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Research article

Carbon and nitrogen stable isotope variations in leaves of two grapevine cultivars (Chasselas and Pinot noir): Implications for ecophysiological studies

Jorge E. Spangenberg [a,*], Marc Schweizer [a], Vivian Zufferey [b]

[a] Institute of Earth Surface Dynamics (IDYST), University of Lausanne, CH-1015, Lausanne, Switzerland
[b] Institute of Plant Production Sciences (IPV), Agroscope, CH-1009, Pully, Switzerland

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ABSTRACT

We investigated the within- and between-leaf variability in the carbon and nitrogen isotope composition ($\delta^{13}C$ and $\delta^{15}N$) and total nitrogen (TN) content in two grapevine cultivars (*Vitis vinifera* cv. Chasselas and Pinot noir) field-grown under rain-fed conditions. The within-leaf variability was studied in discs sampled from base-to-tip and left and right regions from the margin to midrib. The intra- and interplant variability was studied by comparing leaves at different positions along the shoot (basal, median, apical). In leaves from both cultivars, a decrease in $\delta^{13}C$ from base to tip was observed, which is in line with an upward gradient of stomatal density and chlorophyll concentration. Less important, but still significant differences were observed between the right and left discs. The leaf TN and $\delta^{15}N$ values differed between cultivars, showed smaller variations than the $\delta^{13}C$ values, and no systematic spatial trends. The intraleaf variations in $\delta^{13}C$, $\delta^{15}N$, and TN suggest that stomatal behavior, CO_2 fixation, chlorophyll concentrations, and the chemical composition of leaf components were heterogeneous in the leaves. At the canopy scale, the apical leaves had less ^{13}C and more ^{15}N and TN than the basal leaves, indicating differences in their photosynthetic capacity and remobilizations from old, senescing leaves to younger leaves. Overall, this study demonstrates patchiness in the $\delta^{13}C$ and $\delta^{15}N$ values of grapevine leaves and species-specificity of the nitrogen assimilation and ^{15}N fractionation. These findings suggest that care must be taken not to overinterpret foliar $\delta^{13}C$ and $\delta^{15}N$ values in studies based on fragmented material as markers of physiological and biochemical responses to environmental factors.

1. Introduction

The carbon stable isotope composition ($\delta^{13}C$) of leaf dry matter from C_3 and C_4 plants primarily reflects C fractionation during photosynthetic carbon dioxide (CO_2) fixation with traces of local environmental conditions such as temperature, light irradiance, CO_2 concentration, water availability, and salinity (Farquhar et al., 1982, 1989; Lawlor and Cornic, 2002). The leaf nitrogen isotope composition ($\delta^{15}N$), on the other hand, is determined by the $\delta^{15}N$ of the nitrogen source and by various physiological and metabolic processes within the plant (Högberg, 1997; Evans, 2001). Therefore, the $\delta^{13}C$ and $\delta^{15}N$ values of plant leaves, either alone or in combination, have been extensively used to study processes in the fields of plant physiology, biochemistry, and ecology and have been used more recently to learn how plants cope with temperature and water stress in their growing environment (e.g., Sparks and Ehleringer, 1997; Robinson, 2001; Van de Water et al., 2002; Peuke et al., 2006; Tcherkez and Hodges, 2008; Craine et al., 2009; Spangenberg et al., 2020). Most of these studies assessed bulk leaf $\delta^{13}C$ variations at the inter- and intraspecies levels, sometimes in combination with $\delta^{15}N$ measurements. Interleaf and intraplant (or intracanopy) differences in $\delta^{13}C$ values in different leaves of the same species may vary by a few units (generally < 2 mUr) in response to height, age, canopy position, and heterogeneity in the leaf microenvironment, such as nonuniform distributions of CO_2 and H_2O concentrations, temperature and light (e.g., Leavitt and Long, 1986; Zimmerman and Ehleringer, 1990; Turney et al., 2002; He et al., 2008; Vitória et al., 2016). Within-leaf ^{13}C

Abbreviations: $\delta^{13}C$, carbon stable isotope composition (mUr); $\delta^{15}N$, nitrogen isotope composition (mUr); EA/IRMS, elemental analysis/isotope ratio mass spectrometry; TOC, total organic carbon (wt.%); TN, total nitrogen (wt.%).

* Corresponding author.
E-mail address: Jorge.Spangenberg@unil.ch (J.E. Spangenberg).

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discrimination is less studied than whole-leaf ^{13}C discrimination. The few studies using particular leaf regions (i.e., top, middle, bottom) or longitudinal segments along the leaf venation have shown small intraleaf variations in $\delta^{13}C$ values in both C_3 and C_4 plants (Affek et al., 2006; Kodama et al., 2011; Meinzer and Saliendra, 1997; Gao et al., 2015; Lightfoot et al., 2016; Schleser, 1990). The measured intraleaf $\delta^{13}C$ differences were generally < 1 mUr, and not higher than the intraplant ranges of whole-leaf $\delta^{13}C$ values. However, the nonuniform ^{13}C discrimination within leaf blades may have important implications for environmental physiology. It may be more critical when $\delta^{13}C$ values of fossil leaves are used to reconstruct ancient atmospheric CO_2 concentrations and $^{13}C/^{12}C$ isotope ratios. Most of these studies had not had access to well-preserved leaves, and the $\delta^{13}C$ data derived from fragmented material (e.g., Beerling et al., 1993; Van de Water et al., 1994; Beerling and Jolley, 1998; Noble et al., 2016). A nonuniform within leaf $\delta^{13}C$ pattern may lead to biased estimates of the atmospheric CO_2 concentrations and related (paleo)physiological and (paleo)environmental parameters.

The $\delta^{15}N$ values of plant materials are more complicated, much less understood and more rarely used than their carbon isotope compositions. The total nitrogen (TN) content in leaves depends on the relative contributions of newly absorbed N and remobilized N from plant reserves, including older leaves, woody tissue, and roots (Evans, 2001). Therefore, the isotopic composition of the N sources (NH_4^+, NO_3^-, organic N) and the ^{15}N vs. ^{14}N fractionation associated with the internal transformations during uptake, translocation, assimilation, remobilization, and reallocation during plant development determine the leaf $\delta^{15}N$ values (e.g., Högberg, 1997; Robinson et al., 2000; Kolb and Evans, 2002). The $\delta^{15}N$ values of the leaf TN may reflect how these factors affect the nitrogen metabolism in plants. Furthermore, critical environmental factors such as water availability and temperature, which influence N mineralization, NH_3 volatilization, and denitrification processes, may change the $\delta^{15}N$ of the source N in soil solutions (Högberg, 1997). Therefore, interleaf variation in the $\delta^{15}N$ values within and among plants may be indicators of heterogeneity in local environmental conditions, including the soil nutrient availability during leaf growth. In-plant leaves, the N isotope ratios will vary with the $\delta^{15}N$ values of the N in the soil solution that is available for uptake by the plants, the relative contribution of nitrogen reduction by roots or leaves, and the reallocation of assimilated nitrogen from roots or other organs, including older leaves (e.g., Evans, 2001; Tcherkez and Hodges, 2008; Tcherkez, 2011). Multiple nitrogen assimilation, loss, resorption, and reallocation events may cause intraleaf variations in the N content and isotope composition (Evans, 2001).

