



Research paper

Do atmospheric CO₂ concentration increase, climate and forest management affect iWUE of common beech? Evidences from carbon isotope analyses in tree rings

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Beech is one of the most important forest tree species in Europe, hence possible adverse factors affecting its physiology and productivity can have strong ecological and economic impacts. In this context, four beech forests along a latitudinal gradient from southern Apennines to middle European lowlands were selected for chronological determinations of carbon isotope composition ($\delta^{13}\text{C}$) in tree-ring cellulose. The main objectives of this study were to assess (i) the effect of climate on the carbon signature of tree-ring cellulose ($\delta^{13}\text{C}$); (ii) the physiological response to recent CO₂ concentration increment and to climatic variation; and (iii) the relationship between intrinsic water-use efficiency (iWUE, here the average long-term ratio of net photosynthesis to stomatal conductance) and growth of trees in different sites since 1950. Our results demonstrated that site climatic conditions peculiarly affect $\delta^{13}\text{C}$. In northern sites, a climatic control of summer precipitation and temperature on stomatal conductance was demonstrated by their opposite correlations with $\delta^{13}\text{C}$, negative and positive, respectively. Furthermore, an 'earliness effect' was suggested by a significant relationship between spring temperature and $\delta^{13}\text{C}$ in the coldest sites and by a positive one between winter temperature and $\delta^{13}\text{C}$ in the warmest ones. In all the study sites, during the maturity phase, a positive correlation between the increment of CO₂ and iWUE was observed, due to an active response of trees to CO₂ increment. This increment of CO₂ was the main driver of the long term increasing trend of iWUE, resulting by an active response of trees to CO₂ fertilization. Moreover, precipitation mostly influences positively and negatively the inter-annual variations of iWUE of the southernmost and northernmost sites, respectively. Overall, we observed a mean increment of 40% of iWUE. Moreover, the sensitivity of iWUE to the increase of CO₂ was different between the northernmost and southernmost sites. Increasing iWUE was correlated to growth in the two sites during the release phase and we hypothesize a positive effect of silvicultural treatments.

Keywords: adaptation, climate change, *Fagus sylvatica* L., forest management, mitigation, stable carbon isotopes.

Introduction

Changing temperature and precipitation patterns and increasing concentration of atmospheric CO₂ are likely to drive significant modifications in both natural and managed forests (Kirilenko and Sedjo 2007). At the beginning of the 21st century, the global

temperature was about 0.8 °C higher than at the beginning of the previous century (1880–1920 mean), and two-thirds of the warming has occurred only since 1975 (Hansen et al. 2010). Also, the climatic scenario that we are following forecasts the highest climatic variation as demonstrated by Bombi et al. (2017).

In the 21st century, the Mediterranean Basin could be the most sensitive among the European regions to climate change due to global warming (Schröter et al. 2005, European Environment Agency 2012).

Since the 1970s, extreme climatic events (e.g., drought periods) have become more frequent (McCabe and Palecki 2006), and natural forest productivity has declined in the areas with low precipitation, low temperature and low nitrogen deposition (Spiecker et al. 1996, Lindner et al. 2014). Moreover, drought and heat stress, which are becoming more frequent, are causing tree and forest mortality world-wide (Allen et al. 2010, Anderegg et al. 2012). As demonstrated by a study on the main European tree species, climate change will cause a reduction of the forest economical value by 2100, ranging between 14% and 50% (Hanewinkel et al. 2012).

In Europe, common beech (*Fagus sylvatica* L.) is among the most important native tree species, with a wide distribution and covering different climatic zones. In southern European beech stands, growth declines related to climate were observed in north-east Spain (Jump et al. 2006) and in central Italy (Piovesan et al. 2008). Most of the current climate projections for Central Europe predict increasing temperatures, accompanied by an increase in the frequency and the duration of intense summer droughts (Lindner et al. 2010, 2014). Under such changing environmental conditions, beech's sensitivity to low water availability (Ellenberg 1996) and long drought periods (Fotelli et al. 2002) can negatively affect its physiological performance, growth and competitive strength (Peuke et al. 2002).

Carbon, oxygen and hydrogen are the main constituents of wood. The variability in the isotope composition of each of these elements can offer physiological information on a plant's sensitivity to environmental trends (McCarroll and Loader 2006). Because of the heterotrophic link between wood growth and leaf photosynthesis, carbon isotope discrimination ($\Delta^{13}\text{C}$) in tree rings reflects the ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a) during the vegetation season, thus reflecting the balance between the CO_2 influx through stomata and its drawdown by photosynthetic carboxylation (Farquhar et al. 1989, Brugnoli and Farquhar 2000).

The first source of variation of carbon isotope composition ($\delta^{13}\text{C}$) in tree rings is due to the varying $\delta^{13}\text{C}$ value of atmospheric CO_2 , which has been changing since industrialization due to the massive release of isotopically light CO_2 from fossil fuel combustion (McCarroll and Loader 2006). Other sources of $\delta^{13}\text{C}$ variation occur at several steps along the path from foliar CO_2 uptake to wood formation and they are controlled by several physiological and environmental factors (McCarroll and Loader 2006).

A first fractionation step against ^{13}C is due to the binary diffusion of CO_2 in air through the stomata into the intercellular air spaces. This fractionation effect accounts for about $-4.4\text{\textperthousand}$ and it is caused by the fact that $^{12}\text{CO}_2$ molecules are less impeded than $^{13}\text{CO}_2$ ones. A second, relevant fractionation ($-27\text{\textperthousand}$) occurs during the photosynthesis carboxylation by Rubisco, which prefers $^{12}\text{CO}_2$. As a result, $\delta^{13}\text{C}$ in plant tissues is invariably depleted

relative to that of atmospheric CO_2 (Francey and Farquhar 1982, Ehleringer and Monson 1993).

We measured $\delta^{13}\text{C}$ on extracted alpha-cellulose of the tree rings because (i) the cellulose of each tree ring is without ambiguity linked to that specific growth period because cellulose forms the cellular framework during xylem formation; (ii) the isolation of cellulose reduces problems related to possible inter-annual fluctuations in the lignin to cellulose ratio; and (iii) the extraction of cellulose can be performed with a great level of homogeneity during the purification process (McCarroll and Loader 2006).

The variation of carbon isotope discrimination ($\Delta^{13}\text{C}$) in tree-ring cellulose records the long-term changes in the gas exchange metabolism of trees, providing insights on trees' responses to increasing atmospheric CO_2 concentrations. Changes in intrinsic water-use efficiency (iWUE) calculated as the ratio between photosynthesis and stomatal conductance are recorded in the shift of $\Delta^{13}\text{C}$ of the annual growth rings produced during each growing season (Duquesnay et al. 1998). Experimental results demonstrated the plants' ability to increase their iWUE as CO_2 levels rise (Morison 1993, Picon et al. 1996, Saurer et al. 2014). However, short-term studies of plants in growth chambers may not well predict the response of mature trees growing in the natural ecosystem, as trees are able to adapt their physiological behavior over time to gradually increasing CO_2 concentrations (Peñuelas et al. 2008, Voelker et al. 2016). Indeed, several studies have revealed that trees can vary in their responses to increasing atmospheric CO_2 concentrations (Duquesnay et al. 1998, Waterhouse et al. 2004, Fu et al. 2017). Moreover, the response to environmental changes of species distributed over large geographic ranges and contrasting environmental conditions is related to their genetically determined adaptedness and phenotypic plasticity (Lauteri et al. 2004, Peuke and Rennenberg 2004, Bolte et al. 2016, Cocozza et al. 2016, Hajek et al. 2016).

Hence, as demonstrated by many studies focused on other species (Silva et al. 2010, Voelker et al. 2014, Urrutia-jalabert et al. 2015), it is fundamental to study the physiological response of different populations growing in different environmental conditions. Our capability to develop proper management strategies is fundamental for developing adaptation and mitigation measures to global changes (D'Amato et al. 2011). In this context, we selected four beech sites located along a latitudinal gradient from the southern Apennines to middle European lowlands and measured the carbon isotope composition of tree-ring cellulose with the main objectives to assess: (i) the effects of climate on the carbon signature of tree-ring cellulose; (ii) the physiological responses to recent CO_2 concentration increment and to climatic variation; and (iii) the relationship between iWUE and productivity.

Materials and methods

Sites

Four study sites were selected between south of Italy to central of Germany, covering a range of 1300 km with different climatic

regimes (Figure 1), covering a great part of the latitudinal range of the species. Therefore, the sites are representative of environmental variability across vast parts of the biogeographic beech distribution. Stands and soil characteristics are shown in Table 1.

Zoolithenhöhle (ZOO) forest is located in southeast Germany ($49^{\circ}46'45''\text{N}$, $11^{\circ}16'58''\text{E}$) at 450 m above sea level (a.s.l.). It is a mixed beech, spruce and Scots pine forest (64%, 26% and 10% of the total basal area, respectively). The sampled trees were located in the beech dominant area of the forest. This stand is representative of many other midland German forests, which are essentially man made, following the large scale reforestation throughout the 19th century. The bedrock is limestone and the soil, according to FAO, is classified as Cambisol.

