Carbon and oxygen isotope ratios in wood constituents of *Pinus halepensis* as indicators of precipitation, temperature and vapour pressure deficit

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ABSTRACT

Carbon and oxygen isotope compositions ($\delta^{13}C$, $\delta^{18}O$) in tree rings have been shown to bear relevant climatic signals. However, little is known about the interrelationship between both isotopes in wood constituents for species from other than relatively wet climates. We hypothesized that in a species adapted to temporary droughts (e.g. *Pinus halepensis* Mill.) the signal derived from $\delta^{18}O$ in precipitation would be hidden by the strong variability in leaf transpirative enrichment. To test this assumption, we compared the effect of precipitation, temperature and vapour pressure deficit (VPD) on $\delta^{18}O$ and $\delta^{13}C$ along 23 sites covering the ecological range for this species. We extracted the cores from the south side of four to six adult dominant trees per aspect (north/south) within each site. For each aspect and site, fragments of the period 1975–1999 were pooled and milled to a fine powder. To further test the postulated need for cellulose purification in the assessment of climatic information, we studied these relationships in whole and extracted wood, holocellulose and lignin. In all wood fractions, $\delta^{13}C$ was related to annual precipitation [$r = -0.58$ ($P < 0.01$) to -0.78 ($P < 0.001$)] and VPD [$r = 0.53$ ($P < 0.01$) to 0.57 ($P < 0.01$)]. In contrast, for $\delta^{18}O$ only holocellulose showed consistent relationships with climatic data, being strongly significant for VPD [$r = 0.66$ ($P < 0.001$)]. However, it was unrelated to modelled $\delta^{18}O$ in precipitation, confirming that transpirative enrichment (driven by VPD) dampened the source signal in *P. halepensis*. The relationships between $\delta^{13}C$ and $\delta^{18}O$ were generally poor, regardless of the wood constituent, suggesting that although both variables were somewhat related to transpirative demand, they were relatively independent. This was further confirmed by building stepwise models using both isotopes to predict annual and seasonal precipitation [$r^2 = 0.34$ ($P < 0.01$) to 0.68 ($P < 0.001$)], temperature [$r^2 = 0.15$ ($P < 0.05$) to 0.37 ($P < 0.01$)] and VPD [$r^2 = 0.31$ ($P < 0.01$) to 0.55 ($P < 0.001$)]. We concluded that, even when partially describing the same climate variables, the information underlying the two isotopes can be regarded as complementary.

1. Introduction

Carbon and oxygen isotope ratios from tree rings offer the possibility of high-resolution records of past climate. Although both isotopes have been related to similar environmental variables, the processes leading to such relationships differ substantially between them. The carbon isotope composition ($\delta^{13}C$) of plants is isotopically depleted in ^{13}C with respect to atmospheric CO_2, which is the carbon source for photosynthesis. The instantaneous values of $\delta^{13}C$ in C$_3$ plants are determined by the ratio of intercellular to atmospheric partial pressure of CO_2 (p_i/p_a), reflecting the balance between diffusion in the intercellular space (mostly through stomata) and carbon fixation by the carboxylating enzyme. Consequently, $\delta^{13}C$ in dry matter constitutes an integrated

record of the plant's water status during growth (Farquhar et al., 1982). $\delta^{13}C$ in wood is therefore expected to integrate the effect of different environmental variables on the water status of trees. Indeed, $\delta^{13}C$ has been related to various climatic variables, such as humidity or temperature (Anderson et al., 1998; Hemming et al., 1998), and several indicators of water availability (Dupouey et al., 1993; Saurer et al., 1997a; Hemming et al., 1998; Ferrio et al., 2003a).

The association between oxygen isotopes and environmental variables, however, is less clear. The oxygen isotope composition ($\delta^{18}O$) of plant tissues reflects the variation in (1) $\delta^{18}O$ in source water, (2) evaporative enrichment of leaf water due to transpiration and (3) biochemical fractionation during the synthesis of organic matter (Yakir, 1992; Farquhar and Lloyd, 1993). Generally, the $\delta^{18}O$ of source water is strongly dependent on that of rain water ($\delta^{18}O_R$). $\delta^{18}O_R$ is mainly determined by the temperature of droplet formation, being higher with higher

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temperatures (Dansgaard, 1964). Other factors, such as altitude or precipitation, also affect $\delta^{18}O_R$ (Barbour et al., 2001). The level of enrichment of leaf water above source water ($\Delta^{18}O_e$) has been modelled as follows (Dongmann et al., 1974; Farquhar and Lloyd, 1993):

$$\Delta^{18}O_e = \varepsilon^* + \varepsilon_k + \left(\Delta^{18}O_v - \varepsilon_k\right)(e_a/e_i) \qquad (1)$$

where ε^* is the proportional depression of vapour pressure by the heavier $H_2{}^{18}O$, ε_k is the diffusion fractionation through stomata and leaf boundary layer, e_a and e_i stand for vapour pressure in atmosphere and intercellular space, respectively, and $\Delta^{18}O_v$ is the oxygen isotope composition of water vapour in the atmosphere (relative to source water). According to this model, plants growing at a higher humidity (i.e. higher e_a) are expected to have lower $\Delta^{18}O_e$ (for further details see Farquhar and Lloyd, 1993). Within the same environmental conditions, plants with higher stomatal conductance (i.e. lower leaf temperature, which reduces e_i) are also expected to show smaller $\Delta^{18}O_e$. Leaf water enrichment is passed on to the organic molecules formed in the leaf by exchange of oxygen atoms between carbonyl groups and water (Sternberg et al., 1986). However, in the case of cellulose, which is the main component of wood, about 50% of the enrichment signal (derived from sucrose) is further exchangeable with xylem water. Such exchange is a determinant for the observed relationship between $\delta^{18}O$ of tree-ring cellulose and $\delta^{18}O_R$, as it enhances the source-water signal, softening the effect of leaf-level enrichment (Saurer et al., 1997b; Anderson et al., 1998; Barbour et al., 2001).

