Testing a dual isotope model to track carbon and water gas exchanges in a Mediterranean forest

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Due to climate change, drier summers have been observed over the last ten years in Mediterranean areas. Increasing drought levels may have a different weight in influencing the stomatal versus photosynthetic activity of forests, altering the water-use efficiency (*i.e.*, WUE, the amount of carbon gain per water lost) and, consequently, the global carbon balance. By combining leaf gas exchanges and leaf carbon/oxygen isotope measurements, we tested under Mediterranean conditions a semi-quantitative dual isotope model to track adjustments in stomatal conductance (g_s) and maximum CO_2 assimilation at saturating light (A_{max}) in response to changes in air and soil water availability. The experiment was established at Allumiere site (Rome, Italy) over the course of two consecutive years. There, we modified the amount of precipitation reaching the soil on water depleted (D) and watered (W) replicate plots (~100 m^2) of an *Arbutus unedo* L. forest using a system of rain gutters and sprinklers, respectively. Changes in soil water availability affected g_s and A_{max} in parallel. As an application of the model, we found that, in response to reduced air and soil water availability, constant carbon ($\delta^{13}C$) and increasing oxygen ($\delta^{18}O$) isotope values were consistent with a parallel decline of either g_s and A_{max}. As a result of parallel decline, WUE did not differ or only slightly differed between treatments, in contrast with most of the studies that found a wide-spread increase of WUE in response to enhanced drought. This study highlights the potentiality of the dual isotope model to provide insights of forest ecosystem functioning in Mediterranean environments.

Keywords: Carbon assimilation, Drought, Isotope model, *Arbutus unedo*, Stomatal conductance, Water-use efficiency.

Introduction

In a Mediterranean-type climate, forest ecosystems are typically subjected to high temperature and scarce soil water availability during most of the summer. Moreover, due to the impact of climate change, more extreme drought periods are expected for the coming decades as a consequence of reduced precipitation during summers (IPCC 2007, Sarris et al. 2007). Under such conditions, gas exchanges will be largely limited by the availability of water for transpiration (Rambal et al. 2003). Partial stomatal closure is one of the key mechanisms adopted by trees to save water and to avoid cavitation risks (Tyree & Sperry 1989, Irvine et al. 1998, Cinnirella et al. 2002).

In turn, climate change may result in a negative impact on plant carbon assimilation through the increase of stomatal limitation to photosynthesis (Jarvis & Davies 1998, Lawlor & Cornic 2002). Thus, by the adjustments of stomatal conductance (g_s) and maximum CO_2 assimilation (A_{max}) in response to increasing drought will depend the amount of carbon that can be assimilated by trees. Additionally, these adjustments will determine the ability of Mediterranean forests to counterbalance the negative effect of increasing drought on growth and, consequently, on the gross primary production (GPP - Rambal et al. 2003). In semi-arid environments, the shape and the values of A_{max} and g_s will also determine the intrinsic water-use efficiency (WUE_{int}), a component of the long-term water-use efficiency (WUE_T, the amount of carbon gain per water lost). WUE_{int} is also a key parameter for deriving the productivity and the amount of carbon assimilated by forests (Ripullone et al. 2004).

Previous models were based on the simplifying assumption of a constant linear relationship between g_s and A_{max}, with a simple dependence upon vapour pressure deficit (VPD - Wong et al. 1979). However, other studies have shown that this relationship is influenced by other environmental stresses such as soil moisture (Ball et al. 1987, Leuning 1995). Non-stomatal limitations (*i.e.*, mesophyll conductance and biochemical reactions - Rubisco, etc.) may be involved even under mild-moderate water stress (Flexas & Medrano 2002, Grassi & Magnani 2005), thus changing the A_{max} and g_s relationship (and consequently the WUE).

The use of stable carbon ($\delta^{13}C$) isotope as a powerful tool for investigating the balance between A_{max} and g_s (see isotope theory) has grown steadily during the past two decades. The positive relationship between WUE and $\delta^{13}C$ arises through their independent linkages to the ratio of internal to ambient CO_2 concentrations (c_i/c_a - Farquhar et al. 1989, Guehl et al. 1995). To differentiate between the changes in $\delta^{13}C$ driven by A_{max} or g_s, Scheidegger et al. (2000) proposed the incorporation of $\delta^{18}O$ in a dual quantitative model. In fact, $\delta^{18}O$ is affected by transpiration rates, which is closely correlated with g_s (Barbour & Farquhar 2000).

The broader purpose of this study was to test in the Mediterranean environment a dual isotope conceptual model (Scheidegger et al., 2000) to infer CO_2 and H_2O gas exchange activities of *Arbutus unedo* forest. To this aim, $\delta^{13}C$ and $\delta^{18}O$ in combination with direct measurements of leaf gas exchanges have been employed to give insight into the adjustments of g_s and A_{max} resulting from different vapor pressure deficit (VPD) and soil water availability; this latter was changed by an experimental manipulation of the amount of precipitation reaching the soil. Furthermore, based on the observed variations in g_s-A_{max} and $\delta^{13}C$, the effects of water restriction on WUE were also examined.

