

Carbon isotope natural abundance ($\delta^{13}\text{C}$) in grapevine organs is modulated by both water and nitrogen supply

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ABSTRACT

Our objective was to investigate the effect of water and nitrogen (N) availability on the carbon isotope composition of leaf blades, canes and ripe berry must (juice) in field grown grapevines.

In two consecutive years, the combination of two irrigation treatments [Irrigation (I): 70 % of crop evapotranspiration (ET_c) and no irrigation (NI)] and three rates of ammonium nitrate [0 (N_0), 60 (N_{60}) and 120 (N_{120}) kg/ha N] were applied to two separate vineyards planted with cv. Xinomavro and cv. Cabernet Sauvignon respectively in a randomised complete block design. Carbon isotope composition ($\delta^{13}\text{C}$) of leaf blades, stem water potential (Ψ_s), and leaf gas exchange were measured at berry set, bunch closure, veraison and maturity during each growing season. $\delta^{13}\text{C}$ of bulk berry must at maturity and of dormant canes was also measured. Leaf $\delta^{13}\text{C}$ and cane $\delta^{13}\text{C}$ decreased with water supply, but increased with N fertilisation. Must $\delta^{13}\text{C}$ was lower in the irrigated N_0 and N_{60} vines, whereas irrigation treatments did not differ under the N_{120} rate. The relative weight of irrigation and fertilisation effect on leaf $\delta^{13}\text{C}$ varied across samplings: N application accounted for a higher proportion of leaf $\delta^{13}\text{C}$ variance during the early stages of berry growth, whereas irrigation induced higher variance of leaf $\delta^{13}\text{C}$ after veraison. The weight of the irrigation effect on cane $\delta^{13}\text{C}$ was higher from that of fertilisation.

Discrimination mechanisms against ^{13}C in grapevine could be mediated by both N fertilisation and irrigation. The well-documented explanation of $\delta^{13}\text{C}$ variation based on isotopic effects during CO_2 diffusion through leaf stomata and carboxylation driven by water conditions may not apply to all cases, since additional discriminating processes against ^{13}C associated with CO_2 transfer from the intercellular spaces to the carboxylation sites may be affected by nitrogen supply.

The study provides evidence for the first time that carbon isotope composition of leaf blades, canes and berry must is modulated by nitrogen supply. Interpretations of carbon isotope natural abundance in field grown grapevine tissues should therefore consider both water and nitrogen availability.

KEYWORDS

Carbon isotope composition, ^{13}C discrimination, nitrogen fertilisation, irrigation, leaf, cane, must

INTRODUCTION

Chemical elements like carbon, nitrogen, oxygen, sulfur, and hydrogen, which form part of most of the structural elements of living organisms, have more than one stable - non-radioactive - isotopes. The isotopic composition ($\delta^{xx}E$) of a material regarding a heavier isotope (^{xx}E) of a chemical element (E) is defined in relation to an internationally accepted standard. Because the absolute ratios (R) of heavier to lighter isotopes that occur in nature are usually very small, ($\delta^{xx}E$) is expressed as per mill as follows (Dawson *et al.*, 2002):

$$\delta^{xx}E(\text{‰}) = \left[\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right] \text{ equation 1}$$

where R_{sample} and R_{standard} are the isotope ratios R of the material and standard respectively.

In many physical, chemical and biological processes, isotopic effects take place and discrimination or fractionation (usually against the heavier isotope) can be observed, where the lighter isotopes are used more than the heavier ones. This leads to significant variations in isotope composition from the source to the products of a chemical process, or along a biochemical path, in living plant cells. Therefore, the variation in isotope composition can provide information on the process or processes that cause this variation. The study of stable isotopes at their naturally occurring levels (natural abundance) has emerged as a powerful tool for understanding the relationships between plants and their environment (Dawson *et al.*, 2002).

Plants generally contain less ^{13}C than ^{12}C , the two stable carbon isotopes occurring in nature, compared to atmospheric carbon, as a consequence of the discriminations and fractionations which occur during both CO_2 intake and assimilation in leaf photosynthesis (Michener and Lajtha, 2008). In C3 plants specifically, carbon isotope discrimination is determined by several fractionation processes which occur during the diffusion of gaseous CO_2 from the free atmosphere, through boundary layers (canopy and leaf boundary layers) and stomata, to the intercellular air spaces. Subsequently, fractionation occurs during dissolution and liquid phase diffusion to the sites of carboxylation, and during carboxylation itself (Brugnoli and Farquhar, 2000). Among several mathematical models that describe all these fractionations, that of Farquhar *et al.* (1982) has been widely tested and its simplified version

(Equation 2) is often used. This simplified version assumes minimal effects of discrimination associated with the CO_2 transfer from the intercellular spaces to the carboxylation sites and defines a linear relationship between $\delta^{13}\text{C}$ with the C_i/C_a ratio as follows:

$$\delta^{13}\text{C}_p = \delta^{13}\text{C}_a - \alpha - (b - \alpha) \frac{C_i}{C_a} \text{ (Equation 2)}$$

Equation 2 explains the higher leaf $\delta^{13}\text{C}$ commonly found in C3 plants under water deficit conditions, which impose diffusional limitations to carbon intake and assimilation, and the subsequent decrease of the C_i/C_a ratio causes lower discrimination against the ^{13}C isotope (Farquhar *et al.*, 1989). In field grown grapevines with restricted water availability, increased $\delta^{13}\text{C}$ values have been reported for bulk dry matter (BDM) of main leaves (Souza *et al.*, 2005; Koundouras *et al.*, 2008; Pou *et al.*, 2008; Bchir *et al.*, 2016), canes (Glenn *et al.*, 2010), purified sugars of mature berries (van Leeuwen *et al.*, 2001; Gaudillère *et al.*, 2002; Des Gachons *et al.*, 2005; Zufferey *et al.*, 2018), and bulk berry must at maturity (Gómez-Alonso and García-Romero, 2010; Herrero-Langreo *et al.*, 2013). In addition, many studies on grapevines have reported positive relationships between photosynthetic water-use efficiency and leaf carbon isotope composition (Souza *et al.*, 2005; Tomás *et al.*, 2012; Bchir *et al.*, 2016), but these relationships were not always consistent.

Apart from water availability, plant $\delta^{13}\text{C}$ may change due to variations of N availability and many studies on plant species other than grapevines, have shown that $\delta^{13}\text{C}$ values of plant bulk dry matter can increase when nitrogen availability increases (Bender and Berge, 1979; Hogberg *et al.*, 1995; Livingston *et al.*, 1999; Ripullone *et al.*, 2004; Choi *et al.*, 2005). Explanations for these responses are mostly based on the stimulating effects of increasing N availability on carboxylation efficiency or CO_2 net assimilation rate (Hogberg *et al.*, 1995; Ripullone *et al.*, 2004). However, some of these studies point to the possible effects of N availability on mesophyll conductance to explain the variation $\delta^{13}\text{C}$ values of plant bulk dry matter (Livingston *et al.*, 1999; Choi *et al.*, 2005). Despite this extensive research, relatively few data exist for $\delta^{13}\text{C}$ and N availability in grapevines. Gaudillère *et al.* (2002) did not find any effects of nitrogen availability on berry purified sugars at maturity, whereas recently

published work (Brillante *et al.*, 2020) reported a strong positive relationship between leaf blade N concentration at anthesis and berry must $\delta^{13}\text{C}$ at maturity. However, this relationship was evident only on data pooled from two vineyards that were planted with a winegrape (Cabernet-Sauvignon) and a table grape variety (Crimson Seedless), whereas there was no significant relationship between leaf N and berry must $\delta^{13}\text{C}$ within each of the vineyards.

Considering that both nitrogen and water availability may influence the carbon isotope composition of plants and the knowledge gap that exists on any possible effects of N availability in grapevines under varying water supply, our study aimed to investigate the responses of $\delta^{13}\text{C}$ measured in the bulk dry matter of main leaf blades and canes, and in the bulk berry must, to N fertilisation and water application. To fulfill this objective, a classical field response trial was conducted in two commercial winegrape vineyards for two consecutive seasons.

MATERIALS AND METHODS

1. Vineyard site and experimental design

This study was conducted during the growing seasons of 2009 and 2010 in two 18-year-old vineyards located in the Goumenissa region of north Greece (40°52' N, 22°29' S). The vineyards were planted with the red winegrape varieties Cabernet-Sauvignon (CS) and Xinomavro (XM), both *Vitis vinifera* L. cultivars, and grafted onto the 1103 Paulsen rootstock. Both vineyards had the same vine spacing (2.2 m and 1.3 m between and within row respectively) and a north to south row orientation. The vines were spur-pruned to 12 buds per vine and trained to a bilateral Royat system with three fixed trellising wires. Soil had a clay loam and a sandy clay loam texture in the CS and XM vineyard respectively, with an average effective rooting depth of between 60 to 90 cm for both vineyards. Climatic conditions were generally similar for both growth seasons in terms of average temperature and total precipitation for the April to September period; the average temperature was 21.2 and 21.8 °C for 2009 and 2010 respectively, whereas precipitation for the same period was 226.2 mm for 2009 and 227.0 mm for 2010.