Traditionally, tree-ring analyses have been performed on purified cellulose. However, recent works have shown strong relationships between the isotopic composition ($\delta^{13}C$ or $\delta^{18}O$) of cellulose and other wood fractions, suggesting that this time-consuming process might be obviated (Borella et al., 1998; Barbour et al., 2001). On the other hand, although the association between either $\delta^{13}C$ or $\delta^{18}O$ and environmental variables has been widely studied, the information available about the interrelation between both isotopes in wood cellulose is still scarce (Saurer et al., 1997a; Anderson et al., 1998), and we are not aware of such studies for other wood fractions. Moreover, the results from these works, performed in temperate areas, cannot necessarily be extrapolated to Mediterranean species, as they are affected by different environmental constraints and may respond in a different way to the same climatic variables. This might be the case for the species chosen in this study, Aleppo pine (*Pinus halepensis* Mill.), which is highly sensitive to changes in water availability as an adaptive strategy to the great interannual and seasonal variability of Mediterranean climates. Thus, it is able to reduce drastically growth and water expense during drought by stomatal closure, recovering rapidly when water becomes available (Borghetti et al., 1998; Ferrio et al., 2003a). We hypothesized that, in a species with such responsive stomata, the potential $\delta^{18}O_R$ signal in wood tissues would be hidden by the strong variability in transpirative enrichment. Consequently,

$\delta^{18}O$ would be more closely related to tree water status (and thus to $\delta^{13}C$ variation) than to the thermal signal retained in $\delta^{18}O_R$. To assess this hypothesis, we compared the effect of climatic variables on wood $\delta^{13}C$ and $\delta^{18}O$ over the range of environmental conditions where this species can be found. On the other hand, to further test the postulated need for cellulose purification, we aimed to characterize possible changes in the climatic signal present in both $\delta^{13}C$ and $\delta^{18}O$ during the extraction process. The main novelty of our approach lies in the simultaneous examination of both isotopes, as this may help to understand the relationships among the isotope signatures present in different wood components and their implications for environmental studies.

2. Materials and methods

2.1. Plant material and sample preparation

We sampled wood cores (5 mm diameter) of *P. halepensis* Mill. from 23 sites in the East Iberian Peninsula (Western Mediterranean Basin), selected to be representative of the natural ecological range for this species (see Fig. 1). We extracted the cores from the south side of four to six adult dominant trees per
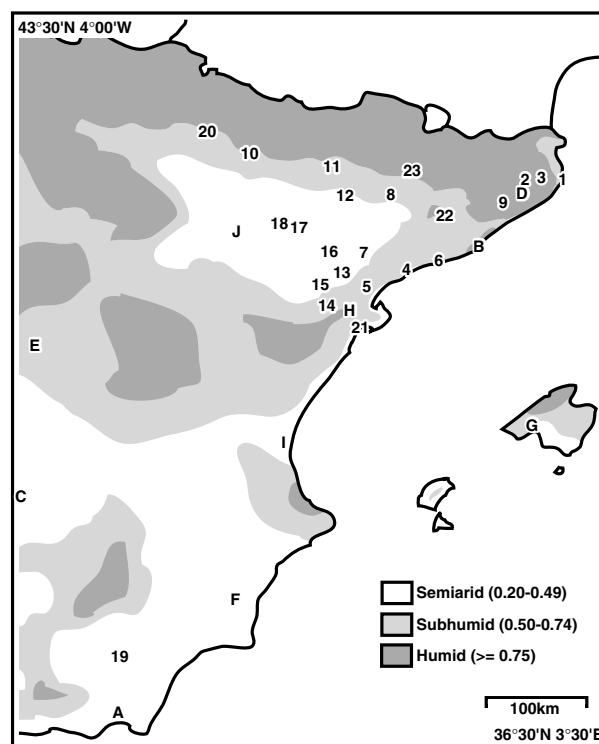

Fig 1. Geographical distribution of the reference sites (Table 1) used to test the accuracy of climatic models and the sampling sites for *Pinus halepensis* wood cores (Table 2). Main bioclimatic regions in the studied area are also indicated, according to the UNESCO humidity index (total precipitation/Penman evapotranspirative demand). Redrawn from Spanish Ministry of the Environment (2000).

aspect (north/south) within each site. Samples were oven-dried at 60 °C for 48 h before being polished to allow tree-ring dating. We selected a fragment of the cores, corresponding to the period 1975–1999. Fragments from the same site and aspect (23 sites × 2 aspects = 46) were then pooled and milled to a fine powder.

2.2.   Carbon and oxygen isotope analysis

For the removal of extractives and lignin we adapted the method of Leavitt and Danzer (1993) for holocellulose purification. We divided the samples into three subsets: the first set of samples was analysed for isotope content without previous treatment (whole wood); the second one after extractives (resins, soluble carbohydrates and other mobile compounds) were removed (extracted wood); and the third one after the removal of lignin (holocellulose). We weighed 90–110 mg dry mass (dried in an oven at 60 °C for 24 h) into individual pouches made of glass-fibre filter paper (No 30 Schleicher & Schuell, Dassel). The pouches were cinched up with 4-inch nylon cable ties with a number inscribed for identification, and reinforced with waxed dental-flossing tape. We distributed the pouches among four Soxhlet extractors (250 ml) filled with a 2:1 toluene/ethanol mixture and run overnight (16 h) at 70 °C. After shutdown and cooling, samples were air-dried for 1–2 h before returning them to the Soxhlet filled with 100% ethanol (16 h at 70 °C). At this point, samples became free of resins and other solvent-extractable substances. After cooling and air-drying (1–2 h), we put the samples in the Soxhlet and extracted them with deionized water for 6 h at 100 °C. Then we took away half of the pouches ("extracted wood"). The rest of pouches (still wet) were transferred to a 2000 ml Erlenmeyer flask filled with 1000 ml deionized water, 5 g sodium chlorite (NaClO$_2$, technical grade) and 1.5 ml glacial acetic acid, keeping the flask at 70 °C for 12 h. After four additions of sodium chlorite and acetic acid, at 12 h intervals, we rinsed the samples until the conductivity of the supernatant was less than 0.5 μS cm^{-1}. All samples were oven-dried (60 °C, 48 h) and weighed for the quantification of holocellulose. δ^{13}C and δ^{18}O in whole wood, extracted wood and holocellulose were determined by mass spectrometry at Isotope Services, Inc. (Los Alamos, NM, USA) and Serveis Científico-Tècnics (University of Barcelona, Spain), respectively. The isotopic composition of lignin was estimated gravimetrically (see e.g. Gray and Thompson, 1977; Borella et al., 1998; Barbour et al., 2001) from the above measurements and the dry-weight ratio between holocellulose and extracted wood (M_C/M_{ew}) using:

$$\delta_L = [\delta_{ew} - \delta_C (M_C/M_{ew})]/[1 - (M_C/M_{ew})] \tag{2}$$

where δ_L, δ_{ew} and δ_C stand for the isotopic composition (δ^{13}C or δ^{18}O) of lignin, extracted wood and holocellulose, respectively.