Pian Del Cansiglio (VEN1) is located in Veneto Region (north Italy, $46^{\circ}03'26''\text{N}$, $12^{\circ}01'56''\text{E}$) at 1100–1200 m a.s.l. Management of the stand is carried out by the National Forest Service of Italy. It is included in the Natural Biogenetic Reserve of Pian Parrocchia-Campo di Mezzo (established in 1977). The total area is 667 ha and the dominant tree species is *Fagus sylvatica* L. Soil is classified according to the IUSS working group WBR (2006), as Haplic Luvisols (ACID) on calcareous sediments.

Collelongo (ABR1) is located in the Abruzzi region (central Italy; coordinates $41^{\circ}50'58''\text{N}$, $13^{\circ}35'17''\text{E}$) at 1560 m a.s.l. Soil depth varies from 40 to 100 cm and is classified as a Humic Alisol (Chiti et al. 2010) on calcareous sediments. In the site since 1993, an eddy-covariance tower measuring the exchange of CO_2 , H_2O and energy was installed (Valentini et al. 1996) and is part of the FLUXNET and ILTER international networks.

Pian di Limina (CAL1) is located in Calabria Region (south Italy; coordinates $38^{\circ}25'38''\text{N}$, $16^{\circ}10'47''\text{E}$) at 1100 m a.s.l. Soil is classified as Haplic Podzol on igneous intrusive rocks (granites). The site is part of ILTER international networks.

Meteorological data

The availability of reliable meteorological series is crucial to evaluate the role of climate in driving significant modifications in forests. However, global climatological datasets frequently lack representativeness at the local scale, mainly in remote sites as forests often are. We therefore tried to reconstruct the climate variability for the specific locations of the sampled sites in a more accurate way. Specifically, site representative temperature and precipitation series were constructed by means of the anomaly method (New et al. 2000, Mitchell and Jones 2005), as described in Brunetti et al. (2012). The procedure is based on the assumption that the spatio-temporal structure of a meteorological variable over a given area can be described by the superimposition of the climatological normals over a given reference period (i.e., the climatologies, characterized by remarkable spatial gradients), and the deviations from them (i.e., the anomalies, linked to climate variability, generally characterized by higher spatial coherence). The two fields can be reconstructed independently from each other, based on different datasets (high

spatial density and limited temporal coverage for the climatologies, and low spatial density but long temporal coverage and accurate quality assessment for the anomalies) and different interpolation techniques: climatologies were reconstructed by estimating a local temperature (precipitation)–elevation relationship (Brunetti et al. 2014, Crespi et al. 2017), anomalies were reconstructed using weighted averages of high-quality neighboring series from the merging of the dataset of Brunetti et al. (2006) and Simolo et al. (2010). Finally, the two fields were superimposed to obtain monthly temperature and precipitation series in absolute values for each sampling site. Regarding the German site (ZOO) we used data collected by the nearest meteorological station, named Bamberg ($49^{\circ}52'\text{N}$, $10^{\circ}54'\text{E}$), with similar climatic conditions.

Tree sampling and dendrochronological analysis

At each site, co-dominant and mature trees of apparently similar age were sampled and two increment cores at breast height were collected from each tree, using an increment borer. Cores were placed into groove boards, wrapped with string to prevent twisting and left to air to dry. Once the cores were dry, they were prepared for tree ring analysis using standard dendroecological methods (Stokes and Smiley 1996). After surfacing, tree-ring width was measured at 0.01 mm resolution using a LINTAB (Rinntech, Heidelberg, Germany) measuring table equipped with a Leica MS5 stereoscope (Leica Microsystems, Wetzlar, Germany) and TSAP-Win software. Crossdating of the tree-ring data was verified using the Program COFECHA, which assesses the quality of crossdating and measurement accuracy of tree-ring series using the segmented time-series correlation technique (Holmes 1983). Hence, we built site chronologies (Table 2) for describing growth patterns and choosing trees for isotope analysis. Tree ring width series were converted into tree basal area increment (BAI) according to the following standard formula:

$$\text{BAI} = \pi(r_n^2 - r_{(n-1)}^2),$$

where r is the radius of the tree and n is the year of tree-ring formation. To examine the mean growth trend of the trees, BAI for each year was averaged over all individuals. We used BAI as indicator of site productivity because BAI is more correlated to volume increment (Phipps and Whiton 1988).

Cellulose extraction, $\delta^{13}\text{C}$, $\Delta^{13}\text{C}$ and iWUE calculation

For cellulose extraction, we selected five cores per site showing a significant similarity with site chronology (Gleichläufigkeit (GLK) >60, Table 2). This number of samples is generally considered sufficient for isotope studies (Leavitt 2010). Cellulose extraction was conducted according to Boettger et al. (2007) on single tree rings for the analysis period spanning from 1950 to 2013.

Carbon isotope composition of cellulose homogenized subsamples was calculated according to Farquhar et al. (1982) as

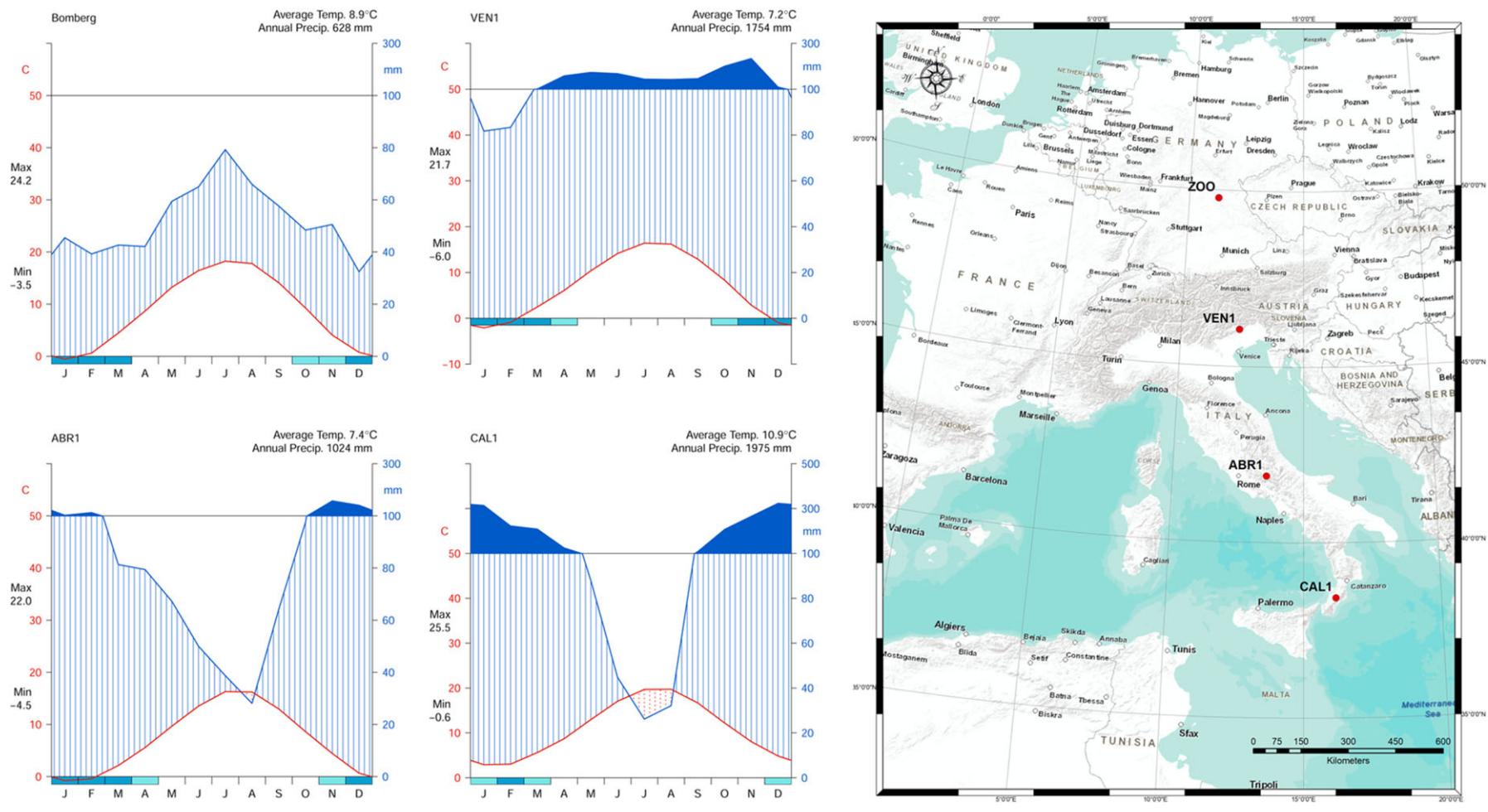


Figure 1. Left panel: Walter Lieth climate diagrams of Bamberg, VEN1, ABR1 and CAL1. Max: mean of the maximum temperatures of the warmest month. Min: mean of the minimum temperatures of the coldest month. Right panel: geographical locations of study sites.