In each vineyard, three blocks were delineated, each containing a 2×3 grid of 6 plots with

6 vines per plot. The plots were separated with four guard vines along the row and with a guard row from each side. The combination of two irrigation levels [Irrigation (I): 70 % of crop evapotranspiration (ET_c) and no irrigation (NI)] and three rates of ammonium nitrate [0 (N_0), 60 (N_{60}) and 120 (N_{120}) kg/ha N] was randomly assigned to each block. A randomised complete block design was applied with six treatments (2 irrigation levels × 3 N fertiliser rates × 3 blocks) resulting in 18 plots per vineyard. Only the mean of each plot was used in the statistical analysis of the results.

Drip irrigation started at berry set (growth stage E-L 27, according to the modified Eichhorn-Lorenz system) in both seasons and was continued at weekly intervals, according to the estimates obtained from the potential evapotranspiration measured by an automated weather station located in the XM vineyard. Ammonium nitrate (34-0-0) was applied to the soil surface at budburst in both years of the study.

2. Carbon isotope composition and N content of leaf blades, canes and berry must

Carbon isotope composition ($\delta^{13}\text{C}$) and total nitrogen content were measured in the bulk dry matter (BDM) of leaf blades, sampled at four time points (BS, BC, VE and MT, corresponding to the growth stages of berry set, bunch closure, veraison and maturity respectively) during each growing season. The leaf tissues were dried at 65 °C and ground to fine powder with a rotary mill and a 0.20 mm mesh. From the final sample preparation, 2.8±0.1 mg, enclosed in SC0009 8×3 mm (SerCon Ltd, Gateway, Crewe, UK) capsules, was passed to an automated combustion elemental analyzer interfaced with a continuous-flow isotope ratio mass spectrometer (IRMS; PDZ Europa, Cheshire, UK).

For $\delta^{13}\text{C}$ in cane BDM, twelve canes from each plot were randomly selected at dormancy. From the middle of each cane, a section containing a latent bud and the internode immediately below the bud was used for $\delta^{13}\text{C}$ determination following the same procedure as for leaf blades.

At technological maturity (late September of each year for both varieties), all clusters from each plot were collected, then transferred to the laboratory and a sample of 200 berries from all parts of each cluster was randomly selected and hand-pressed for must extraction. After

immediate must clarification (0.2 NTU), 5 μL of bulk must (BJ) was directly injected to the IRMS for must $\delta^{13}\text{C}$ determination.

In all cases, the PDB standard was used and $\delta^{13}\text{C}$ was calculated according to Equation 1.

3. Stem water potential

Stem water potential (Ψ_s) was measured at the BS, BC, VE, and MT stages, using a pressure chamber, according to Choné *et al.* (2001). In each measurement set, four leaves per plot from the inner part of the canopy were enclosed in plastic bags and covered with aluminum foil for at least 90 min before measurement to allow equilibration of Ψ_s . The measurements were taken at solar noon (between 12:30 and 13:00) under clear sky conditions.

4. Gas exchange

The same days when sampling for $\delta^{13}\text{C}$ and for Ψ_s measurements in each growth stage, net assimilation rate (A_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) were determined in four mature sunlit leaves, adjacent to Ψ_s leaves, in each plot with a portable gas exchange system LCi (ADC BioScientific Ltd, Hoddesdon, UK). The measurements were performed within the same time window of Ψ_s measurement. Intrinsic water-use efficiency (WUE_i) was estimated as the A_n/g_s ratio. The mean of the four measurements of each plot was used in the statistical analysis.

5. Statistical analysis

In each of the two experimental vineyards, a randomised complete block design, replicated in three blocks and repeatedly measured over the two years, was used to evaluate the effects of two irrigation levels and three fertilisation rates on vine properties. To estimate the fixed and random effects and their size, a linear mixed model with the restricted maximum likelihood method for the estimation of covariance parameters was used. For leaf data that were collected repeatedly within each growing season, the growth stage at each sampling was incorporated as an additional factor into the mixed model. Vineyard, year, irrigation, fertilisation and growth stage were considered as fixed effects, whereas blocking as random effects. Since each vineyard was planted with a different variety, the “vineyard” effects in our

experimental design represent the combined effects of vineyard location (soil, for example) and of variety. The least significant difference test was used to detect differences between the means of the fixed effects at $p < 0.05$. The relationships between the measured variables were evaluated by linear correlation and regression analysis. Data analysis was conducted using Statistical Analysis System software, version 9.3 (SAS Institute, Cary, NC).

RESULTS

1. Stem water potential, leaf nitrogen and gas exchange

Irrigated vines of both vineyards had higher values of stem water potential (Table 1). Ψ_s decreased from the BS to MT stage, and it decreased more steeply between BC and VE (Table 1). The greater differences in Ψ_s between the irrigation treatments were observed at the VE and MT stages [Table 1; data previously published in Taskos *et al.* (2015)]. No difference in Ψ_s was observed among N treatments. Both fertilised treatments in CS and the N_{120} treatment in XM had higher concentrations of leaf N (Table 1). Leaf N concentration declined progressively from BS to MT stage (Table 1). Irrigation had no effect on leaf N content (Table 1).

Irrigation had significant effects on gas exchange of single leaves in the XM vineyard only, where the irrigated vines had higher rates of stomatal conductance and net assimilation, but lower WUE_i (Table 1). However, leaves of both cultivars presented higher net assimilation and stomatal conductance rates at BS and BC compared to VE and MT samplings (Table 1). Contrary to irrigation, the influence of N addition on gas exchange was small (Table 1). However, significant relationships were observed between leaf N content and A_n , g_s , WUE_i , C_i (Figure 1) in both vineyards: with increasing leaf N, net assimilation and stomatal conductance rate increased, whereas intrinsic water use efficiency decreased (Figure 1). The relationship between A_n and leaf N was stronger in the CS vineyard than in the XM vineyard, while the opposite was observed for the C_i values (Figure 1). These relationships were better described by quadratic regression models, except for C_i in the XM vineyard.

2. Carbon isotope composition

In both vineyards, water application and N fertilisation affected leaf carbon isotope composition, but in different directions; leaf $\delta^{13}\text{C}$ values were lower in the irrigated vines compared to the non-irrigated vines, but were higher in the CS fertilised treatments and in the N_{120} XM vines compared to the non-fertilised vines (Table 1). The N_{120} CS and XM vines had consistently higher leaf $\delta^{13}\text{C}$ than N_0 vines, irrespective of growth stage (Figures 2B, D). On the contrary, the irrigated vines had lower leaf $\delta^{13}\text{C}$ only after the BC and VE stages (Figures 2A, C) in the CS and XM vineyards respectively, because of a significant interaction between irrigation and the growth stage ($F = 9.3$, $p < 0.0001$). Leaf $\delta^{13}\text{C}$ values also varied with growth stage, mainly between the BS and BC stages, when $\delta^{13}\text{C}$ values decreased in both vineyards (Table 1), except for non-irrigated XM vines (Figure 2C). The size of the effect of N fertilisation and irrigation on leaf $\delta^{13}\text{C}$ depended on growth stage (Figure 3A, B): N application accounted for a higher proportion of leaf $\delta^{13}\text{C}$ variance at the BS and the BC stages, whereas irrigation induced higher variance of leaf $\delta^{13}\text{C}$ at the VE (only CS) and MT stages.

As in the case of the leaf carbon isotope ratio, different responses of cane $\delta^{13}\text{C}$ to irrigation and N fertilisation were observed in both vineyards: the values increased with N supply but decreased with irrigation (Table 1). Berry must $\delta^{13}\text{C}$ was lower in the irrigated vines of both varieties (Table 1). However, due to significant interaction between irrigation and N fertilisation ($F = 8.98$, $p = 0.0018$), irrigated and not-irrigated treatments did not differ in their must $\delta^{13}\text{C}$ values under the higher nitrogen rate (Figure 4).

In both vineyards and irrespective of irrigation and fertilisation treatment, bulk berry must at maturity was more enriched with ^{13}C compared to leaves and canes (Table 1). The size of the effect of irrigation on must and cane $\delta^{13}\text{C}$ was higher compared to that of fertilisation in both vineyards (Figures 3C, D), and the mixed model accounted for a higher variance of $\delta^{13}\text{C}$ in the must and canes than in leaves (Figures 3C, D). The leaf $\delta^{13}\text{C}$ variance explained by the leaf N content was higher at the BS and BC stages, whereas Ψ_s explained most of leaf $\delta^{13}\text{C}$ variance at the VE and MT stages (Figures 5A, B). Similar responses were observed for cane $\delta^{13}\text{C}$ variance (Figures 5E, F). Only Ψ_s accounted

significantly for must $\delta^{13}\text{C}$ variance, at all stages in XM (Figure 5D) and after the BC stage in CS (Figure 5C).