Very few studies have reported on the combined variations in intraplant values of both $\delta^{13}C$ and $\delta^{15}N$ in leaves of different ages and in different plant development stages; these studies have generally been performed in different geographical settings and under different environmental conditions (Werth et al., 2015; Vitória et al., 2018; Li et al., 2019). To the best of our knowledge, no published studies have reported on intraleaf $\delta^{15}N$ patterns. We recently recorded shifts in interplant $\delta^{13}C$ and $\delta^{15}N$ values and epicuticular lipids in leaves from the same position in the canopy, i.e., the median zone, in two grapevines (*Vitis vinifera* L. cvs. Chasselas and Pinot noir) of the same age grown in the field under the same environmental conditions (i.e., CO_2 concentration, soil type, N source, temperature, irradiance) but under different water treatments (Spangenberg et al., 2020). We demonstrated that the leaf $\delta^{13}C$ and $\delta^{15}N$ values and the total epicuticular fatty acid concentrations reflect the plant water availability over a time period and can therefore be used as indicators of early water-stress in vineyards.

This study aimed to explore potential spatial variations in the total organic carbon and nitrogen concentrations (TOC and TN in wt.%) and the stable isotope ratios of C and N within leaves and within plants in rain-fed Chasselas and Pinot noir grapevines. The specific objectives were to assess the leaf TOC, TN, $\delta^{13}C$, and $\delta^{15}N$ patterns along (1) the intraleaf longitudinal direction, from base to tip; (2) the intraleaf transverse direction, from midrib to margin; and (3) the interleaf and intraplant directions at different within-shoot positions (i.e., in the basal, median, and apical leaves). The results provide chemical data regarding the compositional patterns and related metabolic processes within individual leaves and between leaves from different positions in the plant canopy. We discussed the possible mechanisms explaining the spatial patchiness of the leaf $\delta^{13}C$ and $\delta^{15}N$ values. The results will shed light on the species specificity of the intra- and interleaf variabilities in grapevines.

2. Materials and methods

2.1. Plant material and leaf samples

The leaf samples were obtained from plants of the grapevine cultivars Chasselas (white grape) and Pinot noir (red grape) in the 2014 growing season at the experimental station of the Swiss Institute of Plant Production Sciences (Agroscope) at Leytron (46°11′N; 7°12′E, 525 m above sea level, canton of Valais, Switzerland) (Zufferey et al., 2017, 2018). Chasselas (clone 14/33-4) and Pinot noir (clone 9–18) shoots were grafted onto *Vitis berlandieri x Vitis riparia* cv. Kober 5BB rootstock. The plants were 20 years old at the time of sampling. The vines were planted in the Guyot training system and pruned to six shoots per plant (Zufferey et al., 2017). The plants were grown under rain-fed field conditions and experienced the same soil water availability, soil nitrogen levels, light, and temperature. Leaves were collected from each cultivar between 10:00–15:00 h on a sunny day during the flowering phenological stage (20 June 2014, day 171 of the year, DOY 171), when the new leaves were fully expanded and autotrophic. The gas exchange parameters, including the net photosynthetic rate (A, in μmol CO_2 m^{-2} s^{-1}), transpiration rate (E, in mmol H_2O m^{-2} s^{-1}), stomatal conductance (g_s, in mmol CO_2 m^{-2} s^{-1}), and mesophyll resistance (r_m, in bar mol^{-1}) and the predawn leaf water potential (Ψ_{pd}, in MPa) were measured on DOY 166 and 169, respectively, in fully expanded and well-exposed leaves in the median part of the shoot as described in Spangenberg et al. (2020). The results of these measurements are provided in Supplementary Table S1. The soil nitrogen, which existed predominantly in the form of nitrate or ammonia, was the only N source for the plants; the soil TN content ranged between 0.08 and 0.15 wt%, and the $\delta^{15}N$ values ranged between 3.62 and 4.92 mUr (Spangenberg et al., 2020).

The sampled leaves were undamaged and visibly healthy, showing no surface debris or apparent signs of degradation. We collected three leaves from the median shoot zone of one randomly chosen plant per cultivar to study the intraleaf patterns of the $\delta^{13}C$, TN, and $\delta^{15}N$ values (Fig. 1A). The intraplant leaf $\delta^{13}C$, TN, and $\delta^{15}N$ variations were studied in composite leaf samples of four leaves collected at three different positions along the shoot (basal, median, apical) in order to include leaves of different ages and development stages: mature (55–60 days old), intermediate (40–45 days old), and young (25–30 days old) leaves (Fig. 1B). The leaf initiation rate (leaf appearance) and developmental age were determined by using the plastochron index (Erickson and Michelini, 1957). The length of the reference leaf blade in grapevine was chosen at 30 mm (Schultz, 1992). The number of days between leaf appearance (blade approximately 30 mm long) and flowering (DOY 170) corresponded to the leaf age. The intraplant sampling was replicated for three randomly chosen vines per cultivar. Leaf sampling was performed by cutting the base of the petiole using scalpel and forceps cleaned with an organic solvent. The leaves were briefly rinsed in tap water, any dust and adhering materials were removed with quartz wool (preheated at 500 °C for >4 h) and the leaves were then rinsed with deionized water (DIW) and Milli-Q water (MQW, DIW purified with a Direct-Q UV 3 Millipore® System, Merck, Darmstadt, Germany). The cleaned leaves were carefully flattened, wrapped in preheated aluminum foil and stored in a chilled icebox before being transported to the UNIL-IDYST (Institute of Earth Surface Dynamics of the University of Lausanne) laboratories. In the laboratory, the leaves were stored

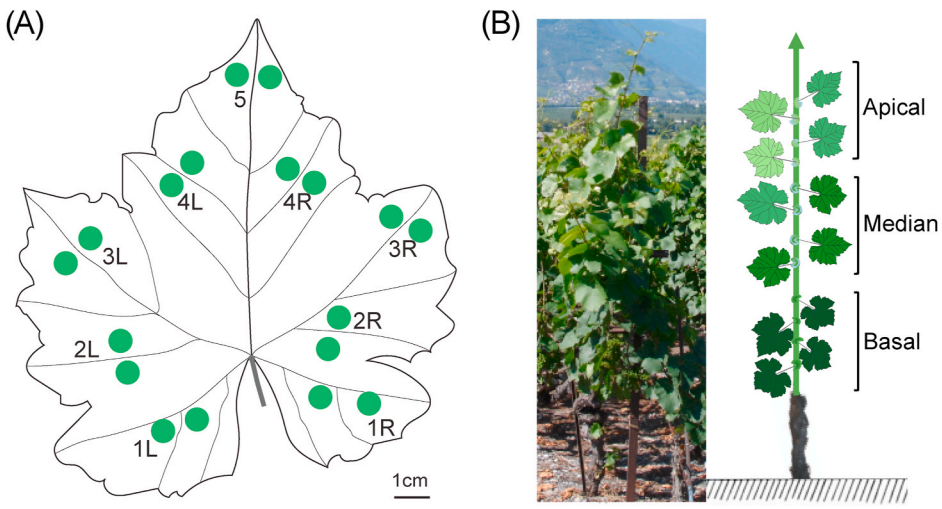

Fig. 1. Studied leaf material from grapevines cultivars Chasselas and Pinot noir. (A) Leaf discs were sampled between the secondary venations between the base and tip and between the midrib and margin on the left and right of the midrib (L, R). The sample sites are numbered from the base upwards. The upper L and R discs were combined to have sufficient analysis material. (B) Whole leaves of different age and development stages were sampled at three different position along the shoot: basal, median, and apical. The intraplant sampling was replicated for three randomly chosen vines per cultivar.

horizontally at +4 °C for within-leaf subsampling within 48 h of field sampling and at −20 °C before being prepared for the intraplant variability study.