Table 1. Forest structure and soil characteristics. Basal area (BA, $\text{m}^2 \text{ha}^{-1}$) is the sum of cross section areas (at 1.30 m) of stem trees in one hectare; N_trees (n ha^{-1}) is the number of trees per hectare; AVG_diameter (cm) is the diameter corresponding to the mean BA; AVG_height (m) is the height corresponding to tree with AVG_diameter; θ_{fc} (% volume) is the field capacity defined as the soil moisture content after soil has been thoroughly wetted to saturation and allowed to drain for 2–3 days (Reynolds et al. 2000); θ_{wp} (% volume) is the wilting point defined as the soil moisture content below which plants wilt during the day and cannot recover overnight (Reynolds et al. 2000); AWC (% volume) represents the available water content, expressed as the difference between θ_{fc} and θ_{wp} . θ_{fc} and θ_{wp} were estimated using soil calculator of the Colorado State University (<https://www.nrel.colostate.edu/projects/century/>).

Site	BA	N_trees	AVG_diameter	AVG_height (m)	θ_{fc}	θ_{wp}	AWC
ZOO	44.29	567	33.4	28.5	34	13	21
VEN1	39.1	341	38.2	28.2	46	25	21
ABR1	44.2	740	27.5	23.0	21	7	14
CAL1	44.5	270	45.8	30.2	28	15	13

Table 2. Characteristics of the sampled trees: N_chrono, number of trees used in site chronology; Range_chr, max and minimum sampled trees diameter (cm); Age_Chrono, mean age of the sampled trees (years) with standard deviation in brackets; EPS_Chrono, Expressed Population Signal of site chronology (according to Wigley et al. 1984, in bold EPS ≥ 0.85); N_iso, number of trees used for isotopic analysis; GLK_sel, represents the range of the GLK values of the cores selected for cellulose extraction; Range_iso, max and minimum sampled trees diameter used for isotopic analysis (cm); Range_Height_iso, max and min height of trees used in the isotopic analysis; Age_iso, mean age of the sampled trees (years) with standard deviation in brackets; EPS $_{\delta^{13}\text{C}}$, the Expressed Population Signal of $\delta^{13}\text{C}$ series (according to Wigley et al. 1984, in bold EPS ≥ 0.85).

Site	N_chrono	Range_chr	Age_Chrono	EPS_Chrono	N_iso	GLK_sel	Range_iso	Range_Height_iso	Age_iso	EPS $_{\delta^{13}\text{C}}$
ZOO	10	38–100	128 (± 18)	0.90	5	74–82	40–46	31–33	136 (± 16)	0.85
VEN1	18	37–56	117 (± 18)	0.90	5	76–80	41–56	28–32	112 (± 16)	0.78
ABR1	20	25–54	108 (± 9)	0.89	5	70–85	40–53	28–29	107 (± 9)	0.88
CAL1	15	38–57	91 (± 9)	0.85	5	64–76	44–52	27–29	88 (± 9)	0.72

$$\delta^{13}\text{C}_{\text{sample}} = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{VPBD}}} - 1 \right] \times 1000,$$

where $^{13}\text{C}/^{12}\text{C}$ is the measured stable isotope ratio of the samples and of the international standard (Vienna-Pee Dee Belemnite, VPDB), respectively. We thereafter calculated $\Delta^{13}\text{C}$, C_i and iWUE. The $\Delta^{13}\text{C}$ (carbon isotopic discrimination) of the trees was calculated as

$$\Delta^{13}\text{C} = \frac{(\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{plant}})}{(1 - \delta^{13}\text{C}_{\text{plant}})},$$

following Farquhar et al. (1982) and using published values for air $\delta^{13}\text{C}$ from ice core measurements, direct atmospheric measurements and inferred values from C4 plants isotopic analyses (McCarroll and Loader 2006). $\Delta^{13}\text{C}$ is related to the C_i over C_a ratio by the following equation:

$$\Delta^{13}\text{C} = a + (b - a) \times \left(\frac{C_i}{C_a} \right),$$

where a is the discrimination against $^{13}\text{CO}_2$ during CO_2 diffusion through stomata ($a = 4.4\%$) and b is the discrimination associated with carboxylation ($b = 27\%$) (Farquhar et al. 1982).

According to the first Fick's Law:

$$A = g_{\text{CO}_2} \times (C_a - C_i)$$

where A is the net photosynthesis, measured as CO_2 uptake, and g_{CO_2} is the leaf conductance to CO_2 , and given that $g_{\text{H}_2\text{O}}$, the leaf conductance to water vapor is $1.6 g_{\text{CO}_2}$, $\Delta^{13}\text{C}$ can be finally related to the ratio $A/g_{\text{H}_2\text{O}}$ (iWUE) (Osmond et al. 1980) by the following equation:

$$\Delta^{13}\text{C} = a + (b - a) \times \left(\frac{1 - 1.6A}{C_a g_{\text{H}_2\text{O}}} \right).$$

We further determined the C_i , using the Francey and Farquhar (1982)'s equation:

$$C_i = C_a \times \frac{(\delta^{13}\text{C}_{\text{plant}} - \delta^{13}\text{C}_{\text{air}} + a)}{(a - b)}.$$

Finally, we calculated iWUE as

$$\text{iWUE} = C_a \times \frac{(b - \Delta^{13}\text{C})}{[1.6 \times (b - a)]}.$$

We compared the iWUE patterns of each site with the three scenarios of responses of trees to changes in C_a suggested by Sauer et al. (2004): (i) $C_i = \text{constant}$, (ii) $C_i/C_a = \text{constant}$ and (iii) $C_a - C_i = \text{constant}$. Those scenarios differ in the degree to which C_i responds the increase in C_a : (i) not at all, (ii) in a proportional way (regulation of stomatal conductance and/or photosynthetic capacity) and (iii) at the same rate (neither stomatal nor extra-stomatal adjustments).

We assumed that C_a and C_i are the only non-constant factors that control the isotope fractionation occurring at the leaf level. Consequently, in the iWUE calculation we did not consider two aspects, i.e., the mesophyll conductance of CO_2 (Seibt et al. 2008) and post-photosynthetic processes that may potentially affect tree-ring α -cellulose $\delta^{13}\text{C}$ (Gessler et al. 2014, Frank et al. 2015).

Statistical analysis

Regression analyses were used to identify significant relations among the different variables considered in the study, normality was tested using the Shapiro–Wilk test and the constant variance test was performed. Correlations were calculated using the Pearson Method and significance was fixed to P -value <0.05 . T -test and Mann–Whitney Rank Sum test were used to compare two groups in case of a normal distribution of data and non-normal distribution, respectively. ANOVA was used to compare groups with a normal distribution and the Kruskal–Wallis ANOVA on ranks in case of groups without a normal distribution. The differences between sites were tested by the post hoc Tukey's test.

In order to remove the effect of the atmospheric CO_2 increase and to enhance the climatic signal we applied the pre-industrial correction (PIN) suggested by McCarroll et al. (2009). The correction is based on the physiological response of trees to increased atmospheric concentrations of carbon dioxide, so following the six steps of the procedure we calculated $\delta^{13}\text{C}_{\text{pin}}$.

The relationship of $\delta^{13}\text{C}_{\text{pin}}$ with climate variables at each site was calculated using bootstrapped correlations, and the statistical significance was fixed at P -value ≤ 0.05 . In the analysis, we considered the monthly maximum average temperature and monthly sums of precipitation from June of the previous year to September of the current year of growth.

ANCOVA was used to evaluate the different responses to atmospheric CO_2 concentration increment between sites. The

differences between sites were tested by the post hoc Tukey's test. Analyses were performed for the whole study period (1950–2013) and after 1975. We decided to split the analysis using 1975 as threshold for two reasons: first, after this year, we observed a higher increment of mean temperature as in the study of Hansen et al. (2010) (Figure 2); second, after 1975, all trees at the study sites had reached the maturity phase, hence the isotope series were free of any possible distortion due to juvenile effects and were better comparable.

We applied the communality analysis (CA) to a Multiple Linear Regression ($i\text{WUE} = aT_{\text{mean}} + bC_a + c\text{PP}$, where T_{mean} is the mean temperature, C_a is the atmospheric CO_2 concentration, PP is the annual sum precipitation) to disentangle the effects of atmospheric CO_2 concentration and climate on iWUE. Communality analysis shares the explained variance into pure and joint effects of predictors, in order to assess the relative contribution of each predictor to the explained variance of the response variable (Huang et al. 2017). We applied the analysis on both raw data and first-order difference data to test the effect of long-term trends and inter-annual fluctuations, respectively (Tsonis et al. 2015). Statistical analyses were performed using R 3.0.1 (R Development Core Team 2013) using the packages bootRes (Zang and Biondi 2013), HH (Heiberger and Holland 2015) and yhat (Nimon et al. 2013). SigmaPlot was used for graphs.

