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SHORT COMMUNICATION

Isotopic comparison and correlation of $\delta^{13}C$ between bulk wood and cellulose of *Vitis vinifera* L.

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ABSTRACT

This study aims to compare the $\delta^{13}C$ isotopic signal between bulk wood and α-cellulose in wood samples from the main trunk of *Vitis vinifera* L. to verify whether α-cellulose extraction is necessary for ecophysiological studies in this species. A pool of samples from different cultivars and provenances was analysed. The wood samples were obtained from cross sections of the main trunk of the plants, from where the annual growth rings were anatomically recognised, dated to the year of formation, and then separated. Each ring comprised both early- and latewood portions. For each sample, a part was saved as bulk wood and another part was destined for α-cellulose extraction. The $\delta^{13}C$ isotopic signal in both types of samples was performed on a Vario Micro Cube elemental analyser coupled to a continuous flow mode to an isotope ratio mass spectrometer. A least squares regression was used to verify the correlation between the two variables. The results showed that the correlation coefficient of the isotopic signal for both types of samples was 0.86, and the slope of the regression line was not significantly different from one. Those results indicated that it is acceptable to use bulk wood instead of α-cellulose for $\delta^{13}C$ isotopic studies in *Vitis vinifera*. This study is the first to compare isotopic $\delta^{13}C$ signals between bulk wood and α-cellulose in grapevines. Therefore, this study constitutes a starting point to explore dendrochemical techniques based on the analysis of the $\delta^{13}C$ content in *Vitis vinifera* wood, with the aim of deepening the ecophysiological knowledge of the grapevine in relation to water economy strategies and the links with climate variability and change.

KEYWORDS: carbon isotope ratio, dendrochemistry, growth rings, *Vitis vinifera* L

INTRODUCTION

The analysis of stable carbon isotopes in plants is a powerful tool that allows for obtaining information on ecophysiological processes (Gessler *et al*., 2014). It has been proven that by analysing the isotopic ratios between ^{12}C and ^{13}C in grape must, it is possible to obtain a continuous integrator of the plant water status throughout the ripening period (Gaudillère *et al*., 2002). This technique is based on the discrimination produced during the CO_2 fixation process (the Calvin–Benson cycle) by the enzyme ribulose 1,5-diphosphate. This enzyme has a higher affinity for ^{12}C, the most abundant stable carbon isotope in the atmosphere (Brillante *et al*., 2018). When the stomata close and gas exchange with the outside of the leaf and the substomatal chamber ceases, discrimination in ^{13}C fixation with respect to ^{12}C decreases. This is because the $^{13}C/^{12}C$ ratio ($\delta^{13}C$) increases within the substomatal chamber and photosynthetic products become enriched in ^{13}C (Farquhar *et al*., 1980). Previous studies have concluded that water deficit is the main factor affecting this isotopic ratio (Brillante *et al*., 2018). Currently, in *Vitis vinifera* L., there are numerous studies on the $\delta^{13}C$ mainly carried out on berry, must and wine samples with various objectives. Among them, the evaluation of the effects of irrigation and plant water status, the identification of the isotopic signal as a function of the content of sugars and organic acids or isotopic characterisations of the cultivar are commonly found in the literature (Brillante *et al*., 2018; Costinel *et al*., 2011; Damiano *et al*., 2019; Gaudillère *et al*., 2002; Gómez-Alonso and García-Romero, 2009; Santesteban *et al*., 2010; Tardaguila *et al*., 1997). The studies on the aforementioned sample types have the limitation that they are bounded to the year in which the berry or wine was developed.

The study of stable carbon isotope ratios in growth rings is widely used to understand the incidence of environmental conditions on the physiology and development of woody plants (Harlow *et al*., 2006; Schleser *et al*., 1999). For this reason, the analysis of $\delta^{13}C$ contained in growth rings (dendrochemistry) is recognised as a valuable environmental proxy, reflecting climatic signals on a local, regional, and even hemispheric scales (Verheyden *et al*., 2005; Andreu *et al*., 2008). Therefore, unlike analysis in berries, must or wine, the isotopic analysis of $\delta^{13}C$ contained in growth rings would provide information on the impact of climate on plant development and growth throughout its life. In the case of climbing plants, Santesteban *et al*. (2010), Tyminski (2013), De Micco *et al.* (2018), and Roig-Puscama *et al*. (2021) have demonstrated the potential of dendrochronology to analyse various aspects of plant physiology, including average productivity, berry sugar production, vine health, rootstock interaction and relations with the edaphic environment, among others. Therefore, $\delta^{13}C$ measurements can be expected to provide metric and historical data on ecophysiological aspects of vineyards, which can be useful for generating management strategies and predictive models of wine yield and quality in the context of climate variability and change processes.