2.2. Sample preparation

For the intraleaf study, discs from nine regions of the leaf, located between the secondary veins between the base and the tip and between the midrib and margin on both sides of the midrib, were excised using a perforator with a 10 mm internal diameter (Fig. 1A). The uppermost left and right discs near the tip were combined in order to provide enough material for the replicate carbon and nitrogen isotope analysis. The material from each leaf region was placed in a glass vial, freeze-dried, and ground to a fine powder under liquid nitrogen in a cleaned agate mortar. For the intraplant study, the frozen leaves were freeze-dried, cut into small pieces with scissors and forceps cleaned with organic solvent, and ground to a fine powder. The powders from the four leaves per canopy zone were combined and homogenized to produce the composite basal, median and apical samples for each plant (Fig. 1B). The leaf powders were stored in borosilicate vials with screw caps at −20 °C before analysis.

2.3. Bulk stable carbon and nitrogen isotope analysis

The TOC and TN concentrations, and the carbon and nitrogen isotope compositions ($\delta^{13}C$, $\delta^{15}N$) of the powdered leaf samples were determined by elemental analysis/isotope ratio mass spectrometry (EA/IRMS), using a Carlo Erba 1108 (Fisons Instruments, Milan, Italy) elemental analyzer connected via a ConFlo III split interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated under continuous helium (He) flow (Spangenberg and Zufferey, 2018). The C and N isotope analyses were performed separately with EA/IRMS using aliquots of different weights (the aliquot size for $\delta^{15}N$ was approximately × 50 bigger than that for $\delta^{13}C$). The stable isotope compositions were reported in the delta (δ) notation as variations in the molar ratio (R) of the heavy isotope to light isotope of the element E ($^{h}E/^{l}E$, i.e., $^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample relative to an international standard:

$$\delta^{h}E_{sample/standard} = \frac{R_{sample}}{R_{standard}} - 1$$

The standard for $\delta^{13}C$ is the Vienna Pee Dee Belemnite limestone (VPDB), and that for $\delta^{15}N$ is the molecular nitrogen in air (Air-N_2). We used the Urey unit (Ur) for the delta values, as recommended by the International Union of Pure and Applied Chemistry (IUPAC); one milliUrey (mUr) is equivalent to one per mil (‰) (Brand, 2011). For the calibration and normalization of the measured isotopic ratios to the international scales (the VPDB-LSPVEC lithium carbonate scale for $\delta^{13}C$, the Air-N_2 scale for $\delta^{15}N$), a four-point (for $\delta^{13}C$) and three-point (for $\delta^{15}N$) calibrations were used with international reference materials (RMs) and UNIL in-house standards (details in Spangenberg and Zufferey, 2019). The quality (i.e., repeatability, intermediate precision, and accuracy) of the EA/IRMS analyses was assessed by separately replicate analyses (n = 3–6) of the leaf samples and RMs; the standard deviations were smaller than 0.05 and 0.1 mUr for $\delta^{13}C$ and $\delta^{15}N$, respectively. The TOC and TN contents (in wt.%) were determined from the EA/IRMS peak areas, with a repeatability better than 0.1 wt%.

2.4. Statistical analysis and data presentation

Statistical analysis was performed using the SPSS software package V25.0 (IBM SPSSInc., Chicago, IL, USA). The values reported in the text are the mean ± standard deviation (SD) for replicate (n) analysis or mean ± standard error (SE) for biologically independent replicates. Graphics were prepared using DeltaGraph (version 7.1.3, Red Rock Software Inc., Salt Lake City, UT, USA) and Adobe Illustrator 202 (version 24.0.3, Adobe Systems Inc., CA, USA). The data were tested for homogeneity of variance (F-test), and the comparisons between the means of each group were performed by paired-samples Student's t-tests with the significance level set at $P < 0.05$.

3. Results

3.1. Intra-leaf variations in TOC contents and $\delta^{13}C$ values

The TOC concentration (wt.% TOC) of the leaf discs ranged from 41.22 to 44.76 wt% (with a mean ± SD of 42.82 ± 0.77 wt%, n = 27) for Chasselas and from 40.78 to 43.55 wt% (42.13 ± 0.66 wt%, n = 27) for Pinot noir. The intraleaf $\delta^{13}C$ values covered a similar range for both cultivars: 28.85 to −26.68 mUr (−27.21 ± 0.44 mUr, n = 27) for Chasselas leaves and −28.34 to −26.68 mUr (−28.05 ± 0.47 mUr, n = 27) for Pinot noir leaves (Fig. 2 and Supplementaty Table S2). These C isotope ratios are typical for C_3 plants, which fix atmospheric CO_2 via the Calvin-Benson pathway and produce organic compounds with $\delta^{13}C$ values between −37 and −20 mUr (Kohn, 2010). The lower $\delta^{13}C$ values occurred in the top region of the leaves (the tip), and higher values occurred in the base region; the $\Delta^{13}C_{B/T} = \delta^{13}C_{Base} - \delta^{13}C_{Tip}$ values were between 0.02 and 1.20 mUr for both sides (left and right from midrib to margin) in Chasselas (0.52 ± 0.42 mUr) and Pinot noir (0.73 ± 0.30 mUr) leaves. These base-to-tip differences were not significant at the

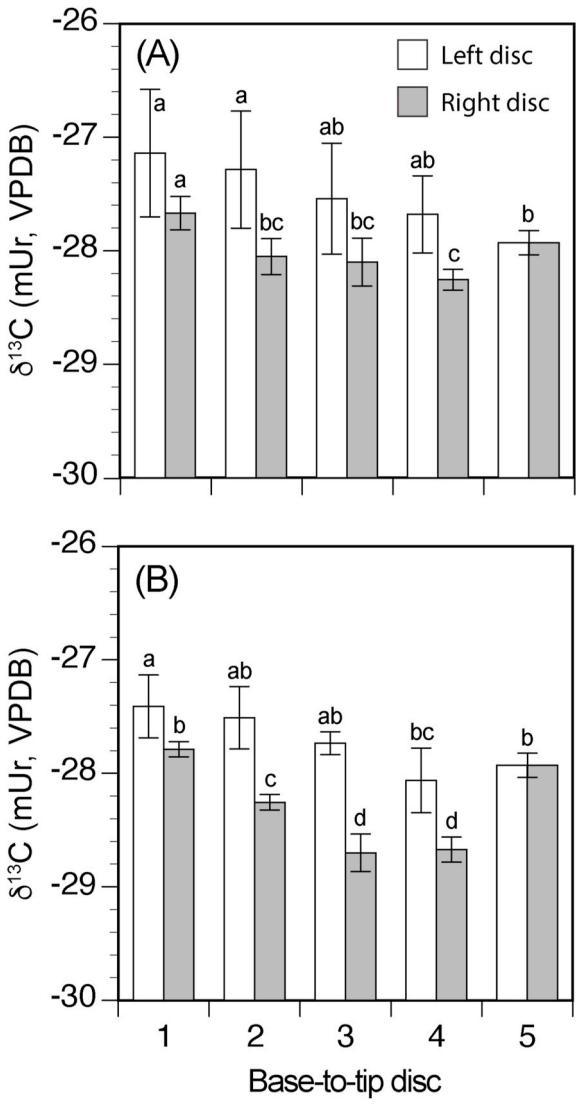

Fig. 2. Within-leaf variability in the carbon isotope composition ($\delta^{13}C$ in mUr vs. VPDB) in Chasselas (A) and Pinot noir (B) grapevines. The sampling pattern is shown in Fig. 1A. The error bars represent the standard error (SE) of the mean from three biological replicates. Columns with different lower-case letters are significantly different according to Student's *t*-test ($P < 0.05$).