In both vineyards must and cane $\delta^{13}\text{C}$ values were positively correlated with the $\delta^{13}\text{C}$ values in leaves at the MT stage ($r = 0.69$ & 0.61 respectively, $p < 0.001$ in CS; $r = 0.50$ $p < 0.01$ & 0.74 $p < 0.001$ respectively in XM).

Discussion

1. Water and nitrogen status

The increased leaf N content in the CS fertilised treatments and in the XM N_{120} treatment (Table 1) is consistent with previous studies (Keller *et al.*, 2001) and indicates a modification of vine N status. The progressive decline of leaf N from the BS stage towards the MT stage (Table 1), can be explained by N translocation from the aging basal leaves to younger actively growing organs (Keller *et al.*, 2001). Regarding water status, according to van Leeuwen *et al.* (2016), irrigated vines were under weak water deficit after VE, while the non-irrigated vines were under moderate to severe water deficit after veraison [data previously published in Taskos *et al.*, (2015)]. The decline of Ψ_s values between BC and VE (Table 1) indicates a corresponding depletion of available water reserves in the soil of both vineyards.

2. Gas exchange in relation to the water and nitrogen status of vines and leaf age

In grapevines, increasing soil water deficit causes a chain of gas exchange responses: stomata closure and a subsequent reduction in g_s and of CO_2 available in the chloroplasts, as observed in the non-irrigated XM vines (Table 1). According to their g_s values, XM vines (Table 1) were under mild water stress after the VE stage (Cifre *et al.*, 2005). According to Chaves *et al.* (2010), some varieties maintain higher g_s during the day for similar predawn water potential under field conditions. Thus, despite the alteration of vine water status, the smaller effects of irrigation on CS gas exchange (Table 1) could be attributed to varietal differences. However, the two varieties were not replicated in our experimental design. Consequently, the “variety” effects may as well represent in fact “location” effects.

Although nitrogen application had a negligible influence on single leaf gas exchange (Table 1),

the positive relationship between leaf N content and A_n , g_s , and C_i (Figure 1A, B, C, D, E, F) indicates that gas exchange responded to the variation in leaf N content. However, in the CS vineyard, A_n was more tightly coupled to leaf N content (Figure 1A) than in XM (Figure 1B), while the opposite was observed for g_s (Figure 1C, D) and C_i (Figure 1E, F). The increasing trend of C_i with leaf N could indicate a decline in mesophyll conductance and adjustments in C_i/C_c

balance (Flexas *et al.*, 2012). Evidence for the regulation of leaf gas exchange responses to N fertilisation at the mesophyll level has been provided by Keller *et al.* (2001) for field grown grapevines. The inverse relationship of WUE_i with leaf N in both vineyards can be explained by the faster increase in g_s compared to A_n of up to approximately 2.5 % of leaf N (Figures 2A, B, C, D). This finding indicates that any stomatal limitations to photosynthesis were minimal at

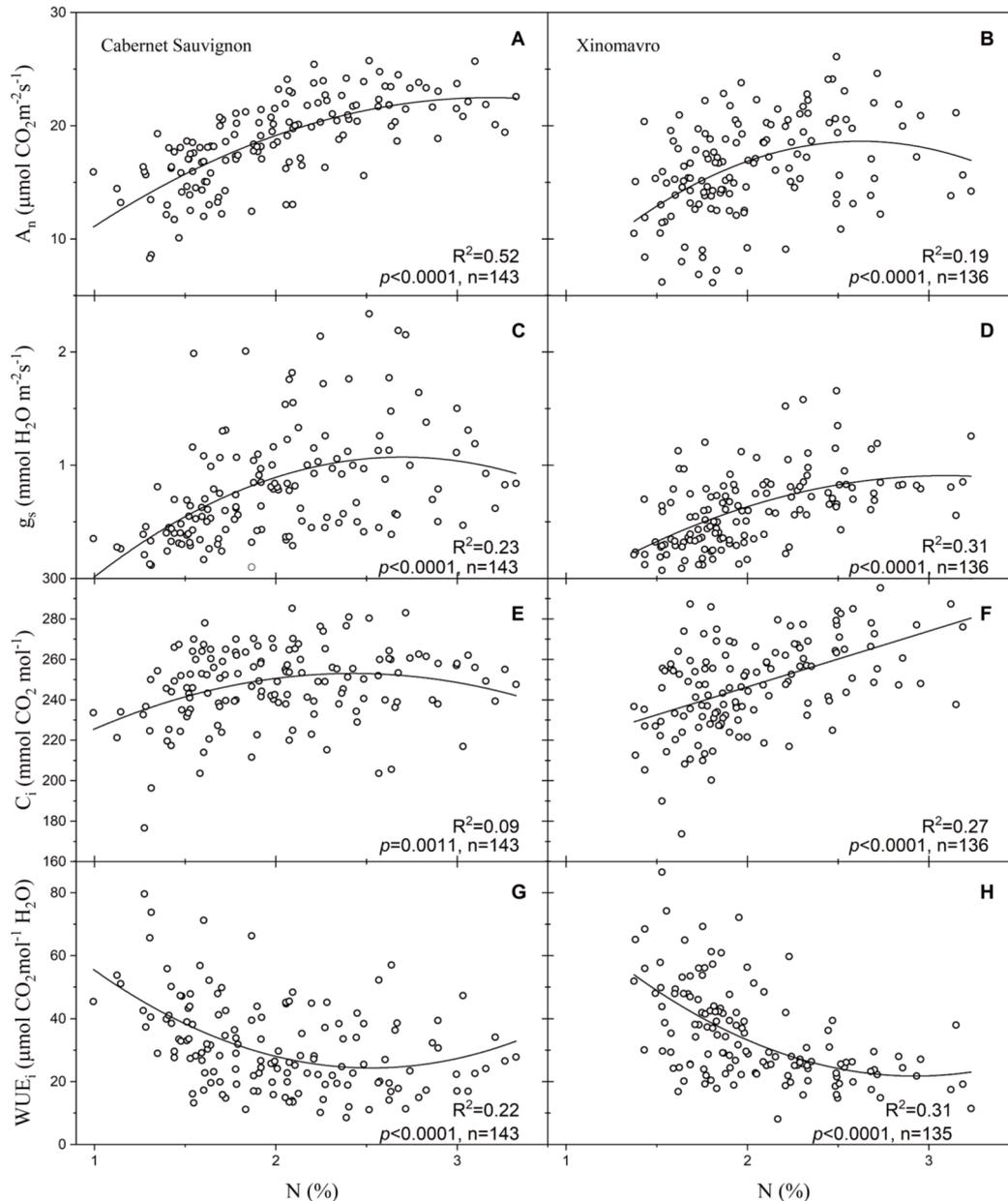


FIGURE 1. Relationship of total nitrogen content (N) in leaf blade dry matter with net assimilation (A_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and intrinsic water-use efficiency (WUE_i) of single leaves in the two vineyards

Growth stage: BS = berry set; BC = bunch closure; VE = veraison; MT = maturity

Data are plot means at each growth stage for two years.

high leaf N content and contradicts the stimulating, but inconsistent, influence of N fertilisation on WUE_i reported by previous studies (Brueck, 2008).

3. Leaf blade carbon isotope composition in relation to water status and leaf age

The higher values of leaf $\delta^{13}C$ in the non-irrigated grapevines of both vineyards (Table 1)

indicate that water deficit led to an enrichment of leaf BDM with ^{13}C . This is a common response of leaf $\delta^{13}C$ to decreasing soil water content in C3 plants, because water deficit imposes diffusional limitations on carbon intake and assimilation. In such conditions, the C_i/C_a ratio decreases, because stomatal conductance decreases at a faster rate compared to net assimilation, leading to a lower discrimination against the ^{13}C isotope (Farquhar *et al.*, 1989).