Isotope theory

Carbon and oxygen isotopes

The stable isotope technique has been revealed to be an important tool in identifying medium and long-term effects of environmental factors on CO_2 and H_2O gas exchanges in plants. The carbon isotope composition ($\delta^{13}C$) of leaf organic matter reflects the fractionation processes occurring during

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the diffusion of $^{12}CO_2$ and $^{13}CO_2$ through stomatal pores and photosynthetic assimilation. In C_3 plants, discrimination against ^{13}C is linked to photosynthesis *via* c_i/c_a, the ratio of intercellular (c_i) to atmospheric (c_a) CO_2 concentrations as described by Farquhar et al. (1982 - eqn. 1):

$$\delta^{13}C_{leaf}=\delta^{13}C_{atm}-a-(b-a)\cdot(c_i/c_a)$$

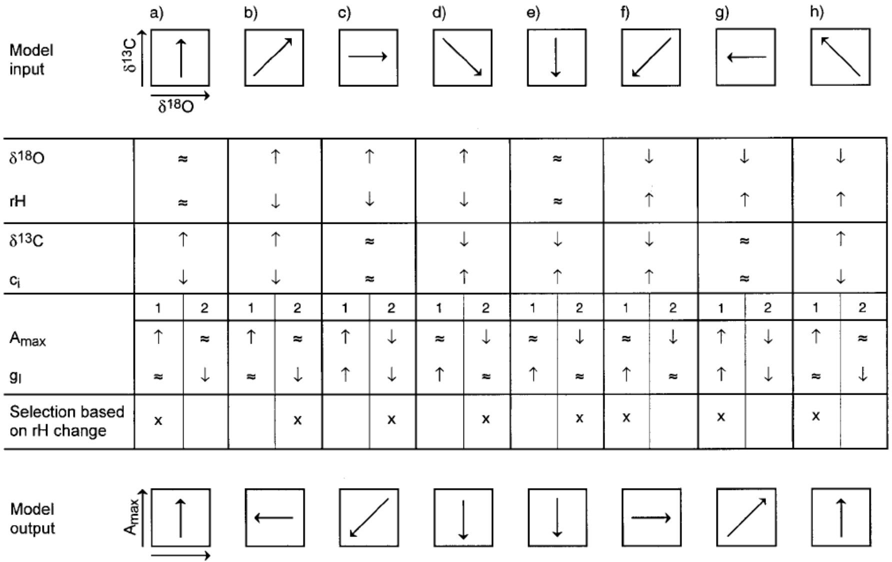
where $\delta^{13}C_{leaf}$ and $\delta^{13}C_{atm}$ are the carbon isotope compositions of leaf organic matter and atmospheric CO_2, respectively, and *a* and *b* are fractionation factors occurring during diffusion of CO_2 through stomata pores (-4‰) and enzymatic C-fixation by Rubisco (-27‰), respectively. According to equation 1, a lower c_i/c_a ratio results in an increased $\delta^{13}C_{leaf}$ due to a lower discrimination (Δ) against $^{13}CO_2$. Environmental factors such as water availability and irradiance can cause variations of c_i/c_a, mainly through their effects on both stomatal conductance (g_s) and photosynthetic activity (Farquhar et al. 1989).

$\delta^{13}C$ has largely been used as a proxy for long-term water-use efficiency (WUE, the amount of carbon gained per water transpired) of C_3 plants (Farquhar et al. 1989), thanks to their independent linkages to c_i/c_a (eqn. 2):

$$WUE=(1-\Phi)\cdot c_a(1-c_i/c_a)/1.6\,VPD$$

where VPD is the vapour pressure difference between the intercellular spaces and the atmosphere and Φ is the fraction of carbon respired by the plants. However, a simpler concept of WUE is often used (*i.e.*, intrinsic water-use efficiency (WUE_{int} = A/g_s, the ratio of CO_2 assimilation to stomatal conductance) or instantaneous water-use efficiency (WUE_{inst} = A/E, the ratio of assimilation to leaf transpiration) when the leaf-to-air vapour pressure difference is known (Farquhar et al. 1989). These are both components of long-term WUE. Although it successfully captures WUE trends, $\delta^{13}C$ fails to account for factors responsible for variations in WUE. These variations can be the result of changes in g_s or A. For example, an increase in $\delta^{13}C$, interpreted as a reduction in c_i and an improvement in WUE in the Farquhar model, can be the result of either: (1) reduced g_s (at constant A); or (2) increased A (at constant g_s).