Results

Chronological and geographical variations in carbon isotope composition and discrimination

Results of $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ are reported in Table 3 that shows descriptive statistics of the parameters for the whole study period and the two sub-periods. At ZOO, $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ mean values were $-25.0\text{\textperthousand}$ and $17.1\text{\textperthousand}$, respectively. Considering the period 1950–75, we assessed average $\delta^{13}\text{C}$ equal to

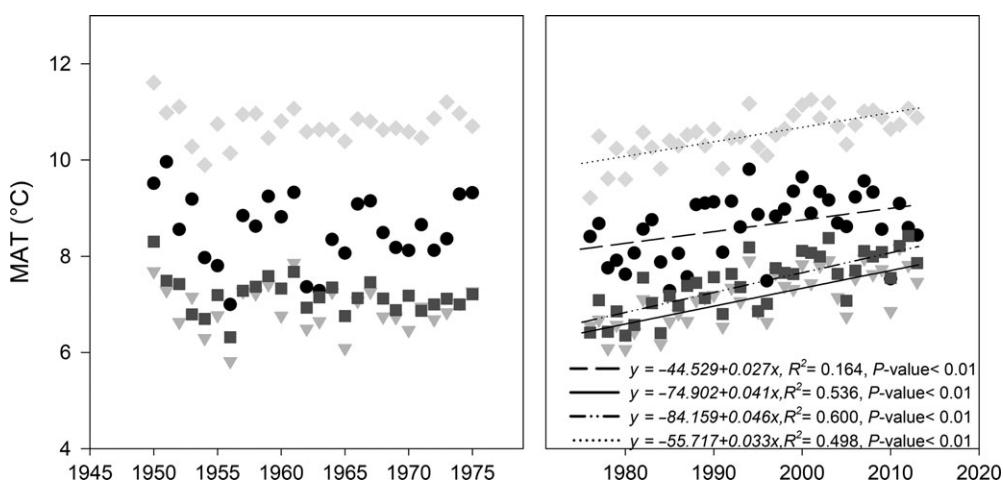


Figure 2. Temporal variation of mean annual temperature (MAT) at study sites from 1950 to 1975 (left panel) and from 1976 to 2013 (right panel); black points and dashed line = ZOO, triangles and continuous line = VEN1, squares and dashed and dotted line = ABR1, diamonds and dotted line = CAL1. Significant trends are represented by lines with the coefficient of determination and statistical significance.

$-24.8\text{\textperthousand}$ and $\Delta^{13}\text{C}$ as $17.3\text{\textperthousand}$. Since 1976, which was an extreme drought year, mean values of $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ were $-25.1\text{\textperthousand}$ and $16.9\text{\textperthousand}$, respectively. Comparing the two sub-periods, we did not find significant differences of $\delta^{13}\text{C}$. On the contrary, carbon isotope discrimination showed significant differences ($t = 3.142$, P -value <0.01).

In all the Italian sites, considering the two sub-periods, we found high significant differences in $\delta^{13}\text{C}$ (always P -value <0.01), but no differences in $\Delta^{13}\text{C}$. Our results showed differences in average $\delta^{13}\text{C}$ ($H = 79.473$, P -value <0.01) and $\Delta^{13}\text{C}$ ($H = 126.168$, P -value <0.01) among the sites.

Relationships between $\delta^{13}\text{C}_{\text{pin}}$ and climatic conditions

After the PIN correction, the $\delta^{13}\text{C}_{\text{pin}}$ patterns result more stable in the Italian sites, while in ZOO we observed an increase of $\delta^{13}\text{C}_{\text{pin}}$ values before 1975, and a decline thereafter (Figure 3).

Correlations between climate variables (T_{max} and PP) and $\delta^{13}\text{C}_{\text{pin}}$ are reported in Figure 4. In ZOO, $\delta^{13}\text{C}_{\text{pin}}$ was negatively correlated with temperature of April and summer precipitation, but positively correlated with the max temperature of July and August of the current growth year. In VEN1, $\delta^{13}\text{C}_{\text{pin}}$ was negatively correlated with precipitation in current summer and April temperature, but a positive correlation was found with July temperature (Figure 4).

In ABR1, there were negative effects on $\delta^{13}\text{C}_{\text{pin}}$ related to maximum temperature in April of the current year and positive effects with the previous June. Precipitation of previous and current June and previous July was negatively correlated to $\delta^{13}\text{C}_{\text{pin}}$, but previous October showed positive correlations.

In the southern site (CAL1), January temperatures had a significant positive effect, while previous September had negative effects on $\delta^{13}\text{C}_{\text{pin}}$. As in ABR1, current June precipitation showed a negative correlation with $\delta^{13}\text{C}_{\text{pin}}$.

Table 3. $\delta^{13}\text{C}$ (\textperthousand) and $\Delta^{13}\text{C}$ (\textperthousand) in each site at different periods. Avg is the average values of all the years of the period, std.dev is the standard deviation of the period, Max is the maximum value of the reference period, Min is the minimum value of the reference period. The statistics are calculated on five trees per year.

Site	Period	$\delta^{13}\text{C}$				$\Delta^{13}\text{C}$			
		Avg	std. dev	Max	Min	Avg	std. dev	Max	Min
ZOO	1950–2013	-24.98	0.76	-22.66	-26.79	17.1	0.6	18.29	15.00
VEN1	1950–2013	-25.27	0.62	-24.43	-26.79	17.3	0.3	18.01	16.68
ABR1	1950–2013	-24.05	0.68	-22.96	-25.43	16.2	0.4	17.01	15.30
CAL1	1950–2013	-25.13	0.61	-24.00	-26.46	17.2	0.3	18.04	16.49
ZOO	1950–75	-24.76	0.42	-24.05	-25.64	17.3	0.5	18.29	16.42
VEN1	1950–75	-24.75	0.23	-24.43	-25.35	17.3	0.3	18.00	16.87
ABR1	1950–75	-23.52	0.41	-22.96	-24.55	16.1	0.4	17.01	15.38
CAL1	1950–75	-24.65	0.39	-24.00	-25.37	17.2	0.4	18.04	16.58
ZOO	1976–13	-25.13	0.90	-22.66	-26.79	16.9	0.6	18.07	15.00
VEN1	1976–13	-25.63	0.54	-24.51	-26.79	17.4	0.3	18.01	16.68
ABR1	1976–13	-24.41	0.60	-23.36	-25.43	16.2	0.4	16.91	15.30
CAL1	1976–13	-25.46	0.50	-24.51	-26.46	17.2	0.3	17.91	16.49

iWUE responses to atmospheric CO_2 concentration increment

In all sites, we observed a negative relationship between atmospheric CO_2 concentration and $\delta^{13}\text{C}$. However, we observed a different trend in ZOO as compared with the other sites (Figure 5A1), where an increase of $\delta^{13}\text{C}$ in the stand after 1975 occurred (Figure 5A2). On the contrary, during the period 1976–2013, all sites showed a decreasing trend in $\delta^{13}\text{C}$ (Figure 5A3). During this stand development phase, the ZOO site responded differently than both VEN1 (z -value = 2.958, P -value = 0.01) and CAL1 (z -value = 3.866, P -value <0.01).

Carbon isotope discrimination showed a parabolic pattern in ZOO and VEN1 (Figure 5B1). Initially, both sites showed a decreasing trend (Figure 5B2), after which $\Delta^{13}\text{C}$ values increased. During the maturity phase of the stands, no relation was found in CAL1 and the response of $\Delta^{13}\text{C}$ to atmospheric CO_2 concentration was different between ZOO and VEN1 (z -value = -2.977, P -value <0.01) (Figure 5B3).

During the whole study period, C_i increased with increasing C_a in all sites (Figure 5C1), but only at VEN1 was the relationship significant before 1975 (Figure 5C2). In all sites, we observed a C_i increment after 1975 and differences in the response of ZOO with both VEN1 (z -value = -2.815, P -value = 0.02) and CAL1 (z -value = -3.702, P -value = 0.001).

According to the three scenarios described by Saurer et al. (2004), iWUE (Figure 6) is the result of an active response (constant C_i/C_a) to changing atmospheric CO_2 concentrations in VEN1 and CAL1. In ZOO and ABR1, we observed at the beginning of the series an independent response to atmospheric CO_2 concentration increase (C_i constant), than the response tended to become active in both sites (Figure 6).