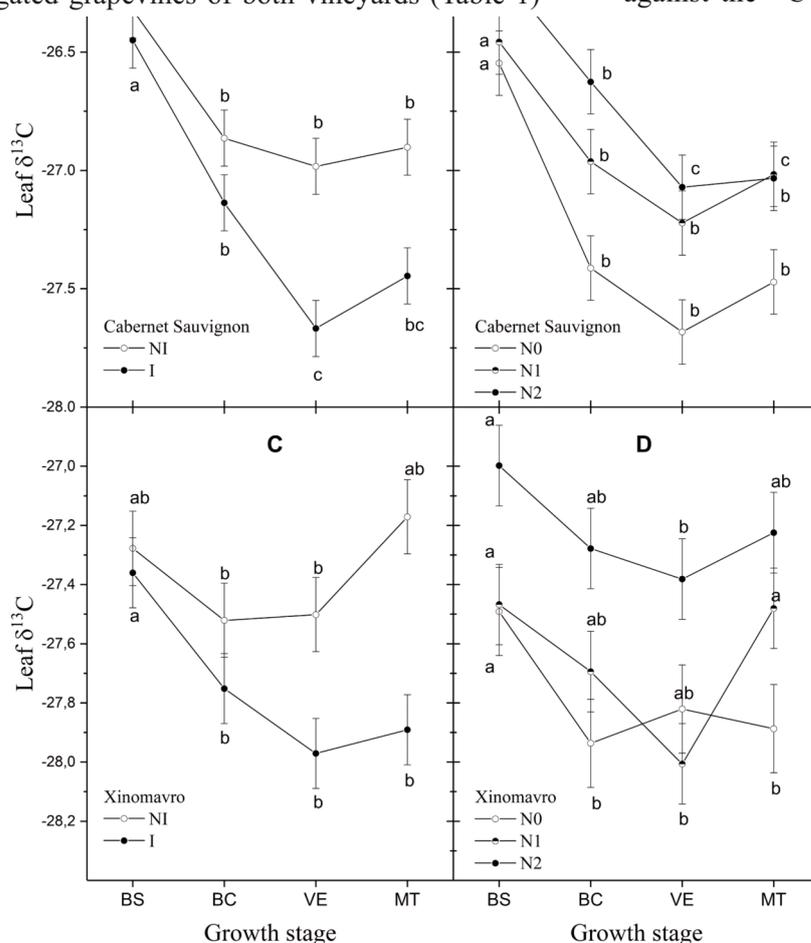


FIGURE 2. Effects of nitrogen fertilisation and irrigation on leaf nitrogen carbon isotope composition according to vineyard and growth stage.

Nitrogen fertilisation: N₀, unfertilised; N₁, 60 kg/ha N; N₂, 120 kg/ha N.

Irrigation: NI = non-irrigated; I = irrigated at 70 % of E_{tc} .

Growth stage: BS = berry set; BC = bunch closure; VE = veraison; MT = maturity.

Within factor levels in each vineyard, lowercase letters indicate significant differences between growth stages at the 0.05 probability level.

The higher g_s and A_n rates at the BS and BC stages (Table 1) can be attributed to leaf age effects, because both these variables have been reported to vary as a function of leaf age, most probably due to non-stomatal effects (Knoll and Redl, 2016).

This mechanism of water status influence on leaf $\delta^{13}\text{C}$ could also explain the larger differences in leaf $\delta^{13}\text{C}$ between the irrigation treatments at the VE and MT stage (Figure 2) when water stress was more intense for the non-irrigated vines in comparison to the BS and BC stages [data previously published in Taskos *et al.* (2015)]. Although similar responses of main leaf BDM $\delta^{13}\text{C}$ have been reported for field grown grapevines with restricted water availability (Souza *et al.*, 2005; Koundouras *et al.*, 2008; Pou *et al.*, 2008; Bchir *et al.*, 2016), leaf $\delta^{13}\text{C}$ in grapevines does not always respond to water deficit. For example, Poni *et al.* (2009) did not observe any irrigation effects on $\delta^{13}\text{C}$ of grapevine main leaves, collected at maturity from the apical nodes of the shoot, although a significant amount of their growth had been completed under water stress conditions.

These inconsistent responses may be a consequence of the inherent limitations of measuring $\delta^{13}\text{C}$ in leaf BDM. Indeed, $\delta^{13}\text{C}$ of

plant or leaf BDM gives an assimilation-weighted integration of the ratio C_i/C_a at increasing integration times that can extend to the plant's life, but only when the influence of factors acting simultaneously - but in different directions - on $\delta^{13}\text{C}$ (such as developmental stage, chemical composition, fractionations during transport of carbohydrates, aging and senescence), is negligible (Brugnoli and Farquhar, 2000). Thus, the interpretation of leaf $\delta^{13}\text{C}$ solely in terms of gas exchange responses to water availability may not be appropriate for all situations. Our data support this hypothesis, because vine water status, as indicated by stem water potential, accounted for less than 30 % of leaf $\delta^{13}\text{C}$ variance (Figures 5A, B) and this variance was lower compared to the size of irrigation treatment effects (Figures 3A, B). Even when leaf $\delta^{13}\text{C}$ was regressed on Ψ_s using data averaged across years and growth stages, at best Ψ_s accounted for 44 % and 41 % of leaf $\delta^{13}\text{C}$ variance in the CS and XM vineyard

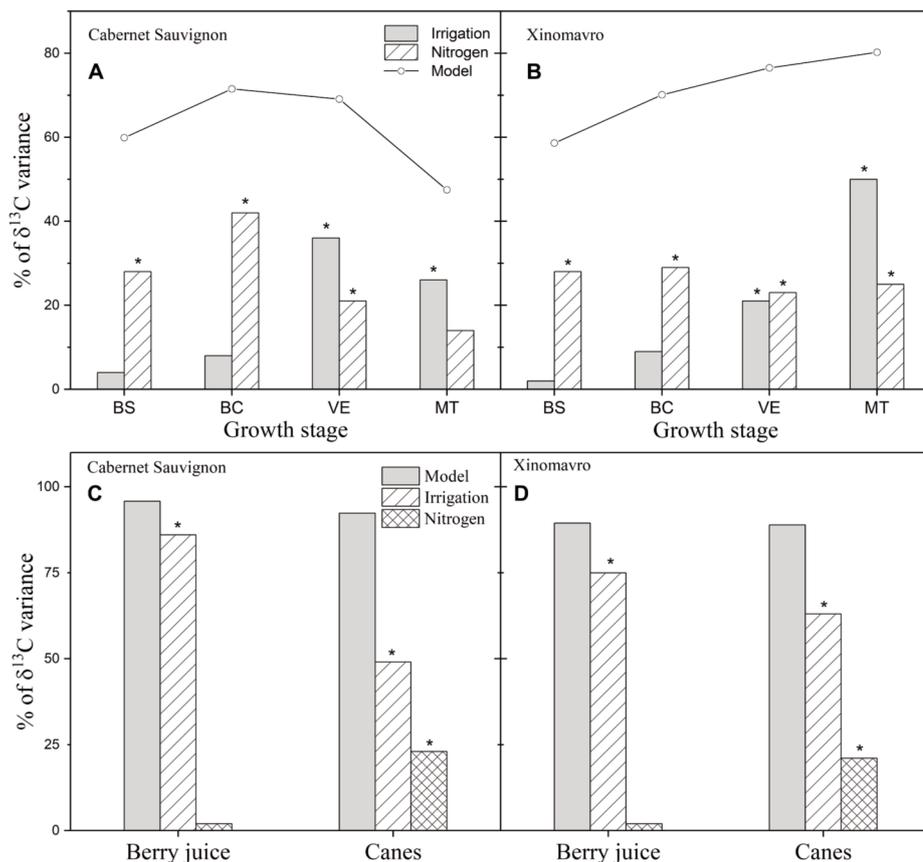


FIGURE 3. Size of effect (% variance) of irrigation and nitrogen fertilisation on carbon isotope composition ($\delta^{13}\text{C}$) of leaves (A, B) and berry juice and canes (C, D) by vineyard. Growth stage (A, B) applies only to leaves. Model represents total variance explained by the mixed model. Growth stage: BS = berry set; BC = bunch closure; VE = veraison; MT = maturity. Asterisks indicate significant effects at the 0.05 probability level.

respectively (data not shown). Therefore, within the confinements of this study, our findings indicate that we cannot consider $\delta^{13}\text{C}$ of the basal main leaf BDM as a reliable indicator of vine water status on any time scale. This conclusion supports the findings of other studies on the topic, as reviewed by Santesteban *et al.* (2015).

In C3 plants, photosynthetic WUE positively correlates with $\delta^{13}\text{C}$, because both these conceptually different variables are linked to the C_i/C_a ratio. Thus, when water availability decreases and diffusional limitations to carbon uptake and assimilation appear, the C_i/C_a ratio decreases while both photosynthetic WUE and $\delta^{13}\text{C}$ increase (Farquhar *et al.*, 1989). Positive relationships between WUE_i and leaf carbon isotope composition $\delta^{13}\text{C}$ in grapevines have been reported by many studies (Souza *et al.*, 2005; Tomás *et al.*, 2012; Bchir *et al.*, 2016), but they have not always been consistent. In our study, leaf $\delta^{13}\text{C}$ and WUE_i were not correlated on any time scale (data not shown). This inconsistency in the relationship of WUE_i with leaf $\delta^{13}\text{C}$ could be partly attributed to their different variation over time: WUE_i not only varies diurnally and spatially within the grapevine canopy, but also seasonally, because of environmental and physiological changes due to leaf ageing (Medrano *et al.*, 2015), whereas $\delta^{13}\text{C}$ in leaf or plant BDM provides an estimation

of the C_i/C_a ratio over the entire period during which the carbon forming that dry matter was fixed (Brugnoli and Farquhar, 2000). Consequently, the relationship of leaf $\delta^{13}\text{C}$ with C_i/C_a at later developmental stages depends on the carbon fixed during the earlier, heterotrophic, stages of leaf development (Terwilliger *et al.*, 2001). It seems that the constraints stated in the previous paragraph for the interpretation of $\delta^{13}\text{C}$ in leaf BDM also apply to the reported inconsistency of the relationship between leaf $\delta^{13}\text{C}$ and on WUE_i .