The oxygen isotope composition ($\delta^{18}O$) of leaf organic material can be used for distinguishing between possible causes of variation in $\delta^{13}C$, thanks to the link of $\delta^{18}O$ with the isotopic fractionation of water during transpiration in leaves. During transpiration, molecules of water containing lighter isotopes ($H_2{}^{16}O$) tend to diffuse faster from the site of evaporation to the atmosphere. In this way, water becomes enriched in the heavier isotopes of ^{18}O, compared to water coming from the soil. The oxygen isotopic composition of leaf water at the sites of evaporation (Δ_E) is expressed as follows (Craig & Gordon 1965, Dongmann et al. 1974, Farquar & Lloyd 1993 - eqn. 3):



	a)		b)		c)		d)		e)		f)		g)		h)	
δ18O	≈		↑		↑		↑		≈		↓		↓		↓	
rH	≈		↓		↓		↓		≈		↑		↑		↑	
δ13C	↑		↑		≈		↓		↓		↓		≈		↑	
ci	↓		↓		≈		↑		↑		↑		≈		↓	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Amax	↑	≈	↑	≈	↑	↓	≈	↓	≈	↓	≈	↓	↑	↓	↑	≈
gl	≈	↓	≈	↓	↑	↓	↑	≈	↑	≈	↑	≈	↑	↓	≈	↓
Selection based on rH change	x			x		x		x		x	x		x		x	

Fig. 1 - The conceptual isotope model from Scheidegger et al. (2000): scheme of the eight scenarios from a) to h) based on all the likely $\delta^{13}C$-$\delta^{18}O$ combinations (model input). The changes are shown by the arrows. Relative humidity (rH) is derived from $\delta^{18}O$, while c_i is derived from $\delta^{13}C$ (symbols: ↑, ≈ and ↓ represent increase, no response and decrease in levels, respectively). For each scenario there are two possible cases, indicated as 1 and 2, with corresponding changes in A_{max} and g_s. The model output at the bottom gives relative A_{max} and g_s based on the rH changes.

$$\Delta_e=\epsilon^*+\epsilon_k+(\Delta_V-\epsilon_k)\cdot e_a/e_i$$

where Δ_V is the oxygen isotopic composition of water vapour in the air, ε^* is the temperature-dependent fractionation associated with the lower vapour pressure of $H_2{}^{18}O$ compared to that of $H_2{}^{16}O$, ε_k is the kinetic fractionation during evaporative water diffusion through the stomata and boundary layer, and e_a/e_i are the vapour pressures in the atmosphere and intercellular air spaces, respectively.

Thus, according to this equation, the degree of leaf water enrichment depends on the rH. The latter represents the evaporative driving force, and a reduced rH causes an increase of $\delta^{18}O$ in the leaf water. This enrichment is then expected to be reflected in the organic matter (De Niro & Epstein 1979, Yakir 1992, Farquhar et al. 1998, etc). $\delta^{18}O$, determined in this manner, has often been found to be negatively correlated with g_s (Barbour & Farquhar 2000, Grams et al. 2007).

Linking $\delta^{13}C$ and $\delta^{18}O$ in a dual isotope conceptual model

The dual isotope conceptual model proposed by Scheidegger et al. (2000) represents a simplified tool to infer A_{max} and g_s from the variation of $\delta^{13}C$ and $\delta^{18}O$ in plants. Fig. 1 shows all combinations of $\delta^{13}C$ and $\delta^{18}O$ values in eight likely scenarios where the outputs of the model (*i.e.*, g_s and A_{max}) are selected on the basis of changes in rH. Different environmental conditions can cause higher (↑), lower (↓) or similar (≈) $\delta^{13}C$ and $\delta^{18}O$ values. As an example, we refer to scenario b) where both $\delta^{13}C$ and $\delta^{18}O$ values increased. We know from the increase in $\delta^{18}O$ that rH must have increased, while from the increasing $\delta^{13}C$, we infer a decreasing c_i. This reduction is explained by two possible cases: (1) A_{max} ↑ and g_s ≈ or (2) A_{max} ≈ and g_s ↓. Because plants in dry air tend to close their stomata, we choose case (2) as it is physiologically more plausible than case (1). Thus, $\delta^{13}C$ reflects variation in c_i, while $\delta^{18}O$ is affected by variation in rH. This variation, in turn, drives changes in transpiration rates and g_s.

Materials and methods

Study site and experimental design

The research was carried out in a drought-prone 50 ha of Mediterranean macchia. The site was dominated by the coppice *A. unedo*, which covers 65% of the surface. The study site, environmental conditions and stand characteristics are detailed in Tab. 1.

Two different levels of volumetric soil water content (SWC, volume of water per

Tab. 1 - Site, environmental conditions and stand characteristics. (1): Soil Taxonomy; USDA Soil Survey Staff 1999.

Location	Allumiere, Lazio, Italy
Latitude	42° 11’
Longitude	11° 56’
Altitude (m a.s.l.)	180
Slope	Uniform
Soil type	Andsols$^{(1)}$
Soil depth (cm)	31
pH	4
Annual rainfall (mm) 1951 - 2005	919
Summer rainfall (mm) 1951 - 2005	120
Annual temperature (°C) 1951 - 2005	13.6
Age of trees (years)	25
Tree density (trees ha^{-1})	4070
Stem diameter at 1.3 m aboveground (cm)	5
Height (m)	5
Leaf area index ($m\ m^{-2}$)	5.5
Total basal area ($m^2\ ha^{-1}$)	19.6

volume of soil, multiplied by 100) have been imposed by an alteration of the amount of precipitation reaching the soil. This was done during the summers of 2004 and 2005 (June to August) on three replicated plots (~100 m^2). A mean value of 7% in SWC was obtained by partial rain exclusion (-20%), using a system of pipes suspended about 1.8 m above the forest floor (water-depleted plots, D). A mean value of 14% in SWC was obtained by adding water through a sprinkler net to simulate rain events (watered plots, W). A 10% threshold of SWC, established in a pre-treatment experiment, represented the dry (below) and well-watered (above) conditions (Ripullone et al. 2009).

