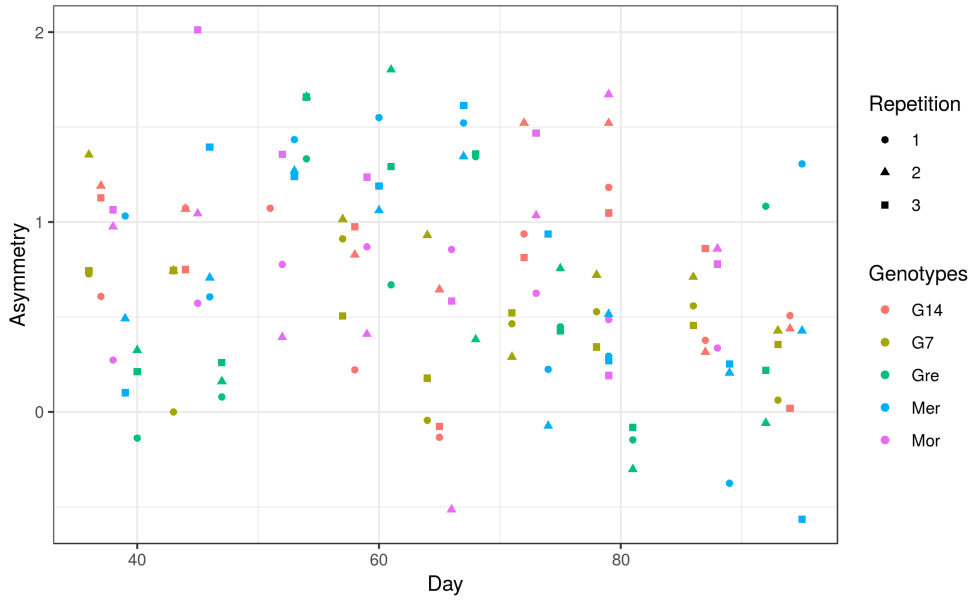


Figure 7: Calculated flattening of berry number distribution depending on sampling date for all genotypes and repetitions.

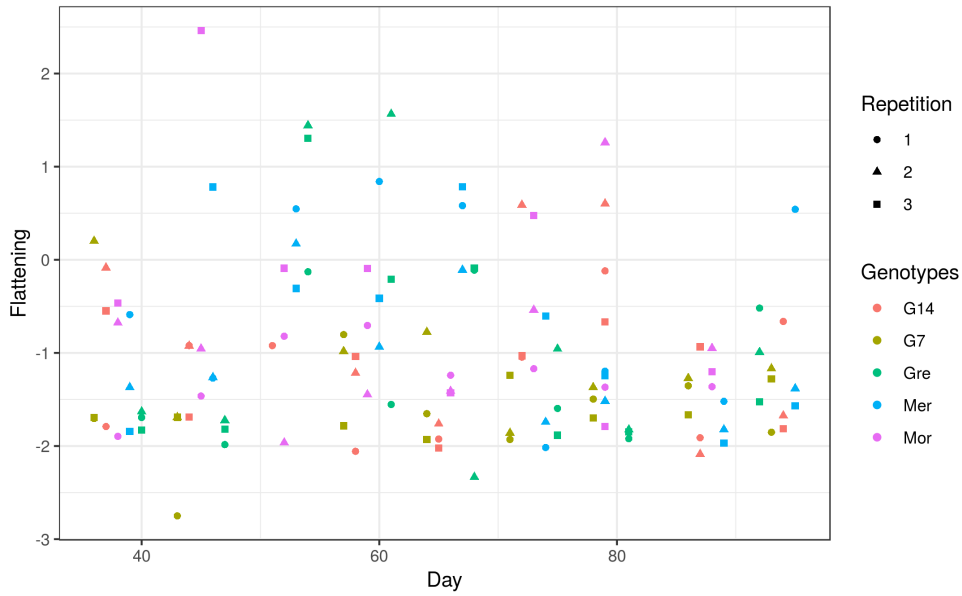


Table 3: Head of the asymmetry and flattening table of berry number distribution calculated for each sampling date and each genotype.

Sampling day	Repetition	Asymmetry	Flattening	Genotype
37	1	0.61	-1.79	G14
44	1	1.07	-0.92	G14
51	1	1.07	-0.92	G14
58	1	0.22	-2.06	G14
65	1	-0.13	-1.92	G14
72	1	0.94	-1.05	G14
79	1	1.18	-0.12	G14
87	1	0.38	-1.91	G14
94	1	0.51	-0.66	G14
37	2	1.19	-0.08	G14
44	2	1.07	-0.92	G14

Statistical analysis performed with this table shows no significant effect of the genotype, repetition or interaction on asymmetry and flattening. Since there is no statistical difference between genotypes, the hypothesis of a higher asynchrony in low sugars content genotypes can be rejected. Repetitions being identical showed that genotypes are equally asynchronous.

For heterogeneity, same approach was used in order to compare distributions of berry average weight per baths and date. For the asymmetry parameter, just the interaction was significant (P-

value < 0.05), making the interpretation impossible. Flattening on it side presented no significant effects. In this order genotypes had same heterogeneity in berry volume.

Others parameters described also same pattern as describe in chapter I, thus no major differences were observed. This permit also to say that berry growth of G7 and G14 are following a normal pattern of development, with just different concentration and water import.

Second conclusion

After a run of statistical analysis on the asymmetry and flattening parameters, no effect was found significant for each parameter, except interaction for the flattening parameter for heterogeneity. Differences observed for those parameters between genotypes are very small and not significant, so the asynchrony and heterogeneity can't explain the low sugar concentration observed in low sugar accumulator genotypes, Morrastel, G7 and G14.

Also, berry accumulation of others metabolites showed same pattern as presented in chapter I. Then proof was made that G7 and G14 presented a normal pattern of ripening.

Having this in mind (the existence of the low sugars content trait and the fact that the asynchrony/heterogeneity are not responsible for it), all parameters from pooled berries has been review. The only way left to determine the reality of this trait is to down-scale measurement to single berries and to find comparable key development stages.

III.3. The key developmental stages during berry development

As seen in previous sections, the low sugars content trait can't be explained by an uncommon berry population structure (asynchrony/heterogeneity) so it is really a feature affecting the development of all individual berries which are melted in the global population harvested. However, the berry density sorting procedure was inefficient in identifying how the relation between growth, or water, and solute accumulation was modified in these low sugar cultivars. In particular, it failed in detecting the sugar concentration at the particular moment when phloem unloading in berry stopped, that can be taken as an objective definition of physiological ripe stage. To overcome this difficulty, analysis were down-scaled to the single berry, following the paradigm developed by Shahood (2017).

Materials and Methods

Meteorological data

Data were obtained from Historique-météo.net website (www.historique-meteo.net) which is collecting the data of the INRA station of Pech-Rouge (France).

Plant material

Berries were sampled from outdoor vines at the INRA of Pech-Rouge, Gruissan, France in 2016 and 2017 (43.14' N latitude and 3.14'' W longitude, elevation 6m above sea level). This place is characterised by a semi-arid Mediterranean climate (Giorgi & Lionello, 2008). The vineyard was managed though drip irrigation to keep the predawn leaf water potential (ΨPD) higher than 0.5MPa. For 2016, G14 genotype, deriving from a cross between *Muscadinia rotundifolia* and Vitis vinifera (Escudier et al., 2017; Ojeda et al., 2017) was compared to Grenache. For 2017, G7, G14 and G5 genotypes (Escudier et al., 2017; Ojeda et al., 2017) were compared to Grenache, Merlot, and Morrastel.

Sampling methods

Single berries were randomly sampled by cutting the pedicel the nearest possible from the berry, still to minimise the impact of this organ for the volume measurement and to limit juice losses.

During sampling, at least 50 berries were sampled per genotype and quickly transported to the laboratory in plastic bags in a cool box.

Firmness

Berry firmness was analysed with a digital penetrometer called Penelaup (Abbal et al. in 1992). This allows the calculation of the regression coefficient between a force and a displacement, expressed in $g.mm^{-1}$. The diameter is also given by the machine with the berry weight. After firmness measurement, berries were frozen at -20°C for further biochemical analyses.

Chemical analysis

Berry weight was measured using an Ohaus® scale (PA224 model) precise at +/-0.0001g (OHAUS, *www.us.ohaus.com*). Then 4 times berry fresh weight of 0.25 N HCl were added to sample. Seeds were removed in 2017 samples. After 48 hours of incubation, 3 microtubes were prepared.

Primary metabolites - The extract was diluted 11 times with 8.3 10^{-3} N acetic acid (internal control) + 16.4 10^{-3} N sulphuric acid. After centrifuging at 18,500 g during 5 min at 20°C, supernatants were directly injected for HPLC to separate glucose, fructose, malic and tartaric acids through a Biorad aminex-HPX87H column according to Bories et al. (2011) with slight modifications (60°C and 0.6 $ml.min^{-1}$ rate flow).

Micro-elements - The extract was juice diluted 10 times using water and frizzed at -30°C for storing. Before analysis, samples were firstly defrozen at room temperature and homogenised by shacking. After 3 min centrifugation at 12000 rpm (20°C), 10 µl clear supernatant juice was injected via a Waters® 717 (Waters, *www.waters.com*) injector in the HPLC through a Waters® IC-Pak Cation M/D 3.9x150 mm column (20°C) using HNO_3 as mobile phase (0.004 N). Waters® 600 pump was set as 1ml/min rate flow. Data are then obtain using a Shimadzu® CDD-10A conductimeter (Shimadzu, *www.shimadzu.fr*) and analysed using Waters® EMPOWER-3 software. Potassium, Magnesium, Ammonium and Calcium concentrations are then provided as data.

Osmoticums analysis

Osmoticum measurement where performed using a freezing point osmometer (Osmomat 3000, Gonotec, *www.gonotec.com*). Data obtain by the machine is in $mOsm.kg^{-1}$.

Statistical analysis

R-software version 3.4.3 was used for statistical analysis (R Core Team, 2017). Packages used for analysis were "lsmeans" (Version 2.27-2), "multcompView" (Version 0.1-7), "ordinal" (Version 2015.6-28), "car" (Version 2.1-4), "RVAideMemoire" (Version 0.9-68), "ggplot2" (Version 3.0.0), "rootSlove" (Version 1.7).

To test G, E and GxE interaction effects, a two-way ordinal regression (using a symmetric threshold) was performed on data before a type II ANOVA analysis in order to normalise them. Then, when the effect was significant, associated post-hoc test (Compact letter display of pairwise comparisons, using a Tukey adjustement) was used to classify data by significance. Also, Pearson correlations, mean and standard deviation were used. A Wilcoxon Rank Sum and Signed Rank Tests were also performed for only one parameter analysis.

Graphical representations

Graphics were performed using R-software version 3.4.3 (R Core Team, 2017). Data represented are direct measurement or transformed data (mean value, standard deviation value, slope of a line, Pearson correlation) using a sliding box of defined size.

Results and discussion

1. Kinetics of berry growth and metabolites accumulation

Since the softening date of each approximatively 1000 individual berry couldn't be reasonably determined, sugar concentration was used as a proxy for the advancement of ripening, being aware that obviously, in a growing volume, there is virtually no chance that concentration simply linearly increases with time. The major underlying assumption is that each unitary berry volume would follow the same developmental rules, regarding firmness, growth and concentration of major

solutes, regardless of berry individual volume and exposition in the canopy. The purpose of this chapter is to compare the evolution of different analytical parameters with respect to sugar concentration in low content genotypes (G5, G7 and G14) and usual genotypes (Merlot, Morrastel and Grenache). Only 2017 genotypes were used in order to compare genotypes with same growing conditions.