Variation of leaf $\delta^{13}\text{C}$ during ontogenesis has been well documented for several herbaceous species (Salmon *et al.*, 2011) and grapevines (Bchir *et al.*, 2016), but without a clear trend in the latter case. Our data show that the BDM of basal leaves became depleted in their ^{13}C content from the BS up to the BC stage in XM and up to VE in CS (Table 1), and that the magnitude of $\delta^{13}\text{C}$ change between these growth stages was comparable or higher to the change caused by irrigation and N fertilisation (Table 1, Figure 2). This depletion of leaf BDM happened in parallel with increasing leaf age from BS to MT, since at all samplings $\delta^{13}\text{C}$ was measured in leaves from the basal shoot zone. However, we cannot conclusively attribute this decrease in leaf $\delta^{13}\text{C}$ to increasing leaf age because of the significant interaction between irrigation and growth stage effects (Figures 2A, C), which shows that any

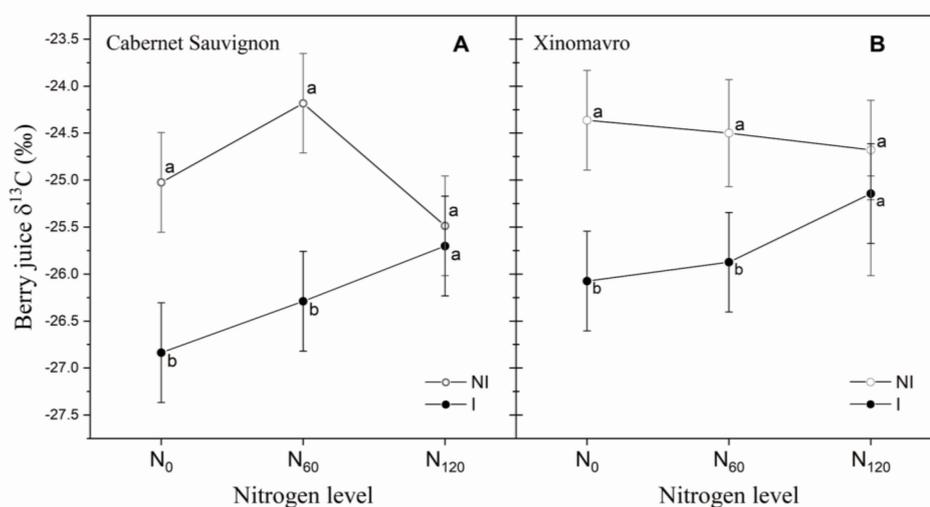


FIGURE 4. Effects of irrigation and nitrogen fertilisation on the carbon isotope composition ($\delta^{13}\text{C}$) of berry juice according to vineyard, factor level and growth stage.

Data are treatment means.

Nitrogen fertilisation: N₀ = unfertilised; N₁ = 60 kg/ha N; N₂ = 120 kg/ha N.

Irrigation: NI = non-irrigated; I = irrigated at 70 % of E_t .

Within nitrogen levels in each vineyard, different lowercase letters indicate significant differences between irrigation treatments at the 0.05 probability level.

leaf age effects depended on water availability. It is generally difficult to disentangle ontogenetic from environmental effects on plant $\delta^{13}\text{C}$, which occur at the same time in field conditions (Salmon *et al.*, 2011). We conclude that the growth stage effects on leaf $\delta^{13}\text{C}$ in Table 1 might represent complex interactions between the vine water status variation and leaf ontogenetic changes, further complicating the interpretation of $\delta^{13}\text{C}$ in leaf BDM. This topic could be the subject of future investigations.

4. Leaf blade carbon isotope composition and nitrogen status

Many studies in other plant species have shown that $\delta^{13}\text{C}$ values in plant BDM may increase when nitrogen availability is improved (Bender and Berge 1979; Ripullone *et al.*, 2004). However, to the best of our knowledge, the higher leaf $\delta^{13}\text{C}$ values in the CS fertilised treatments and in the N₁₂₀ XM vines (Table 1) are the first such data to be reported on this topic for grapevines.

The leaf $\delta^{13}\text{C}$ responses to N supply have been commonly attributed to the higher carboxylation efficiency or to higher assimilation rate under increased N supply (Hogberg *et al.*, 1995; Ripullone *et al.*, 2004). Essentially, these explanations are based on the simplified version of the model of Farquhar *et al.*, (1989) formulated in Equation 2, which defines a linear relationship between $\delta^{13}\text{C}$ and the C_i/C_a ratio, assuming minimal effects of discrimination associated with the CO₂ transfer from the intercellular spaces to the carboxylation sites (Brugnoli and Farquhar, 2000). However, this assumption may not apply to all conditions, since differences in mesophyll conductance can be large enough to account for the plant $\delta^{13}\text{C}$ variations, because photosynthetic carbon isotope discrimination is sensitive to the C_c/C_a ratio (Seibt *et al.*, 2008). In our study, an explanation for the higher leaf $\delta^{13}\text{C}$ values in the fertilised treatments cannot be solely based on the simplified model of Equation 2, because such an increase would require the ratio C_i/C_a to decrease with increasing N availability, while WUE_i should increase; instead, the opposite was observed (Figures 1E, F, G, H). However, a positive linear relationship between the A/C_i ratio and leaf N ($R^2 = 0.38$, $p < 0.0001$, $n = 144$) was observed in the CS vineyard. Therefore, although carboxylation efficiency was not measured, any possible effects of nitrogen supply

on leaf $\delta^{13}\text{C}$ through corresponding effects on carboxylation efficiency cannot be precluded, at least in the CS vineyard and during the early growth stages when stomatal conductance rate was not limiting for photosynthesis (Table 1). Additionally, our data and discussion on the relationship of leaf N with gas exchange suggest that photosynthetic carbon isotope discrimination may be regulated in a different manner in the main leaves of the two vineyards/cultivars.

We conclude that it is necessary to consider the additional discriminating processes against the ^{13}C isotope during carbon assimilation in the leaf (as denoted in the detailed model of Farquhar *et al.* (1982)) in order to explain the N fertiliser isotope effects. Several studies in other species also state this view. For example, Choi *et al.* (2005) found that nitrogen fertilisation in loblolly pines increased leaf $\delta^{13}\text{C}$, most probably because of reduced mesophyll conductance and less likely because of increased carboxylation efficiency. In another study, the decreasing leaf $\delta^{13}\text{C}$ values in nitrogen-stressed white spruce plants were attributed to an increase in CO₂ concentration within the chloroplasts following an increase in mesophyll conductance for CO₂ (Livingston *et al.*, 1999). In such investigations, when gas exchange data are compared with carbon isotope composition data of leaves with relatively long lifespans, consideration should be given to the conditions that prevailed when a particular leaf's cellulosic carbon was actually assimilated, and to the time scales governing adjustments in C_i/C_a and subsequent changes in foliar $\delta^{13}\text{C}$ values (Meinzer *et al.*, 1992). Finally, as already discussed, $\delta^{13}\text{C}$ measured in plant BDM only gives an assimilation-weighted integration of the ratio C_i/C_a if other factors that contribute to leaf $\delta^{13}\text{C}$ variation have minimal influence (Brugnoli and Farquhar, 2000); for example, nitrogen shortage increases the starch content of grapevine leaves (Grechi *et al.*, 2007). Thus, it is possible that the indirect effects of N supply on leaf $\delta^{13}\text{C}$ are a result of modifications in leaf chemical composition and mainly in the non-structural fraction of the total leaf carbon pool.

5. Within season variation of water and nitrogen status effects on leaf $\delta^{13}\text{C}$

Since leaf $\delta^{13}\text{C}$ was measured in leaf BDM, leaf $\delta^{13}\text{C}$ values at each growth stage integrated the isotope effects during the preceding period of leaf development, when the carbon forming the

leaf structural carbohydrates was fixed (Brugnoli and Farquhar, 2000). In addition, part of leaf $\delta^{13}\text{C}$ can be determined by the carbon isotope composition of non-structural carbohydrates like starch and primary photosynthates close to the time of leaf sampling. Therefore, whatever the underlying mechanisms of leaf $\delta^{13}\text{C}$ modulation by nitrogen and water availability, and whatever the extent to which leaf $\delta^{13}\text{C}$ was determined by these two factors, leaf $\delta^{13}\text{C}$ at a specific growth stage will integrate the opposite effects of irrigation and fertilisation during leaf development on leaf structural and non-structural carbohydrates up to that specific stage.