Isotopic analysis in wood is nowadays predominantly based on measurements of α-cellulose material, a component that is dominant in the cell wall. The use of α-cellulose is based on the fact that certain non-structural components, such as resins, organic acids, sugars or gums, can affect the isotopic value of wood (Harlow *et al*., 2006; Taylor *et al*., 2008; Wilson and Grinsted, 1977). Therefore, α-cellulose extraction is proposed as a standardised way to perform isotopic analysis on wood (Loader *et al*., 2003). However, due to the long time required for the α-cellulose extraction and the cost involved, this methodological step is often a limiting factor when many samples must be analysed (D'Alessandro *et al*., 2004; Loader *et al*., 2003; Verheyden *et al*., 2005). In addition, α-cellulose extraction can be more difficult when samples are small (< 1 g), which can occur in grapevine due to their small annual radial growth (Tyminski, 2013).

Studies on different woody plant species have shown that there is a strong positive correlation between the isotopic $\delta^{13}C$ signal of bulk wood and α-cellulose (Borella *et al*., 1998; Guerrieri *et al*., 2017; Harlow *et al*., 2006; Loader *et al*., 2003; Taylor *et al*., 2008), suggesting that isolation of α-cellulose may not be necessary for $\delta^{13}C$ analysis. These relationships should always be corroborated for each plant species (Ma *et al*., 2011).

At present, no indicators have been developed on the isotopic ($\delta^{13}C$) ratio between bulk wood and α-cellulose material in *Vitis vinifera*. In the scarce antecedents that currently exist on isotopic analyses in grapevine wood (Brook *et al*., 2020), the studies were carried out directly on whole wood without having made a previous comparison with α-cellulose. This could lead to errors in the calculation of derived physiological indicators since the presence of gums or resins could produce an important bias. Therefore, it is necessary to consider whether the isotopic variability between bulk wood and cellulose remains highly correlated and constant throughout the life of the plant. This information would facilitate more consistent ecophysiological interpretations and the understanding of how the climate influences the growth and development of the vine, crucial aspects in the management of these plants in the face of the challenges posed by climate change. The main goal of this contribution is to compare the values of $\delta^{13}C$ both in bulk wood and α-cellulose of *Vitis vinifera*.

MATERIAL AND METHODS

Samples of 3 cultivars from two different vineyards were used. One of the vineyards, made up of 10-year-old Malbec and Bonarda cultivars, is located in Mendoza, Argentina (33°46'20.29'' S; 69°9'14.62'' W; 1100 m altitude). The other vineyard is located in the region of Burgundy, France (47°16'44.45'' N; 4°58'52.44'' E; 295 m altitude), composed of 6-year-old Chardonnay cultivar plants. During the 2019 dormant season, wood samples were obtained from the main trunks at 0.4 m height: in the Argentine vineyard by using a 6" Pressler increment borer of 5.15 mm in diameter, and in the French vineyard, by cross-cutting sections of the main stems.

From each plant/individual, 3 replicates were extracted and pooled into a single sample.

Once the annual growth rings were anatomically identified, they were separated with a cutting knife under a binocular microscope. The rings of the different samples were then pooled according to their corresponding calendar age, resulting in a set of composite samples, each one including the rings formed in the same year for all plants (Ma *et al*., 2011). For the French vineyard samples, the growth rings corresponding to the 2018 growing season were sectioned, while for the Argentine vineyards, those rings corresponding to the 2017–2018 austral growing season were sectioned. Subsequently, the wood samples were dried for 24 hours at 60 °C and ground with a mixer mill (MM 400 Retsch®, Germany).

For each ring sample, a portion of the wood material was kept as bulk wood, while the rest was used for α-cellulose extraction. To extract α-cellulose, the methodology of Brendel *et al*. (2000) for small samples (≥ 100 mg) was followed. The procedure consisted of weighing 100 mg of ground wood in 10 mL Pyrex™ tubes. Subsequently, under a fume hood, acid digestion of each sample was performed with acetic and nitric acid in a thermostatic bath at 120 °C for 20 min. Once cooled, ethanol was added, and the samples were centrifuged. The supernatant was then removed, and the pellets were washed sequentially with ethanol, deionised water, and acetone.