Figure 8 represents changes in berry firmness with respect to sugar concentration. Softening is largely documented as the first sign of the onset of ripening (Coombe, 1984; Robin et al., 1997; Castellarin et al., 2015). Curves presented show the same pattern as described by Castellarin et al. (2015) and Robin et al. (1997), with a rapid decrease in firmness (factor 10) just before 135 mM sugars (except Morrastel who's going to 250 mM). Then after 250 mM sugars, no differences between genotypes were detectable.

Figure 8: Berry firmness ($g.mm^{-1}$) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

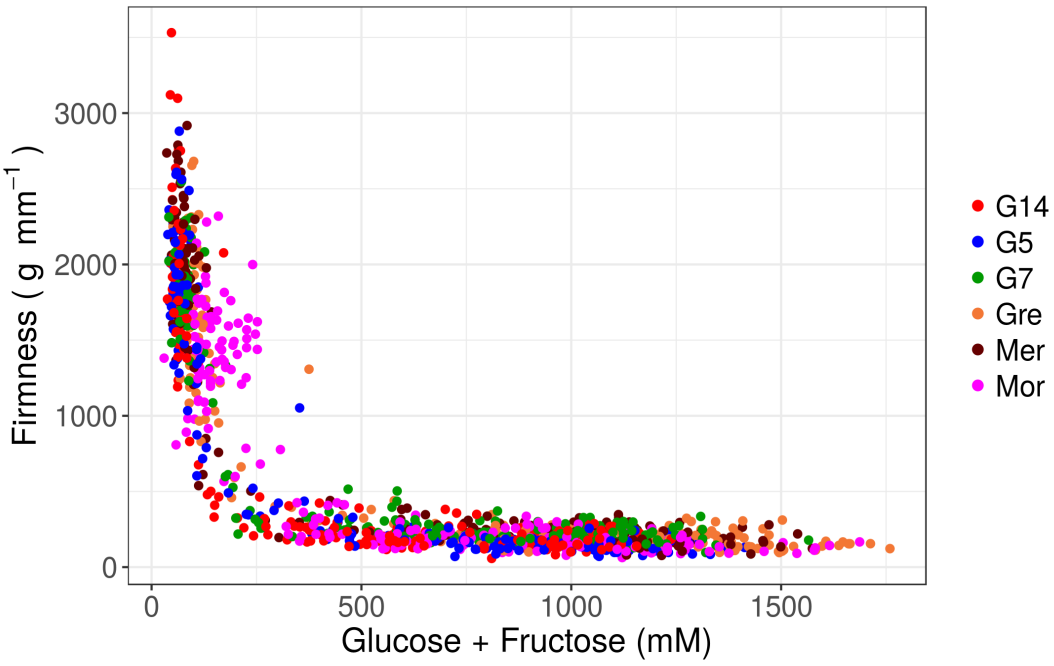


Figure 9 represents the berry weight with respect to sugar concentration. First, heterogeneity in berry weights for same sugar concentration appeared to be as important as described by Shahood (2017), making difficult to determine at which sugar concentration growth ceases. Also, average weights of genotypes are different, showing diversity for this trait as previously reported (Bigard et al., 2018). At the beginning of sugars accumulation, it appeared that berry weight remains constant up to at least 250 mM sugars. This confirms the delay between berry growth and softening already described by Coombe (1984) and Shahood (2017).

Figure 9: Evolution of berry weight (g) with respect to sugars concentration (Glucose + Fructose; mM) for 2017 experiment.

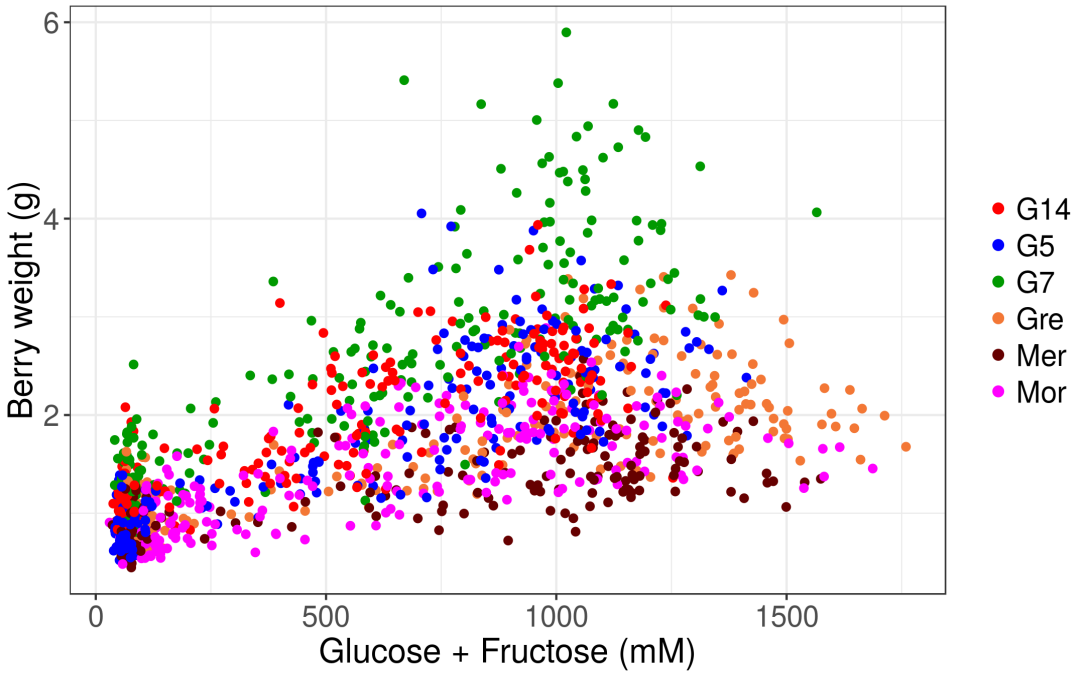


Figure 10 represents the changes in tartaric acid concentration with respect to sugar concentration. During green stages, the first hypothesis is that tartaric acid is accumulated in parallel with sugars, in this case maximum tartaric acid concentrations for genotypes are between 200 à 300 meq.L^{-1}. The second hypothesis is that berries located on the line passing through the origins should in fact just be more or less diluted. Above 135 mM glucose plus fructose, maxima lie between 150 and 240 meq.L^{-1}, due to tartaric acid dilution at the end of green growth phase (Champagnol, 1984; Terrier & Romieu, 2001). One should remark that during sugars accumulation, G5, G7 and G14 displayed lower tartaric acid concentrations than Merlot, Morrastel and Grenache, suggesting a greater dilution of this acid and possible difference in cell size at the end of green growth phase, in low sugar cultivars. Finally, at 1 molar sugars or before, tartaric acid concentrations reached a minimum

and were diluted by a factor of 2 (or more), compared to the concentrations observed during the softening period. Above this minimum, several cultivars showed an increase in tartaric acid concentration, probably related to final withering (considering the amount of this constant acid during maturation (Rösti et al., 2018)) as the corresponding points also lie in a line passing through the origin.

Figure 10: Berry tartaric acid concentration (meq.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

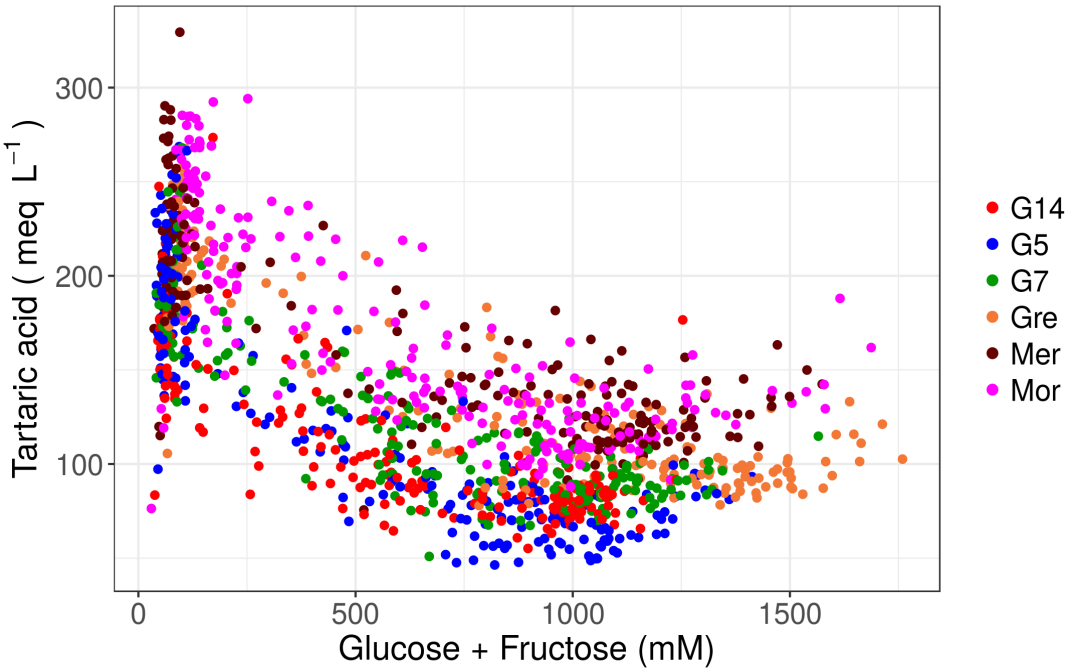


Figure 11 represents the changes in berry malic acid concentration with respect to sugar concentration. Maxima of malic acid concentration of the respective genotypes were between 300 and 600 meq.L^{-1}. Also, for this acid compared to tartaric one, berry heterogeneity seemed to be lower at maximum berry concentration and during sugar accumulation. Noticeably, the maxima of G7 and G5 were higher than Morrastel and G14, which were higher than Merlot and Grenache, malic acid globally showing the opposite tendency to tartaric acid when comparing low sugar cultivars to the usual ones. This may confirm that once tartaric acid synthesis decreases in the young berry, it is more diluted by the continuation of malic acid synthesis, what can be the result of earlier stop of tartrate synthesis, or greater cell growth in low sugar cultivars. Whatever, after this maximum, malic acid concentration decreased rapidly until 800 mM sugars was reached, as observed in Shahood (2017). This graphic showed that G5, G7 and G14 displayed lower malic acid concentrations than Merlot, Morrastel and Grenache after 600 mM sugar concentration but this

trend change after 1100 mM sugars. We can also quote that some berries were totally depleted in malic acid above 750 mM sugars (especially G5). It also seemed that G7 was consuming or diluting faster its malic acid compared to sugars accumulation. However, the very global trends of malic acid consumption compare to sugars accumulation were similar in all genotypes analysed, with roughly 1 M sugars importation for 500 meq.L^{-1} malic acid loss (except G7).