Considering that any significant water deficit before the BS stage was unlikely under the experimental conditions, the stronger effect of N fertilisation at the BS and BC growing stages (as shown in Figures 3A, B) represents a higher contribution of nitrogen availability to leaf $\delta^{13}\text{C}$ variation, but only in the context of minimal influence of water availability. The opposite was observed at the VE and MT stages when water status effects on leaf $\delta^{13}\text{C}$ modulation prevailed (Figures 3A, B; Figures 5A, C). That means that a water deficit at the early stages of leaf development could have equally impacted leaf $\delta^{13}\text{C}$ at the BS and BC stages. Furthermore,

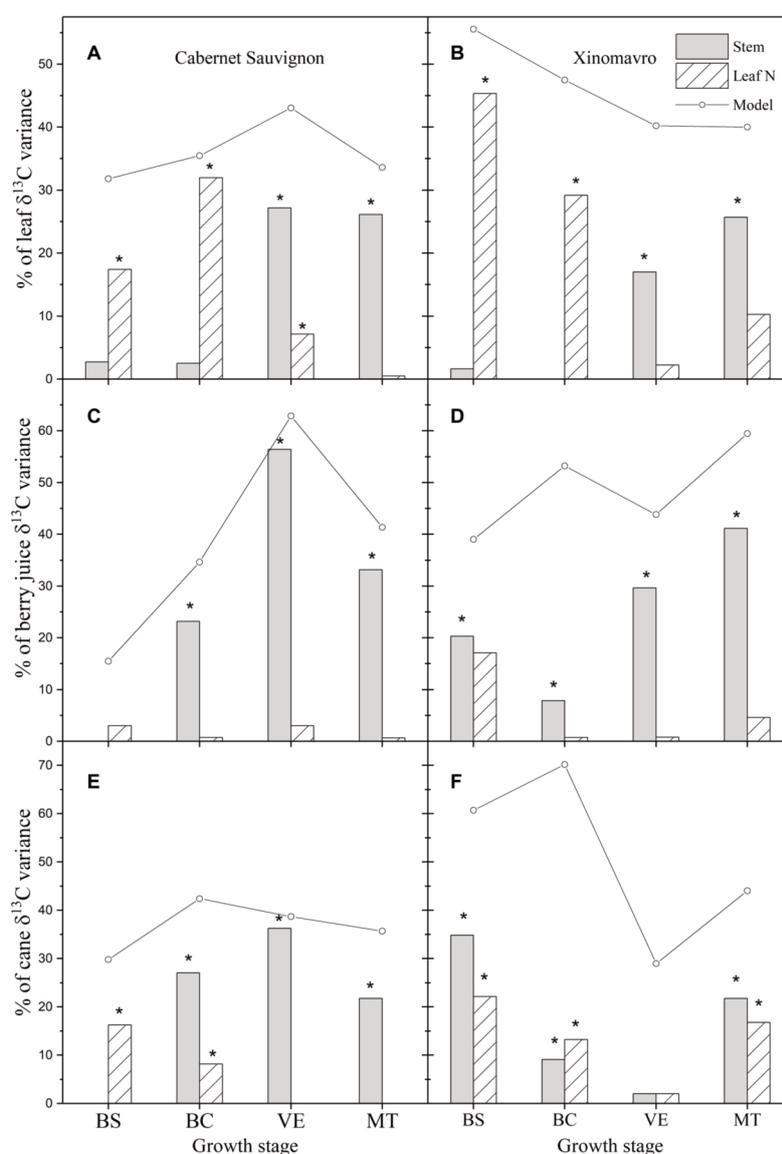


FIGURE 5. Effect sizes of stem water potential (Stem) and leaf nitrogen (Leaf N) on carbon isotope composition ($\delta^{13}\text{C}$) of leaf blades, must and canes according to growth stage and vineyard. Growth stage: BS = berry set; BC = bunch closure; VE = veraison; MT = maturity. Asterisks denote significant effects at the 0.05 probability level.

carbohydrate allocation in grapevine leaves continues well past the time needed for completing lamina expansion (Poni *et al.*, 1994), and the amount of structural carbohydrates does not change much after the completion of leaf development. Thus, the carbon isotope composition of the non-structural fraction of leaf carbon may have increasingly contributed to leaf $\delta^{13}\text{C}$ close to the later grapevine stages. Consequently, the increased differences in leaf $\delta^{13}\text{C}$ between the irrigation treatments (Figure 2) at the VE and MT stages are probably the result of the more significant irrigation effects on the non-structural fraction of leaf carbon.

We can conclude that the variation in size of the effects from the BS to the MT stage (as shown in Figures 3A, B and in Figures 5A, B) corresponds to a variation in time of the relative contribution of water and nitrogen status on leaf $\delta^{13}\text{C}$ in response to the specific experimental conditions from the beginning of the growing season. These findings lead to the question of whether leaf BDM $\delta^{13}\text{C}$ can provide information - via proper sampling and experimental setup - on the interplay between water and nitrogen in modulating leaf $\delta^{13}\text{C}$ at a growth season scale. In this context, future research could focus on leaf $\delta^{13}\text{C}$ responses to conditions of early water deficit or late nitrogen supply. However, as water and nitrogen accounted for no more than half of the total variance (Figures 3A, B), other factors known to affect BDM $\delta^{13}\text{C}$ should also be considered. Such experiments should strive for higher variation of water and nitrogen availability.

6. Carbon isotope ratio in berry must and canes

Carbon isotope composition of leaf soluble sugars provides a reliable estimation of C_i/C_a variation induced by environmental stresses, such as drought or salinity (Brugnoli and Farquhar, 2000). In water deficit conditions, diffusional limitations reduce the C_i/C_a ratio leading to an enrichment of primary photosynthates with ^{13}C (Farquhar *et al.*, 1989). This is why the carbon isotope composition of the purified hexose fraction of berry sugars at maturity provides a consistent and integral measure of vine water status during berry ripening (van Leeuwen *et al.*, 2001; Gaudillère *et al.*, 2002; Des Gachons *et al.*, 2005; Zufferey *et al.*, 2018). In addition, $\delta^{13}\text{C}$ of bulk berry must at harvest correlates strongly with $\delta^{13}\text{C}$ of purified

berry sugars (Gaudillère *et al.*, 2002), and thus also provides an efficient measure of vine water status during the berry maturation period (Gómez-Alonso and García-Romero, 2010; Herrero-Langreo *et al.*, 2013). Therefore, the higher $\delta^{13}\text{C}$ values for the bulk must of the non-irrigated vines (Table 1) are consistent with the higher water deficit they were subjected to during the VE and MT stages. The irrigation treatment means (Table 1) fall within the range of $\delta^{13}\text{C}$ values as reviewed by Santesteban *et al.* (2015) for berry must, indicating moderate to severe water deficit for the non-irrigated grapevines.

Although well-established knowledge exists on the effects of water deficit on the carbon isotope composition of berry must, data for any corresponding effects on vine nitrogen status are scarce and inconsistent. Gaudillère *et al.* (2002) did not observe any significant influence of nitrogen status, or any interaction between nitrogen and water status on the $\delta^{13}\text{C}$ of berry purified sugar in a study in which variation in leaf nitrogen was indirectly induced by different soil tillage treatments; however, as the authors pointed out, the leaf nitrogen range of their data was narrower than that in our study. In agreement with these findings, and under the conditions of our study, the variation in must $\delta^{13}\text{C}$ at harvest was not explained by nitrogen regime at any of the four growth stages (Figures 5C, D). However, in a one year study, Brillante *et al.* (2020) found a strong positive relationship between leaf blade N concentration at anthesis and berry must $\delta^{13}\text{C}$ at maturity, but only when the data from two different vineyards were pooled; the authors attributed the absence of a similar relationship within each vineyard to the narrower leaf N variation in comparison to the pooled data. On the other hand, we observed a significant interaction between irrigation and N fertilisation (Figure 4), which shows that the water supply effects on bulk juice $\delta^{13}\text{C}$ depended on the fertiliser rate in both vineyards: while N rate increased, bulk juice $\delta^{13}\text{C}$ values of the unirrigated and the irrigated treatments tended to converge. Whatever the underlying mechanisms, we expect these effects to interfere with interpretations of berry must carbon isotope composition in terms of water availability only in cases of high nitrogen variation and availability. Nevertheless, it should be noted that by veraison most of the added N will have been allocated to or incorporated into various vine

organs. Thus, in order to better study the effect of N supply on must $\delta^{13}\text{C}$, a late (e.g., at VE) N treatment would be necessary.