After 1975, when all the stands reached maturity, ZOO and CAL1 (z -value = -3.708, P -value <0.01) and ZOO and VEN1

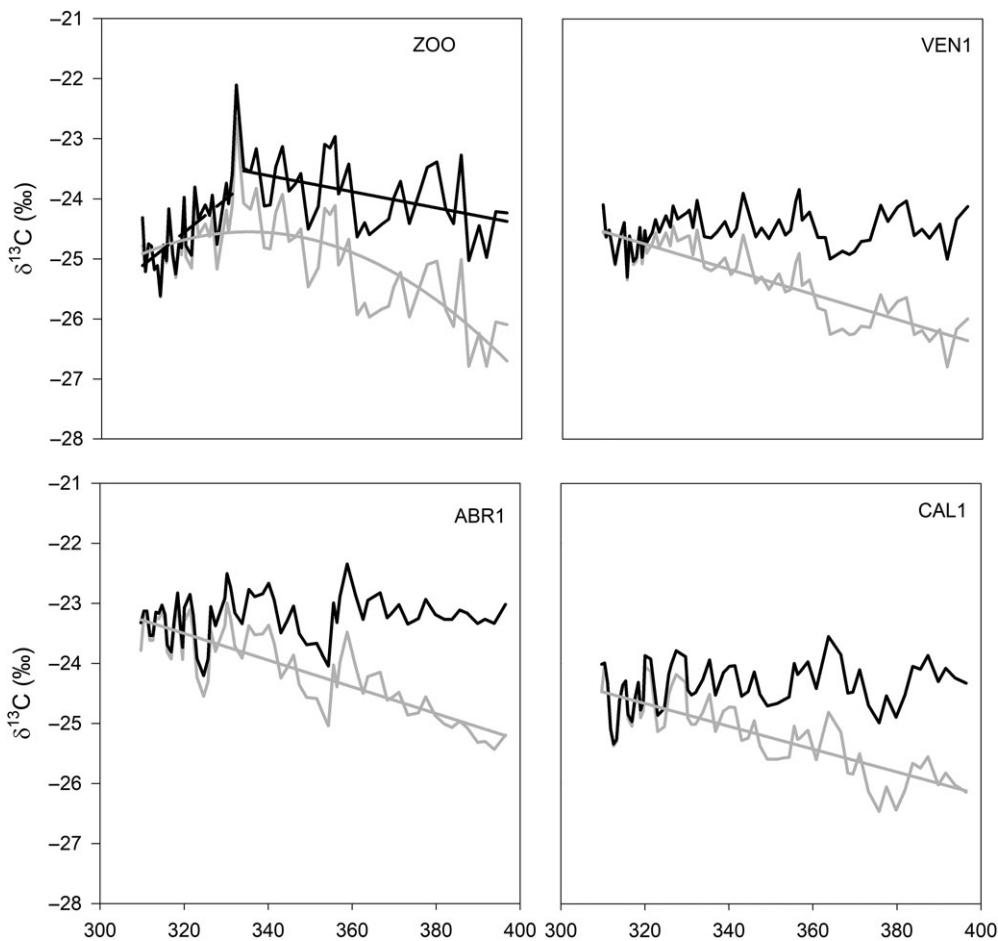


Figure 3. $\delta^{13}\text{C}$ (light gray) and $\delta^{13}\text{C}_{\text{pn}}$ (black) patterns in each site. Standard deviation bars for each year were omitted for a clear representation.

(z -value = 2.818, P -value = 0.02) showed different responses of iWUE to increasing atmospheric CO₂ concentrations.

Disentangling CO₂ and climate effects on iWUE

As demonstrated by the commonality analysis the models explained a great amount of iWUE variance (Figure 7).

In ZOO, the model ($i\text{WUE} = -0.65 T_{\text{med}} + 0.27 C_a - 0.02 \text{PP}$) explained 62.2% of the iWUE variance, and the pure effects of T_{med} , C_a and PP are 0.2%, 44.6% and 9.9%, respectively. For the first difference order data, the model ($i\text{WUE} = -0.21 T_{\text{med}} + 0.27 C_a - 0.02 \text{PP}$) explained 24.6% of iWUE variance during 1950–2013, and the main effects are related to precipitation (21.2%)

In VEN1, the model explained 78.5% of iWUE variability ($i\text{WUE} = -0.12 T_{\text{med}} + 0.22 C_a - 0.002 \text{PP}$), with T_{med} , C_a and PP accounting purely for 0.0, 45.6 and 2.2%, respectively. Considering the first-order data, the model explained 13.2% ($i\text{WUE} = 1.2 T_{\text{med}} + 0.99 C_a - 0.001 \text{PP}$), and T_{med} , C_a and PP affect iWUE variability of 6.8, 3.3 and 4.7%, respectively.

In ABR1, the only effect considering the raw data is related to C_a and its interactions with the other variables, with the ability of the model ($i\text{WUE} = -0.82 T_{\text{med}} + 0.25 C_a - 0.0001 \text{PP}$) to explain

73% of the iWUE variance. Considering, the inter-annual variability ($i\text{WUE} = -0.42 T_{\text{med}} + 0.003 C_a + 0.50 \text{PP}$) is explained at 54.4% of iWUE variance exclusively by precipitation.

In CAL1, we found similar results to ABR1. Indeed, the explained variance of iWUE (74.2%), considering the raw data, is due to the C_a pure effects and its joint effects with climatic conditions ($i\text{WUE} = -0.26 T_{\text{med}} + 0.24 C_a + 0.0004 \text{PP}$). The inter-annual variation of iWUE is explained by 53% by precipitation ($i\text{WUE} = -0.18 T_{\text{med}} - 0.0004 C_a + 0.54 \text{PP}$).

Interaction between radial tree growth and iWUE

The mean temporal growth patterns of the study sites are shown in Figure 8. In ZOO, BAI series typically showed an early growth suppression phase from 1870 until 1950, then a rapid increase of BAI (release phase). Afterwards, growth remained stable along the maturity phase (until 2013). Dendrochronological results of VEN1 indicated three phases in forest development: a short early growth suppression from 1870 to 1900 before a rapid increase of growth (release phase) until 1940 and a stable maturity phase up to 1970, then a BAI decline was observed after a small increase.

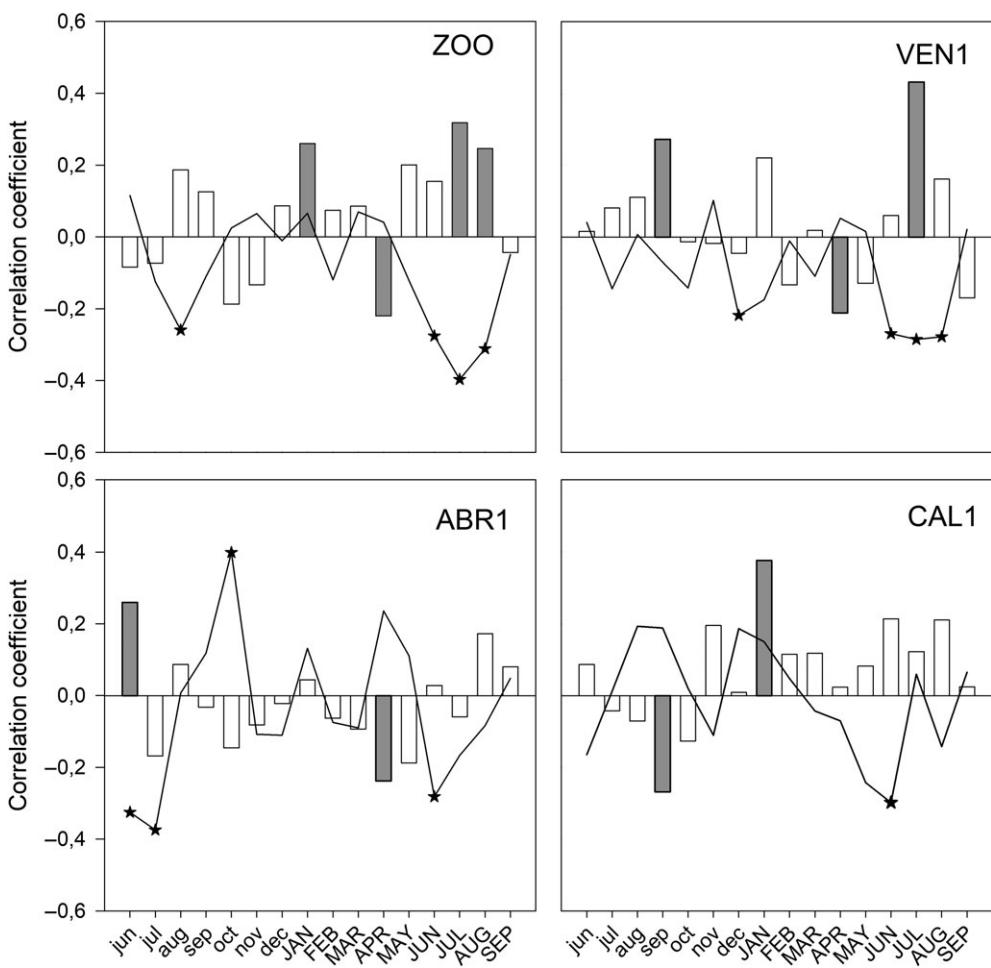


Figure 4. Relationships between monthly climatic parameters of current (uppercase) and previous year (lowercase) and $\delta^{13}\text{C}_{\text{pin}}$. Correlation coefficients were calculated using bootstrapped correlations, and the statistical significance was fixed at P -value ≤ 0.05 . Vertical bars represent correlation coefficients between $\delta^{13}\text{C}_{\text{pin}}$ and monthly max temperature, gray bars are significant. Light gray line represents correlation coefficients between precipitation and $\delta^{13}\text{C}_{\text{pin}}$, black stars represent significant coefficients.