Figure 11: Berry malic acid concentration (meq.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

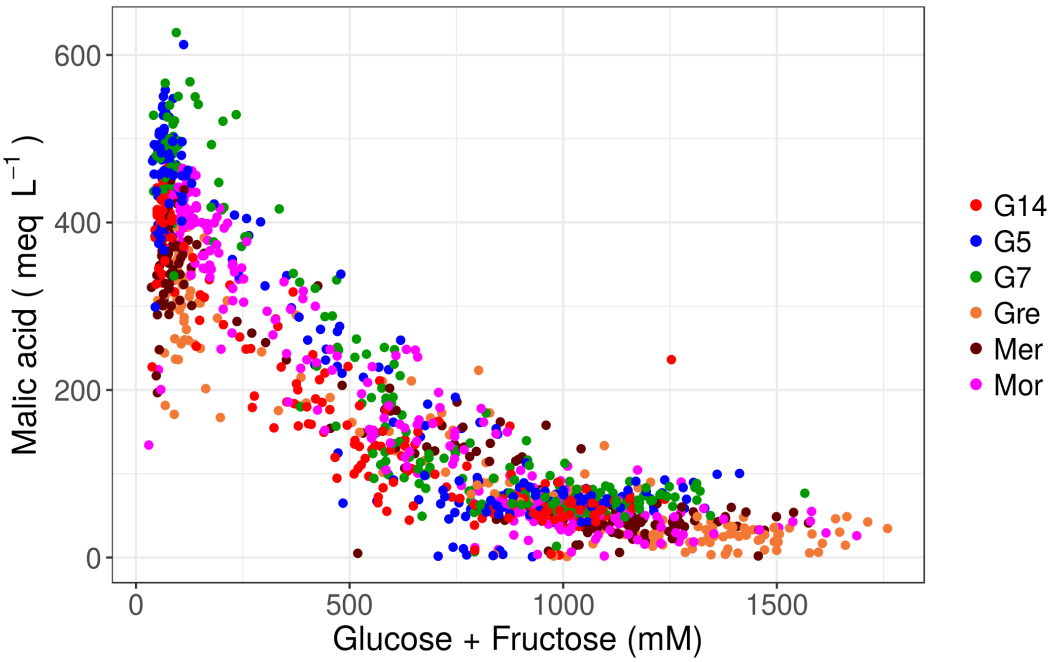


Figure 12 represents berry potassium concentration according to it sugar concentration. At the beginning of sugars accumulation, potassium concentrations in all genotypes were around 40 mmol.L^{-1}. Then, it concentration slightly increased up to 55 mmol.L^{-1} before 800 mM sugars. After 800 mM sugars, potassium concentrations increased faster in all genotypes, but Grenache. Finally, G5, G7 and G14 seemed to have an higher skin/volume potassium concentration ratio higher than Merlot, Morrastel and Grenache.

Figure 12: Berry potassium concentration (mmol.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

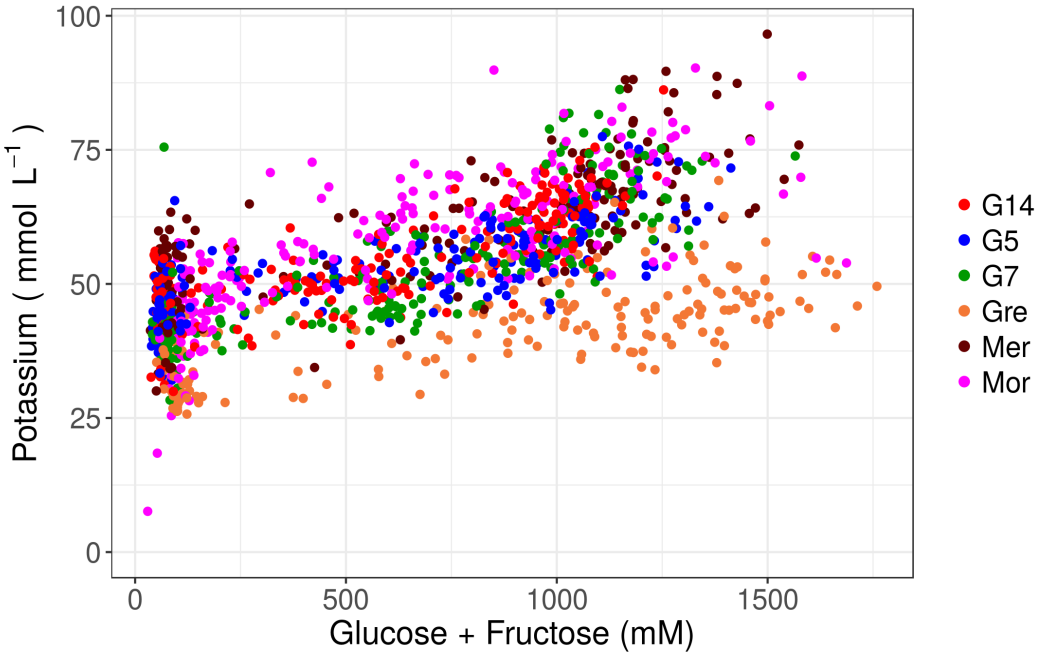


Figure 13 represents berry magnesium concentration according to it sugar concentration. No major evolution during ripening was observable, despite an upward trend.

Figure 13: Berry magnesium concentration (mmol.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

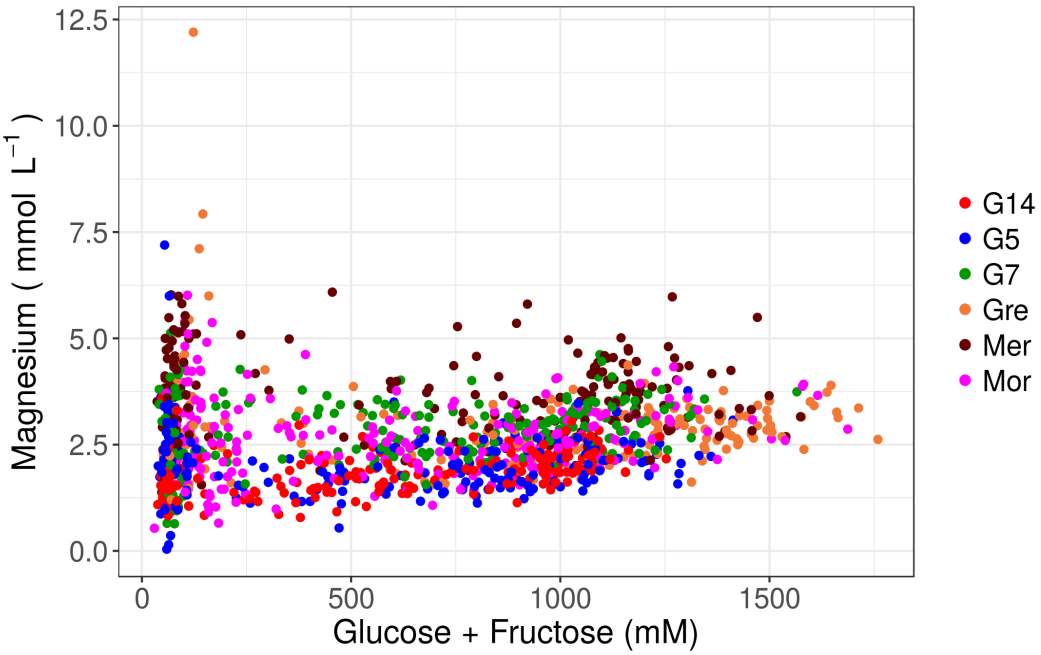


Figure 14 represents berry ammonium concentration according to it sugar concentration. Differences between genotypes at the onset of ripening were observable. Considering only genotypes on the same experimental plot (excluding Grenache), G5, G7 and G14 seemed to be higher in ammonium concentrations at the onset of ripening than Merlot and Morrastel (except some berries of Morrastel). Then, trends were to decrease up to 800 mM sugars, with 80% loss for some genotypes. After 800 mM sugars, no differences were presented.

Figure 14: Berry ammonium concentration (mmol.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.

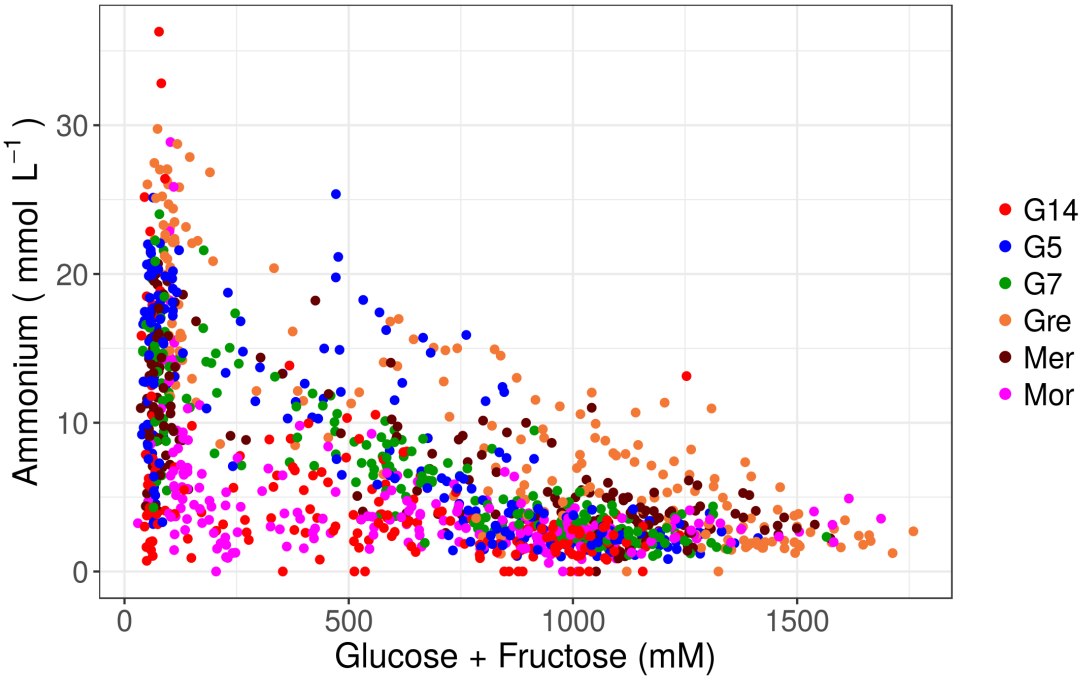
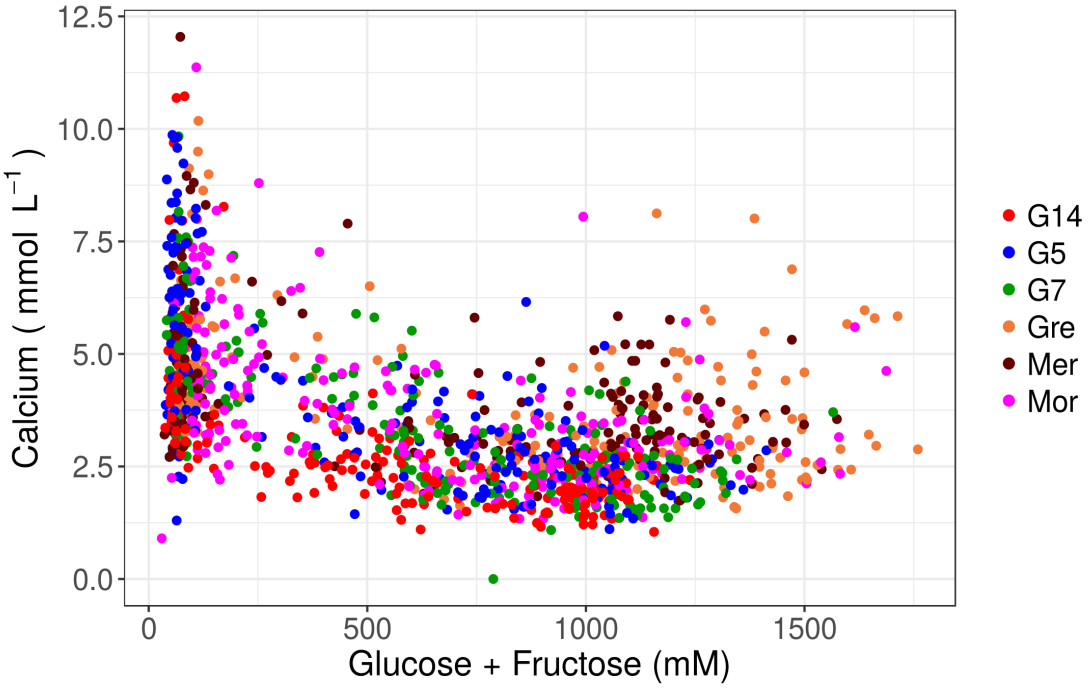


Figure 15 represents berry calcium concentration according to it sugar concentration. At the onset of ripening, maxima for genotypes were between 5 and 10 mmol.L^{-1}. Then, concentrations decreased to a minimum around 800 mM sugars. This loss in calcium could indicate a xylem back-flow after the onset of ripening

Figure 15: Berry calcium concentration (mmol.L^{-1}) compared to sugars (Glucose + Fructose; mM) for 2017 experiment.