Water relations and gas exchange measurements

SWC was measured within the D and W treatments by probes (Campbell Scientific, INC, Logan, Utah, USA). These probes consisted of two 30 cm long stainless steel rods, fully inserted into the soil at six different locations per replicate. The time domain reflectometry (TDR) method was used to translate the readings in SWC (Topp & Davis 1985).

Eight intensive field campaigns were carried out during 2004 and 2005 to assess plant water status. Predawn leaf water potential (Ψ_{pd}) was measured on six to eight experimental trees per replicate in D and W plots. For each tree we measured five fully expanded leaves with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA).

Maximum CO_2 assimilation at saturating light (A_{max}) and stomatal conductance (g_s) were measured using a portable infrared gas analyzer (LI-6400 Li-cor, Lincoln, NE, USA). Measurements were performed on 10 sunlit leaves of six trees growing in the central portion of each replicated plot to avoid the “edge effect”. Hours between 11:30 am and 15:30 pm on cloudless days were chosen for all measurements. This is when environmental conditions were most stable and when photosynthetic photon flux density (PPFD) was above 1200 µmol m^{-2} s^{-1} (above saturating light conditions for *A. unedo* - data not shown). In order to take into account the effect of vapour pressure deficit (VPD) in driving variation in WUE, we considered the instantaneous WUE (WUE_{inst}), calculated from gas exchange measurements, as the ratio of A_{max} to leaf transpiration rate (E).

Carbon and oxygen isotope analysis

Six samples of non-fully expanded leaves were collected from each replicate plot in five field campaigns (June, July 2004 and June, July, September 2005) to measure carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopes. Leaf samples were collected from the same shoots where gas exchange measurements were performed.

Bulk leaf samples were dried, ground to a fine powder and then weighed in tin capsules, using a value of 0.6-0.8 mg for $\delta^{13}C$ and 1.1-1.3 for $\delta^{18}O$. Bulk leaf material was combusted to CO_2 for carbon isotope analysis in an elemental analyzer (EA-1108, Carlo Erba, Italy), which was connected to a mass spectrometer (Delta-S Finningan MAT, Germany) *via* a variable open split interface (CONFLO II Finnigan MAT, Germany). For determination of $\delta^{18}O$, leaf bulk material was pyrolized (Saurer et al. 1998) to CO with an elemental analyzer (EA-1108, Carlo Erba, Italy), connected to the same mass spectrometer.

$\delta^{13}C$ and $\delta^{18}O$ were calculated as:

$$\delta x = [R_{sample}/R_{standard} - 1] \times 1000$$

where *R* is the isotope ratio and $^{13}C/^{12}C$ or $^{16}O/^{18}O$ refer to the sample and to the standard, respectively. The isotope values are expressed in delta notation, with V-PDB for

Tab. 2 - Variations in vapour pressure deficit (VPD), soil water content (SWC), predawn water potential (Ψ_{pd}), stomatal conductance (g_s), maximum CO_2 assimilation (A_{max}), intercellular CO_2 concentration (c_i), instantaneous water-use efficiency (WUE_{inst}), bulk leaf carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope composition in water depleted (D) and watered (W) plots during the experiment. Values marked with asterisks are significant for P < 0.05.

Date	Plot	VPD (kPa)	SWC (%)	Predawn Ψ (MPa)	g_s (mol $m^{-2}s^{-1}$)	A_{max} (µmol $m^{-2}s^{-1}$)	c_i (ppm)	WUE_{inst} (µmol mol^{-1})	$\delta^{13}C$ (‰)	$\delta^{18}O$ (‰)
Jun-04	D	3.2	13*	-0.54	0.107	5.63	254	1.82	-26.7	29.11
	W	3.3	17*	-0.46	0.113	5.21	257	1.79	-26.9	29.30
Jul-04	D	4.6	12*	-0.97*	0.044*	3.20*	235	2.21*	-26.6	30.37*
	W	4.4	15*	-0.86*	0.078*	5.24*	230	1.81*	-26.8	31.22*
Jan-05	D	0.9	16	-0.07	0.108	6.56*	253	7.20	-	-
	W	0.9	18	-0.05	0.125	7.86*	246	7.30	-	-
Feb-05	D	1.1	17	-0.47	0.084*	6.06	239	6.34*	-	-
	W	1.1	20	-0.45	0.110*	7.05	252	5.52*	-	-
Apr-05	D	4.4	23	-0.57	0.130*	7.92*	267	1.80	-	-
	W	4.7	23	-0.52	0.161*	9.02*	273	1.68	-	-
Jun-05	D	2.9	8*	-0.65	0.121*	7.80	227	2.77*	-25.8*	28.60
	W	3.1	14*	-0.61	0.148*	8.93	235	2.15*	-26.3*	28.70
Jul-05	D	3.3	5*	-0.89*	0.078*	5.84*	216	2.55	-26.1	28.80*
	W	3.1	15*	-0.44*	0.162*	10.11*	228	2.37	-25.9	29.26*
Sep-05	D	2.2	10*	-0.34	0.122*	7.88*	239	3.50	-26.3	27.20*
	W	2.3	21*	-0.41	0.151*	8.95*	238	3.39	-26.5	27.90*

carbon and VSMOW for oxygen. The accuracy of the method was ± 0.2‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{18}O$.