BAI series in ABR1 showed a period of early growth suppression (under coppice cycle) till 1910. A subsequent increase in growth (release phase) lasted till 1978 as an effect of the conversion to a high stand in the 1950s. During maturity phase, BAI showed a little decline from 1980 until 2000.

CAL1 BAI series showed a release phase until 1970 and a rather unstable maturity phase up to 2013.

Our results showed significant positive correlations between iWUE and BAI only in ZOO ($R = 0.692$, P -value < 0.01) and ABR1 ($R = 0.577$, P -value < 0.01).

Discussion

Climatic effects on carbon isotope discrimination

Our findings suggest a strong effect of spring temperature on $\delta^{13}\text{C}_{\text{pin}}$ in ZOO, VEN1 and ABR1. On the other hand, we found no correlations with spring temperature at the warmest site CAL1. This could be due to an inter-sites phenological diversity. Phenology is

highly correlated with temperature, especially in the months preceding seasonal ontogenetic phases (Peñuelas and Filella 2001). Michelot et al. (2012) suggested that in diffuse porous species, such as beech, the onset of xylem production is mainly dependent on leaf photosynthesis. Hence, an early onset of photosynthesis may produce wood with lower values of $\delta^{13}\text{C}$, owing to the abundant soil water availability in spring. Scartazza et al. (2013) observed a wide seasonal variation of soluble sugar $\delta^{13}\text{C}$ in beech, with an enrichment in ^{13}C during the growing season. Moreover, our results evidence the sensitivity of $\delta^{13}\text{C}_{\text{pin}}$ to summer drought. In the northern sites (ZOO and VEN1), this sensitivity is underlined by positive correlations with summer temperature. Porté and Loustau (2001) demonstrated that the inter-annual variability in $\Delta^{13}\text{C}$ observed in tree-ring chronologies of 26-year-old maritime pines is partly explained by variability in summer temperature. Moreover, we observed negative correlations between $\delta^{13}\text{C}_{\text{pin}}$ and whole summer precipitation (ZOO and VEN1) and early summer precipitation (ABR1 and CAL1). Similarly, Roden

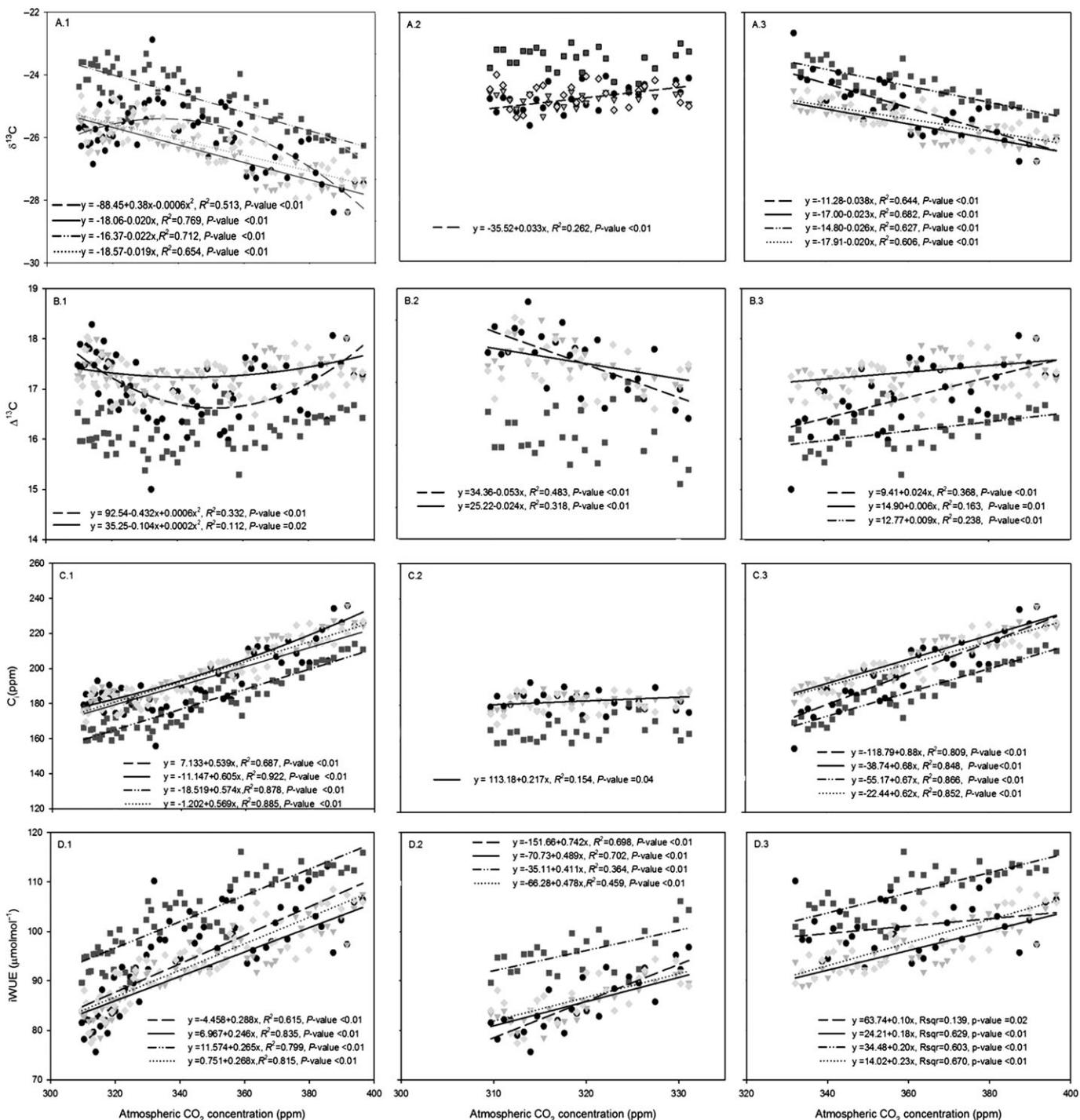


Figure 5. Relationships between $\delta^{13}\text{C}$ (A), $\Delta^{13}\text{C}$ (B), C_i (C) and iWUE (D) during the whole study period (1), from 1950 to 1975 (2) and from 1976 to 2013 (3) with atmospheric CO₂ concentration. Black dot and dashed line = ZOO, triangle and continuous line = VEN1, square and dashed and dotted line = ABR1, diamond and dotted line = CAL1. Significant relationships are represented by lines with the respective equation, coefficient of determination and statistical significance. Standard deviation bars for each year were omitted for a clear representation.

and Ehleringer (2007) observed a negative correlation between summer precipitation and $\delta^{13}\text{C}$ in *Pinus ponderosa*. Furthermore, observations on oak trees growing in the north of France showed a negative correlation between $\Delta^{13}\text{C}$ and July temperature but a positive one linking $\Delta^{13}\text{C}$ with July–August relative humidity (Raffalli-Delerce et al. 2004).

iWUE responses to increasing atmospheric CO₂ concentrations

The tree-ring $\delta^{13}\text{C}$ declining trend observed in our study is in agreement with previous findings (Leavitt and Lara 1994, Kitagawa and Matsumoto 1995, Raffalli-Delerce et al. 2004,