First conclusion

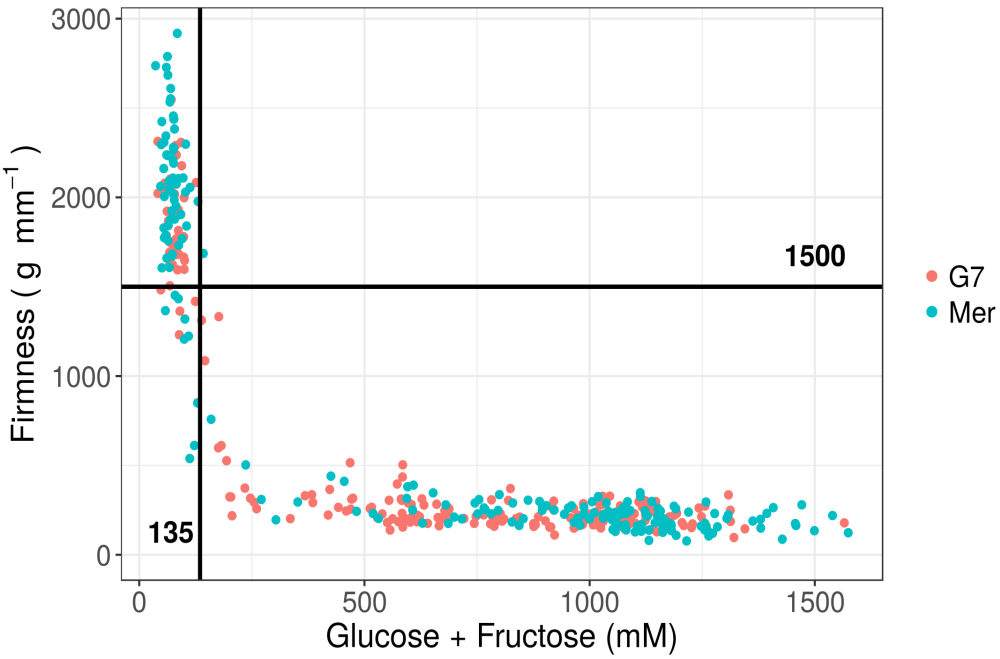
The observation of the raw data did not showed many obvious differences between low sugars genotypes (G5, G7 and G14) and controls (Merlot, Morrastel and Grenache), except malic and tartaric acids and ammonium concentration. Low sugars genotypes (G5, G7 and G14), at the beginning of their growth, would be more prone to accumulate malic acid and thus dilute tartaric acid during growth. The cells would therefore appear larger at the beginning of ripening, which can be tested by comparing DNA concentration in the pericarp (Ojeda et al., 1999). For the rest, grapes seemed to develop in a universal way (Shahood, 2017).

2. Berry composition at specific stages

2.1. The onset of ripening

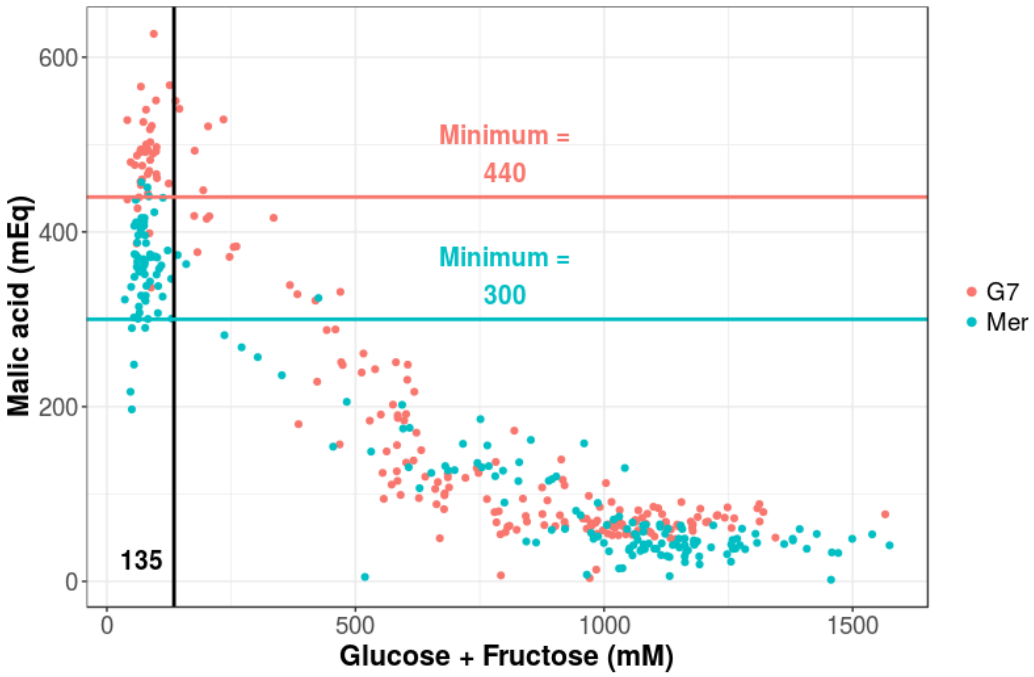
The determination of the first key stage of ripening (berry softening) is not so difficult (Abbal et al., 1992; Robin et al., 1997; Castellarin et al., 2015; Bigard et al., 2018). As described in previous works, first sign of ripening is fruit softening, which occur 1 to 4 days before colour change and berry growth (Coombe, 1984; Huang & Huang, 2001: Chapter 1). Figure 16 represents the changes in berry firmness ($g.mm^{-1}$) for each G7 berry when compared to the concentration in sugars (Glucose + Fructose; mM) in 2017. Green hard berries, just before softening are represented on the left side of this plot when sugar contents are low (Castellarin et al., 2015; Robin et al., 1997). In this plot, a horizontal line was first drawn at 1500 $g.mm^{-1}$, considering that lower firmness berries in 2017 for all genotypes were already soft (For 2016 samples 1000 $g.mm^{-1}$ was selected due to lower firmness results). Then, based on the first line drawn, maximum Glucose + Fructose concentration for berries can be selected for each genotype as represented in Figure 16 (in the case of G7 in 2017, it was at 135 mM sugars).

Figure 16: Berry firmness ($g.mm^{-1}$) compare to sugars (Glucose + Fructose; mM) in G7, Merlot genotypes for 2017 experiment.



Also, green berries, just before softening, berries are reaching a maximum in acid concentration and quantity (Terrier and Romieu, 2001; Conde et al., 2007; Keller et al., 2015). The sugar concentration at softening is reported on figure 17 showing the evolution of malic acid. It appears that malic acid breakdown starts as soon as berry have soften, contrary to observations made on climatic chambers preventing T° higher than 12/22 °C (Rienth et al., 2016), but in accordance with Coombe's and Shahood's results. Berries above a certain concentration of malic acid were empirically selected, in order to exclude too young green hard berries that did not already reached the peak in malic acid as indicated by a horizontal line (figure 16). For example, with G7 in 2017, only berries with a malic acid concentration higher than 440 mEq were selected. The same approach was performed on other genotypes giving minimum malic acid concentration of 420 mEq for G5, 320 mEq for G14, 300 mEq for Merlot, 260 mEq for Grenache, 330 mEq for Morrastel in 2017 and 230 mEq for G14, 180 mEq for Grenache in 2016 (not shown).

Figure 17: Berry malic acid (mEq) compare to sugars (Glucose + Fructose; mM) in G7, Merlot genotypes for 2017 experiment.



This two steps selection procedure allowed obtaining berries that were reasonably at the maximum of malic acid just before softening. We have selected only 31 up to 209 berries for G7 in 2017. For G5 44 up to 210 berries were selected, for G14 40 up to 210, for Merlot 53 up to 195, for Grenache 28 up to 195 and for Morrastel 13 up to 225 in 2017. For 2016 Grenache 12 up to 134 berries were

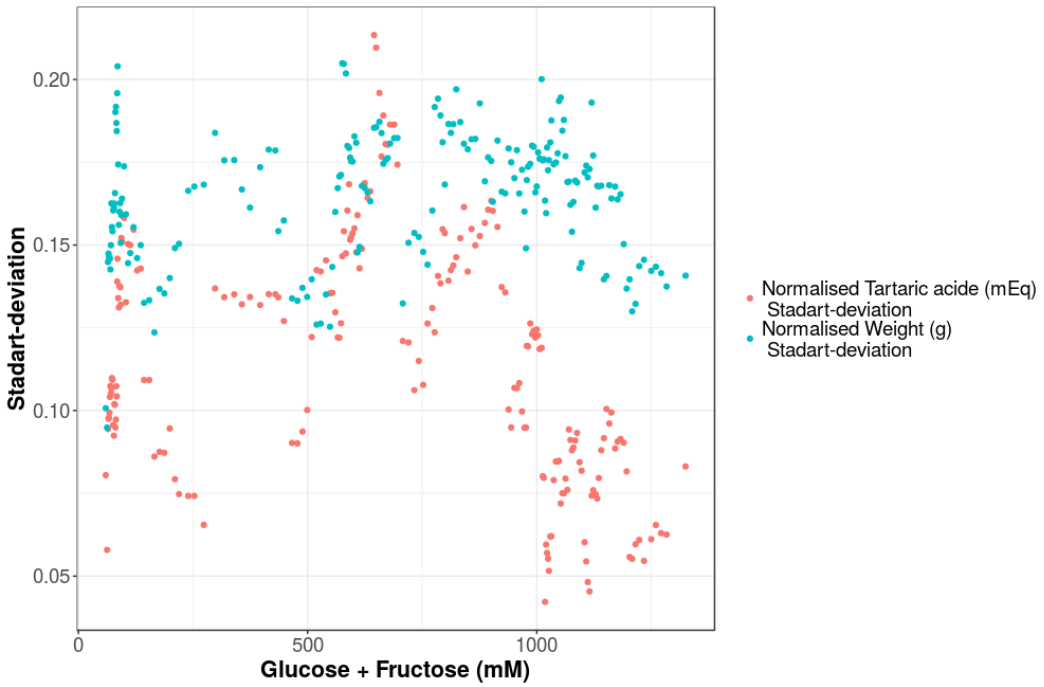
selected and 27 up to 165 for G14 (not shown). This selection will allow calculating average volume and composition of synchronised berries at the onset of ripening (Table 4).

2.2. Maximum berry volume as an indicator of physiological maturity

Maximum volume stage, that permit to give an objective definition of physiological maturity (Terrier et al., 2001), is excessively difficult to determine because there is no morphological indication excepted that berry volume starts to decrease (Coombe, 1984; Huang & Huang, 2001; Bigard et al., 2018). In this respect, the growth pattern of every single should be known, through daily diameter measurements or image analysis, which is impossible to check with pertinent periodicity on distant vines. In addition, due to heterogeneity and asynchrony, it is not relevant to compare two different berries (Pagay & Cheng, 2010; Böttcher et al., 2011; Dai et al., 2011; Kuhn et al., 2013; Doumouya, 2014; Reshef et al., 2017; Shahood, 2017). Several mathematical treatments were experimented to characterise this phenological stage on the previous set of crude data.