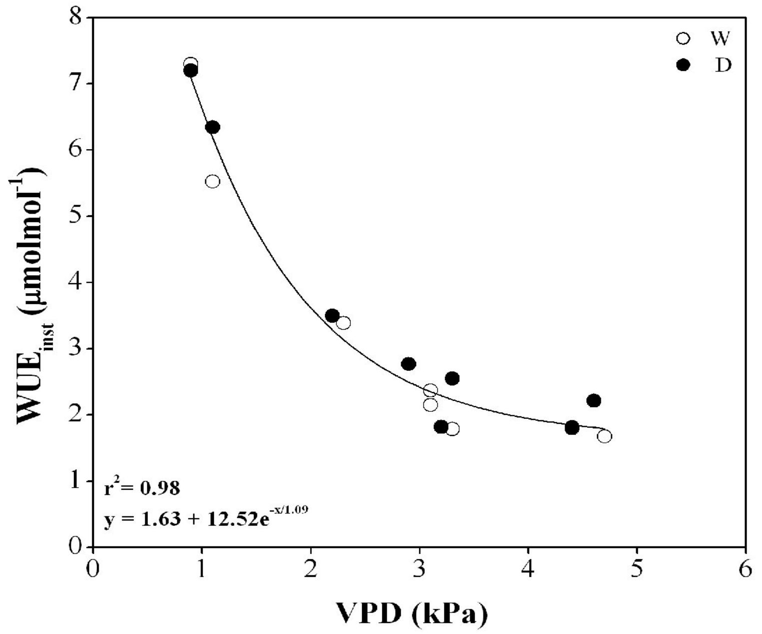


Fig. 2 - Correlation of instantaneous water-use efficiency (WUE_{inst}) and vapour pressure deficits (VPD) in water depleted (D) and watered (W) plots during the experiment.

Statistical analysis

Values of treatments (W, D) are presented as the mean ± standard error and compared using the Student-Newman-Keuls test. Statistical significance was defined as $P \leq 0.05$ and $P \leq 0.01$. Linear regressions were analysed using Pearson correlation coefficients. All statistics were computed with the SPSS statistical package (SPSS, Chicago, IL).

Results

Carbon and water relations

As a consequence of treatments, significant differences in soil water content (SWC) emerged between water depleted (D) and watered (W) plots, although such differences were higher in 2005 than 2004 (Tab. 2).

In general, predawn water potentials (Ψ_{pd}) reflected the SWC conditions during the two years of the experiment (Tab. 2). Significant differences in Ψ_{pd} were observed in the summer of 2004 and 2005 between D and W plots ($P < 0.05$), demonstrating the effectiveness of the applied treatments, even if the mean values for D plots never exceeded -1.0 MPa. Minimum Ψ_{pd} in the D treatment was similar in both years.

Leaf vapour pressure deficit (VPD) showed a bimodal pattern with a minimum in winter-spring and a maximum in summer, although July 2004 (4.8 kPa) was drier than July 2005 (3.5 kPa - Tab. 2).

Drier conditions during the summer had a strong effect on gas exchange activities. Significant reductions of stomatal conductance (g_s) and maximum CO_2 assimilation (A_{max}) were observed over the seasons, in response to changes in water availability (Tab. 2). g_s reached the maximum value (0.16 mol m^{-2} s^{-1}) in spring 2005 when environmental conditions were most favourable, whilst the lowest minimum rate was recorded in July 2004 (0.04 mol m^{-2} s^{-1}) under air and soil humidity stress. Despite the same Ψ_{pd}, the difference in g_s measured between July 2004 and 2005 was 0.3 mol m^{-2} s^{-1}. This was likely due to differences in VPD recorded between the two dates.