2.3.   Meteorological data and δ^{18}O in precipitation

Meteorological data for the period 1975–1999 were supplied by the Instituto Nacional de Meteorología and the Confederación Hidrográfica del Ebro. We took monthly values of precipitation (in mm) and temperature (in °C). Annual means of δ^{18}O in rain water ($\delta^{18}O_R$) and vapour pressure have been successfully modelled on a global scale by Barbour et al. (2001) from annual precipitation (P_{an}), mean annual temperature (T_{an}) and altitude (Z, in m). However, annual means are probably imperfect indicators of the great differences in climate seasonality found among Mediterranean sites. Thus, we built alternative models to predict vapour pressure on a monthly basis. We gathered from the Global Network of Isotopes in Precipitation (GNIP) database (IAEA, 2001) monthly values of precipitation (P_m), temperature (T_m), vapour pressure (VP) and $\delta^{18}O_R$ from 52 sites across the Mediterranean region. We calibrated the model using monthly means for each site, and taking only the sites with data available for the whole year. The resulting model for VP is as follows [$r^2 = 0.94$ ($P < 0.001$), $n = 312$, 26 sites]:

$$\ln(VP) = 6.34 + 0.047T_m + 0.93(P_m/1000) - 0.22(Z/1000). \tag{3}$$

We used the data from the sites not included in the calibration to validate this model, obtaining a strong correlation between predicted and measured values [$r = 0.93$ ($P < 0.001$), $n = 102$]. Moreover, calculating annual means for 10 sites located around the studied area (Fig. 1) we obtained better results using eq. 3 than with the global model from Barbour et al. (2001) (see Table 1). Taking monthly means of day-time temperature ($T_{day} = 1/3$ average minimum temperature $+2/3$ average maximum temperature), we the obtained day-time saturation vapour pressure (VP$_{sat}$) on a monthly basis (Jones, 1992):

$$VP_{sat} = 613.75 \exp\left(17.502 \frac{T_{day}}{240.97 + T_{day}}\right). \tag{4}$$

Finally, we estimated monthly means of day-time vapour pressure deficit (VPD) by subtracting VP from VP$_{sat}$.

In the same way, we obtained a monthly model for $\delta^{18}O_R$ [$r^2 = 0.52$ ($P < 0.001$), $n = 360$, 30 sites]:

$$\delta^{18}O_R = 0.42T_m - 0.007T_m^2 - 26.8(P_m/1000)$$
$$+ 112(P_m/1000)^2 - 0.046\sqrt{Z} - 8.26. \tag{5}$$

On a monthly basis, the estimates of $\delta^{18}O_R$ were less accurate than for VP [$r = 74$ ($P < 0.001$), $n = 128$ for validation sites]. Nevertheless, when annual means were calculated, the results obtained with the global model (Barbour et al., 2001) were still improved, as shown in Table 1.

2.4.   Statistical analysis

The data were subjected to analysis of variance (ANOVA) to assess the effect of chemical extraction, site and aspect on stable

Table 1. Site description and oxygen isotope composition in precipitation ($\delta^{18}O_R$; rainfall-weighted annual mean) for 10 reference sites used to assess the accuracy of vapour pressure (VP$_{an}$, annual mean of vapour pressure) and $\delta^{18}O_R$ models described in eqs 3 and 5. Lat., latitude; Long., longitude; Z, altitude; P_{an}, mean annual precipitation; T_{an}, mean annual temperature; VPD$_{an}$, annual mean of day-time vapour pressure deficit; Obs., observed values; Global, predicted values using the global, yearly models developed by Barbour et al. (2001); MED, predicted values using our models for the Mediterranean region, built on a monthly basis. Correlation coefficients between predicted and observed values are also indicated

Reference site	Lat.	Long.	Z (m)	P_{an} (mm)	T_{an} (°C)	VP$_{an}$ (Pa) Obs.	Global	MED	VPD$_{an}$ (Pa) Obs.	MED	$\delta^{18}O_R$ (‰) Obs.	Global	MED
A, Almería	36°51′	02°23′E	21	182	19.3	1503	1387	1451	945	825	−3.7	−5.0	−4.3
B, Barcelona	41°23′	02°07′E	90	593	16.0	1347	1196	1277	676	757	−5.0	−5.7	−5.1
C, Ciudad Real	38°59′	03°55′W	682	383	15.9	1061	1195	1125	1163	891	−6.9	−6.7	−6.4
D, Girona	41°54′	02°45′E	129	562	15.0	1310	1142	1207	650	754	−4.7	−6.2	−5.5
E, Madrid	40°25′	03°41′W	655	433	14.5	956	1114	1051	1009	915	−6.4	−7.1	−6.4
F, Murcia	38°00′	01°10′W	62	287	18.9	1253	1363	1439	1239	988	−4.6	−5.1	−4.6
G, Palma	39°33′	02°37′E	3	311	18.6	1600	1346	1427	779	876	−4.9	−4.9	−4.4
H, Tortosa	40°49′	00°29′E	48	496	18.1	1345	1320	1417	1035	963	−4.9	−5.0	−4.6
I, València	39°28′	00°23′W	13	477	18.9	1504	1366	1464	902	878	−5.1	−4.6	−4.3
J, Zaragoza	41°39′	01°01′W	263	301	15.8	1153	1187	1207	1006	969	−5.5	−6.4	−5.2
r (pred *vs* obs)							0.75**	0.85***		0.83***		0.75**	0.85***

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

isotope compositions. Principal components analyses (PCA) and simple correlations were used to assess the relationships between the variables. We built stepwise linear regressions to predict site variables from oxygen and/or carbon isotopes in different wood fractions, but the selection of variables for the model was further restricted in such a way that only the best-fitting wood fraction per isotope (carbon or oxygen) was allowed to enter the model. This was done to quantify the existence of complementary responses to climate variables between stable carbon and oxygen isotopes. Unless otherwise stated, differences were considered statistically significant for $P < 0.05$.