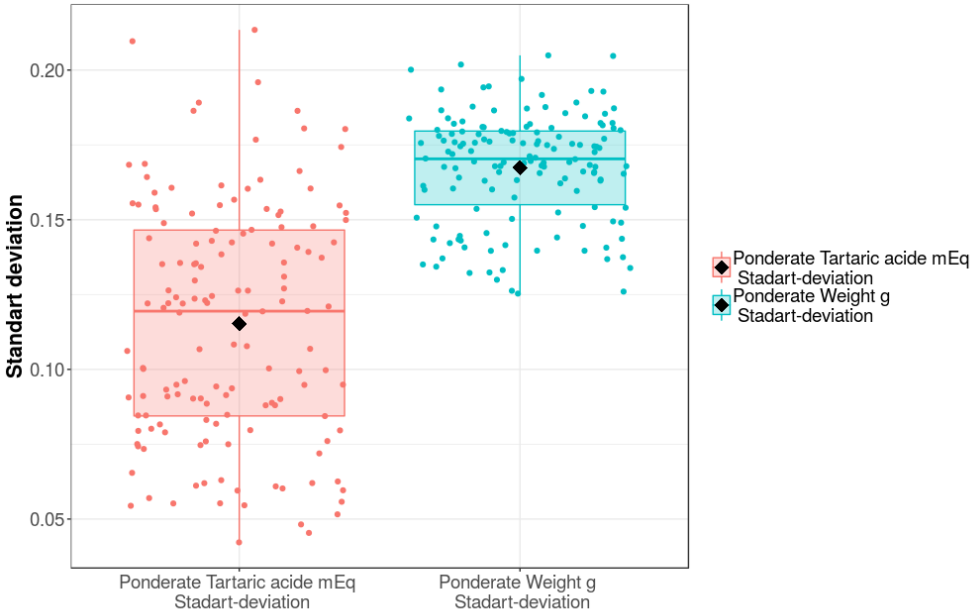
First step was to test heterogeneity between berry growth and the one estimated by tartaric acid dilution, considering that this acid is constant in quantity during fruit development (Rösti et al., 2018). To do so, berry features were smoothed using a sliding interval of 10 berries. The analysis begins with the 10 lowest concentrations of glucose and fructose and ends, step by step, with the 10 highest concentrations. Standard deviation divided by mean was calculated on each step. Barycentre of glucose plus fructose concentration was also given as output. This analysis was performed on each genotype and year. Values obtained for G5 genotype are represented as an example in the following graph (Figure 18).

Figure 18: Standard-deviation of normalised tartaric acid concentration dilution and berry weight compare to weighted mean sugars (Glucose + Fructose; mM) using a sliding box of 10 berries for the G5, Merlot genotypes during 2017 experiment.



In this graph (figure 18), standard-deviation of normalised tartaric acid dilution appears to be lower than that of normalised weight. Wilcoxon Rank Sum and Signed Rank Tests showed that for all genotypes in 2017, normalised tartaric acid displayed lower standard-deviations than normalised weight although; no differences appeared for G14 in 2016. Figure 19 is a boxplot representing the values represented in figure 18 (without data under 400 mM sugars) as an illustrative example.

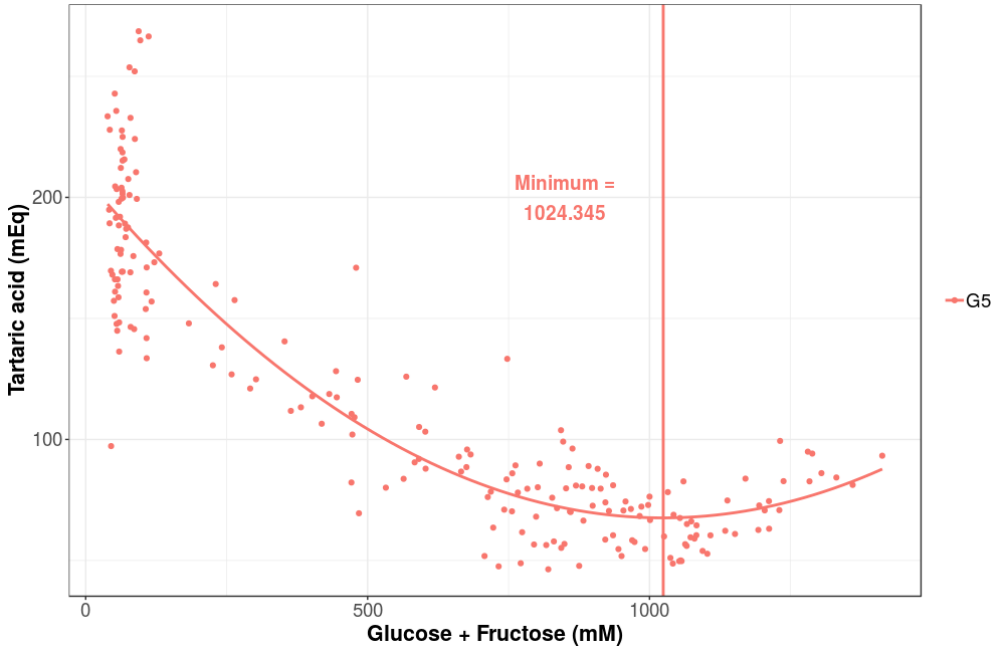
Figure 19: Boxplot of the standard-deviation of normalised tartaric acid concentration and berry weight compared to weighted mean sugars (Glucose + Fructose; mM) scope using a sliding box of 10 berries for the G5 genotype during 2017 experiment. Black trapezoid point represents the average.



We can conclude that tartaric acid dilution appeared as a more suitable indicator than berry weight for characterising berry growth kinetics.

To find the glucose plus fructose concentration when phloem unloading in berries definitively stops, tartaric concentration was first analysed using a quadratic regression (figure 20) and finding its minimum. Figure 20 presents this minimum for G5 in 2017, at 1024 mM glucose plus fructose.

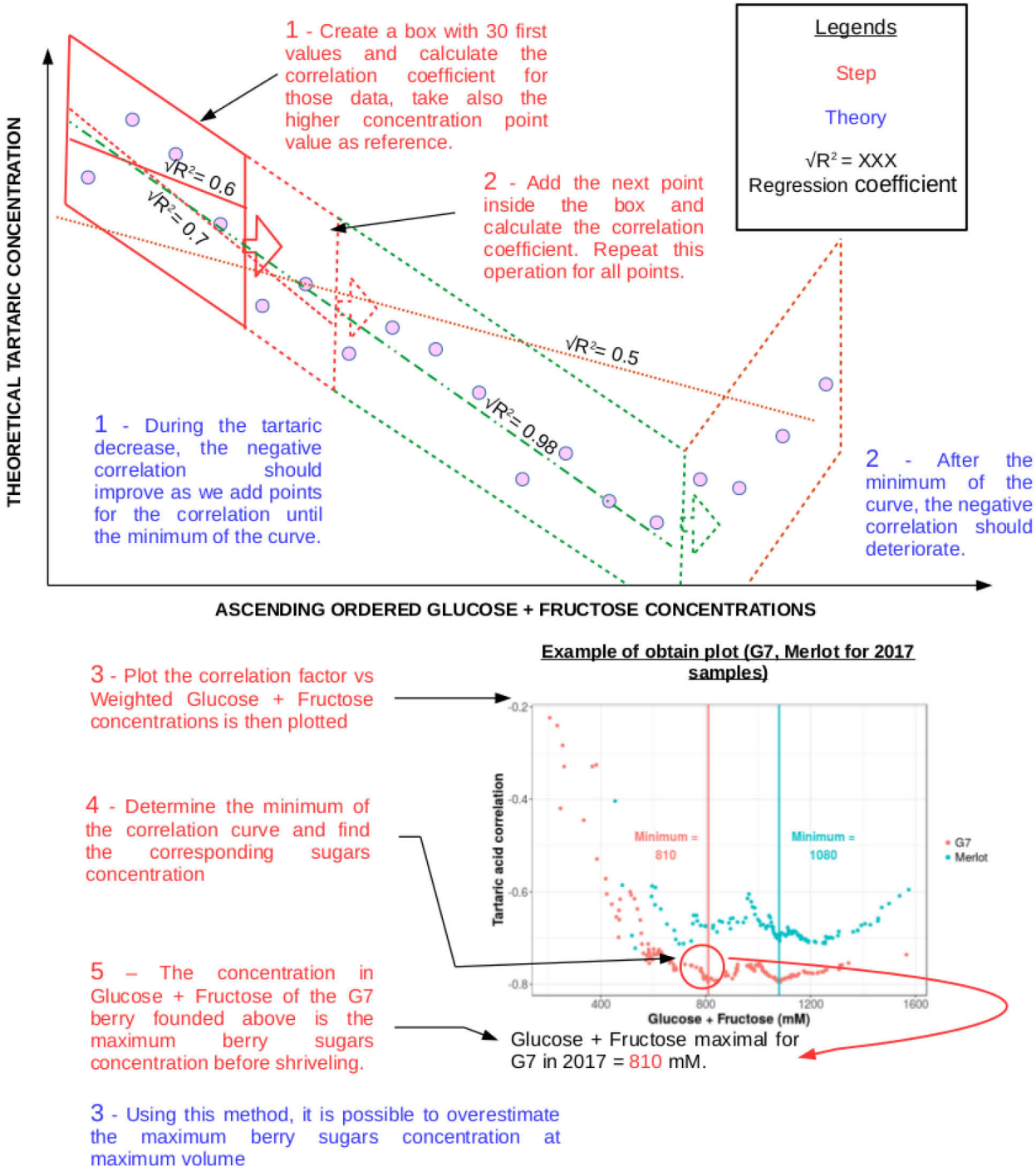
Figure 20: Berry tartaric acid (mEq) compared to sugars (Glucose + Fructose; mM) in G5 genotype for 2017 experiment. The curve is the associated quadratic regression reaching a minimum at the vertical line the corresponding glucose plus fructose value).



The curve fitting presented in figure 20 was the best regression possible to fit this data but this empirical quadratic function does not take into account present knowledge on berry development. First, at the very beginning of sugar accumulation, during a few days, berry size should not increase (Coombe, 1984; Huang & Huang, 2001; Shahood, 2017), but in the figure the fit is decreasing since the beginning. Also, once berry growth started, Shahood (2017) suggests that berry volume increases in proportion with sugar accumulation. Visual examination of figure 20 suggests that berry growth could arrest largely before 1000 mM glucose plus fructose. Moreover, there is a fundamental mathematical incongruity in trying to find a discontinuity (ie sudden arrest of phloem unloading, shift from growth to shrivelling) using a continuous function.

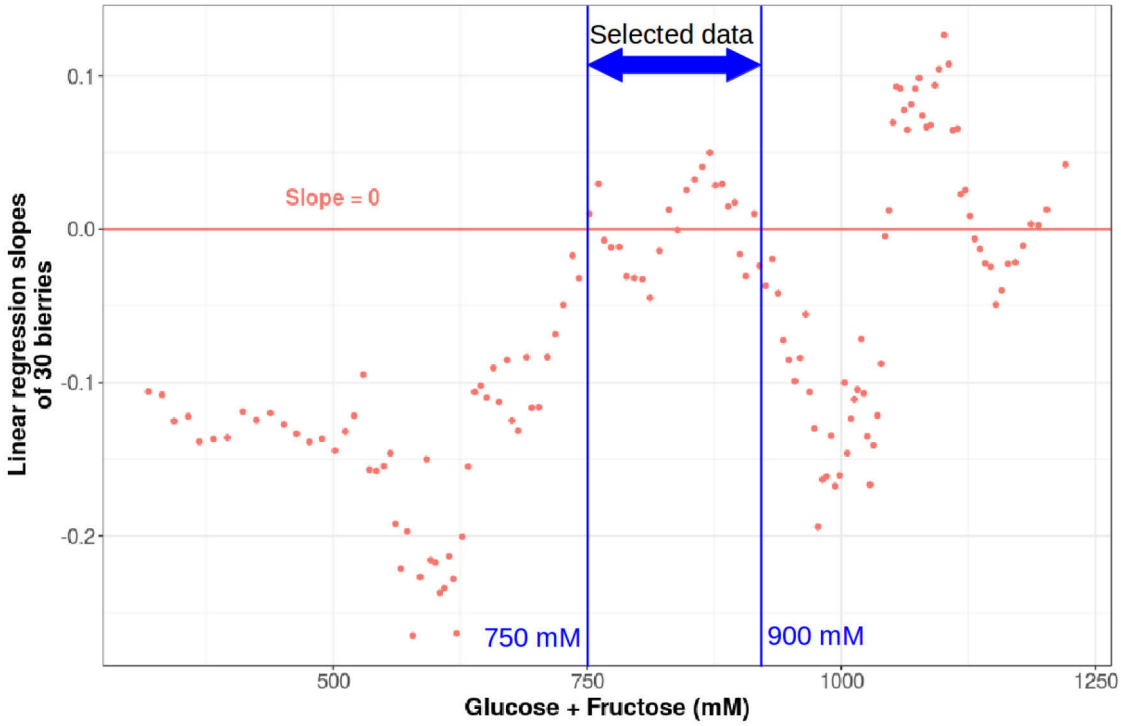
Two different approaches were thus tested to identify this maximum volume which should correspond to the minimum tartaric acid concentration (Rösti et al., 2018). The correlation between tartaric acid and sugars should be improved when adding points from the beginning of the growing period to maturity, and then deteriorate at the onset of shrivelling. Also, adjusting linear regressions on 30 samples following the onset of sugars accumulation, there should be a time when slopes of regressions are equal or near zero. To test the first hypothesis, berries under 125 mM glucose plus fructose were deleted from the analysis, then analysis were performed as explained for figure 21 using a box of 30 berries.