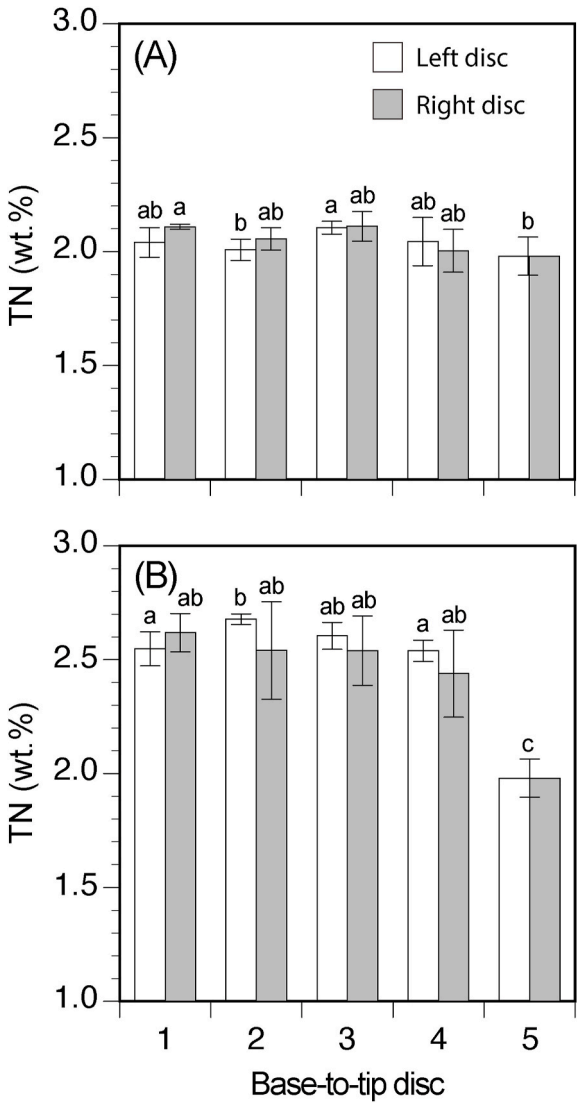

Fig. 3. Within-leaf variability in the total nitrogen content (TN in wt.%) in Chasselas (A) and Pinot noir (B) grapevines. The sampling pattern is shown in Fig. 1A. The error bars represent the standard error (SE) of the mean from three biological replicates. Columns with different lower-case letters are significantly different according to Student's *t*-test ($P < 0.05$).

95% confidence level (Fig. 2). We observed differences in the $\delta^{13}C$ values in the transverse direction, between the corresponding left and right discs at the same longitudinal position from the midrib (discs one to four, L and R sides, Fig. 2). Generally, the right discs had lower $\delta^{13}C$ values than the left disks. These differences ($\Delta^{13}C_{L/R} = \delta^{13}C_{Left} - \delta^{13}C_{Right}$) ranged from −0.03 to 1.12 mUr (0.61 ± 0.34 mUr, $n = 12$) for Chasselas and from 0.17 to 1.20 mUr (0.67 ± 0.31 mUr, $n = 12$) for Pinot noir, and were generally significant at a confidence interval of 95% in the middle to upper leaf regions (i.e., discs two to four) (Fig. 2 and Supplementary Table S2).

3.2. Intraleaf variations in TN contents and $\delta^{15}N$ values

Chasselas leaves contained less nitrogen than Pinot noir leaves (2.05 ± 0.07 and 2.57 ± 0.11 wt%, respectively, $P < 0.05$, $n = 27$) (Fig. 3 and Supplementary Table S2). The TN content was relatively uniform within leaves in the longitudinal and transverse directions, except in the uppermost (tip) discs of the Pinot noir leaves, which had significantly less TN than the discs from the middle and base of the leaves (Fig. 3). The discs from the Chasselas leaves had average $\delta^{15}N$ values ≈ 0.8 mUr higher than those for Pinot noir (3.17 ± 0.36 and 2.32 ± 0.32 mUr, respectively, $P < 0.05$, $n = 27$) (Fig. 4 and Supplementary Table S2). The within- leaf ranges of the $\delta^{15}N$ values reached 1.54 mUr for Chasselas, and 1.14 mUr for Pinot noir. For the Chasselas leaves, the longitudinal base-to-tip difference in $\delta^{15}N$ values ($\Delta^{15}N_{B/T} = \delta^{15}N_{Base} - \delta^{115}N_{Tip}$) ranged between −0.60 and 0.35 mUr (−0.11 ± 0.39 mUr, $n = 6$); in the transverse direction, the difference between the $\delta^{15}N$ values of the left and right discs ($\Delta^{15}N_{L/R} = \delta^{15}N_{Left} - \delta^{15}N_{Right}$) ranged between −1.00 and 0.50 mUr (−0.06 ± 0.45 mUr, $n = 12$). On average, there were no significant trends of the disc $\delta^{15}N$ values in either the longitudinal or transverse direction (Fig. 4). For the Pinot noir leaves, the $\Delta^{15}N_{B/T}$ values ranged between −1.09 and 0.18 mUr (−0.44 ± 0.54 mUr, $n = 6$), and the $\Delta^{15}N_{L/R}$ values ranged between −1.14 and 0.61 mUr (−0.22 ± 0.53 mUr, $n = 12$). Overall, some of the differences between disc $\delta^{15}N$ values in the longitudinal and transverse directions were significant at $P < 0.05$; the tip discs had significantly higher $\delta^{15}N$ values than the discs from the middle and base of the leaves (Fig. 4).