3. Results and discussion

3.1. $\delta^{13}C$ and $\delta^{18}O$ values in wood and wood fractions

As expected, we found significant changes in $\delta^{13}C$ and $\delta^{18}O$ during removal of extractives and lignin from wood to obtain holocellulose (Table 2). On the contrary, we did not find any significant effect of sampling aspect on either $\delta^{18}O$ or $\delta^{13}C$, so we decided to work with site means (north and south-facing slopes combined) throughout the study. $\delta^{13}C$ of whole wood ($\delta^{13}C_W$) and extracted wood ($\delta^{13}C_{eW}$) differed significantly, and both had clearly lower values than that of holocellulose ($\delta^{13}C_C$), with a mean difference of 1.2 ‰ and 1.0 ‰, respectively. Unlike for $\delta^{13}C$, there were no differences between $\delta^{18}O$ of whole wood ($\delta^{18}O_W$) and extracted wood ($\delta^{18}O_{eW}$), but $\delta^{18}O$ of holocellulose ($\delta^{18}O_C$) was about 4 ‰ higher. Similar results have been reported for $\delta^{13}C$ (Leavitt and Danzer, 1993; Borella et al., 1998) and $\delta^{18}O$ (Gray and Thompson, 1977; Barbour et al., 2001) vari-

ations in several conifers. The effect of holocellulose purification on $\delta^{13}C$ was constant across sites, whereas there was a significant interaction with site for $\delta^{18}O$, probably caused by the sensitivity of $\delta^{18}O_C$ to climate variables, that was not reflected in $\delta^{18}O_W$ or $\delta^{18}O_{eW}$, as we will discuss in Section 3.2. On the other hand, our results suggest that, in contrast to $\delta^{13}C$, the sources of variability for $\delta^{18}O$ differed strongly between the main fractions of wood (lignin and holocellulose). $\delta^{13}C$ in lignin ($\delta^{13}C_L$) was strongly positively related to $\delta^{13}C_C$ (Table 3) despite being 2.5 ‰ lower on average. Conversely, $\delta^{18}O_C$ and lignin $\delta^{18}O$ ($\delta^{18}O_L$) differed considerably (8.7 ‰) and, unlike $\delta^{13}C$ values, were negatively correlated. This observation offers further evidence that, for carbon and oxygen, the origin of differences in isotope composition between lignin and holocellulose is totally independent. Changes in $\delta^{13}C$ may be due to chemical fractionation during lignin biosynthesis (Benner et al., 1987) and/or seasonal differences in the amount of carbon directed to the synthesis of cellulose or lignin precursors (Förster et al., 2000). However, the main source of variation in $\delta^{13}C$ for C$_3$ plants is shared by all wood components, and it appears to be fractionation during carbon fixation. This explains the observed correspondence between $\delta^{13}C_C$ and either $\delta^{13}C_L$, $\delta^{13}C_{eW}$ or $\delta^{13}C_W$, in agreement with previous works (Mazany et al., 1980; Borella et al., 1998). On the contrary, the negative correlation between $\delta^{18}O_C$ and $\delta^{18}O_L$ is more controversial, and both negative (Gray and Thompson, 1977) and positive (Barbour et al., 2001) relationships have been reported. In both studies, $\delta^{18}O_L$ was estimated gravimetrically, as was done in our study, and thus we could discard the errors associated with this method as the cause of such differences. Moreover, such errors would have affected $\delta^{13}C_L$ and $\delta^{18}O_L$

Table 2. Site description and mean values (north- and south-facing slopes combined) for $\delta^{13}C$ and $\delta^{18}O$ (‰) in whole wood ($\delta^{13}C_W$, $\delta^{18}O_W$), extracted wood ($\delta^{13}C_{eW}$, $\delta^{18}O_{eW}$), holocellulose ($\delta^{13}C_C$, $\delta^{18}O_C$) and lignin ($\delta^{13}C_L$, $\delta^{18}O_L$). Lat., latitude; Long., longitude; Z, altitude; P_{an}, mean annual precipitation; T_{an}, mean annual temperature; VPD_{an}, annual mean of day-time vapour pressure deficit; $\delta^{18}O_R$, estimated $\delta^{18}O$ of precipitation (rainfall-weighted annual mean)

Site	Lat.	Long.	Z (m)	P_{an} (mm)	T_{an} (°C)	VPD_{an} (Pa)	$\delta^{18}O_R$	$\delta^{13}C_W$	$\delta^{13}C_{eW}$	$\delta^{13}C_C$	$\delta^{13}C_L$	$\delta^{18}O_W$	$\delta^{18}O_{eW}$	$\delta^{18}O_C$	$\delta^{18}O_L$
1	41°56′	03°15′E	185	693	15.2	805	−5.7	−24.9	−24.7	−23.9	−26.2	26.7	26.4	30.1	18.6
2	41°58′	02°49′E	44	732	15.4	778	−5.3	−24.2	−24.0	−23.2	−25.3	27.2	27.9	30.6	23.2
3	42°01′	03°00′E	190	809	14.1	723	−6.0	−24.7	−24.4	−23.6	−25.4	28.1	27.7	30.3	24.2
4	41°09′	01°17′E	148	478	15.8	812	−5.0	−24.0	−23.7	−22.8	−25.2	27.9	28.9	31.4	24.1
5	41°01′	00°36′E	180	490	15.2	807	−5.4	−23.8	−23.6	−22.7	−25.1	28.2	28.2	31.3	22.8
6	41°12′	01°39′E	106	511	15.9	849	−5.0	−23.8	−23.7	−22.7	−25.2	27.1	28.2	29.7	25.9
7	41°20′	00°30′E	80	395	14.8	826	−5.2	−22.7	−22.7	−21.6	−24.7	28.4	29.3	32.4	23.5
8	41°55′	01°01′E	525	551	12.7	791	−6.4	−24.4	−24.2	−23.1	−25.9	28.2	29.4	31.3	26.6
9	41°47′	02°26′E	208	753	15.1	807	−5.8	−23.7	−23.7	−22.7	−25.1	27.0	27.9	29.5	25.7
10	42°19′	00°45′W	520	519	13.7	841	−6.3	−23.6	−23.5	−22.3	−25.1	28.0	28.0	31.2	23.5
11	42°09′	00°15′E	625	551	12.9	814	−6.5	−24.6	−24.2	−23.3	−25.0	28.3	28.0	31.9	24.2
12	41°54′	00°26′E	518	423	12.5	788	−6.3	−24.0	−23.8	−22.7	−25.2	27.5	27.1	30.9	21.4
13	41°10′	00°23′E	363	427	14.7	898	−5.9	−23.4	−23.1	−22.1	−24.9	28.0	27.6	32.2	20.7
14	40°53′	00°12′E	630	563	13.2	788	−6.4	−24.3	−24.1	−23.1	−25.1	27.9	27.5	31.5	23.3
15	41°03′	00°06′E	359	361	15.9	997	−5.3	−22.8	−22.7	−21.7	−24.8	28.1	28.8	31.7	22.8
16	41°23′	00°17′E	125	385	14.5	827	−5.3	−23.2	−22.8	−21.9	−24.3	28.8	29.7	32.1	26.1
17	41°41′	00°12′W	356	362	14.9	897	−5.5	−23.3	−22.9	−22.3	−24.5	27.9	28.4	31.2	23.0
18	41°50′	00°32′W	380	407	13.5	801	−5.8	−23.2	−22.7	−21.6	−24.0	27.7	27.5	30.7	23.7
19	37°26′	02°20′W	900	337	17.8	1220	−5.6	−22.7	−22.6	−21.5	−24.2	28.0	28.2	34.6	18.9
20	42°28′	01°15′W	360	561	13.2	743	−6.2	−25.0	−24.9	−23.7	−26.5	27.2	26.4	31.5	19.6
21	40°41′	00°34′E	201	562	16.7	917	−5.2	−24.3	−23.8	−23.0	−25.2	27.6	28.7	35.0	17.8
22	41°36′	01°43′E	230	417	14.0	695	−5.4	−23.6	−23.4	−22.1	−25.7	27.2	27.0	30.7	20.7
23	42°05′	01°20′E	480	610	13.2	773	−6.2	−25.0	−24.4	−23.4	−25.8	27.1	26.7	29.6	22.7
Mean				517	14.6	835	−5.7	−23.9	−23.6	−22.7	−25.2	27.7	28.0	31.4	22.7
SD				133	1.4	106	0.5	0.7	0.7	0.7	0.6	0.5	0.9	1.4	2.4