Figure 21: Schematic representation of the protocol used to test hypothesis one.



The second hypothesis was tested using a similar sliding interval approach than before but rejecting at each different step the lowest concentration berry, and calculating the slope instead than the regression coefficient, on each successive interval. Barycentre glucose plus fructose concentration using weight were also taken as output of the analysis. Figure 22 represents a run of this analysis on 30 berries representing coefficients obtained with associated sugar concentrations.

Figure 22: Graphical representation obtained after a run of the second analysis on G7 in 2017. Linear regression slopes are represented in function of corresponding weighted sugar concentration (Glucose + Fructose; mM).



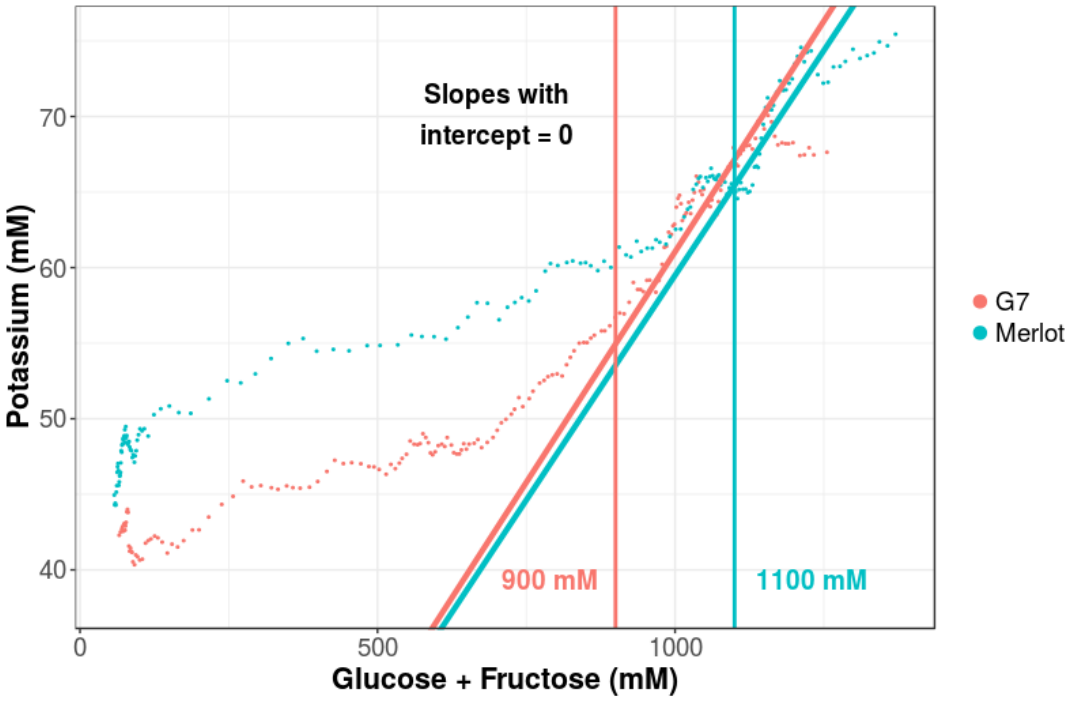
As represented in figures 21 and 22, a range of sugar concentration can be estimated at ripe stage. Similar analyses were performed on all genotypes. G7 sugar concentration at physiological maturity ranged between 750 and 900 mM, G5 one between 780 and 880 mM, G14 in 2017 between 720 and 800 mM and in 2016 between 640 and 660, Grenache in 2017 between 960 and 1070 mM, Merlot between 1060 and 1100 and finally Morrastel between 920 and 1020 mM. G14 which is the only genotype repeated during two different years showed differences between years.

Those differences are due to two factors: Firstly berry sizes were higher in 2017 than 2016 (p-Value = $9.73\ 10^{-9}$). Also, sugars accumulated in a 1 kg theoretical berry at ripe stage were different (p-Value = $5.97\ 10^{-9}$), with higher accumulation in 2017. This result suggests that sugar concentration at ripe stage is highly depending on water availability (berry size) and climate conditions (sugars loading (Etienne et al., 2013)). But in our case the G14 vine was irrigated during both years 2016 and 2017, in order to maintain leaf water potential (Ψ_{PD}) above 0.5 MPa. Water availability could thus be considered as not limiting during berry growth. In this case, only climate conditions couldn't be controlled and seems to have an impact on sugars accumulation and berry growth as

previously reported (Kriedmann, 1968; Lakso & Kliewer, 1975; Bindi et al., 2001b; Edwards et al., 2017; Arrizabalaga et al., 2018).

In these conditions, one may conclude that this approach to find berry maximum volume, relative to maturity, is relevant enough to yield pertinent results. Moreover, when plotting potassium in function of sugars and smoothing using a sliding box given barycentre values (Figure 23), we can observe that experimental points above 900, 1100 mM sugars reach an asymptote passing to the origin, as expected on shrivelling berries concentrating both sugar and potassium just upon loosing water. This plot gives independent indications that berry shrivelling starts after 900 mM for G7 in 2017.

Figure 23: Graphical representation of barycentre potassium (mM) concentration in function of average weighted sugars concentration (Glucose + Fructose; mM) using a box of 20 berries on G7, Merlot in 2017. Red, blue asymptotes are passing to the origin.



3. The low sugars content trait

The two key stages of development corresponding to the onset and the arrest of massive unloading of sugars in berries will be used to see differences between low sugars content genotypes and regular varieties.

3.1. The onset of ripening

First, all traits were compared at veraison stage (as define above) using statistical tests on selected berries only. Results showed that there is no interaction between year (climate) and genotype (genetically speaking) except for fructose concentration (mM), glucose/fructose ratio, berry weight and calcium concentration, limiting the interpretation of those factors. Genotypic effect was found significant for all parameters analysed. Year of sampling, corresponding to climate condition, was significant for most trait analysed at this stage (exceptions were berry weight and glucose/fructose ratio), showing the strong impact of climate on different berry attributes as previously mentioned by (Jones, 2004; Mira de Orduña, 2010; Sadras & Moran, 2012; Ollat et al., 2017; Arrizabalaga et al., 2018).

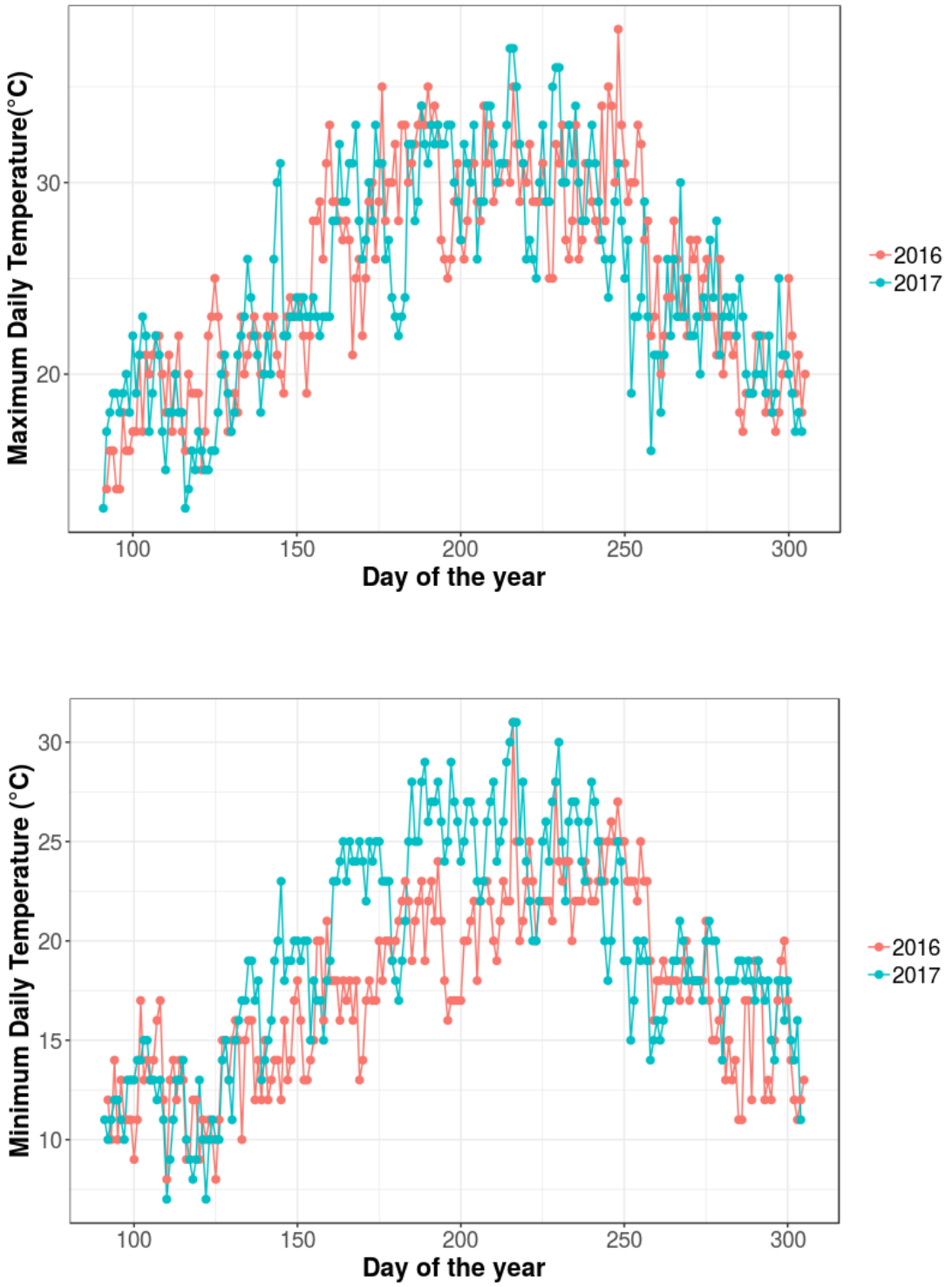
At this stage, berry weight was found significantly lower for Merlot, Morrastel and G5 than Grenache and G14 (both years) which were significantly lower than G7. The green berry weight actually seems determined at a genetic level. The osmotic potential estimated with sugars plus acids plus cation was lower in Grenache and G14 than in Merlot which was intermediary, and statistically higher in Morrastel, G7 and G5. 2016 values were also higher. Without cations, which may be more concentrated in the peel than in the flesh (Duchène & Chardonnay, 1992; Conde, 2007), osmotic pressure appeared significantly higher in G7, G5 and Morrastel than G14, Merlot and Grenache. Berry size at veraison did not correlate with osmotic pressure indicating that genotypes may differ in cell wall or peel extensibility or/and cells number (Ojeda et al., 1999). Berry deformability failed in detecting differences between genotypes, only 2016 was less firm than 2017.