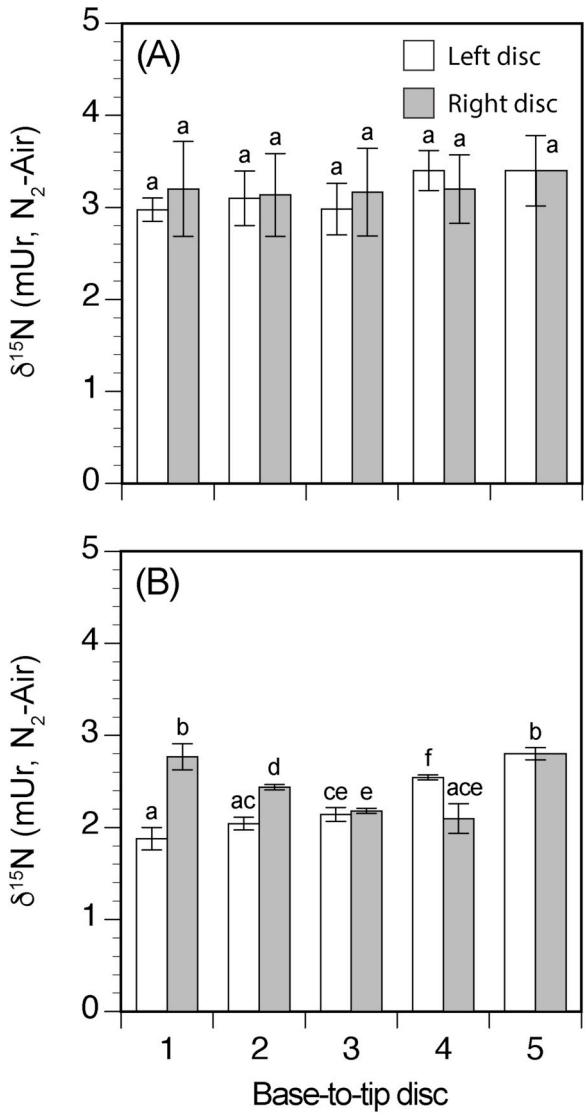

Fig. 4. Within-leaf variability in the nitrogen isotope composition ($\delta^{15}N$ in mUr vs. Air-N_2) in Chasselas (A) and Pinot noir (B) grapevines. The sampling pattern is shown in Fig. 1A. The error bars represent the standard error (SE) of the mean from three biological replicates. Columns not sharing a lower-case letter are significantly different according to Student's *t*-test ($P < 0.05$).

3.3. Intra- and interplant variations in leaf $\delta^{13}C$ values

Significant intraplant and interplant variations were observed in the $\delta^{13}C$ values of the composite samples from different positions in the canopy (i.e., the basal, median, and apical shoot zones) in the three plant replicates. The intraplant variations were estimated as the difference between the $\delta^{13}C$ values of the old basal and young apical leaves in the same plant ($\Delta^{13}C_{Basal/Apical} = \delta^{13}C_{Basal} - \delta^{13}C_{Apical}$) (Fig. 5A, D and Supplementary Table S3). In both cultivars, the apical leaves had nonsignificant lower average $\delta^{13}C$ values than the basal leaves, but significant in single pants. The $\Delta^{13}C_{Basal/Apical}$ ranged from 0.36 to 0.79 mUr (average ± SE, 0.67 ± 0.15 mUr, $n = 3$) for Chasselas and from 0.51 to 1.17 mUr for Pinot noir (0.78 ± 0.20 mUr, $n = 3$). The SDs of the replicate $\delta^{13}C$ measurements were small (<0.23 mUr, Supplementary Table S3), indicating that the within plant $\Delta^{13}C_{Basal/Apical}$ was significant at $P < 0.05$. These intraplant variations in grapevine are well within the range of a few mUr (<2 mUr) reported in the literature, mainly for forest trees (e.g., He et al., 2008; Vitória et al., 2016). We used the differences in the $\delta^{13}C$ values of the basal, median, and apical leaves between replicate plants to estimate the interplant patterns (Fig. 5A, D, and Supplementary Table S3). For both cultivars, higher average $\delta^{13}C$ values with less variation were in the old basal leaves (average ± SE, $n = 3$, −26.63 ± 0.62 mUr for Chasselas, −25.89 ± 0.34 mUr for Pinot noir), and lower $\delta^{13}C$ values with greater variation were observed in the young apical leaves (−27.29 ± 0.74 mUr for Chasselas, −26.67 ± 0.52 mUr for Pinot noir). The median leaves had intermediate $\delta^{13}C$ values (−26.75 ± 0.66 mUr for Chasselas, −26.48 ± 0.31 mUr).

3.4. Intra- and interplant variations in leaf TN content and $\delta^{15}N$ values

The intra- and interplant variations in leaf $\delta^{15}N$ values showed the opposite trend to the variations in the $\delta^{13}C$ (Fig. 5B, E, C, D, and Supplementary Table S3). For both cultivars, the basal leaves had lower TN levels and lower $\delta^{15}N$ values (generally significant at $P < 0.05$) than apical leaves. The within-plant TN values (i.e., $\Delta TN_{Basal/Apical} = TN_{Basal} - TN_{Apical}$) ranged between −0.52 and −0.13 wt% (−0.31 ± 0.11 wt%, $n = 3$) for Chasselas, and between −0.72 and −0.54 wt% (−0.64 ± 0.05 wt %, $n = 3$) for Pinot noir (Fig. 5B, E). The $\delta^{15}N$ values in the basal leaves were significantly lower than those in the apical leaves, with $\Delta^{15}N_{Basal/Apical}$ ($\delta^{15}N_{Basal} - \delta^{15}N_{Apical}$) values ranging between −1.29 and −0.90 mUr range (−1.07 ± 0.12 mUr, $n = 3$) for Chasselas and −2.15 and −1.10 mUr (−1.78 ± 0.34 mUr, $n = 3$) for Pinot noir (Fig. 5C, F). The interplant variations in the TN content were higher in basal (by up to 0.50 wt% for Chasselas, 0.63 wt% for Pinot noir) than apical leaves (by up to 0.25 wt% for Chasselas, 0.36 wt% for Pinot noir). The $\delta^{15}N$ showed the opposite trend, with lower interplant variations in basal (by up to 0.60 mUr for Chasselas, 0.70 mUr for Pinot noir) than in apical leaves (by up to 0.89 mUr for Chasselas, 1.29 mUr for Pinot noir). In the Chasselas leaves, there was a significant correlation between TN and TOC values ($r = 0.52$, $P < 0.001$, $n = 27$), but no other significant relationships were found between leaf composition parameters (data not shown).

4. Discussion

4.1. Intraleaf heterogeneity in ^{13}C discrimination

The two grapevine cultivars analyzed (*Vitis vinifera* cvs. Chasselas and Pinot noir) showed differences in within-leaf carbon isotope composition in both the longitudinal direction between the leaf base and the tip ($\Delta^{13}C_{B/T} < 1.20$ mUr), and the transverse direction, on the left and right side of the leaf from the margin to the midrib ($\Delta^{13}C_{L/R} < 1.24$ mUr). Previous studies have reported ^{13}C discrimination increasing ($\delta^{13}C$ values becoming more negative) from base to tip in the leaves of C_3 and C_4 plants. The studied C_3 plants include beech (*Fagus sylvatica*, $\Delta^{13}C_{B/T} < 0.3$ mUr, Schleser, 1990) and cotton (*Gossypium hirsutum*, $\Delta^{13}C_{B/T} < 0.6$ mUr, Farquhar and Gan, 2003). The studied C_4 plants include corn (*Zea mays*, $\Delta^{13}C_{B/T} = 1.1$ mUr by Sasakawa et al., 1989; 0.4 mUr, Affek et al., 2006), sugarcane (*Saccharum* ssp., 0.6 mUr, Meinzner and Saliendra 1997), and millet (*Setaria italica*, 2.1 mUr, Lightfoot et al., 2016). Intraleaf variations in $\delta^{13}C$ values were also reported from fossil leaves. In fossil leaves from the early–middle Eocene (57–36 million years ago, Ma), Messel Pit in Germany intraleaf $\delta^{13}C$ differences were measured within the basal, central, and apical regions and averaged 2.2 mUr for *Laurophyllum lanigeroides* leaves, and 1.4 mUr for *Rhodomyrtophyllum sinuatum* leaves (Grein et al., 2010). These authors also showed intraleaf variability in the leaves of extant plant families, averaging 1.2 mUr for Lauraceae and 1.4 mUr for Myrtaceae. Apical regions generally had lower $\delta^{13}C$ values than basal regions. The base of a fossil *Dictyozamites* sp. leaf recovered from the ca. 110 Ma (Early Cretaceous) sediments of the Bhuj Formation in western India had a $\delta^{13}C$ value 1 mUr higher than the top (Chakraborty et al., 2011).