For isotopic analyses, 1 mg of bulk wood and 1 mg α-cellulose of each sample were weighted. Three replications of each sample were used. Carbon isotopic analyses were performed on a Vario Micro Cube elemental analyser coupled in a continuous flow mode to an isotope ratio mass spectrometer (IsoPrime, Elementar). USGS40 L-Glutamic acid ($\delta^{13}C_{VPDB}$ = -26.39 ± 0.04 ‰) and IAEA-600 Caffeine ($\delta^{13}C_{VPDB}$ = -27.77 ± 0.04 ‰) were used as internal standards. The $\delta^{13}C$ values were reported in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite (VPDB) international reference.

1. Statistical analyses

A simple ANOVA was used to calculate the difference between the isotopic values of bulk wood and α-cellulose samples (Fisher's LSD test, $p \leq 0.05$). The relationship between $\delta^{13}C$ values of bulk wood and α-cellulose (onwards $\delta^{13}C_{bulk\ wood}$ and $\delta^{13}C_{\alpha\text{-cellulose}}$, respectively) was calculated by linear least squares regression analysis (LLSR), placing $\delta^{13}C_{\alpha\text{-cellulose}}$ values as the dependent variable and $\delta^{13}C_{bulk\ wood}$ values as the regressor variable. Subsequently, Student's *t*-test was applied for the slope of the LLSR ($p \leq 0.05$). The analyses were performed with InfoStat software (version 2018, Universidad Nacional de Córdoba, Argentina).

RESULTS

In grapevine wood, α-cellulose represents 44.3 % (± 0.012 %) with respect to bulk wood (w/w). The $\delta^{13}C$ values of bulk wood and α-cellulose are shown in Table 1. The mean $\delta^{13}C_{bulk\ wood}$ value was -27.35 ± 0.11 ‰, while the $\delta^{13}C_{\alpha\text{-cellulose}}$ value was slightly higher, reaching -26.49 ± 0.11 ‰.

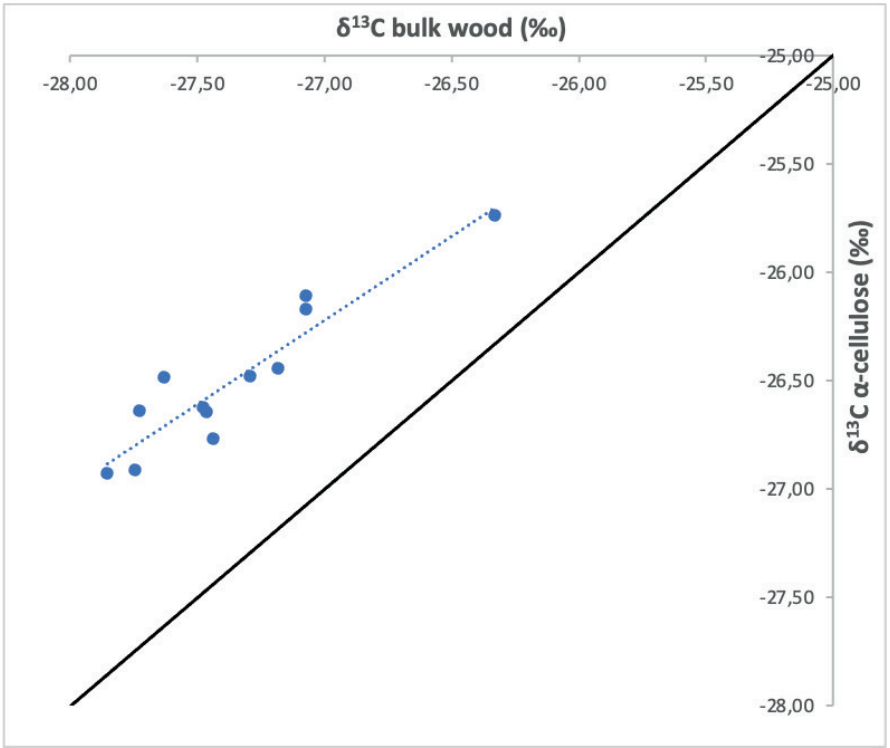

FIGURE 1. Values of $\delta^{13}C$ (‰) of bulk wood versus $\delta^{13}C$ (‰) of the corresponding α-cellulose.