All parameters were tested individually (for average and standard-deviation of each genotype, refer to table 4). For glucose concentration, Grenache and Morrastel were significantly higher than others. G7 and Merlot were significantly higher than G14 with G5 in intermediary position. Glucose concentrations were correlated to glucose plus fructose (0.98; significant). Also fructose concentrations correlated with sugar concentration (0.77; significant). Glucose plus fructose were

significantly highest in Morrastel and Grenache, G14 were the lowest one. For malic acid concentration, G7 and G5 were significantly higher than G14 and Morrastel which were significantly higher than Grenache and Merlot with significant lower values in 2016.

Malic acid is known to be correlated with T° (Mira de Orduña, 2010; Etienne et al., 2013; Rienth et al., 2016; Arrizabalaga et al., 2018). Figure 24 represents climatic data collected in the experimental centre of INRA Pech-Rouge in 2016 and 2017. With maximum daily T° (°C) and minimum one and 2017 were the year with the warmest spring. This observation is then going on an abnormal way, so it is maybe due to others factors than temperature (Rienth et al., 2016). Even so, Malic acid concentration at the onset of ripening trait was the first one that allowed distinguishing low sugar content genotypes from regular varieties eccepted Morrastel which appeared to be in the first group as well. Tartaric acid concentration showed that G14 was the lowest, then G5, G7 and Grenache were in the middle range and Morrastel, Merlot were significantly higher. 2016 was presenting lower concentrations at veraison stage. In total, acidity (malic acid plus tartaric acid) were significantly higher in G5, G7 and Morrastel than G14, Grenache and Merlot.

Figure 24: Maximum and minimum daily temperature (°C) in Gruissan (France) during 2016 and 2017.



Cations were also analysed separately. Results obtained with Potassium, the major cation (Storey, 1987; Mira de Orduña, 2010), were hard to interpret due to the interaction between year and genotype. Despite this, it seems that 2016 berries were lower in concentration and that Grenache

was the lowest in concentration. Calcium showed no differences in 2017 genotypes and higher values in 2016. Magnesium was significantly higher in Merlot than others except Morrastel, with G14 being the lowest. For ammonium, Grenache showed the highest concentration then G5, G7, Merlot were significantly higher than G14 except Morrastel.

Malic acid concentration was found highly correlated to the sum of malic and tartaric acid and the total acidity, with 0.93 and 0.90 correlations respectively (p-Value < 0.05). Malic acid being accumulated around 2 times more than tartaric acid at this stage (Champagnol, 1984), this result is not surprising. Glucose was highly correlated to the sum of glucose plus fructose (0.98; p-Value < 0.05), but with ratio between 2 and 10 (Varandas et al., 2004), this correlation sowed that glucose was in higher concentration than fructose in green berries. For cations, correlations between calcium and potassium concentrations (0.83; p-Value < 0.05), between magnesium and potassium concentrations (0.71; p-Value < 0.05), between calcium and magnesium concentrations (0.75; p-Value < 0.05), between calcium and ammonium concentrations (0.75; p-Value < 0.05) and between magnesium and ammonium concentrations (0.77; p-Value < 0.05) were found. Those correlations indicate than concentrations on each cation seem to be regulated by each other at green stage, which was interestingly similar at ripe stage for *Vitis vinifera* diversity (Chapter II.2).

In this experiment, no special precaution was taken in order to prevent sucrose hydrolysis by invertase following berry crushing (Davies & Robinson, 1996; Takayanagi & Yokotsuka, 1997). In this way, and by the possible fact that parents of G5, G7 and G14 are table grape cultivars, sucrose can be accumulated in their cells as osmoticum during enlargement (Shiraishi et al., 2012; Davies et al., 2012).

This analysis confirmed that genetic variation exerts critical control on green berry characteristics (Lakso & Kliewer, 1975; Houel et., 2013, 2015; Arrizabalaga et al., 2018; Bigard et al., 2018). Results showed that at this stage, the concentrations in major cations are correlated, showing maybe a genetic common control of the concentration of every cation in grape vine berry cells, which was suggested by the diversity analysis (Chapter II.2). The berry sampling protocol for this stage appeared to be relevant because not so much parameters presented a significant interaction during the analysis. Single berry analysis combined with a precise strategy of sampling provided a consistent tool for genetic and physiological studies dealing with berries at the onset of ripening (Coombe, 1984; Shahood, 2017).

3.2. Physiological ripe stage

As above, berries selected at physiological maturity were analysed for berry attributes. Analysis revealed a constant significant genotypic effect on all these parameters and also a year effect in some cases. GxE interactions were found for osmotic potential (sugars plus acids plus cations), ammonium, glucose plus fructose and glucose concentrations.

For berry weight, G7 and G14 had the biggest berries. G5 and Grenache were following and Merlot, Morrastel displayed the smallest fruits. On overall, 2016 berries were significantly smaller than in 2017. Merlot showed the higher osmotic potential, followed by Grenache and Morrastel and then G5, G7 and G14. This suggests that "Low sugar content" genotypes need less osmotic pressure to enlarge cells and upload water. Statistical analysis revealed that G5 ripe berry are softer than other studied genotypes, Merlot displaying the more firm fruits at ripe stage. G14 and Morrastel were belonging to both groups.

Merlot showed higher fructose concentration than others, Grenache and Morrastel belonging to the next group followed by G5, G7 and G14. 2016 presented lower values than 2017. G5, G7 and G14 also displayed lower glucose/fructose ratios than Merlot, Morrastel and Grenache. The sum of both sugars suggested the same as fructose concentration. Malic acid concentration was higher in G7 and G5 without statistically significant differences except between G7 and Merlot. Tartaric acid concentrations were significantly higher in Merlot, Morrastel and Grenache than G5, G7 and G14 varieties. This result is in agreement with the observations done at green stage, supposing that G5, G7 and G14 cells were maybe bigger at least at the onset of ripening.

Cations were also analysed as for previous developmental stage. Potassium was found significantly higher in Merlot and Morrastel than G5, G7 and Grenache. G14 was not statistically significantly different from Grenache. 2016 potassium concentrations were higher than in 2017. Calcium concentration was found significantly higher in Merlot than G7 and G14, the others were on the average. Magnesium concentration was found significantly the highest in Merlot. G7, Grenache and Morrastel were in the following group being significantly higher than G14 and G5. Ammonium concentration was low in G14 and Morrastel as compared to Grenache and G7 which were high. All data average and standard deviation are represented in the following table 4 for 2017. At ripening stage, values were in the same concentration range as reported in the literature (Storey, 1987; Mpelasoka et al., 2003; Rogiers et al., 2017).

Correlation calculations showed different results than during green stage. Interesting positive correlation (0.76; p-Value < 0.05) was found between sugars and tartaric acid concentration which means that higher sugars berries tend to have higher tartaric concentration. The sum of both acids concentration was correlated to total acidity (0.90; p-Value < 0.05) showing they critical in the acidity determination as previously reported (Lakso & Kliewer, 1975). For cations, calcium and magnesium as well as magnesium and potassium concentrations were correlated, with 0.77 and 0.71 respectively (p-Values < 0.05). This is in agreement with previous observations (Chapter II.2). Altogether, these data suggest a possible common control of those three cations.

Table 4: Berry average and standard-deviation for each genotype and each factor for 2017 data.

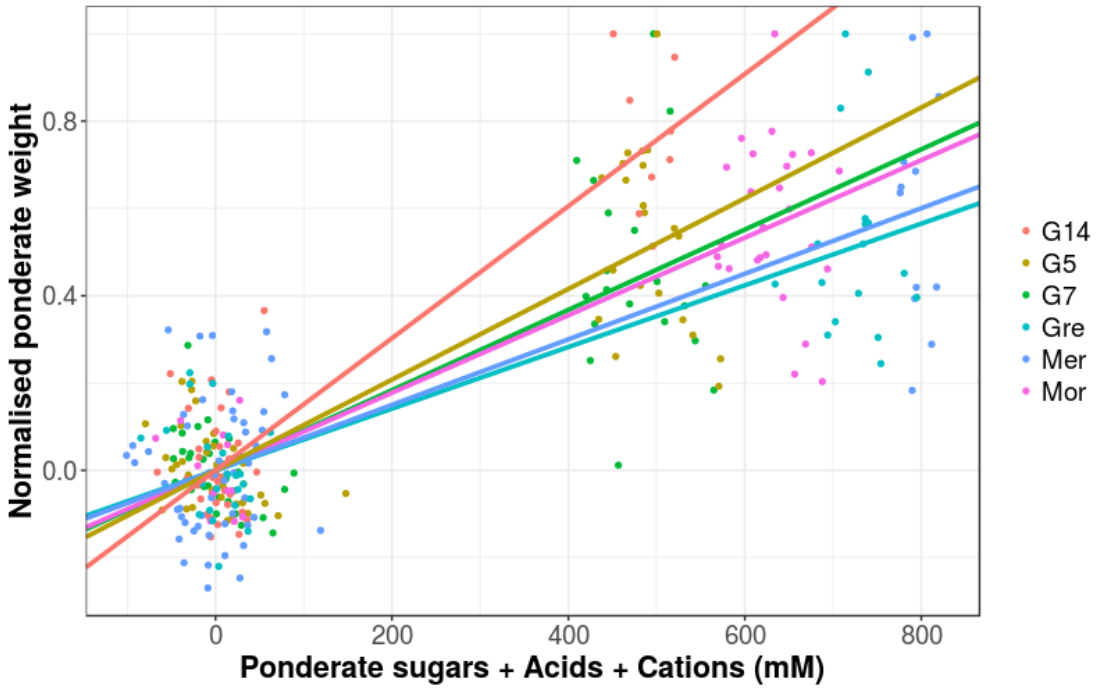
Genotype	Stage	Berry weight (g)	Standard-deviation	Texture (mm.g^{-1})	Standard-deviation	Tartaric acid (meq.l^{-1})	Standard-deviation	Malic acid (meq.l^{-1})	Standard-deviation	Glucose (mmol.l^{-1})	Standard-deviation	Fructose (mmol.l^{-1})	Standard-deviation	Ammonium (mmol.l^{-1})	Standard-deviation	Potassium (mmol.l^{-1})	Standard-deviation	Magnesium (mmol.l^{-1})	Standard-deviation	Calcium (mmol.l^{-1})	Standard-deviation
G14	Verasison	1.1	0.2	2055.5	481.4	174.2	22.8	404.5	37.1	44.7	7.9	16.7	3.6	9.4	7.8	46.6	5.9	1.7	0.6	4.6	1.6
G5	Verasison	0.9	0.2	2041.3	294.0	197.7	34.0	492.1	37.8	49.8	12.8	16.5	4.5	14.7	5.5	44.8	6.4	2.3	1.3	5.7	2.1
G7	Verasison	1.5	0.3	1893.1	267.1	198.4	29.8	498.8	39.3	61.8	12.5	18.9	4.4	13.8	4.6	41.4	7.9	2.7	1.3	5.1	1.7
Grenache	Verasison	1.1	0.2	1964.7	314.4	218.0	29.7	335.1	34.9	82.8	15.8	16.8	3.8	21.9	4.8	30.5	3.2	2.7	2.0	5.1	1.4
Merlot	Verasison	0.8	0.2	2130.5	323.3	227.4	34.3	369.0	41.5	56.7	12.9	18.8	7.2	13.2	4.1	47.3	7.9	3.8	1.1	5.3	1.8
Morrastel	Verasison	0.8	0.2	1768.7	160.4	242.9	26.4	409.9	33.1	85.8	10.7	24.4	4.0	9.9	5.6	40.7	9.0	3.0	1.2	5.0	1.5
G14	Maximum volume	2.6	0.3	206.5	111.8	81.8	11.8	61.6	30.5	391.8	11.8	381.5	20.8	2.5	1.5	56.5	7.6	2.0	0.4	2.1	0.9
G5	Maximum volume	2.3	0.5	149.3	27.9	72.9	16.8	69.7	42.9	416.5	14.1	418.4	14.8	4.6	3.6	55.7	4.8	2.0	0.5	2.8	1.1
G7	Maximum volume	3.1	0.9	216.0	55.9	92.9	16.3	79.6	34.4	418.5	20.3	401.8	19.8	4.0	1.4	53.7	4.9	2.7	0.5	2.4	0.9
Grenache	Maximum volume	2.3	0.5	212.2	32.5	107.7	19.6	51.3	29.0	541.0	23.5	482.7	12.5	6.5	3.1	45.3	5.0	2.8	0.6	3.2	0.9
Merlot	Maximum volume	1.6	0.3	223.2	45.5	125.5	15.3	47.6	10.8	561.6	6.9	514.8	6.5	3.3	1.6	65.3	5.9	3.8	0.7	3.8	1.0
Morrastel	Maximum volume	2.0	0.4	185.5	58.3	116.8	18.3	55.2	25.3	503.2	17.7	468.6	19.2	2.2	1.1	63.5	7.4	2.7	0.6	2.7	1.3