in the same way, and they displayed very different behaviours. The most probable origin for these contrasting results lies in the exceptional climatic range used by Barbour et al. (2001), including over 19 ‰ of variation in $\delta^{18}O_R$ (1.5 ‰ in our work). Although the sources of variation for $\delta^{18}O_C$ are well described, little is known about $\delta^{18}O_L$ (Barbour et al., 2001). Assuming some exchange of oxygen atoms between xylem water ($=\delta^{18}O_R$) and lignin (as in cellulose), we would expect a positive relationship between $\delta^{18}O_C$ and $\delta^{18}O_L$ over a wide range of $\delta^{18}O_R$, but not if variability in $\delta^{18}O_C$ and $\delta^{18}O_L$ cannot be explained by $\delta^{18}O_R$, as seems to be our situation (see Section 3.2).

3.2. *Effect of environmental variables on $\delta^{13}C$ and $\delta^{18}O$*

A PCA weights plot (Fig. 2) provided an initial insight into the association trends for $\delta^{13}C$ and $\delta^{18}O$ along with the environmental variables P_{an}, T_{an} and VPD_{an}. $\delta^{13}C$ of the different wood fractions clustered together and were opposed to P_{an} over the axis of the first component. This agrees with the good correspondence among $\delta^{13}C$ of different wood fractions (Table 3)

and the expected negative relationship between $\delta^{13}C$ and plant water status. In contrast, the performance of $\delta^{18}O$ varied substantially among fractions. According to their relative positions in the biplot, $\delta^{18}O_C$ was related to changes in both VPD_{an} and T_{an}, whereas $\delta^{18}O$ of other wood constituents was not clearly related to any environmental variable, being relatively independent of $\delta^{18}O_C$ variation. Surprisingly, estimated $\delta^{18}O_R$ was only significantly related to $\delta^{18}O_{eW}$, but not to $\delta^{18}O_C$ (Table 4). The same pattern was found when looking at correlations with estimated seasonal means of $\delta^{18}O_R$ (data not shown). However, the highest correlation with $\delta^{18}O_{eW}$ [$r = 0.49$ ($P < 0.05$), $n = 23$] was found in summer, when modelled $\delta^{18}O_R$ showed the poorest correlation with measured $\delta^{18}O_R$ [$r = 0.49$ ($P < 0.01$), ranging from 0.71 ($P < 0.001$) to 0.84 ($P < 0.001$) for the other seasons, $n = 30$]. Such a relationship might be an artefact, and could be explained by the strong negative relationship found between the lignin to cellulose ratio (L/C) and estimated $\delta^{18}O_R$ (Table 4). As lignin is isotopically lighter than cellulose, a depletion in lignin content is expected to increase both $\delta^{13}C_{eW}$ and $\delta^{18}O_{eW}$. Indeed, correlation coefficients between $\delta^{18}O_R$ and
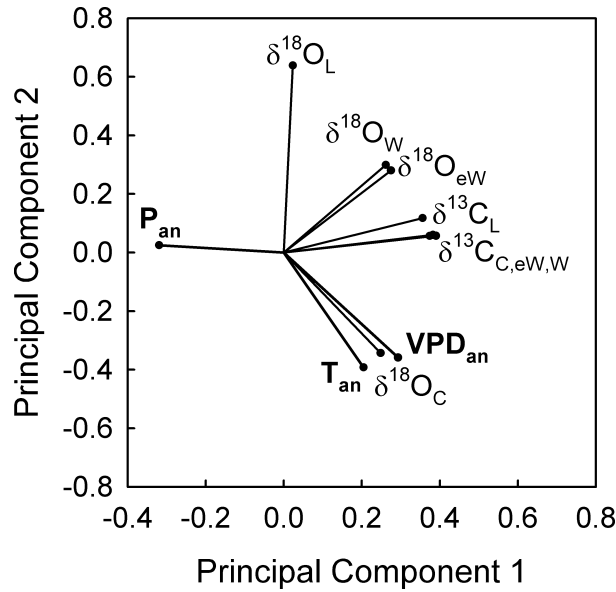
Fig 2. Component weights plot of the principal components analysis including the three climatic variables studied and site means for $\delta^{13}C$ and $\delta^{18}O$ in whole wood ($\delta^{13}C_W$, $\delta^{18}O_W$), extracted wood ($\delta^{13}C_{eW}$, $\delta^{18}O_{eW}$), holocellulose ($\delta^{13}C_C$, $\delta^{18}O_C$) and lignin ($\delta^{13}C_L$, $\delta^{18}O_L$). P_{an}, mean annual precipitation; T_{an}, mean annual temperature; VPD$_{an}$, annual mean of day-time vapour pressure deficit. The two components plotted explained 54% and 19% of total variance, respectively.