Environmental factors and the nutritional status of the cells, including heterogeneity in irradiance and light absorption, stomatal conductance, enzyme levels, water availability, and salinity, may induce

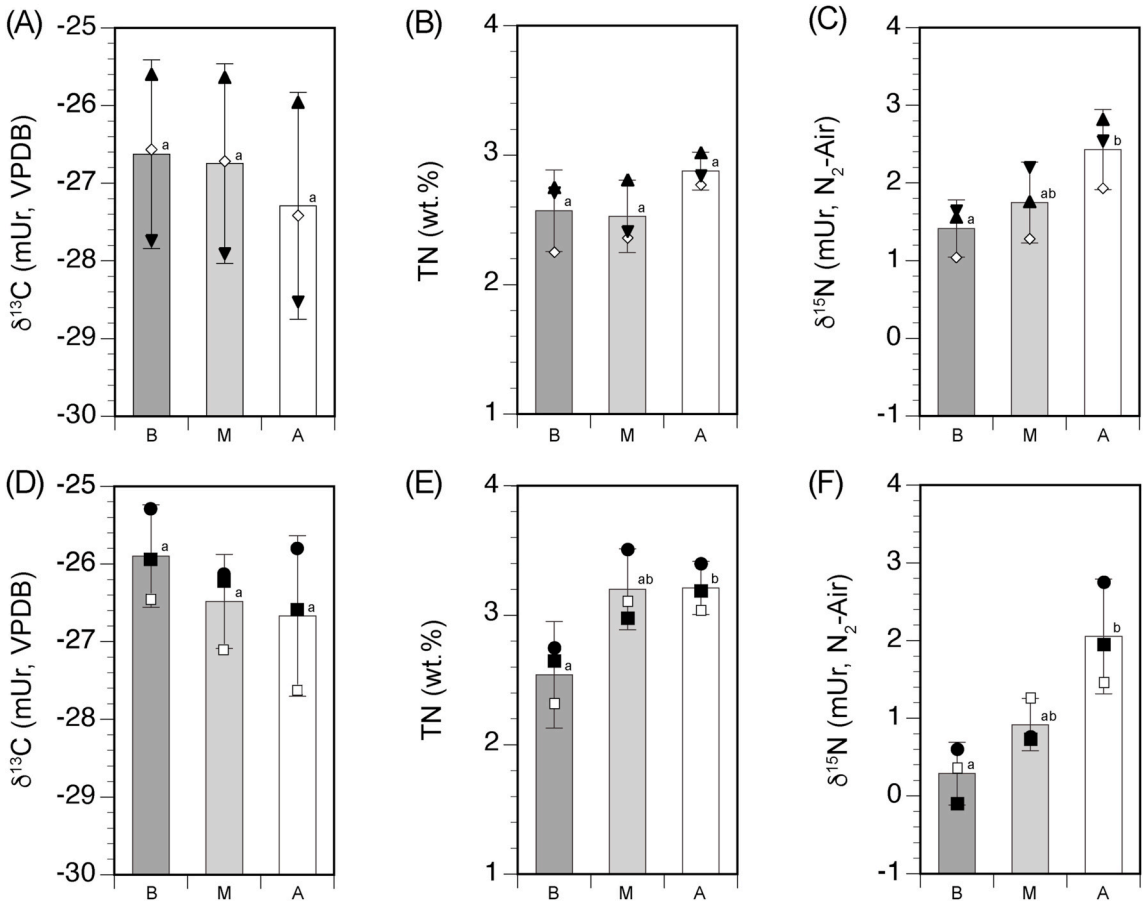

Fig. 5. Intra- and interplant variability in the isotopic composition ($\delta^{13}C$, $\delta^{15}N$) and nitrogen content in leaves of Chasselas (upper plots) and Pinot noir (lower plots) grapevines. Composite leaf samples from individual plants were obtained by grinding and homogenizing four leaves from the basal, median, and apical shoot zones. This was replicated for three randomly chosen plants per cultivar in the experimental blocks. Columns and bars represent mean ± standard error (SE) of the mean of the three biological replicates. The different symbols correspond to the plant replicates. Columns with different lower-case letters are significantly different according to Student's *t*-test ($P < 0.05$).

spatial variability in the within-leaf carbon isotope composition. Stomatal aperture is critical in the control of leaf transpiration and photosynthesis. The stomatal conductance (g_s) and intercellular CO_2 concentration (c_i) are higher in the apical regions of leaves than in the basal regions (Farquhar and Gan, 2003; Affek et al., 2006; Nardini et al., 2008), leading to a base-to-tip increase in photosynthetic activity and carbon isotope discrimination ($\Delta^{13}C$). Therefore, the longitudinal base-to-tip ^{13}C-depletion in leaves could be explained by the increasing g_s. Additionally, the temporal and spatial progression in c_i and photosynthetic activity during leaf development and senescence may also contribute to a base-base-to-tip decrease in $\delta^{13}C$ values (increasing $\Delta^{13}C$), as the longitudinal axis is the dominant growth direction of the leaf (Affek et al., 2006).

The mechanistic reason for the transverse variability in $\delta^{13}C$ values between the left and right leaf regions from margin to midrib remains unexplained. The variability may be related to nonuniform stomatal closure and chlorophyll distribution within leaves as well as to spatial heterogeneities in the leaf chemical composition. The nonuniform distribution of g_S and the distinctive behavior of small groups of stomata, often referred to as "stomatal patchiness", lead to nonuniform gas exchange and photosynthetic rates over the leaf surface (Terashima, 1992; Weyers and Lawson, 1997; Mott and Buckley, 2000). Stomatal patchiness in grapevine leaves was first described by Düring and Stoll (1996). When heterogeneous stomatal opening occurs, c_i and photosynthetic assimilation become nonuniform, which would lead to intraleaf variations in the $\Delta^{13}C$ and $\delta^{13}C$ value (Farquhar et al., 1989). Under similar source CO_2, humidity, and leaf nitrogen conditions, the stomatal limitation on photosynthesis increases with increasing irradiance, i.e., c_i decreases with increasing light level, resulting in higher $\delta^{13}C$ values (Ehleringer et al. 1986). The $\delta^{13}C$ values in the leaves of the Panamanian orchid *Catasetum viridiflavum* increased by up to 4 mUr under different light levels (Zimmerman and Ehleringer, 1990). Spatial changes in the amount of irradiance on the leaf blade may cause stomatal patchiness (Eckstein et al., 1996; Düring and Loveys, 1996), nonuniform carbon assimilation rates, and spatial variations in $\Delta^{13}C$ (Meinzer and Saliendra, 1997). Additionally, the high g_s in the apical leaf regions can increase the photosynthetic efficiency of sunflecks on leaf surfaces. In fact, triggered by fluctuating light pulses, the highly hysteretic g_s response to sunflecks (i.e., stomatal closing and opening rates that are not in phase with the light pulse) could account for a significant (additive) positive impact on carbon assimilation during successive sunflecks in plant canopies (Zimmerman and Ehleringer, 1990; Pearcy and Sims, 1994; Pieruschka et al., 2010). In this experiment, the grapevine leaves were fully expanded and sun-exposed; however, small intra-leaf differences in light level (see Fig. 1B), and microhabitat environmental variability (i.e., temperature, humidity, CO_2 partial pressure) should not be overlooked. The effects of the factors that influence intraleaf heterogeneity, particularly stomatal patchiness, may be accentuated in plants under water and/or salinity stress (Sharkey and Seemann, 1989, Haefner et al., 1997; Guàrdia et al., 2012). Specifically, for grapevines, it was shown that patchy stomatal behavior reduced the uptake of CO_2 into the leaf in water- and salt-stressed plants (Downton et al., 1988, 1990; Düring and Stoll, 1996). The studied grapevine leaves came from non-water-stressed plants ($\Psi_{pd} = -0.11 \pm 0.02$ MPa for Chasselas, $\Psi_{pd} = -0.09 \pm 0.02$ MPa