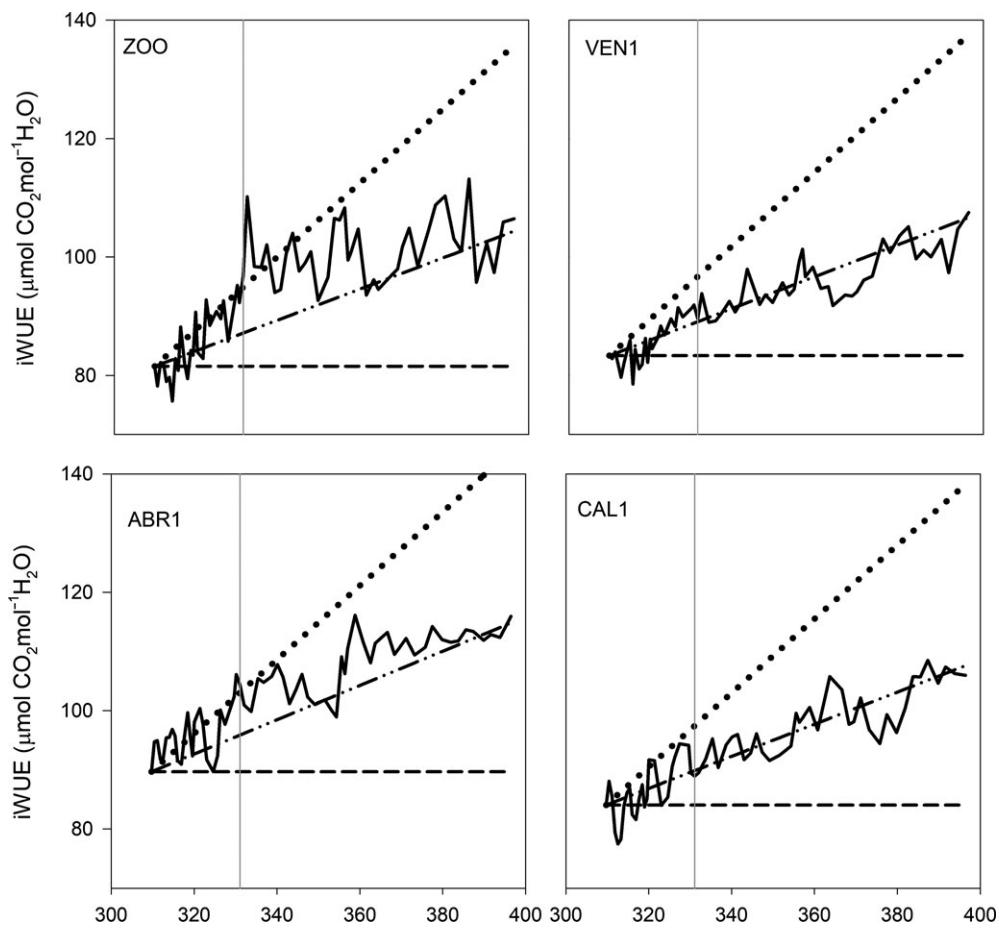


Figure 6. Intrinsic water-use efficiency (iWUE) (black line) in relation to the three possible different scenarios based on the theoretical response of trees to rising atmospheric CO₂:C_i constant (dotted lines), C_i/C_a constant (dashed and dotted lines), C_a – C_i constant (dashed lines). The gray vertical line represents the atmospheric CO₂ concentration of 1975.

McCarroll et al. 2009) reporting negative trends since the start of the industrial period (~ AD 1850). This is probably not just related to climate changes, but likely depends on plant physiological responses to anthropogenic changes in the atmospheric CO₂ concentration (Treyte et al. 2009), and on the incorporation of isotopically lighter carbon primarily released by the burning of fossil fuels (Freyer and Belacy 1983). Indeed, after PIN correction (McCarroll et al. 2009), based on the physiological response of trees to increased atmospheric concentrations of CO₂, $\delta^{13}\text{C}_{\text{pin}}$ patterns were more or less stable, except for ZOO where we found increasing and decreasing trends before and after 1975, respectively.

In this study, we observed a negative relationship between $\delta^{13}\text{C}$ and C_a but positive ones between C_a, $\Delta^{13}\text{C}$ and C_i when the stands reached the maturity phase, similar to what was reported for Spanish beech forests (Peñuelas et al. 2008). Before the maturity phase, absent or weak correlations were found (see Figure 5A2). This is probably due to a confounding age effect, which is related to two main factors: changes in micro-environmental variables during stand maturation and physiological changes linked to tree structural development (Peñuelas et al. 2008).

The iWUE increased by an average of 39% over the second half of the 20th century as expected from the fast build-up of atmospheric CO₂. The magnitude of change is in agreement with previous reports that found an increase of iWUE during the same period by using historical records in both leaves and tree rings (Peñuelas et al. 2008, Frank et al. 2015). Over the whole period of study (1950–2013), iWUE increased by approximately 50% in ZOO, 37% in VEN1, 30% in ABR1 and 40% in CAL1, in agreement with many studies investigating chronological responses in tree-ring $\delta^{13}\text{C}$ and iWUE (Feng and Epstein 1995, Bert et al. 1997, Duquesnay et al. 1998, Peñuelas et al. 2008). In other studies on coniferous trees, absent responses have been reported (Marshall and Monserud 1996).

According to Saurer et al. (2004), our results suggest that in ZOO and ABR1, iWUE response to atmospheric CO₂ increase was independent from C_a (C_i constant), as observed in other studies (Brienen et al. 2011, Huang et al. 2017), then the responses became active (C_i/C_a constant). These results suggest that there might be a dynamic leaf gas exchange strategy as a response to increasing atmospheric CO₂ (Voelker et al. 2016), derived by different environmental factors.

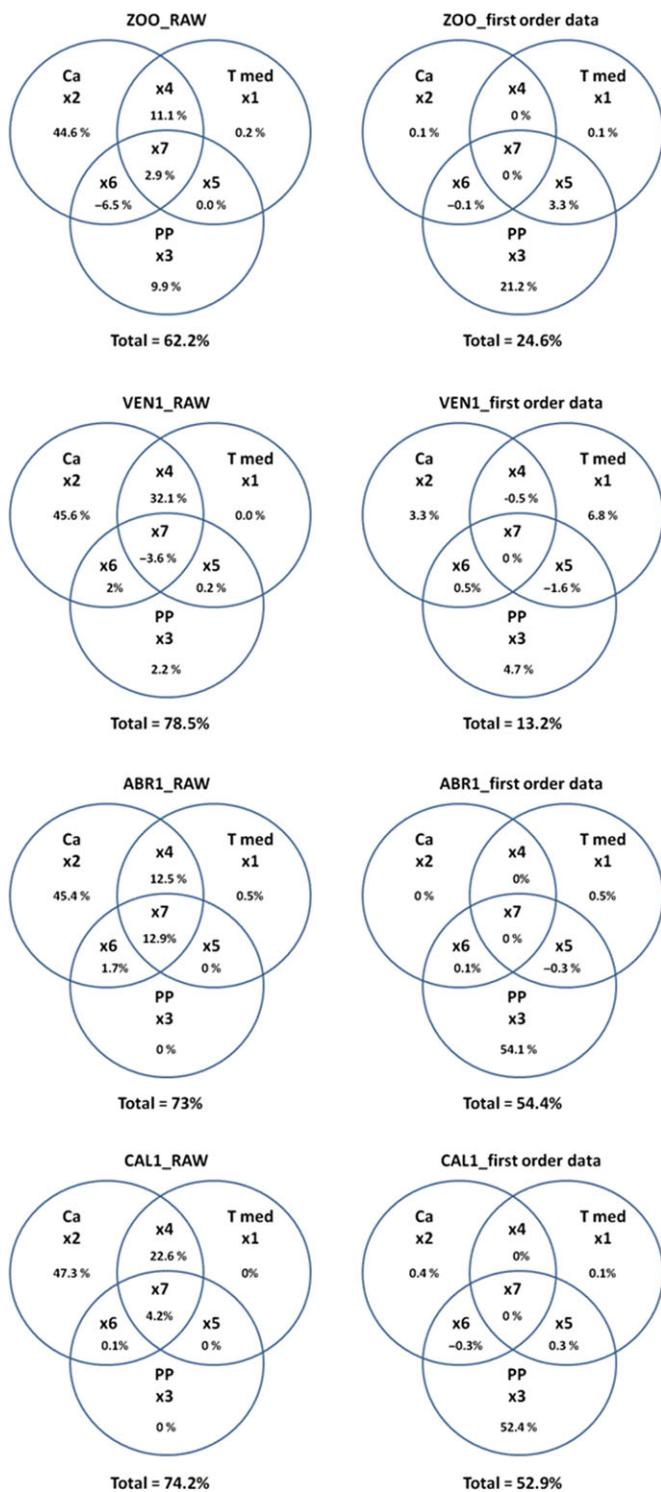


Figure 7. Variations of iWUE explained by the commonality analysis (CA) for the raw and first-order difference data in each site. The CA led to seven fractions of explained variations for iWUE: the pure effect of T_{med} (x_1), the pure effect of C_a (x_2), the pure effect of PP (x_3), the joint effect of C_a and T_{med} (x_4), the joint effect of T_{med} and PP (x_5), the joint effect of C_a and PP (x_6) and the joint effect of T_{med} , C_a and PP (x_7). The percentages represent the explained variance of each singular fraction.

Since 1950, the responses to atmospheric CO_2 increase were active in VEN1 and CAL1, as found in other studies performed in moist sites (Giammarchi et al. 2016).

Moreover, after 1975 when all stands reached the maturity stage and temperature increased faster, we found the highest difference in response of iWUE to variation of C_a between the northernmost and southernmost sites. We cannot differentiate between possible contributions of genetic adaptation and/or phenotypic plasticity to the phenomenon. However, intra-specific genetic diversity is the result of the species' biogeographical history (Robson et al. 2012). As for many other forest species, beech distribution range was reduced during the last glaciation to southern refugia around the Mediterranean and in the Balkans (Magri et al. 2006, Magri 2008, Gömöry et al. 2010). Post-glacial recolonization into central Europe took place from the southeast (Giesecke et al. 2006). Several studies reported a high genetic differentiation among populations (Jump and Peñuelas 2007, Gömöry et al. 2010) and inbreeding within populations, evidencing the capacity of beech towards a relatively fast local adaptation (Jump et al. 2007).