Except for final concentration in sugars at the end of physiological ripening, all genotypes displayed similar pattern of fruit development. This suggests that cells can growth at lower osmotic pressure in low sugar content genotypes. This decorrelation between primary metabolite accumulation and growth offer options for further studies to mitigate some effect of climate change (table 4).

3.3. Relations Pressure - Growth

As seen previously, relationships between berry growth and osmotic pressure can be a factor explaining low sugars content trait. With previously presented data, the relative increase in berry weigh since veraison was calculated for each genotype. Same was done with osmoticum (sugars plus acids plus cations). Finally, slopes of linear regressions were extracted in order to compare the amount of osmoticum (sugars plus acids plus cations) needed to have 1 percent of berry growth (figure 25) for each genotype. Results are shows in table 5.

Figure 25: Weighted normalised weights compared to weighted osmoticum (sugars plus acids plus cations; mM) for 2017 data. Corresponding slopes are represented. Mer for Merlot, Mor for Morrastel and Gre for Grenache.



Growth was also analysed using berry growth ratio between physiological ripe stage and green stage. Due to the fact that we didn't followed individual berry growth, each ratio between each berry at different stage were calculated using both weight and tartaric acid as growing indicator. Corresponding averages are also listed in table 5.

Table 5: Berry growth and it relation with osmoticum for 2017 data.

Genotype	Osmoticum (mmol) necessary for 1% growth	Weight increment	Standard-deviation	Tartaric increment	Standard-deviation
G5	9.6236	2.8	0.9	2.9	0.8
G7	10.8757	2.3	0.8	2.2	0.5
G14	6.6071	2.4	0.5	2.2	0.4
Grenache	14.1525	2.6	0.8	2.1	0.4
Merlot	13.3190	2.0	0.6	1.8	0.3
Morrastel	11.2552	2.1	0.6	2.1	0.5

Data shown that low sugar contents genotypes displayed a ripening growing rate higher than 2 and that a lower concentration of osmoticum was needed to reach the same size increment (column 1). These results are in agreement with previous observations about the specificities of these genotypes for relationships between osmotic potential and berry growth. As we did not observe any specific signature for turgor, this suggests that low sugar contents genotypes may display structural cell wall peculiarities impacting cell extensibility (Nunan et al., 2001). Enzymatic activities may also play a role in those differences, even with no differences in calcium concentrations at softening (Marín-Rodríguez et al., 2002; Marín-Rodríguez et al., 2003). Alternatively, cell may just be larger, but extensibility similar. Number of cells and cell growth analysis can also provide relevant information on this trait (Ojeda et al., 1999).

Conclusions and perspectives

Conclusions

This study allowed to objectivize the low sugars concentration trait at the individual berry scale and to propose possible mechanisms for its genetic origins. Moreover, beyond this specific trait, it strengthens the most recent concept in berry development and primary metabolisms. Of course, it raises new methodological and biophysical questions regarding the relation between berry growth, metabolites accumulation and fruit pericarp structure.

Today, most research programs are using samples composed of pooled berries to characterize berry growth and metabolism. However, there is a basic incongruity in characterizing precise stage of development in a non-synchronous population of fruits. The dynamic structure of this population must of course be taken into account but they are virtually no comprehensive data on this aspect. Thus, we have chosen the simplest model of berry development, assuming that berries just differ in cell number without any difference in the fate of these cells during ripening; which implicitly means that the water balance is not modified from one berry to another.

For detecting the onset of ripening, there is increasing evidences that berry softening is a much pertinent indicator than color change in the literature. During ripening, the asynchrony and heterogeneity of berry development was already illustrated by density sorting, hoverer, we are among the first who tried to extract the osmoticum-growth path from the berries synchronized by this type of sorting. It appeared that organic acid and anthocyanins concentrations displayed significant plasticity with respect to sugar concentration. Concerning anthocyanins, it is known since Robin et al. (1997). However, berry density sorting procedure led to an equivocal view on the relation between growth, or water, and solute accumulation, late berries being less acidic than early ones at same sugar concentration. Whatever, as showed by image analysis, berry population average volume can't charicterise the maximum volume stage, because of the considerable heterogeneity in berry. Finally, techniques used above permit to appreciate berry asynchrony and heterogeneity but data showed that average berry population analysis are not precise enough to characterize berry growth. They also showed that neither asynchrony nor heterogeneity could explain differences in berry solute and water accumulation between classical genotypes and low sugar content ones. To improve accuracy, analyses were then downscaled to single berry but a volume difficulty remains. Actually, the pertinent indicator is size increment of each berry compared with its volume at softening.

Low sugar concentration genotypes (G5, G7 and G14) were not different than *Vitis vinifera* varieties (Grenache, Merlot and Morrastel) for asynchrony and heterogeneity factors. Single berry analysis put forward that phloem unloading stops at lower osmotic pressure in the pericarp of low sugar concentration genotypes although similar or even greater growth. Do these cultivars differ in their parietal composition and turgor during ripening, which is known to vary from one grape variety to another in relation to firmness (Nunan et al., 2001)? Whatever, even at the onset of ripening, they were more malic and less tartaric acid in low sugar concentration genotypes. This unexpected observation indicates that the trait could be controlled by much earlier events than the sudden acceleration of cell wall turnover during ripening. If not circumstantial, the simplest hypothesis is that differences in cell size programmed since the first growth phase would lead to the low sugar concentration phenotype. This opens up new perspectives for research, but our attempts to correlate low sugar concentration character with the firmness or macroscopic turgidity of the berries were hampered by the lack of a biomechanical berry model in the literature.

An originality of our work has been to take into account cations together with anions and sugars. Cations showed similar trends in all genotypes and a correlation was found between calcium, potassium and magnesium concentration, excluding they act in the low sugar concentration trait.

Determining whether this characteristic has been inherited from *M. rotundifolia*, genetically distant from *Vitis vinifera*, would provide new contextual elements to determine possible responsible mechanisms. Phenotypically distant *Vitis vinifera* varieties including a microvine segregating population displayed a range of climate adaptation attributes (Chen et al., 2015) and a huge diversity of fruit growth during ripening (Chapter II; Bigard et al., 2018). Sugar concentration at physiological ripe stage also presented a high diversity. It was negatively correlated to malic acid content, but this was not observed in low sugar concentration genotypes and may have resulted from sampling artifacts since it was not validated at the single berry scale. Regarding cations, the impact of the environment was decisive and more stringent than for sugars. Calcium, potassium and magnesium concentrations were correlated to each other, suggesting a common control for accumulation. It is thus difficult to conclude that common mechanisms would explain the low sugar concentration character in pure *V. vinifera* accession and those introgressed for *M. rotundifolia* resistance genes.

Perspectives

Berry heterogeneity and asynchrony were found not different within the five genotypes studied in a similar environment, but it seems pertinent to generalize this result. Informations on such parameters would be relevant to decipher the precise growth pattern of emblematic varieties.

Establishing berry growth in relation with osmoticum accumulation is a particularly tedious task. It would now be appropriate to follow continuously berry growth at single berry level. Sampling at known growth increments is needed to validate the major assumption that each unitary berry volume would follow the same developmental rules, regardless of berry individual volume and exposition in the canopy. On large populations, automated image analysis would allow to phenotype a statistically relevant number of berries at precise growth stage. A non-destructive analysis of sugar concentration would be useful using NIR or better MIR remote sensing. Chlorophyll fluorescence was also related to the sugar content but it is questionable whether in fact, berry growth was measured.

Regarding cell size and expansion in the pericarp, first idea would be to compare berry growth with its DNA content, as it seems difficult to get a statistically relevant number of microscopic observations (Ojeda et al., 1999). However, confocal microscope observations showed that the pericarp cells have extremely variable volumes, with a very small number of them occupying most of the volume.

Detailed analysis of celluloses, pectins and parietal proteins is nowadays necessary using up to date analytical tools to study putative differences in cell wall structure and proteins (Nunan et al., 2001; Zepeda et al., 2018). Enzyme activity can also be controlled as well as berry skin extensibility (Coombe, 1984; Nunan et al., 2001; Marín-Rodríguez et al., 2003; Terrier et al., 2005). Models for the reological behaviour of berries according to their size, cell count and parietal firmness should also be implemented. As such, two naive models are opposed: that of the elastic sphere of homogeneous composition (Castellarin et al., 2016), and that of the elastic sphere filled with incompressible liquid (Bernstein & Lustig, 1981). It should be also determined whether blocking growth following the induction of sugar loading still prevent phloem unloading as it did in jacketed berries before softening.

Another strategy would consist on studying the segregation of the low sugar concentration trait in the aim of finding QTLs (Quantitative Trait Loci). Genetics can help to find genes possibly involved on the different process cited above. Analysis on the diversity generated on previous crossing showed that all parameter of berry development can segregate (Bigard et al., 2018). However the analysis performed was not detailed on individual berries and present results show that this variability is overestimated. Present work on 6 genotypes confirms Shahood (2017) results on Syrah, Pinot Noir and Zinfandel. Two populations have been collaboratively created crossing G5 and G14 (Escudier et al., 2017; Ojeda et al., 2017) with the V3 microvine (Chaib et al., 2010). Female (selected by micro-satelites) and anomalies plants (albinos) were discarded to obtain two population of 76 plants (V3 x G14) and 78 plants (V3 x G5) both in two repetitions. Tools developped during this thesis and Shahood's one (2017), allowed to phenotype berries just before softening and at theoretical maximum volume stage. Genetic maps were obtained using GBS (Genotyping By Sequencing), with *ApeK1* enzyme fragmentation and sequencing with Illumina Hiseq. Two parental maps were created and QTLs analysis just began under the direction of Dr. Agnès Doligez. At the time of present report, only one population has been analysed for a single year. Data already showed interesting results, as the significant QTL of maximum green stage tartaric acid concentration (36% character explanation) on V3 x G14 population in 2017.

Targeted or un-targeted transcriptomic studies could provide relevant informations on the origin of the low sugar content trait. Present results make obvious which sampling protocol and berry developmental stage must be addressed first (Rienth et al., 2016). Actually RNA seq experiments coupled with QTLs analysis was successful in previous studies on grapevine mutants (Fernandez et al., 2010, 2013, 2014).

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