Table 4. Correlation coefficients between site parameters and either $\delta^{13}C$ or $\delta^{18}O$ in whole wood ($\delta^{13}C_W$, $\delta^{18}O_W$), extracted wood ($\delta^{13}C_{eW}$, $\delta^{18}O_{eW}$), holocellulose ($\delta^{13}C_C$, $\delta^{18}O_C$) and lignin ($\delta^{13}C_L$, $\delta^{18}O_L$), as well as with dry-weight lignin to cellulose ratio (L/C). P_{an}, mean annual precipitation; T_{an}, mean annual temperature; VPD$_{an}$, mean annual vapour pressure deficit; $\delta^{18}O_R$, estimated $\delta^{18}O$ of precipitation (rainfall-weighted annual mean)

	Altitude	P_{an}	T_{an}	VPD$_{an}$	$\delta^{18}O_R$
$\delta^{13}C_W$	0.02	−0.72***	0.43*	0.56**	0.45*
$\delta^{13}C_{eW}$	0.04	−0.75***	0.42*	0.57**	0.45*
$\delta^{13}C_C$	0.11	−0.78***	0.33	0.53**	0.36
$\delta^{13}C_L$	0.16	−0.58**	0.32	0.55**	0.25
$\delta^{18}O_W$	0.17	−0.46*	−0.04	0.25	−0.03
$\delta^{18}O_{eW}$	−0.18	−0.35	0.32	0.32	0.42*
$\delta^{18}O_C$	0.31	−0.46*	0.46*	0.66***	0.17
$\delta^{18}O_L$	−0.19	0.11	−0.30	−0.31	−0.05
L/C	−0.56**	0.20	−0.54**	−0.16	−0.74***

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

driving force for transpiration) was stronger than with T_{an} (Table 4), further suggesting that $\delta^{18}O_C$ variations were more closely related to transpirative demand than to the temperature signal stored in $\delta^{18}O_R$. In contrast, other authors found, over a similar range of $\delta^{18}O_R$, good correlations between $\delta^{18}O_R$ and $\delta^{18}O_C$ in tree rings of *Fagus sylvatica* (Saurer et al., 1997b) and *Picea abies* (Anderson et al., 1998). However, both species are less adapted to water stress than *P. halepensis*, and are thus expected to show looser stomatal control. Moreover, Saurer et al. (1997b) obtained a poorer relationship with $\delta^{18}O_R$ at the driest site, in agreement with our assumption that the source water signal in $\delta^{18}O_C$ is easily dampened when water is limiting and VPD increases.

$\delta^{13}C$ in the different wood fractions provided similarly strong relationships with climatic variables (Table 4). P_{an} showed strong negative correlations with $\delta^{13}C_W$, $\delta^{13}C_{eW}$ and $\delta^{13}C_C$, being somewhat weaker for $\delta^{13}C_L$. On the other hand, $\delta^{13}C$ showed positive trends with T_{an} and VPD$_{an}$. For T_{an}, this relationship

either $\delta^{13}C_{eW}$ or $\delta^{13}C_W$ were even greater than with $\delta^{18}O_{eW}$. At this point it should be noted that the relationship between L/C and $\delta^{18}O_R$ has no physiological meaning, being just the consequence of a common influence of temperature and altitude on both variables (Kollmann, 1955; Dansgaard, 1964; Barbour et al., 2001).

The lack of a consistent relationship between $\delta^{18}O$ of wood constituents and $\delta^{18}O_R$ confirms our hypothesis that, for a species with tight stomatal regulation such as *P. halepensis*, a relatively small range of $\delta^{18}O_R$ (1.5‰) is hidden by the stronger variability in $\Delta^{18}O_e$ (see eq. 1). The association of $\delta^{18}O_C$ with VPD$_{an}$ (the

Table 3. Correlation coefficients among site means of $\delta^{13}C$ and $\delta^{18}O$ values for each of the wood fractions studied: whole wood ($\delta^{13}C_W$, $\delta^{18}O_W$), extracted wood ($\delta^{13}C_{eW}$, $\delta^{18}O_{eW}$), holocellulose ($\delta^{13}C_C$, $\delta^{18}O_C$) and lignin ($\delta^{13}C_L$, $\delta^{18}O_L$)

	$\delta^{13}C_W$	$\delta^{13}C_{eW}$	$\delta^{13}C_C$	$\delta^{13}C_L$	$\delta^{18}O_W$	$\delta^{18}O_{eW}$	$\delta^{18}O_C$
$\delta^{18}O_L$	0.12	0.10	0.08	0.21	0.34	0.51*	−0.48*
$\delta^{18}O_C$	0.35	0.38	0.36	0.38	0.49*	0.41*	
$\delta^{18}O_{eW}$	0.54**	0.54**	0.46*	0.52**	0.73***		
$\delta^{18}O_W$	0.47*	0.49*	0.44*	0.57**			
$\delta^{13}C_L$	0.80***	0.87***	0.79***				
$\delta^{13}C_C$	0.97***	0.97***					
$\delta^{13}C_{eW}$	0.98***						

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was only significant for $\delta^{13}C_W$ and $\delta^{13}C_{eW}$, whereas for VPD_{an} we found significant correlations for all wood constituents. As for $\delta^{18}O_R$, the greater sensitivity of whole and extracted wood to T_{an} may be due to the negative relationship observed between L/C and T_{an} (Table 4). As lignin is more depleted in ^{13}C than the remaining wood, the greater lignin content found in colder sites (see e.g. Kollmann, 1955) would have enhanced the depletion in $\delta^{13}C$ associated with lower temperatures (mostly due to lower evaporative demand).