This could explain the higher sensitivity of iWUE to C_a increment of the southern marginal population (CAL1), which is expected to exhibit a better drought adaptation (Rose et al. 2009). A study focused on European chestnut populations growing in mesic and xeric areas showed that a high phenotypic plasticity in iWUE and $\Delta^{13}\text{C}$ is a genetically based prerequisite of drought adaptedness (Lauteri et al. 2004). Such a capability in forest species exposed to climate changes would imply a better fitness, especially for those populations established in drought prone areas, where increasing temperatures are expected to meet decreasing and more erratic precipitation.

Moreover, our CA results suggested that the long-term trend of iWUE is affected by C_a increment with a pure and a joint effect of temperature. In contrast, precipitation mostly influences the short-term variations of the data. Precipitation had a positive effect on iWUE inter-annual variance in the southernmost sites, and a negative impact at the northernmost sites. This difference can be related to regional climatic characteristics. In fact, CAL1 and ABR1 are characterized by dry summers, and precipitation affects soil water availability and air humidity. Hence, it modulates the stomatal conductance and, therefore, photosynthesis, as demonstrated by Fernández de Uña et al. (2016) on *Quercus faginea* growing in Spain and by Hartl-Meier et al. (2015) on beech and larch growing in Austria.

Relationship between growth and iWUE

Despite the increment of iWUE, expected increases in growth rates in response to rising atmospheric CO_2 (Long et al. 2004) were only observed in the ZOO and ABR1 sites. In ZOO and

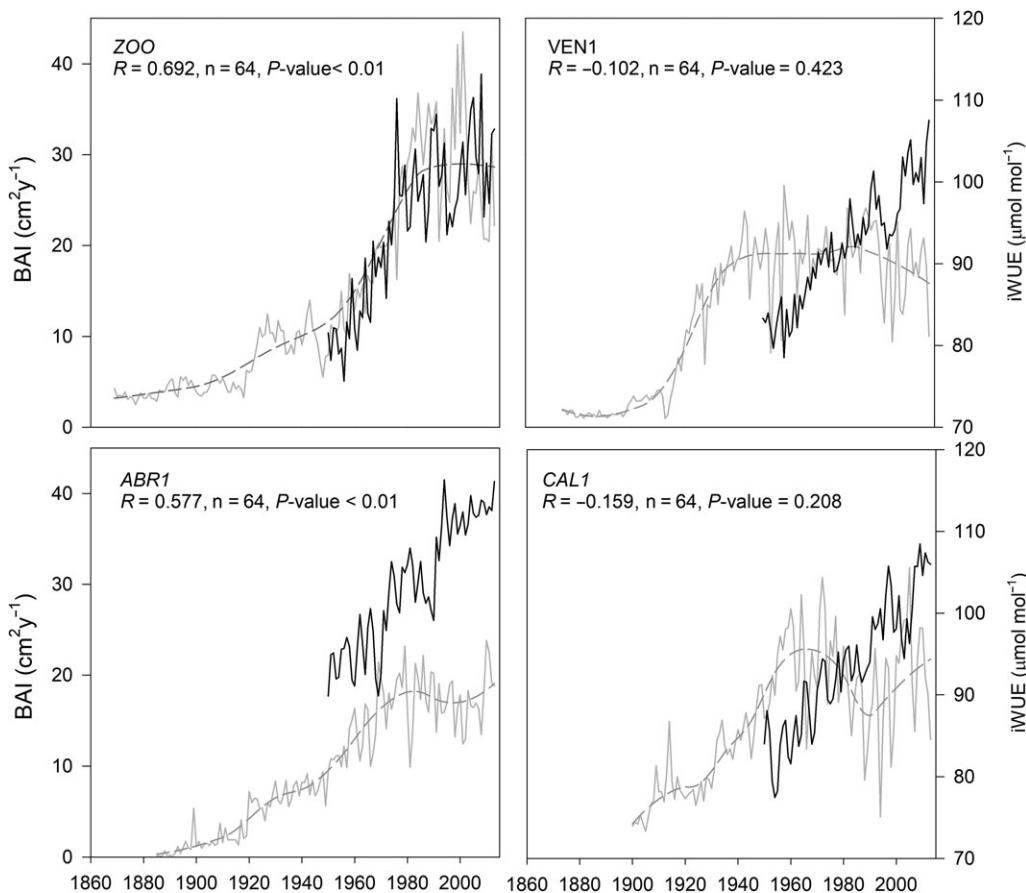


Figure 8. Basal area increment (BAI) at ZOO, VEN1, ABR1 and CAL1. Gray lines represent BAI curves; gray dashed lines are the 50 years smoothing spline. Black lines represent iWUE values.

ABR1, we hypothesize that in addition to the effect of C_a , iWUE could be affected by silvicultural treatment. In particular, in ABR1 the management plan evidenced that a conversion from coppice with standard to high stand occurred immediately after the end of the second World War.

Our hypothesis is that generally the canopy is ‘organized’ to maximize carbon fixation. Thinning, after a drastic decline of stand leaf area index, deeply modified the ratio between shade and light leaves, increasing the amount of light leaves that have higher photosynthetic rates (Matteucci et al. 1999, Scartazza et al. 2016). This higher photosynthetic rate caused the increase of iWUE. Hence, the increase of iWUE due to the photosynthetic component of the ratio is reflected in BAI increment, as evidenced in studies on *Abies pinsapo* (Linares et al. 2009) and in the Shawinigan Lake experimental site (Brooks and Mitchell 2011). Hence, in these two sites, the response of iWUE to atmospheric CO₂ increase, following the C_i constant scenario, is related to an increase of A component of the ratio, not to reduction of g_s as reported by Huang et al. (2017) and Brien et al. (2011). The relationship between thinning and iWUE was explored by other studies that evidenced a reduce in iWUE (McDowell et al. 2003, 2006, Di Matteo et al. 2010, Brooks and Mitchell 2011, Sohn et al. 2013), related to an increase in soil

water availability, through reduced root competition and canopy rainfall interception, thus decreasing drought stress and increasing g_s (Fernández de Uña et al. 2016). Hence, different silvicultural treatments can have different effects on iWUE.

In VEN1 and CAL1, increasing iWUE did not produce growth increments, indicating that other local stress factors overshadowed the expected CO₂-induced growth increase (Poorter and Pérez-Soba 2001, Saurer et al. 2004, Waterhouse et al. 2004, Linares and Camarero 2012). Indeed, a decline in productivity coupled with an increase in iWUE was observed in Norway spruce (Sanders et al. 2016). Climate change and particularly warming-related drought, light competition, nutrient limitation and/or physiological long-term acclimation to elevated CO₂ have been proposed as potential factors that could limit the expected CO₂ fertilization effect (Hyvönen et al. 2007, Levanic et al. 2011).

Conclusions

Site climatic conditions affect carbon isotope discrimination with several ecophysiological implications. At higher latitudes, a climatic control on stomatal conductance by summer precipitation and temperature that affect soil water availability can be suggested.

Moreover, an 'earliness effect' is suggested by a positive relationship between spring temperature and $\delta^{13}\text{C}_{\text{pin}}$ in the coldest sites, where the early onset of photosynthetic activity allows the trees to grow under favorable conditions (i.e., soil water availability).

In all study sites, we observed an increasing temperature since 1975, probably related to the atmospheric CO₂ concentration increment. This increment of CO₂ was the main driver of the long-term increasing trend of iWUE, resulting in an active response of trees to CO₂ fertilization. Moreover, precipitation mostly influences positively and negatively the inter-annual variations of iWUE of the southernmost and northernmost sites, respectively.

In addition, sensitivity of iWUE to increment of atmospheric CO₂ was different between the two extreme sites. This behavior is generally shown by species occupying large geographic ranges that respond to contrasting environmental conditions by genotypic variation and different expression of phenotypic plasticity.

Finally, we hypothesize that the increase of iWUE was correlated with growth in the sites where appropriate silvicultural treatments were performed. Hence, for a better understanding of forest responses in a changing world, we should consider not just climatic drivers but also interactions with forest management. Tree rings and their isotopic signatures can be powerful tools to investigate the past responses of trees to different forest management under the forcing effect of climate changes. In order to clarify this point, a specific experiment comparing isotopic signatures under different management options under controlled conditions (stand structure before and after the treatments) should be launched.

Moreover, $\delta^{18}\text{O}$ analysis may provide additional information on stomatal response (e.g., Grams et al. 2007), which would better highlight the complex physiological mechanisms that control trees' carbon uptake under real site conditions, highlighting the forests' potential in mitigation processes.

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Conflict of interest

None declared.

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