Each values represent a single measurement. The dashed line corresponds to the least square regression ($\delta^{13}C_{\alpha\text{-cellulose}}$ = 0.78 (± 0.10) * $\delta^{13}C_{bulk\ wood}$ - 5.24 (± 2.73); p = 0.05244; n = 12; r^2 = 0.86) and the solid line represent the one-to-one line.

The $\delta^{13}C_{\alpha\text{-cellulose}}$ values differed significantly with respect to the $\delta^{13}C_{\text{bulk wood}}$ values. The mean $\delta^{13}C$ value of the difference between the two types of samples was +0.86 ‰. Because the analysed samples came from a pool of different cultivars and from different geographical locations, the isotopic values and the variation patterns were different. However, the $\delta^{13}C_{\text{bulk wood}}$ and $\delta^{13}C_{\alpha\text{-cellulose}}$ values showed a significant linear relationship ($\delta^{13}C_{\alpha\text{-cellulose}}$ = 0.78 (± 0.10) * $\delta^{13}C_{\text{bulk wood}}$ -5.24 (± 2.73); r^2 = 0.86; Figure 1), where Student's *t*-test indicated that the slope of the LLSR is not significantly different from one (p = 0.05244). The regression confidence interval was 0.56 (lower limit; 95 %) and 1.00 (upper limit; 95 %).

TABLE 1. Carbon isotope composition of bulk wood and α-cellulose from *Vitis vinifera* wood samples, n = 12

	$\delta^{13}C$ (‰)			
	Average	Standard deviation	Max	Min
Bulk wood	-27.35	0.41	-26.33	-27.85
α-cellulose	-26.49	0.35	-25.74	-26.93

DISCUSSION

The fraction of α-cellulose with respect to bulk wood is within the range reported in *Vitis vinifera* by Agrelli *et al*. (2009) and coincides with theoretical values of about 45 % reported for most woody plant species (Guerrieri *et al*., 2017). Regarding $\delta^{13}C$ isotopic values, the difference between bulk wood and α-cellulose values is around +1 ‰, within the range cited by the literature for other woody species, although this value depends on the species under study (Harlow *et al*., 2006; Helle and Schleser, 2004; Loader *et al*., 2003; Macfarlane *et al*., 1999). This difference in isotopic signal between bulk wood and α-cellulose is mainly due to the contribution of the isotopic contents in lignin and other compounds such as gums, resins or sugars present in bulk wood (Verheyden *et al*., 2005). Regression analyses confirmed that there is a high and significant relationship in $\delta^{13}C$ values between bulk wood and α-cellulose in grapevine, indicating that isotopic changes in α-cellulose correspond to changes of similar direction and magnitude with respect to bulk wood. This correlation in $\delta^{13}C$ isotopic values between bulk wood and α-cellulose agrees with previous studies conducted in other woody species of both angiosperms and gymnosperms (Borella *et al*., 1998; Guerrieri *et al*., 2017; Harlow *et al*., 2006; Loader *et al*., 2003; Verheyden *et al*., 2005). However, high correlations may not occur in all woody species (D'Alessandro *et al*., 2004; Ma *et al*., 2011).

The fact that the correlation between the two variables is high suggests that the non-α-cellulosic components present in *V. vinifera* wood contain broadly the same isotopic signal recorded in α-cellulose (Verheyden *et al*., 2005). However, in this study, the correlation between the variables (0.86), as well as the *t*-test for the slope (0.05244), would perhaps have been higher if the bulk wood had been previously subjected to organic solvents using a Soxhlet. In the case of lignin, and according to the literature, it correlates very well with the isotopic signal of α-cellulose (Loader *et al*., 2003; Mazany *et al*., 1980). In this sense, this structural component of the cell wall would not affect the correlation between $\delta^{13}C_{\text{bulk wood}}$ and $\delta^{13}C_{\alpha\text{-cellulose}}$. However, it must be considered that the isotopic composition of wood extracts does not always correlate well with the isotopic signal of α-cellulose, modifying the correlation between both variables (Borella *et al*., 1998; Taylor *et al*., 2008). Therefore, as explained by Verheyden *et al*. (2005), an important requirement in deciding whether to use $\delta^{13}C_{\text{bulk wood}}$ instead of $\delta^{13}C_{\alpha\text{-cellulose}}$ is that the slope of the LLSR is not significantly different from one. The fact that there is a high correlation between the $\delta^{13}C_{\text{bulk wood}}$ and $\delta^{13}C_{\alpha\text{-cellulose}}$ values would be an indicator that it is possible to use bulk wood directly for isotopic interpretations. However, because of the possible modification of the isotopic signal due to wood extracts, α-cellulose values should be predicted from bulk wood by a regression that is significantly linear. This means that if the slope of the LLSR is not significantly different from one, performing α-cellulose extraction becomes unnecessary, allowing the use of bulk wood for isotopic analysis. The results of the present study show that the slope of the regression is not significantly different from one (p = 0.05244), suggesting that the isolation of α-cellulose in grapevines may not be necessary for $\delta^{13}C$ isotopic analysis and, therefore, direct calculation from bulk wood are possible.