Correlation coefficients between seasonal means of climate variables and $\delta^{13}C$ were also consistent among wood components, despite being weaker for $\delta^{13}C_L$ (Fig. 3). Hence, the relationship of $\delta^{13}C$ with precipitation and VPD was significant throughout the seasons, albeit being a bit lower in winter. For temperature, summer was the most influential period on $\delta^{13}C$. The higher sensitivity of $\delta^{13}C$ to summer temperature and VPD is a direct consequence of Mediterranean climates, characterized by a hot dry summer, when VPD strongly determines the water status of plants, and thus $\delta^{13}C$. The generally good sensitivity of $\delta^{13}C$ to precipitation throughout the year may be explained by the particular physiology of the species under study. Unlike in deciduous trees (see e.g. Helle and Schleser, 2004), early wood in *Pinus* species is almost entirely produced from current photosynthate (Dickmann and Kozlowski, 1970; Glerum, 1980) and both early and late wood isotope composition has proven to be related to the environmental conditions in which they were laid down (Leavitt and Long, 1991; Barbour et al., 2002). More-

over, *P. halepensis* has an indefinite growth period, which often spans over the whole year even though it is slowed during the drier weeks of summer as well as in cold winters (Liphschitz and Lev-Yadun, 1986; Borghetti et al., 1998; Lev-Yadun, 2000; Nicault et al., 2001). Consequently, the isotope composition of tree rings in this species has the potential of being representative of the environmental conditions throughout the year. However, differences among sites in the seasonal growth pattern might have added an uncontrolled source of variation to the isotopic values of wood material. In order to check for this effect, we calculated the ratio between early and late wood widths in 11 sites (sites 3, 5, 7, 11, 14, 18, 19, 20, 21, 22 and 23; Table 2) where scanned images of the cores were available. Despite having a considerable range of variation for this ratio across sites (1.2–3.5), it was not significantly related to either $\delta^{18}O$ or $\delta^{13}C$ variation. This suggests that growth variability among sites had little influence on the bulk isotopic values obtained.

Unlike for $\delta^{13}C$, holocellulose purification was required to obtain consistent relationships between climatic variables and $\delta^{18}O$. Although $\delta^{18}O_W$ and $\delta^{18}O_{eW}$ were somewhat related to precipitation and temperature, respectively, such relationships were poor and inconsistent (Table 4, Fig. 3). In contrast, $\delta^{18}O_C$ showed significant relationships with both annual and seasonal means of VPD ($r = 0.58–0.69$), as well as with summer precipitation ($r = -0.63$). As expected from the lack of correspondence between $\delta^{18}O_C$ and $\delta^{18}O_L$, we did not find any significant correlation between climate variables and $\delta^{18}O_L$. As already suggested

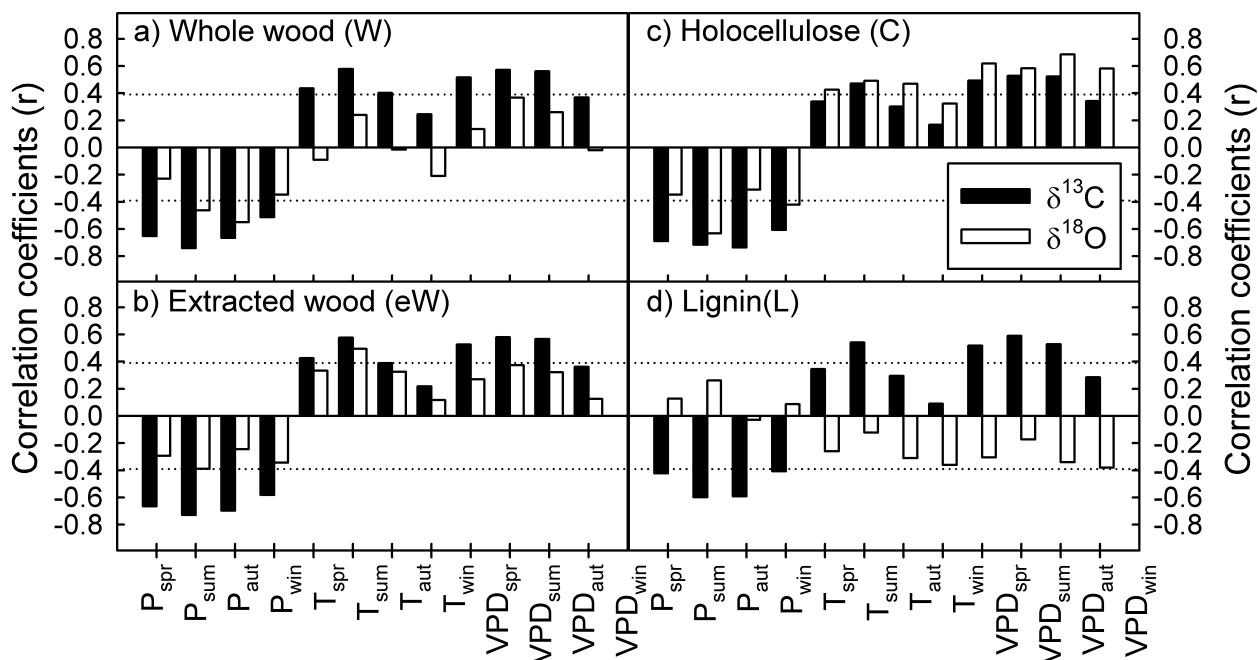*Fig 3.* Correlation coefficients between seasonal means of climatic variables and site means for $\delta^{13}C$ and $\delta^{18}O$ of the different wood fractions. Dotted lines indicate the threshold value for significant correlations ($P < 0.05$). P, T and VPD stand for precipitation, temperature and vapour pressure deficit, respectively. Each seasonal mean is indicated by the following subscripts: spr, spring; sum, summer; aut, autumn; win, winter.

by Barbour et al. (2001), only along extremely wide ranges of $\delta^{18}O_R$ (unrealistic for most tree-ring studies) is it likely that similar information will be obtained from holocellulose and wood or lignin. The suggested sources of variability for $\delta^{18}O_L$ are the isotopic composition of atmospheric O_2 (used in the synthesis of lignin precursors) and the potential exchange with either leaf or xylem water (Barbour et al., 2001). As $\delta^{18}O_L$ and $\delta^{18}O_C$ were negatively correlated, the proportion of oxygen exchanged with water in lignin should have been relatively small; otherwise, they would share the same water-derived $\delta^{18}O$ signal. Thus, the variability in $\delta^{18}O_L$ could be related to variations in O_2 isotopic signature. The bulk isotopic value of atmospheric O_2 is relatively stable around the world (Barbour et al., 2001). However, and despite the high concentration of O_2 in the atmosphere, in closed canopies, or at least within the leaf boundary layer, isotopic composition of air O_2 is affected by the balance between different respiratory processes with contrasting fractionations for O_2 (Farquhar and Lloyd, 1993; Ribas-Carbo et al., 2000; Angert and Luz, 2001; Nagel et al., 2001). Such balance is probably not directly related to the environmental variables monitored here, which would agree with the apparent lack of environmental effects on $\delta^{18}O_L$ variations. Although a discussion of the mechanisms of CO_2 fractionation is far from the aim of this work, further studies to determine the origin of the $\delta^{18}O$ signature in lignin are encouraged, as they might offer a new, integrated source of physiological information (e.g. if the isotopic signal from canopy or leaf O_2 is somehow preserved in lignin).