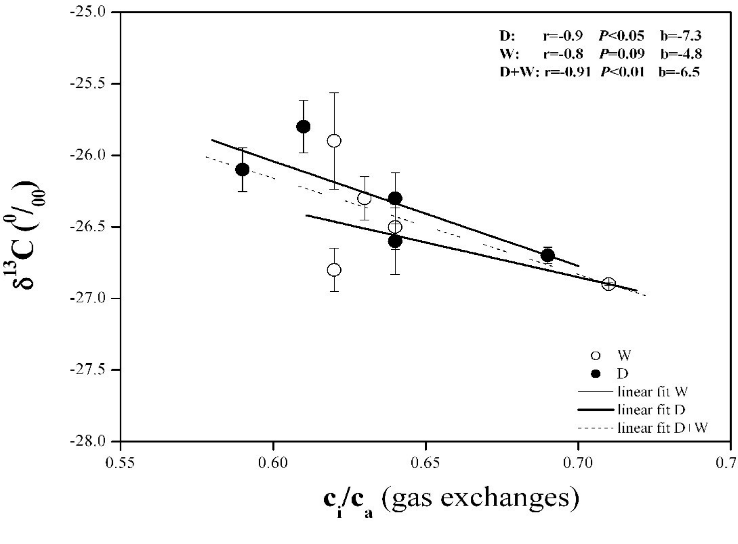


Fig. 3 - Correlation between leaf carbon isotope composition ($\delta^{13}C$) and intercellular to ambient CO_2 concentrations (c_i/c_a) assessed by gas exchange in water depleted (D) and watered (W) plots during the summer in 2004 and 2005. Symbols represent the mean value ± standard error for each date of measurement.

Significant differences in A_{max} between the D and W treatments were observed during the experiment, in particular during the summer drought. However, A_{max} values in D plots did not fall below 60% of those observed in the W treatment (Tab. 2); this was the consequence of not marked drought treatment imposed.

Because of parallel declines in g_s and A_{max}, computed intercellular CO_2 (c_i) concentration and instantaneous water-use efficiency (WUE_{inst}) did not differ between treatments (Tab. 2). A slight increase in WUE_{inst} was observed in D plots during July 2004 and 2005. However, WUE_{inst} showed large fluctuations over the season, mainly related to changes in VPD (Fig. 2).

$^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios in leaf organic matter

The carbon isotope composition ($\delta^{13}C$) was not affected by changes in SWC and VPD during the experiment (Tab. 2), with the exception of June 2005, when only slight and significant differences were shown between D and W plots. This result was consistent with our gas exchange findings, in which little or no changes in intercellular CO_2 concentration (c_i) and WUE_{inst} were found (Tab. 2). Furthermore, $\delta^{13}C$ was negatively correlated with c_i/c_a as derived from leaf gas exchange assessments (Fig. 3). However, this result was significant only for treatment D ($P < 0.05$) and D+W ($P < 0.01$). A significant and positive relationship (r = 0.76, $P < 0.05$) was also found between $\delta^{13}C$ and WUE_{inst} (data not shown).

In contrast, $\delta^{18}O$ was found to be more sensitive to variations in soil water availability than $\delta^{13}C$. In fact, $\delta^{18}O$ differed significantly between D and W plots in July 2004 and July/September 2005 (Tab. 2). A lack of difference in $\delta^{18}O$ values recorded in June 2004 between D and W treatments suggests that full access to water for *A. unedo* trees did not differentiate g_s from the transpiration response.

$\delta^{18}O$ significantly increased in response to reduced stomatal aperture ($P < 0.05$) and to the consequent leaf transpiration rate (Fig. 4). The correlation was not significant when the two treatments were considered separately, but they showed a parallel increase in $\delta^{18}O$ with a reduction of g_s. Both had a similar slope from the linear fit. A significant and negative correlation ($r = -0.67$; $P < 0.05$) was observed between $\delta^{18}O$ and WUE_{inst} (data not shown).

As a test of the dual isotope conceptual model, $\delta^{13}C$ was found not significantly correlated with $\delta^{18}O$ ($r = 0.42$, $P = 0.23$ - Fig. 5, top-left panel). The higher VPD during the period of leaf formation in 2005 led to a parallel increase in $\delta^{18}O$ (more positive values) in both D and W plots. In contrast, $\delta^{13}C$ was less sensitive to VPD changes, showing only

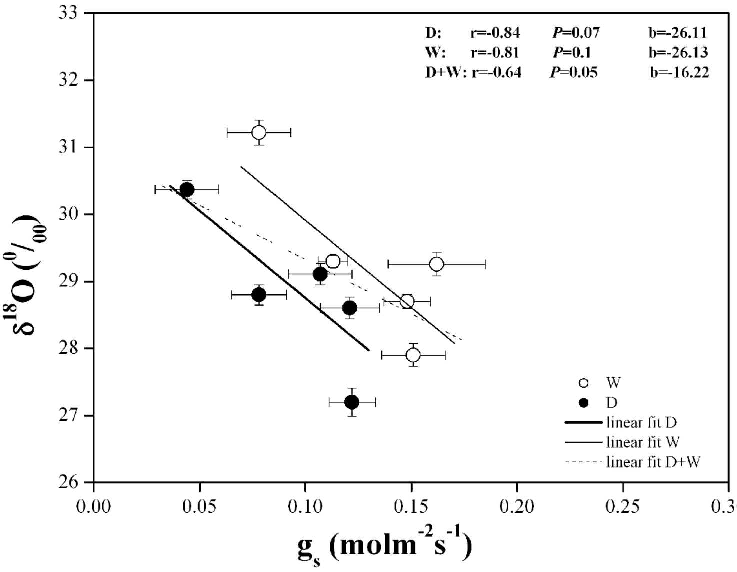


Fig. 4 - Correlation between leaf oxygen isotope composition ($\delta^{18}O$) and stomatal conductance (g_s) assessed by gas exchange in water depleted (D) and watered (W) plots during the summer in 2004 and 2005. Symbols represent the mean value ± standard error for each date of measurement.