for Pinot noir, Supplementary Table S1); however, patchy stomatal conductance that induced a patchy photosynthetic assimilation pattern may be the best explanation for the observed within-leaf longitudinal and transversal heterogeneity of the $\delta^{13}C$ values in the Chasselas and Pinot noir vines.

Finally, the highly compartmentalized leaf blades of grapevine, like those of most woody C_3 plants, have a heterogeneous structure that comprises three different main tissues. These tissues are composed of layers of cells (i.e., palisade and spongy mesophyll cells, vascular tissue in the midrib and secondary veins, and epidermis guard cells) that differ in shapes, cell wall architecture and composition. Cell molecular components, including soluble sugars from current photosynthates, carbohydrates (cellulose, hemicellulose, starch), lignins (polyphenols), pigments, proteins, and lipids, are assembled in different cell types and provide different mechanical properties and physiological functions within (e.g., the rigidity of vein xylem and phloem vs. the elasticity of spongy mesophylls) and between leaves (e.g., guard cells control the size of stomata in sun vs. shade leaves; vascular tissues provide pathways for water and nutrients). Some variability in the $\delta^{13}C$ values of the sampled leaf discs may be accounted for by the different contribution of carbon from the cell molecular components, which have different carbon isotope compositions. Plant metabolites have different $^{13}C/^{12}C$ ratios due to the different postphotosynthetic ^{13}C discrimination patterns that occur during their biosynthesis and the different carbon sources used during heterotrophic metabolism; cellulose and starch were shown to have 3 mUr more ^{13}C than photosynthate and lipids had 8 mUr less ^{13}C, while lignin phenols had 3.5 mUr less ^{13}C than cellulose carbohydrates (e.g., Benner et al., 1987; Hobbie and Werner, 2004; Gilbert et al., 2012). Additionally, the leaf epidermis secretes epicuticular lipids, which help grapevine plants to retain water, and have an isotopic composition that depends on plant development stage, water status, and leaf age (Spangenberg et al., 2020).

4.2. Intra- and interplant patterns of leaf $\delta^{13}C$ values

The intraplant variability in the ^{13}C discrimination among developing leaves may reflect differences in the contribution of carbon translocated from older leaves to younger leaves and that of carbon supplied by plant assimilates, as was discussed for C_3 plants (e.g., wheat, Condon et al., 1992; potato, Jefferies and MacKerron 1997; green tea, Liu et al., 2019; oil palm, Lamade et al., 2009). The observed $\Delta^{13}C_{Basal/Apical}$ in fully expanded grapevine leaves that were well exposed to sunlight at different positions along the shoot can be attributed mainly to a difference in age and the various biosynthetic and translocation processes of compounds in leaves. The $\Delta^{13}C_{Basal/Apical}$ values were significantly higher than zero for both grapevine cultivar (on average 0.67 mUr for Chasselas, 0.78 mUr for Pinot noir). This observation reflects the greater capacity for CO_2 assimilation, carbon fixation, and respiration and the consequently higher ^{13}C discrimination in intact photosynthetic tissues within the young (autotrophic) apical leaves than in those within the old (senescing) basal leaves. This outcome is in line with a previous study reporting greater photosynthetic and photorespiration capacities of young adult leaves than old leaves in plants of grapevine cvs. Riesling and Chasselas (Zufferey et al., 2000). Here we show that the interplant $\delta^{13}C$ variations were low in the mature basal leaves (2.1 and 1.2 mUr for Chasselas and Pinot noir, respectively) and higher in the young apical leaves (2.6 and 1.5 mUr). The interplant differences in the foliar $\delta^{13}C$ values were two times higher than the within-canopy variations and were generally in the range expected for plants of the same species (e.g., Leavitt and Long, 1986; Turney et al., 2002). Some of the variation in ^{13}C discrimination in leaves of the same plant at different canopy positions and in leaves of different plants at approximately the same canopy position can be assigned to some spatial and temporal variability of environmental factors (e.g., temperature, humidity, CO_2 concentration, irradiance; see Fig. 1B) involved in the CO_2 fluxes, plant uptake, and assimilation. We recently demonstrated that the $\delta^{13}C$ values of the median leaves in Chasselas and Pinot noir vines are strongly correlated with the soil water availability at vineyard scale (Spangenberg et al., 2020).

4.3. Intra-leaf heterogeneities in TN content and ^{15}N discrimination

The within-leaf variations in TN and $\delta^{15}N$ values reached 0.46 wt% and 1.5 mUr, respectively, showing no systematic trends in the longitudinal or transverse directions for either grapevine. Heterogeneous nitrogen availability (e.g., Cochetel et al., 2017; Mackie-Dawson, 1999) and multiple nitrogen assimilation, loss, resorption, and reallocation events (Evans, 2001) can cause within-leaf variations in the TN content and nitrogen isotope composition. In the current study, we can exclude the differences in the plant N sources and availability as potential causes of variation. The only N source was soil nitrogen, and the soil in the experimental blocks for both cultivars had similar TN contents and $\delta^{15}N_{TN\text{-}soil}$ (Spangenberg et al., 2020). Furthermore, the generally uniform intraleaf distribution of the TN concentrations of both cultivars suggests unrestricted N availability to the plants. It is important to note that Chasselas leaves contained, on average, ~0.5 wt% less TN and had a $\delta^{15}N$ value ≈ 0.8 mUr higher than Pinot noir leaves (Figs. 3, 4, and 5B, E, C. D). The difference in leaf TN content between cultivars was no longer observed after the Chasselas plants received foliar N fertilization (Spangenberg et al., 2020). This outcome suggests that genotypic differences in the uptake and assimilation of soil nitrogen by plants may explain the differences in the TN contents and $\delta^{15}N$ values of the Chasselas and Pinot noir leaves. However, it is unlikely that a difference in nitrogen uptake by roots caused the variations in leaf TN (e.g., Osone and Tateno, 2005) and $\delta^{15}N$ values between cultivars because both grapevine cultivars were grafted onto the same rootstock (i.e., *Vitis berlandieri x Vitis riparia* cv. Kober 5BB). Genotypic differences in N assimilation pathways and allocation may explain the differences in leaf nitrogen composition shifts between Chasselas and Pinot noir.