3.3. Are $\delta^{13}C$ and $\delta^{18}O$ complementary?

As a further test of the applicability of combining $\delta^{13}C$ and $\delta^{18}O$ isotopes in environmental studies, we built stepwise regression models to estimate climate variables from oxygen and/or carbon isotopes (Table 5). For precipitation, $\delta^{13}C_C$ was the most useful variable, although closely followed by $\delta^{13}C_W$. $\delta^{18}O_C$ was only considered for summer precipitation, where the best model included $\delta^{13}C_W$ and $\delta^{18}O_C$. Conversely, temperature was poorly predicted. For annual means, only $\delta^{18}O_C$ showed some (weak) relationship with temperature. The best fit among temperature models was found in summer, combining $\delta^{13}C_W$ and $\delta^{18}O_C$. On the other hand, VPD was generally related to $\delta^{13}C_W$ and $\delta^{18}O_C$. Indeed, the best predictions were found by combining both variables, except in winter, where $\delta^{18}O_C$ was taken alone. In general, $\delta^{13}C_C$ and $\delta^{13}C_W$ showed similar relationships with precipitation, and $\delta^{13}C_W$ was even better related to temperature and VPD, confirming the idea that purification of holocellulose is not strictly necessary when assessing $\delta^{13}C$ variation in wood. Nevertheless, for individual tree-ring studies, the use of extracted wood may still be necessary, as resins and other extractives are highly mobile and would add noise to the yearly and/or seasonal climatic signal present in cellulose and lignin. Regarding $\delta^{18}O$, only holocellulose offered a reliable climatic inference. Remarkably, although the isotopic signal of $\delta^{18}O_R$ was apparently lost, $\delta^{18}O_C$ still provided independent information from that reflected in $\delta^{13}C$. This was already shown by the poor relationship observed between $\delta^{18}O$ and $\delta^{13}C$ (Table 3, Fig. 2), further suggesting that the sources of variation for both isotopes differ partially. Hence, $\delta^{13}C$ variability can mostly be explained by the stomatal regulation of CO_2 fluxes into the leaf, integrating any environmental variable related to stomatal conductance, e.g. water availability or VPD (Farquhar et al., 1982; Ferrio et al., 2003b). However, it is also affected by changes in photosynthetic activity derived from irradiance or nutritional stresses, among other factors (Yakir and Israeli, 1995; Livingston et al., 1998; Ferrio et al., 2003b). On the other hand, $\delta^{18}O$ may also respond to stomatal conductance (see eq. 1), but it is not affected by photosynthetic activity (Yakir, 1992; Farquhar and Lloyd, 1993; Yakir and Israeli, 1995). Moreover, regardless of its effect on stomatal conductance, VPD has a direct influence on leaf water enrichment as, by definition, it is closely related to the e_a term

Table 5. Stepwise linear regression models to predict annual and seasonal means of climate variables from $\delta^{13}C$ and/or $\delta^{18}O$ in different wood components. VPD, vapour pressure deficit; $\delta^{13}C_W$, $\delta^{13}C_C$, $\delta^{13}C$ in whole wood and holocellulose, respectively; $\delta^{18}O_C$, $\delta^{18}O$ in holocellulose

Season	Precipitation (mm)		Temperature (°C)		VPD (Pa)	
	Model	r^2	Model	r^2	Model	r^2
Annual	$-2646 - 139.6\,\delta^{13}C_C$	0.59***	$0.1 + 0.46\,\delta^{18}O_C$	0.17*	$928 + 58.6\,\delta^{13}C_W$ $+ 41.6\,\delta^{18}O_C$	0.52***
Spring	$-572 - 31.6\,\delta^{13}C_C$	0.45***	$31.2 + 0.77\,\delta^{13}C_W$	0.15*	$684 + 41.7\,\delta^{13}C_W$ $+30.9\,\delta^{18}O_C$	0.43**
Summer	$-260 - 28.6\,\delta^{13}C_W$ $-10.3\,\delta^{18}O_C$	0.68***	$33.6 + 0.87\,\delta^{13}C_W$ $+ 0.31\,\delta^{18}O_C$	0.37**	$2264 + 110\,\delta^{13}C_W$ $+ 58.1\,\delta^{18}O_C$	0.44**
Autumn	$-967 - 50.2\,\delta^{13}C_C$	0.52***	$-1.4 + 0.54\,\delta^{18}O_C$	0.18*	$815 + 62.5\,\delta^{13}C_W$ $+ 48.4\,\delta^{18}O_C$	0.55***
Winter	$-483 - 25.9\,\delta^{13}C_C$	0.34**	–	–	$-652 + 32.8\,\delta^{18}O_C$	0.31**

$^*P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001.$

in eq. 1. Hence, in some cases both isotopes are affected by the same environmental variables (e.g. VPD) but, as the processes underlying such relationships are different, the information derived from each one can still be complementary.

4. Conclusions

According to our findings, δ^{13}C in whole wood may provide similar relationships to climatic variables as those obtained with holocellulose, at least when comparing long-term temporal series from different sites. In contrast, our results point out that for δ^{18}O holocellulose purification is indeed necessary to obtain consistent relationships with climatic variables, at least when working with the usual climatic ranges found in tree-ring studies. On the other hand, $\delta^{18}O_C$ in *P. halepensis* was mostly explained by variations in VPD, being unrelated to $\delta^{18}O_R$, confirming our hypothesis that in a species with strong stomatal regulation the signal of source water ($\delta^{18}O_R$) would be lost due to the great variability in evaporative enrichment at the leaf level. Nevertheless, even in such cases, δ^{18}O may provide climatic information independent of δ^{13}C, as each one reflects in a different way the effect of environmental variables on stomatal conductance and transpirative fluxes. Hence, we found poor correlations between δ^{18}O and δ^{13}C, which leads us to recommend the joint use of δ^{18}O and δ^{13}C in environmental studies, as it seems feasible to recover far more information than by using both isotopes separately. However, our results also suggest that the particular adaptive properties at the species level strongly determine the kind of information provided by δ^{13}C and δ^{18}O. Thus, a careful selection of the material of study (e.g. taking co-occurring species with contrasting physiological performance) would probably increase considerably the environmental information obtained from stable isotopes in tree rings.

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