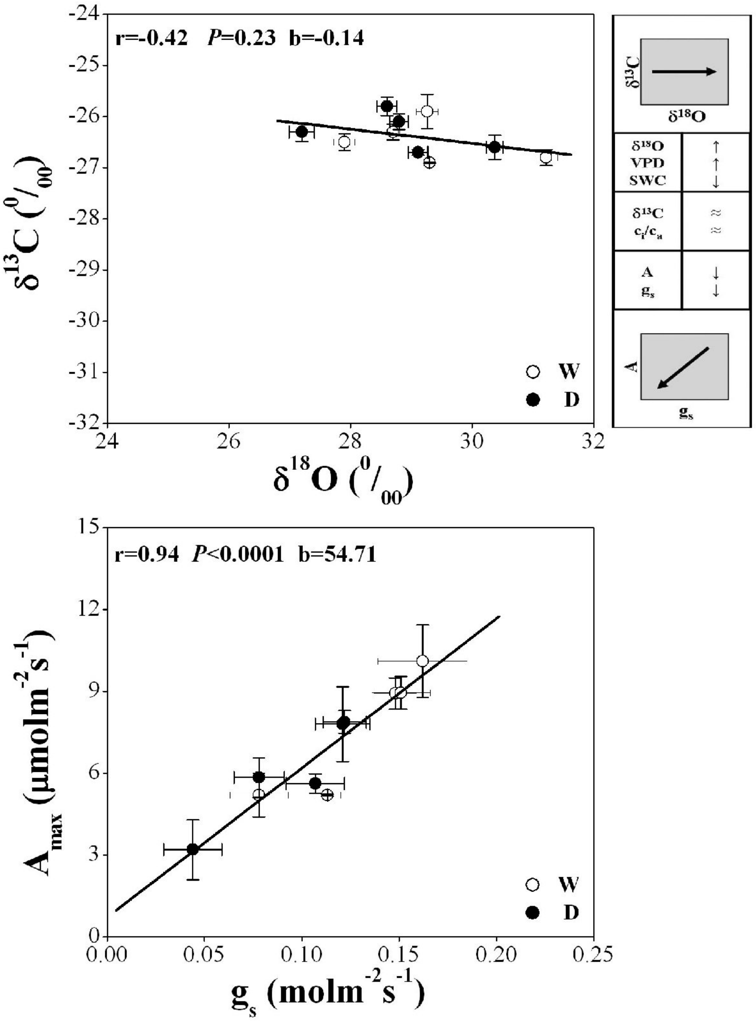


Fig. 5 - The $\delta^{13}C$-$\delta^{18}O$ relationship (top-left panel) in water depleted (D) and watered (W) plots during the summer in 2004 and 2005. The top right panel provides information derived from the $\delta^{13}C$-$\delta^{18}O$ relationship: this result is consistent with case c) in the Scheidegger model with the arrow pointing to right side; the model output of g_s-A_{max} indicates a decrease of either g_s or A_{max}. The bottom panel shows the correlation between A_{max} and g_s from gas exchange measurements. Symbols represent the mean value ± standard error for each date of measurement.

about 1.0‰ of variation. The direction of the arrow in the $\delta^{13}C$-$\delta^{18}O$ relationship matches scenarios *C*) and *G*), indicating that g_s and A_{max} were simultaneously affected by decreasing (scenario *C*) or increasing (scenario *G*).

Discussion

Although the study period was slightly wetter than the area's long-term average (*i.e.*, 1095 mm in 2004 and 935 mm in 2005 - Tab. 2), the summer (June to August) was drier (*i.e.*, 90 mm in 2004 and 80 mm in 2005). Thus, the reduction in precipitation was 25% for the summer of 2004 and 33% for the summer of 2005 when compared to the long term average. This gives further evidence in predicted drier summers for Mediterranean regions (IPCC 2007). Moreover, the reduction of total precipitation (-20%) induced by the experimental manipulation exacerbated the summer drought.

As a result, the enhanced drought affected water and carbon relations, leading to reductions in maximum CO_2 assimilation (A_{max}) and stomatal conductance (g_s) for *A. unedo* forests over the two monitored seasons. This result is in agreement with other studies on the same species (Castell et al. 1994, Gratani & Ghia 2002). Furthermore, variations in directly measured leaf physiological traits, *e.g.*, the intercellular to ambient CO_2 (c_i/c_a) ratio and g_s, were well reflected in the carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope signature measured in bulk leaf material. In particular, our data show that measurements of $\delta^{13}C$ can be used to make time-integrated estimates of c_i/c_a at the tree or stand scale (Farquhar et al. 1989). In addition, the negative correlation between $\delta^{18}O$ and g_s that we observed for D+W is consistent with other studies (Barbour & Farquhar 2000, Barbour et al. 2000, Sullivan & Welker 2007). This indicates that $\delta^{18}O$ values reflect the signal of g_s variations under Mediterranean conditions. Although the correlation was not significant when the two treatments were considered separately, the similar slope and the parallel trend for the linear fit highlights the differences in g_s behaviour in response to different water availabilities. In fact, with $\delta^{18}O$ equal, g_s was lower in D compared to W. This confirms stomatal control of transpiration, imposed by soil water restriction and coupled with high VPD conditions.