According to previous studies, dendrochronology applied to viticulture allowed one to study relationships between wood-derived anatomical variables (e.g., ring widths), with vineyard variables (e.g., productivity) and environmental indicators (e.g., temperature and rainfall) (De Micco *et al*., 2018; Lovisolo and Schubert, 1998; Schultz, 2003). For example, it is possible to indicate how the climatic conditions of a season impact wood growth and productivity, and estimates based on climate forecasts can be generated from them (Maxwell *et al*., 2016). In turn, the analysis of stable isotopes of carbon and oxygen in growth rings is a powerful tool to calculate physiological indicators that allow interpretation of how climate affects plant growth and development (Verheyden *et al*., 2005). As mentioned above, this type of analysis has been widely explored in viticulture by sampling berries, must and wine for various purposes (Brillante *et al*., 2018; Gaudillère *et al*., 2002; Horacek *et al*., 2021). However, this analysis has been very little explored in grapevine growth rings. The main importance of carbon and oxygen stable isotope analysis in grapevine growth rings is the potential to retrospectively assess how physiological processes vary in space and time, with seasonal resolution. In a recent study, image reconstruction using CNN (convolutional neural network) was validated from isotopic and anatomical variables extracted from growth rings of *V. vinifera* cv. Aglianico, resulting in a highly accurate spatial reconstruction of indices related to plant growth and canopy evolution, as well as to water status throughout the growing season. In addition, it was possible to differentiate plant

behaviour at the interannual level with a 4-year retrospective (Brook *et al*., 2020). In the aforementioned study, isotopic measurements of growth rings were performed directly on bulk wood without any wood cleaning (e.g., Soxhlet). The high accuracy achieved in the previous study in the reconstruction of physiological indices from grapevine bulk wood isotopic data validates the high correlation found in the present study.

Despite this, the correlation is likely to improve further if non-structural organic extracts were removed from the bulk wood. The extractive content in *Vitis vinifera* L. wood (separated by Soxhlet and absolute alcohol) is 2.3 % (± 0.27 %) on average. This value is relatively lower than that found in other species, such as *Pseudotsuga menziesii* (12 %; Taylor *et al*., 2008), although greater than the 30 % detected in other woody species (Harlow *et al*., 2006). Therefore, the incidence of these extracts in the isotopic signal of bulk wood is presumably low in grapevines. However, it is likely that correlation and slope significance values would increase if the bulk wood were pre-treated by some method of extraction of non-structural organic compounds (e.g., by Soxhlet), which would allow the removal of gums, resins or sugars, each of them with a particular contribution to the final isotopic value. In addition, many of these compounds may have been formed in previous years and mobilised and deposited in growth rings formed later, producing an alteration in the annual isotopic signal (Harlow *et al*., 2006; Taylor *et al*., 2008). According to Harlow *et al*. (2006), the prior process of removing wood extracts not only reduces the random variation associated with these compounds but also reduces measurement errors resulting from contamination during the sampling and handling processes. Therefore, and as a perspective for future work, it is suggested to compare the isotopic signals of α-cellulose and wood without non-structural organic compounds to corroborate possible differences in the quality of the climatic signal contained in the isotopic series.

Our results indicated that there is a close correlation in stable carbon isotope values ($\delta^{13}C$) between α-cellulose and bulk wood of *Vitis vinifera* L. cvs. Malbec and Bonarda from Mendoza, Argentina, and Chardonnay from Burgundy, France. This indicated that isolation of α-cellulose could be avoided for isotopic analysis and, therefore, derived directly from whole wood. Carbon isotopic measurement directly from bulk wood would allow faster and cheaper dendrochemical and ecophysiological studies in grapevine. Although the results indicate a high correlation between the isotopic values measured in α-cellulose and bulk wood, this behaviour should not be conclusive for all *V. vinifera* cultivars. Therefore, studies on the nature of these differences should be extended to different grapevine cultivars

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