Relatively few data exist on cane BDM $\delta^{13}\text{C}$, and these are related to grapevine genotypic variation in cane $\delta^{13}\text{C}$ as a proxy of WUE (Virgona *et al.*, 2003) and the effect of water deficit (Glenn *et al.*, 2010). The contrasting effects of irrigation and N fertilisation on cane $\delta^{13}\text{C}$ resemble their effects on leaf $\delta^{13}\text{C}$ (Table 1), suggesting that leaf and cane $\delta^{13}\text{C}$ were modulated in a similar manner within both organs. However, water availability contributed more to cane $\delta^{13}\text{C}$ than N fertilisation, because irrigation accounted for more than double the variance of cane $\delta^{13}\text{C}$ than that accounted for by N fertilisation (Figures 3C, D). In addition, the influence of water availability, as indicated by stem water potential, was evident for the BS stage in XM and from BC in CS (Figures 5A, B) when water conditions were not limiting (Table 1). This probably reflects the high sensitivity of shoot growth to water availability (Keller, 2005). Despite its regulation by water availability, cane $\delta^{13}\text{C}$ cannot be considered an indicator of vine water status since stem potential accounted for at best 35 % of its variance (Figures 5E, F); nor can it be considered as an indicator of WUE_i on a seasonal basis, since the average WUE_i of the four samplings and cane $\delta^{13}\text{C}$ were only positively correlated in the second year of the experiment and in the CS vineyard ($R^2 = 0.48$, $p = 0.0008$, $n = 18$, data not shown). Thus, the interpretations of cane $\delta^{13}\text{C}$ in terms of single leaf WUE_i may not be correct.

Canes are lignified shoots that develop and mature during the preceding growing season. Although green shoots can photosynthesise if they contain chlorophyll, their own carbon assimilation activity contributes to their carbon pool much less than the carbon they receive from leaves and other vine parts. However, metabolic processes in various plant organs or during translocation of metabolites from one organ to another can cause carbon isotope fractionations (Cernusak *et al.*, 2013). Therefore, the carbon isotope abundance of canes was modulated by the isotope composition of translocated carbon and any associated fractionations. Additionally, carbohydrate composition is known to vary in response to N and water supply (Holzapfel *et al.*, 2010), thus nitrogen and water status effects may be mediated by cane compositional changes. However, based on our data we cannot make

inferences regarding the relative contribution of these effects and how water and nitrogen availability impacted them. Despite this limitation, the stronger positive relationship between leaf and cane $\delta^{13}\text{C}$ from the later samplings ($r = 0.61$, $p < 0.001$ in CS and $r = 0.74$ $p < 0.001$ in XM at MT) probably represents the more intense allocation of leaf-originated carbon that was fixed during the maturation period (Holzapfel *et al.*, 2010).

CONCLUSIONS

Our findings show that the carbon isotope composition of grapevines was affected by water and nitrogen supply simultaneously, but differently, depending on the developmental stage and tissue (leaf blades, canes and berry must). Although the differences we found between nitrogen fertilisation and irrigation treatments were relatively small (a limitation of our study), to the best of our knowledge, this is the first reported evidence for the modulation of carbon isotope composition by nitrogen fertilisation in grapevines in conditions of varying water availability. According to the data presented, interpretations of carbon isotope natural abundance solely in terms of plant water status might be confounded by nitrogen availability in N-rich soils, especially when measured in leaves and canes. More factorial trials are needed to elucidate the individual effects of water and N on the mechanisms underlying the modulation of natural carbon isotope abundance in grapevines.

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REFERENCES

- Bchir, A., Escalona, J., Gallé, A., Hernández-Montes, E., Tortosa, I., Braham, M., & Medrano, H. (2016). Carbon isotope discrimination ($\delta^{13}\text{C}$) as an indicator of vine water status and water use efficiency (WUE): Looking for the most representative sample and sampling time. *Agricultural Water Management* 167, 11–20, doi: 10.1016/j.agwat.2015.12.018
- Bender, M. & Berge, A. (1979). Influence of N and K fertilization and growth temperature on $^{13}\text{C}/^{12}\text{C}$ ratios of timothy (*Phleum pratense* L.). *Oecologia* 44 (1), 117–118, doi: 10.1007/BF00346409
- Brillante, L., Martínez-Lüscher, J., Yu, R., & Kurtural, S. (2020). Carbon isotope discrimination

- ($\delta^{13}\text{C}$) of grape musts is a reliable tool for zoning and the physiological ground-truthing of sensor maps in precision viticulture. *Frontiers in Environmental Science* 8, doi: 10.3389/fenvs.2020.561477
- Brueck, H. (2008). Effects of nitrogen supply on water-use efficiency of higher plants. *Journal of Plant Nutrition and Soil Science* 171 (2), 210–219, doi: 10.1002/jpln.200700080
- Brugnoli, E. & Farquhar, G. 2000. Photosynthetic Fractionation of Carbon Isotopes. In: Leegood, R.C., Sharkey, T.D., Caemmerer, S. von (Eds.), *Photosynthesis: Physiology and metabolism*. Ed. by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer. *Advances in photosynthesis*, v.9. Kluwer, Dordrecht, London, pp. 399–434.
- Cernusak, L., Ubierna, N., Winter, K., Holtum, J., Marshall, J., & Farquhar, G. (2013). Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist* 200 (4), 950–965, doi: 10.1111/nph.12423
- Chaves, M., Zarrouk, O., Francisco, R., Costa, J., Santos, T., Regalado, A., Rodrigues, M., & Lopes, C. (2010). Grapevine under deficit irrigation: hints from physiological and molecular data. *Annals of Botany* 105 (5), 661–676, doi: 10.1093/aob/mcq030
- Choi, W., Chang, S., Allen, H., Kelting, D., & Ro, H. (2005). Irrigation and fertilization effects on foliar and soil carbon and nitrogen isotope ratios in a loblolly pine stand. *Forest Ecology and Management* 213 (1-3), 90–101, doi: 10.1016/j.foreco.2005.03.016
- Choné, X., van Leeuwen, C., Dubourdieu, D., & Gaudillère, J. (2001). Stem water potential is a sensitive indicator of grapevine water status. *Annals of Botany* 87 (4), 477–483, doi: 10.1006/anbo.2000.1361
- Cifre, J., Bota, J., Escalona, J., Medrano, H., & Flexas, J. (2005). Physiological tools for irrigation scheduling in grapevine (*Vitis vinifera* L.) An open gate to improve water-use efficiency? *Agriculture Ecosystems & Environment* 106 (2-3), 159–170, doi: 10.1016/j.agee.2004.10.005
- Dawson, T., Mambelli, S., Plamboeck, A., Templer, P., & Tu, K. (2002). Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics* 33 (1), 507–559, doi: 10.1146/annurev.ecolsys.33.020602.095451
- Des Gachons, C., van Leeuwen, C., Tominaga, T., Soyer, J.-P., Gaudillère, J.-P., & Dubourdieu, D. (2005). Influence of water and nitrogen deficit on fruit ripening and aroma potential of *Vitis vinifera* L. cv Sauvignon blanc in field conditions. *Journal of The Science of Food and Agriculture* 85 (1), 73–85, doi: 10.1002/jsfa.1919
- Farquhar, G., Ehleringer, J., & Hubick, K. (1989). Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 40 (1), 503–537, doi: 10.1146/annurev.pp.40.060189.002443
- Farquhar, G., O’Leary, M., & Berry, J. (1982). On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* 9 (2), 121–137.
- Flexas, J., Barbour, M., Brendel, O., Cabrera, H., Carriqui, M., Díaz-Espejo, A., Douthe, C., Dreyer, E., Ferrio, J., Gago, J., Gallé, A., Galmés, J., Kodama, N., Medrano, H., Niinemets, Ü., & Peguero (2012). Mesophyll diffusion conductance to CO_2 : An unappreciated central player in photosynthesis. *Plant Science* 193-194, 70–84, doi: 10.1016/j.plantsci.2012.05.009
- Gaudillère, J., van Leeuwen, C., & Ollat, N. (2002). Carbon isotope composition of sugars in grapevine, an integrated indicator of vineyard water status. *Journal of Experimental Botany* 53 (369), 757–763.
- Glenn, D., Cooley, N., Walker, R., Clingeleffer, P., & Shellie, K. (2010). Impact of kaolin particle film and water deficit on wine grape water use efficiency and plant water relations. *HortScience* 45 (8), 1178–1187.
- Gómez-Alonso, S. & García-Romero, E. (2010). Effect of irrigation and variety on oxygen ($\delta^{18}\text{O}$) and carbon ($\delta^{13}\text{C}$) stable isotope composition of grapes cultivated in a warm climate. *Australian Journal of Grape and Wine Research* 16 (2), 283–289, doi: 10.1111/j.1755-0238.2009.00089.x
- Grechi, I., Vivin, P., Hilbert, G., Milin, S., Robert, T., & Gaudillère, J.-P. (2007). Effect of light and nitrogen supply on internal C:N balance and control of root-to-shoot biomass allocation in grapevine. *Environmental and Experimental Botany* 59 (2), 139–149, doi: 10.1016/j.envexpbot.2005.11.002
- Herrero-Langreo, A., Tisseyre, B., Goutouly, J.-P., Scholasch, T., & van Leeuwen, C. (2013). Mapping grapevine (*Vitis vinifera* L.) water status during the season using carbon isotope ratio ($\delta^{13}\text{C}$) as Ancillary Data. *American Journal of Enology and Viticulture* 64 (3), 307, doi: 10.5344/ajev.2013.12125
- Hogberg, P., Johnnisson, C., Hogberg, M., Hogbom, L., Nasholm, T., & Hallgren, J. (1995). Measurements of abundances of ^{15}N and ^{13}C as tools in retrospective studies of N balances and water stress in forests: A discussion of preliminary results. *Plant and Soil* 168-169 (1), 125–133.
- Holzappel, B., Smith, J., Field, S., & Hardie, W. 2010. *Dynamics of Carbohydrate Reserves in Cultivated Grapevines*. In: Janick, J. (Ed.), *Horticultural reviews*. John Wiley & Sons, Inc, Hoboken, NJ, pp. 143–211.
- Keller, M. (2005). Deficit irrigation and vine mineral nutrition. *American Journal of Enology and Viticulture* 56 (3), 267–283.

- Keller, M., Kummer, M., & Vasconcelos, M. (2001). Soil nitrogen utilisation for growth and gas exchange by grapevines in response to nitrogen supply and rootstock. *Australian Journal of Grape and Wine Research* 7 (1), 2–11.
- Knoll, M. & Redl, H. (2016). Gas exchange of field-grown *Vitis vinifera* L. cv. zweigelt leaves in relation to leaf age and position along the stem. *OENO One* 46 (4), 281, doi: 10.20870/oeno-one.2012.46.4.1524
- Koundouras, S., Tsialtas, I., Zioziou, E., & Nikolaou, Brugnoli and Farquhar N. (2008). Rootstock effects on the adaptive strategies of grapevine (*Vitis vinifera* L. cv. Cabernet-Sauvignon) under contrasting water status: Leaf physiological and structural responses. *Agriculture, Ecosystems and Environment* 128 (1-2), 86–96, doi: 10.1016/j.agee.2008.05.006
- Livingston, N., Guy, R., Sun, Z., & Ethier, G. (1999). The effects of nitrogen stress on the stable carbon isotope composition, productivity and water use efficiency of white spruce (*Picea glauca* (Moench) Voss) seedlings. *Plant, Cell & Environment* 22 (3), 281–289, doi: 10.1046/j.1365-3040.1999.00400.x
- Medrano, H., Tomás, M., Martorell, S., Flexas, J., Hernández, E., Rosselló, J., Pou, A., Escalona, J.-M., & Bota, J. (2015). From leaf to whole-plant water use efficiency (WUE) in complex canopies: Limitations of leaf WUE as a selection target. *The Crop Journal* 3 (3), 220–228, doi: 10.1016/j.cj.2015.04.002
- Meinzer, F., Saliendra, N., & Crisosto, C. (1992). Carbon isotope discrimination and gas exchange in coffee arabica during adjustment to different soil moisture regimes. *Functional Plant Biology* 19 (2), 171, doi: 10.1071/PP9920171
- Michener, R.H., Lajtha, K., (2008). Stable isotopes in ecology and environmental science. *Ecological methods and concepts series*, 2nd ed. Blackwell Pub, Malden, MA.
- Poni, S., Bernizzoni, F., Civardi, S., Gatti, M., Porro, D., & Camin, F. (2009). Performance and water-use efficiency (single-leaf vs. whole-canopy) of well-watered and half-stressed split-root Lambrusco grapevines grown in Po Valley (Italy). *Agriculture, Ecosystems and Environment* 129 (1-3), 97–106, doi: 10.1016/j.agee.2008.07.009
- Poni, S., Intrieri, C., & Silvestroni, O. (1994). Interactions of leaf age, fruiting, and exogenous cytokinins in Sangiovese grapevines under non-irrigated conditions. II. Chlorophyll and Nitrogen Content. *American Journal of Enology and Viticulture* 45 (3), 278.
- Pou, A., Flexas, J., Alsina, M., Bota, J., Carambula, C., Herralde, F. de, Galmés, J., Lovisoló, C., Jiménez, M., Ribas-Carbó, M., Rusjan, D., Secchi, F., Tomás, M., Zsófi, Z., & Medrano, H. (2008). Adjustments of water use efficiency by stomatal regulation during drought and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandieri* x *V. rupestris*). *Physiologia Plantarum* 134 (2), 313–323, doi: 10.1111/j.1399-3054.2008.01138.x
- Ripullone, F., Lauteri, M., Grassi, G., Amato, M., & Borghetti, M. (2004). Variation in nitrogen supply changes water-use efficiency of *Pseudotsuga menziesii* and *Populus x euroamericana*; a comparison of three approaches to determine water-use efficiency. *Tree Physiology* 24 (6), 671–679, doi: 10.1093/treephys/24.6.671
- Salmon, Y., Barnard, R., & Buchmann, N. (2011). Ontogeny and leaf gas exchange mediate the carbon isotopic signature of herbaceous plants. *Plant, Cell & Environment* 34 (3), 465–479, doi: 10.1111/j.1365-3040.2010.02256.x
- Santesteban, L., Miranda, C., Barbarin, I., & Royo, J. (2015). Application of the measurement of the natural abundance of stable isotopes in viticulture: A review. *Australian Journal of Grape and Wine Research* 21 (2), 157–167, doi: 10.1111/ajgw.12124
- Seibt, U., Rajabi, A., Griffiths, H., & Berry, J. (2008). Carbon isotopes and water use efficiency: sense and sensitivity. *Oecologia* 155 (3), 441–454, doi: 10.1007/s00442-007-0932-7
- Souza, C. de, Maroco, J., Dos Santos, T., Rodrigues, M., Lopes, C., Pereira, J., & Chaves, M. (2005). Impact of deficit irrigation on water use efficiency and carbon isotope composition (δ C-13) of field-grown grapevines under Mediterranean climate. *Journal of Experimental Botany* 56 (418), 2163–2172, doi: 10.1093/jxb/eri216
- Taskos, D., Koundouras, S., Stamatiadis, S., Zioziou, E., Nikolaou, N., Karakioulakis, K., & Theodorou, N. (2015). Using active canopy sensors and chlorophyll meters to estimate grapevine nitrogen status and productivity. *Precision Agriculture* 16 (1), 77–98, doi: 10.1007/s11119-014-9363-8
- Terwilliger, V., Kitajima, K., Le Roux-Swarthout, D., Mulkey, S., & Wright, S. (2001). Intrinsic water-use efficiency and heterotrophic investment in tropical leaf growth of two Neotropical pioneer tree species as estimated from δ 13C values. *New Phytologist* 152 (2), 267–281, doi: 10.1046/j.0028-646X.2001.00252.x
- Tomás, M., Medrano, H., Pou, A., Escalona, J., Martorell, S., Ribas-Carbó, M., & Flexas, J. (2012). Water-use efficiency in grapevine cultivars grown under controlled conditions: effects of water stress at the leaf and whole-plant level. *Australian Journal of Grape and Wine Research* 18 (2), 164–172, doi: 10.1111/j.1755-0238.2012.00184.x
- van Leeuwen, C., Gaudillère, J., & Trégoat, O. (2001). The assessment of vine water uptake conditions by $^{13}\text{C}/^{12}\text{C}$ discrimination in grape sugar. L'évaluation du régime hydrique de la vigne à partir du rapport isotopique $^{13}\text{C}/^{12}\text{C}$. L'intérêt de sa mesure

sur les sucres du moût à maturité. *Journal International des Sciences de la Vigne et du Vin* 35 (4), 195–205

Virgona, J., Smith, J., & Holzappel, B. (2003). Scions influence apparent transpiration efficiency of *Vitis vinifera* (cv. Shiraz) rather than rootstocks. *Australian Journal of Grape and Wine Research* 9 (3), 183–185, doi: 10.1111/j.1755-0238.2003.tb00268.x

Zufferey, V., Verdenal, T., Dienes, A., Belcher, S., Lorenzini, F., Koestel, C., Rösti, J., Gindro, K., Spangenberg, J., Viret, O., & Spring, J.-L. (2018). The impact of plant water status on the gas exchange, berry composition and wine quality of Chasselas grapes in Switzerland. *OENO One* 52 (4), doi: 10.20870/oenone.2018.52.4.2181