Thus, the reliable correlation observed between $\delta^{18}O$ and g_s suggests that bulk leaf material was a suitable medium to infer the physiological performance of trees. This was previously observed by Sullivan & Welker (2007) and Barbour et al. (2000). While in other studies, the extraction of leaf cellulose was necessary because the $\delta^{18}O$ determined in leaf matter was not correlated with g_s (Grams et al. 2007).

The combination of $\delta^{13}C$ and $\delta^{18}O$ in a semi-quantitative model revealed qualitative variations of A_{max} and g_s across two different soil water regimes. Thus, in this investigation, the dual isotope model proposed by Scheidegger et al. (2000) was modified with the inclusion of SWC and rH (or VPD) as model inputs. Isotopic measurements on bulk leaf material showed an increase in $\delta^{18}O$ and a slight variation in $\delta^{13}C$. Among the eight different $\delta^{13}C$ and $\delta^{18}O$ scenarios proposed in the model, our results matched scenarios *C* and *G* (see isotope theory). This means that c_i remained almost constant, that g_s and A_{max} were simultaneously affected, and that both increased (scenario *G*) or decreased (scenario *C*). Therefore, as air and soil water availability reduced in our experiment, we can assume that both g_s and A_{max} decreased (scenario *C*). The output of the model found a robust confirmation from our gas exchange measurements. In fact, we did not observe variation in c_i between D and W over the two years of the experiment. Furthermore, direct measurements of gas exchange revealed a reduction of both A_{max} and g_s with increasing water stress (from W to D). The information deduced from the carbon and oxygen stable isotopes reflects the long-term integrated information of A_{max} and g_s (because organic matter accumulates over some time) and are consistent with short-term gas exchange measurements on a different time scale.

The down regulation of g_s may have induced a parallel decrease in A_{max}, suggesting that stomata strongly limit carbon assimilation. However, a similar c_i, found in this experiment in D and W plots, indicates that photosynthesis may have been down-regulated and that non-stomatal limitations may have played an important role (Lawlor & Cornic 2002). Through a study of quantitative limitation analysis carried out in July 2004 on the same experimental site, it was shown that non-stomatal limitations largely affected A_{max} (Grassi et al., unpublished data), accounting for 35% of total limitations (9% biochemical - carboxylation and electron transport rate - and 26% resistance to CO_2 from intercellular spaces to carboxylation sites). Several studies have shown evidence that this is far from negligible, and it is often the most important factor under moderate water stress (*e.g.*, Loreto et al. 1992, Warren et al. 2004, Grassi & Magnani 2005).

Although the dual isotope approach has been shown to be a reliable tool to infer the relationship between g_s and A_{max}, it is not able to explain in details the underlying mechanism involved. In fact, under Mediterranean conditions, it is often necessary to assess the contribution of stomatal and non-stomatal limitations in driving changes in A_{max} and g_s and their weight in this ratio that, in turn, affects the WUE.

As a result of the parallel decrease in g_s and A_{max}, we observed little or no differences in either WUE_{inst} and integrated WUE as assessed by $\delta^{13}C$. This result contrasts with most findings in Mediterranean areas, where a significant increase in WUE has been found (Ogaya & Peñuelas 2003, Lloret et al. 2004, etc.). These results derive from a more intense stomatal control of water loss than inhibition of photosynthesis.

The large fluctuations in WUE_{inst} over the two seasons appear to be mainly related to changes in VPD. Thus, VPD is considered an important parameter in driving gas exchanges in Mediterranean environment (Martínez-Vilalta et al. 2003) and in influencing the productivity of forest ecosystems (Law et al. 2001).

Conclusions

The combination of $\delta^{13}C$-$\delta^{18}O$, in a semi-quantitative conceptual model, proved to be a valid tool for investigating the time integrated g_s-A_{max} relationship under Mediterranean conditions. In fact, a constant $\delta^{13}C$ and an increase of $\delta^{18}O$ isotopes in response to reduced air and soil water availability were consistent with a parallel decline of either g_s and A_{max}, as assessed by gas exchange. The good correlations found between $\delta^{13}C$ and c_i or between $\delta^{18}O$ and g_s confirm this result. Furthermore, either instantaneous (from gas exchanges) and integrated WUE (from $\delta^{13}C$ isotopes) data are in agreement in showing that, as a consequence of a parallel decrease of either g_s and A_{max}, soil water restriction had no or slight influence on WUE. VPD was shown to have a larger impact on WUE than SWC. Such a response should result in a negative feature under climate change scenarios that may further reduce the carbon sequestration and the productivity of Mediterranean forests.

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