The within-leaf $\delta^{15}N$ variability can be explained by heterogeneity in nitrogen allocation, which is known to be species-specific (Ripullone et al., 2003; Funk et al., 2013), and, in particular, the nonuniform distribution of N-containing compounds. In leaves, N occurs in soluble inorganic and organic compounds such as nitrate and ammonium (NO_3^-, NH_4^+), amino acids, nucleic acids, proteins, and alkaloids, and in insoluble organic compounds, mainly chlorophyll and membrane-bound proteins (i.e., thylakoid proteins) in the chloroplast (Evans,1989; van Wijk, 2004; Tegeder and Masclaux-Daubresse 2018). These organic compounds have different biosynthetic origins, molecular compositions, and structures, and due to different bond strength (e.g., alkyl carbon-nitrogen bonds, aryl carbon-nitrogen bonds, aromatic carbon-nitrogen bonds) they most likely have different N isotope compositions. Therefore, the heterogeneous spatial distribution of N compounds within leaves can induce the patchiness in $\delta^{15}N$ values, and, probably less importantly, in TN content.

4.4. Intra- and interplant patterns of leaf TN content and ^{15}N discrimination

The TN concentrations in the young apical leaves were higher than those in the old basal leaves for both cultivars ($\Delta TN_{Basal/Apical}$ up to 0.7 wt%; Fig. 5 B, E). The $\delta^{15}N$ values displayed the opposite trend to the $\delta^{13}C$ values, with higher $\delta^{15}N$ values for apical leaves than for basal leaves ($\Delta^{15}N_{Basal/Apical}$ up to 2.15 mUr); intermedia values in median leaves (Fig. 5 C, E). This observation suggests that ^{15}N discrimination occurred through the remobilization and reallocation of stored N. Few previous studies have reported on the intraplant variations in leaf TN content and $\delta^{15}N$ values. For evergreen trees (Picea abies), the opposite trend was reported, with higher $\delta^{15}N$ values in older needles than in younger needles (Gebauer and Schulze, 1991). A dedicated study of the effect of N recycling on the ^{15}N content of new leaves in deciduous shrubs (*Encelia* species) and trees (*Quercus* species) growing under

controlled conditions showed no consistent change in their $\delta^{15}N$ values (Kolb and Evans, 2002). The authors of that study recognized species specificity in the effect of N recycling on foliar $\delta^{15}N$. Similar decreases in TN and $\delta^{15}N$ values with leaf age and degree of senescence, as those shown here for two grapevine cultivars were reported for a free-growing aspen (*Populus tremula*) tree (Keskitalo et al., 2005), and more recently, for Qinghai spruce (*Picea crassifolia* Kom) plants that were in the same stage of development (Li et al., 2019).

We observed in both cultivars that the interplant differences in the TN content were higher in the basal than in the apical leaves and that, in contrast, the differences in the $\delta^{15}N$ values were lower in the basal than in the apical leaves. Unlike the within-leaf patterns, the intra- and interplant variations in foliar TN content and $\delta^{15}N$ values may be due to multiple assimilation events and organ-specific losses of nitrogen in combination with nitrogen resorption and reallocation (Evans, 2001). Notably, the leaf total N and ^{15}N contents may vary with the variations in light availability in their growth environment and degree of senescence between lower and upper leaves. Leaves acclimate to changes in light availability in their growth environment; sunlit leaves have higher specific area, stomatal and mesophyll conductance (g_s and g_m), nitrogen content, and photosynthetic capacity (A_{max}) than shaded leaves (Evans, 1989; Pearcy and Sims, 1994; Schultz, 1995; Evans and Porter, 2001). The amount of nitrogen allocated to leaves is positively correlated with the vertical light gradient through the canopy in order to maximize canopy carbon gain (e.g., Keller et al., 1998; Evans and Porter, 2001; Campany et al., 2016). Additionally, nitrogen is remobilized from senescing old leaves to the young upper leaves within the canopy. Leaf senescence (i.e., autophagy, Havé et al., 2017) is the primary mechanism involved in the internal recycling of nitrogen. It represents a dramatic reversal of metabolic processe in leaves and is marked by the transition from nutrient uptake and assimilation to nutrient remobilization and reallocation (e.g., Hörtensteiner and Feller, 2002; Distelfeld et al., 2014; Havé et al., 2017). Most of the nitrogen available for remobilization comes from the disassembly of the mesophyll chloroplasts, which account for 70–80% of leaf nitrogen, and degradation of the photosynthetic proteins (including Rubisco), nucleic acids, and chlorophyll. Proteolysis of chloroplast proteins starts in the early stages of senescence. Nitrogen is remobilized predominantly as free amino acids and small peptides, and less significantly as NH_4^+, urea, and NO_3^-. The four pyrrole nitrogen atoms in chlorophylls are not exported during leaf senescence; they remain as linear tetrapyrrolic catabolites in the vacuoles of mesophyll cells (Hörtensteiner and Keller, 2002).

5. Conclusion

This study provides evidence of differences in the $\delta^{13}C$ and $\delta^{15}N$ values and nitrogen content within and among leaves of two grapevine cultivars (the *Vitis vinifera* white grape cv. Chasselas and the red grape cv. Pinot noir) at the same plant development stage grown under uniform environmental conditions and with the same soil water availability. We measured significant intraleaf differences in both $\delta^{13}C$ (of up to 1.2 mUr) and $\delta^{15}N$ (of up to 1.5 mUr) values for both cultivars; these differences may be partly explained by patchy stomatal conductance and photosynthetic rates and differences in the chemical composition of the analyzed leaf discs (e.g., vein tissues vs. mesophyll tissues). We observed that apical leaves had lower ^{13}C content (by up to 1.2 mUr) and had higher TN and ^{15}N contents (by up to 2.2 mUr) than basal leaves. These intraplant variations reflect both the higher ^{13}C discrimination in intact photosynthetic tissues in juvenile leaves than in mature leaves and the remobilization and translocation of carbon and nitrogen metabolites from senescent basal leaves to young apical leaves. We demonstrated within-leaves and within-canopy foliar $\delta^{13}C$ and $\delta^{15}N$ heterogeneity in grapevine, a similar pattern may be expected in deciduous plants having highly compartmentalized leaf blades. Furthermore, we observed a strong genotype specificity in the nitrogen metabolism of the grapevine cultivars; this was shown by the significant differences in the nitrogen content and isotope ratios of Chasselas and Pinot noir leaves, which cannot be explained by differences in the (soil) nitrogen source or nitrogen root uptake.

The interplant variations in leaf ^{13}C, ^{15}N, and nitrogen content among grapevines of the same genotype, development stage, and senescence condition growing in the same environment were of the same order of magnitude as those found within leaves and among intraplant leaves (leaves within the same canopy). Therefore, these results suggest that care must be taken not to overinterpret foliar $\delta^{13}C$ and $\delta^{15}N$ levels as markers in ecophysiological studies based on fragmented material. Thus, it is important to use well homogenized composite samples of whole leaves from the same canopy position and light exposure condition from several replicate plants to more fully represent the physiological and biochemical responses of field-grown plants to environmental factors.

Credit author statement

Jorge E Spangenberg: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing-Original draft, Review & Editing. **Marc Schweizer:** Investigation, Formal analysis, Visualization. **Vivian Zufferey**: Methodology, Review & Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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