

# Stomatal and non-stomatal limitations on leaf carbon assimilation in beech (*Fagus sylvatica* L.) seedlings under natural conditions

I. Aranda<sup>1,\*</sup>, J. Rodríguez-Calcerrada<sup>2,3</sup>, T. M. Robson<sup>4</sup>, F. J. Cano<sup>3</sup>, L. Alte<sup>1</sup>  
and D. Sánchez-Gómez<sup>3</sup>

<sup>1</sup> Instituto Nacional de Investigaciones Agrarias y Tecnologías Agroalimentarias, Centro de Investigación Forestal, Carretera a Coruña Km 7.5, E-28040 Madrid, Spain

<sup>2</sup> Centre d'Ecologie Fonctionnelle et Evolutive. CNRS 1919 Route de Mende, 34293 Montpellier Cedex 5, France

<sup>3</sup> Unidad de Anatomía, Grupo de Investigación de Genética y Fisiología Forestal, E.T.S.I. Montes, Universidad Politécnica de Madrid (UPM), Ciudad Universitaria s/n, 28050 Madrid, Spain

<sup>4</sup> Department of Biological and Environmental Sciences, Plant Biology, University of Helsinki, 00014, Finland

## Abstract

Limitations to diffusion and biochemical factors affecting leaf carbon uptake were analyzed in young beech seedlings (*Fagus sylvatica* L.) growing in natural gaps of a beech-wood at the southern limit of the species. Half of the seedlings received periodic watering in addition to natural rainfall to reduce the severity of the summer drought. Plant water status was evaluated by measuring predawn water potential. Basic biochemical parameters were inferred from chlorophyll fluorescence and photosynthesis-CO<sub>2</sub> curves (*A-C<sub>c</sub>*) under saturating light. The curves were established on three dates during the summer months. The main variables studied included: stomatal and mesophyll conductance to CO<sub>2</sub> (*g<sub>s</sub>* and *g<sub>m</sub>* respectively), maximum velocity of carboxylation (*V<sub>cmax</sub>*) and maximum electron transport capacity (*J<sub>max</sub>*). The *g<sub>m</sub>* was estimated by two methodologies: the curve-fitting and J constant methods.

Seedlings withstood moderate water stress, as the leaf predawn water potential ( $\Psi_{pd}$ ) measured during the study was within the range -0.2 to -0.5 MPa. Mild drought caused *g<sub>s</sub>* and *g<sub>m</sub>* to decrease only slightly in response to  $\Psi_{pd}$ . However both diffusional parameters explained most of the limitations to CO<sub>2</sub> uptake. In addition, it should be highlighted that biochemical limitations, prompted by *V<sub>cmax</sub>* and *J<sub>max</sub>*, were related mainly to ontogenic factors, without any clear relationship with drought under the moderate water stress experienced by beech seedlings through the study.

The results may help to further understanding of the functional mechanisms influencing the carbon fixation capacity of beech seedlings under natural conditions.

**Key words:** diffusion limitations; stomatal conductance; mesophyll conductance; photosynthesis; drought; regeneration.

## Resumen

### Limitaciones estomáticas y no-estomáticas en la asimilación foliar de carbono en brinzales de haya (*Fagus sylvatica* L.) bajo condiciones naturales

Las limitaciones a la difusión de CO<sub>2</sub> y los factores bioquímicos que afectan la captura foliar de carbono fueron analizadas en jóvenes brinzales de haya (*Fagus sylvatica* L.) que crecían en un claro de un hayedo en el límite Sur de la distribución de la especie. La mitad de los brinzales recibieron periódicamente un riego adicional a la lluvia caída durante el verano con el objeto de minimizar la sequía estival. El grado de estrés hídrico soportado por las plantas fue evaluado a través de la medida del potencial hídrico al pre-alba ( $\Psi_{pd}$ ). Los parámetros funcionales básicos que condicionan la fijación de carbono fueron inferidos a través de curvas de respuesta bajo luz saturante en las que se relaciona la tasa neta de fotosíntesis (*A*), y la concentración de CO<sub>2</sub> en el interior de los cloroplastos (*C<sub>c</sub>*), en conjunción con medidas de fluorescencia de la clorofila. Dichas curvas se elaboraron en tres momentos a lo largo de los meses de verano, infiriéndose de las mismas: las conductancias estomáticas y del mesófilo al CO<sub>2</sub> (*g<sub>s</sub>* y *g<sub>m</sub>* respectivamente), la velocidad máxima de carboxilación (*V<sub>cmax</sub>*) y la tasa máxima de transporte electrónico (*J<sub>max</sub>*). La *g<sub>m</sub>* fue estimada a partir de dos metodologías: métodos del ajuste de la curva y la *J* constante.

\* Corresponding author: [aranda@inia.es](mailto:aranda@inia.es)  
Received: 02-11-11. Accepted: 27-04-12.

Los brinzales tuvieron que afrontar un estrés hídrico moderado, tal y como se deduce del potencial hídrico medido a lo largo del estudio ( $-0,2$  a  $-0,5$  MPa). Esto supuso una sequía que provocó que  $g_s$  y  $g_m$  disminuyeran solo ligeramente en respuesta al  $\Psi_{pd}$ . