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

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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 (ETc) and no irrigation (NI)] and three rates of ammonium nitrate [0 (N0), 60 (N60) and 120 (N120) 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 ($\delta^{13}C$) of leaf blades, stem water potential ($\Psi_s$), and leaf gas exchange were measured at berry set, bunch closure, veraison and maturity during each growing season. $\delta^{13}C$ of bulk berry must at maturity and of dormant canes was also measured. Leaf $\delta^{13}C$ and cane $\delta^{13}C$ decreased with water supply, but increased with N fertilisation. Must $\delta^{13}C$ was lower in the irrigated N0 and N60 vines, whereas irrigation treatments did not differ under the N120 rate. The relative weight of irrigation and fertilisation effect on leaf $\delta^{13}C$ varied across samplings: N application accounted for a higher proportion of leaf $\delta^{13}C$ variance during the early stages of berry growth, whereas irrigation induced higher variance of leaf $\delta^{13}C$ after veraison. The weight of the irrigation effect on cane $\delta^{13}C$ 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 $\delta^{13}C$ variation based on isotopic effects during CO2 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 CO2 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 ($\delta^{13}E$) of a material regarding a heavier isotope ($^{13}E$) 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, ($\delta^{13}E$) is expressed as per mill as follows (Dawson et al., 2002):

$$\delta^{13}E(\text{‰}) = \frac{[R_{\text{sample}} - R_{\text{standard}}]}{R_{\text{standard}}} \times 1000 \quad \text{equation 1}$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ 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 processes 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 $\delta^{13}C$ with the $C_i/C_a$ ratio as follows:

$$\delta^{13}C_p = \delta^{13}C_a - a - (b - a) \frac{C_i}{C_a} \quad \text{Equation 2}$$

Equation 2 explains the higher leaf $\delta^{13}C$ 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 $\delta^{13}C$ values have been reported for bulk dry matter (BDM) of main leaves (Souza et al., 2005; Koundouras et al., 2008; Pou et al., 2008; Behr 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 Garcia-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; Behr et al., 2016), but these relationships were not always consistent.

Apart from water availability, plant $\delta^{13}C$ may change due to variations of N availability and many studies on plant species other than grapevines, have shown that $^{13}C$ 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 $\delta^{13}C$ values of plant bulk dry matter (Livingston et al., 1999; Choi et al., 2005). Despite this extensive research, relatively few data exist for $\delta^{13}C$ 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 δ13C 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 δ13C 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 δ13C 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 (ETc) and no irrigation (NI)] and three rates of ammonium nitrate [0 (N0), 60 (N60) and 120 (N120) 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 (δ13C) 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 δ13C 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 δ13C 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 δ13C determination.

In all cases, the PDB standard was used and δ13C was calculated according to Equation 1.

3. Stem water potential

Stem water potential (Ψs) 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 Ψs. 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 δ13C and for Ψs measurements in each growth stage, net assimilation rate (An), stomatal conductance (gs), intercellular CO2 concentration (Ci) were determined in four mature sunlit leaves, adjacent to Ψs 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 Ψs measurement. Intrinsic water-use efficiency (WUEi) was estimated as the An/gs 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). Ψs decreased from the BS to MT stage, and it decreased more steeply between BC and VE (Table 1). The greater differences in Ψs 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 Ψs was observed among N treatments. Both fertilised treatments in CS and the N120 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 WUEi (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 An, gs, WUEi, Ci (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 An and leaf N was stronger in the CS vineyard than in the XM vineyard, while the opposite was observed for the Ci values (Figure 1). These relationships were better described by quadratic regression models, except for Ci 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 $\delta^{13}C$ 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 $\delta^{13}C$ than $N_0$ vines, irrespective of growth stage (Figures 2B, D). On the contrary, the irrigated vines had lower leaf $\delta^{13}C$ 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 $\delta^{13}C$ values also varied with growth stage, mainly between the BS and BC stages, when $\delta^{13}C$ 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 $\delta^{13}C$ depended on growth stage (Figure 3A, B): N application accounted for a higher proportion of leaf $\delta^{13}C$ variance at the BS and the BC stages, whereas irrigation induced higher variance of leaf $\delta^{13}C$ at the VE (only CS) and MT stages.

As in the case of the leaf carbon isotope ratio, different responses of cane $\delta^{13}C$ to irrigation and N fertilisation were observed in both vineyards: the values increased with N supply but decreased with irrigation (Table 1). Berry must $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ was higher compared to that of fertilisation in both vineyards (Figures 3C, D), and the mixed model accounted for a higher variance of $\delta^{13}C$ in the must and canes than in leaves (Figures 3C, D). The leaf $\delta^{13}C$ variance explained by the leaf N content was higher at the BS and BC stages, whereas $\Psi_s$ explained most of leaf $\delta^{13}C$ variance at the VE and MT stages (Figures 5A, B). Similar responses were observed for cane $\delta^{13}C$ variance (Figures 5E, F). Only $\Psi_s$ accounted significantly for must $\delta^{13}C$ variance, at all stages in XM (Figure 5D) and after the BC stage in CS (Figure 5C).

In both vineyards must and cane $\delta^{13}C$ values were positively correlated with the $\delta^{13}C$ 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 $\Psi_s$ 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),
TABLE 1. Effects of year, irrigation and nitrogen fertilisation on attributes of single leaf, berry juice and canes in the two vineyards. Growth stage effect was measured only for leaf attributes and the tissue effect only for carbon isotope composition.

| Effect        | Leaf          | Juice         | Cane          | Leaf          | Juice         | Cane          |
|--------------|---------------|---------------|---------------|---------------|---------------|---------------|
|              | N | δ¹³C | Ψₛ | A | gₛ | WUEᵣ | Cᵢ | δ¹³C | δ¹³C | N | δ¹³C | Ψₛ | A | gₛ | WUEᵣ | Cᵢ | δ¹³C | δ¹³C |
| Year         |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| 2009         | 2.14a | -26.87a | -0.95a | 19.7a | 1.07a | 24.0b | 256a | -25.61 | -26.95 | 2.07 | -27.49 | -0.85a | 17.9a | 0.74a | 32.8 | 247 | -24.87 | -27.12 |
| 2010         | 1.90b | -27.07b | -1.00b | 17.6b | 0.57b | 38.5a | 238b | -25.57 | -26.93 | 2.00 | -27.62 | -0.98b | 14.5b | 0.50b | 37.4 | 247 | -25.34 | -27.24 |
| Stage        |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| BS           | 2.65a | -26.39a | -0.76a | 21.4a | 0.83b | 31.7b | 243b | nd    | nd     | 2.59a | -27.32a | -0.62a | 17.6b | 0.77b | 24.7b | 265a | nd    | nd     |
| BC           | 2.13b | -27.00b | -0.78a | 20.6a | 1.25a | 21.1c | 258a | nd    | nd     | 2.04b | -27.64b | -0.74b | 19.3a | 0.93a | 25.8b | 252b | nd    | nd     |
| VE           | 1.78c | -27.33c | -1.16b | 17.5b | 0.77b | 32.4b | 245b | nd    | nd     | 1.82c | -27.74b | -1.12c | 13.6c | 0.42c | 42.3a | 237e | nd    | nd     |
| MT           | 1.53d | -27.17bc | -1.20b | 15.0c | 0.44c | 39.8a | 244b | nd    | nd     | 1.69c | -27.53ab | -1.18c | 14.3c | 0.35c | 47.6a | 234e | nd    | nd     |
| Irrigation   |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| NI           | 2.03   | -26.77a | -1.04b | 18.4  | 0.78a | 33.0  | 245  | -24.90a | -26.61a | 2.06   | -27.37a | -0.99b | 15.6b | 0.52b | 38.8a | 241b | -24.51a | -26.87a |
| I            | 2.01   | -27.18b | -0.91a | 18.9  | 0.87a | 29.5  | 249  | -26.28b | -27.27b | 1.94b   | -27.74b | -0.84a | 16.9a | 0.72a | 31.4b | 253a | -25.70b | -27.49b |
| Nitrogen     |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| N₀           | 1.86c | -27.28b | -0.96a | 18.6ab | 0.85a | 30.0  | 249  | -25.93b | -27.27b | 1.94b   | -27.78b | -0.88a | 16.0  | 0.57  | 36.8  | 246  | -25.22 | -27.33b |
| N₆₀          | 1.99b | -26.91a | -1.00  | 17.6b | 0.72a | 34.8a | 245  | -25.24a | -26.83a | 1.99b   | -27.66b | -0.92a | 15.8a | 0.64a | 33.8a | 250  | -25.19 | -27.33b |
| N₁₂₀         | 2.22a | -26.72a | -0.96a | 19.7a | 0.88a | 29.0  | 248  | -25.59ab| -26.72a | 2.17a   | -27.22a | -0.94a | 16.9a | 0.64a | 34.7a | 245  | -24.91 | -26.88a |
| Tissue       | nd    | -26.97*  | nd    | nd    | nd    | -25.59** | -26.94* | nd    | -27.55* | nd    | nd    | nd    | -25.10** | -27.18* |

N₀ - unfertilised; N₆₀ - 60 kg /ha N; N₁₂₀ - 120 kg /ha N; NI - non-irrigated; I - irrigated at 70 % of ETc; BS = berry set; BC = bunch closure; VE = veraison; MT = maturity. Total nitrogen (N, %); Carbon isotope ratio (δ¹³C, ‰); Stem water potential (Ψₛ, MPa); Net assimilation rate (A, μmol CO₂ m⁻² s⁻¹); Stomatal conductance (gₛ, mol m⁻² s⁻¹ H₂O); Intrinsic water use efficiency (WUEᵣ, μmol CO₂/mol H₂O); Intercellular CO₂ concentration (Cᵢ = mmol /mol CO₂).

Data for leaf N, Ψₛ have previously been published in Taskos et al., (2015); nd = no data

Means within factors and vineyards followed by different lowercase letters are significantly different at the 0.05 probability level according to LSD. For Tissue, means within vineyards followed by different numbers of asterisks are significantly different at the 0.05 probability level according to LSD.
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 WUEi 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 δ13C in the non-irrigated grapevines of both vineyards (Table 1) indicate that water deficit led to an enrichment of leaf BDM with 13C. This is a common response of leaf δ13C to decreasing soil water content in C3 plants, because water deficit imposes diffusional limitations on carbon intake and assimilation. In such conditions, the C/CA ratio decreases, because stomatal conductance decreases at a faster rate compared to net assimilation, leading to a lower discrimination against the 13C 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 Etg.

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 gs and An 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 δ¹³C could also explain the larger differences in leaf δ¹³C 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 δ¹³C 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 δ¹³C in grapevines does not always respond to water deficit. For example, Poni et al. (2009) did not observe any irrigation effects on δ¹³C 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 δ¹³C in leaf BDM. Indeed, δ¹³C 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 δ¹³C (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 δ¹³C 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 δ¹³C variance (Figures 5A, B) and this variance was lower compared to the size of irrigation treatment effects (Figures 3A, B). Even when leaf δ¹³C was regressed on \( \Psi_s \), using data averaged across years and growth stages, at best \( \Psi_s \) accounted for 44 % and 41 % of leaf δ¹³C variance in the CS and XM vineyard.

**FIGURE 3.** Size of effect (% variance) of irrigation and nitrogen fertilisation on carbon isotope composition (δ¹³C) 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 δ13C 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 δ13C, because both these conceptually different variables are linked to the Ci/Ca ratio. Thus, when water availability decreases and diffusional limitations to carbon uptake and assimilation appear, the C/Ca ratio decreases while both photosynthetic WUE and δ13C increase (Farquhar et al., 1989). Positive relationships between WUE, and leaf carbon isotope composition δ13C 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 δ13C and WUE, were not correlated on any time scale (data not shown). This inconsistency in the relationship of WUE, with leaf δ13C could be partly attributed to their different variation over time: WUE, 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 δ13C in leaf or plant BDM provides an estimation of the C/Ca ratio over the entire period during which the carbon forming that dry matter was fixed (Brugnoli and Farquhar, 2000). Consequently, the relationship of leaf δ13C with C/Ca 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 δ13C in leaf BDM also apply to the reported inconsistency of the relationship between leaf δ13C and on WUE,.

Variation of leaf δ13C 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 13C content from the BS up to the BC stage in XM and up to VE in CS (Table 1), and that the magnitude of δ13C 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 δ13C was measured in leaves from the basal shoot zone. However, we cannot conclusively attribute this decrease in leaf δ13C 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 (δ13C) 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 Et. Within nitrogen levels in each vineyard, different lowercase letters indicate significant differences between irrigation treatments at the 0.05 probability level.](image_url)
leaf age effects depended on water availability. It is generally difficult to disentangle ontogenetic from environmental effects on plant δ13C, which occur at the same time in field conditions (Salmon et al., 2011). We conclude that the growth stage effects on leaf δ13C in Table 1 might represent complex interactions between the vine water status variation and leaf ontogenetic changes, further complicating the interpretation of δ13C 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 δ13C 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 δ13C 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 δ13C 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 δ13C and the C/Ca 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 δ13C variations, because photosynthetic carbon isotope discrimination is sensitive to the C/Ca ratio (Seibt et al., 2008). In our study, an explanation for the higher leaf δ13C 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/Ca to decrease with increasing N availability, while WUE should increase; instead, the opposite was observed (Figures 1E, F, G, H). However, a positive linear relationship between the A/C ratio and leaf N (R² = 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 δ13C 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 13C 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 δ13C, most probably because of reduced mesophyll conductance and less likely because of increased carboxylation efficiency. In another study, the decreasing leaf δ13C 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/Ca and subsequent changes in foliar δ13C values (Meinzer et al., 1992). Finally, as already discussed, δ13C measured in plant BDM only gives an assimilation-weighted integration of the ratio C/Ca if other factors that contribute to leaf δ13C 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 δ13C 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 δ13C

Since leaf δ13C was measured in leaf BDM, leaf δ13C 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 δ¹³C 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 δ¹³C modulation by nitrogen and water availability, and whatever the extent to which leaf δ¹³C was determined by these two factors, leaf δ¹³C 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 δ¹³C 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 δ¹³C 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 δ¹³C 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 (δ¹³C) 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 $\delta^{13}C$ close to the later grapevine stages. Consequently, the increased differences in leaf $\delta^{13}C$ 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 $\delta^{13}C$ in response to the specific experimental conditions from the beginning of the growing season. These findings lead to the question of whether leaf BDM $\delta^{13}C$ can provide information - via proper sampling and experimental setup - on the interplay between water and nitrogen in modulating leaf $\delta^{13}C$ at a growth season scale. In this context, future research could focus on leaf $\delta^{13}C$ 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 $\delta^{13}C$ 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, $\delta^{13}C$ of bulk berry must at harvest correlates strongly with $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ depended on the fertiliser rate in both vineyards: while N rate increased, bulk juice $\delta^{13}C$ 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
organisms. Thus, in order to better study the effect of N supply on must δ13C, a late (e.g., at VE) N treatment would be necessary.

Relatively few data exist on cane BDM δ13C, and these are related to grapevine genotypic variation in cane δ13C 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 δ13C resemble their effects on leaf δ13C (Table 1), suggesting that leaf and cane δ13C were modulated in a similar manner within both organs. However, water availability contributed more to cane δ13C than N fertilisation, because irrigation accounted for more than double the variance of cane δ13C 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 δ13C 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 WUEi on a seasonal basis, since the average WUEi of the four samplings and cane δ13C were only positively correlated in the second year of the experiment and in the CS vineyard (R² = 0.48, p = 0.0008, n = 18, data not shown). Thus, the interpretations of cane δ13C in terms of single leaf WUEi may not be correct.

Canes are lignified shoots that develop and mature during the preceding growing season. Although green shoots can photosynthesize 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 δ13C 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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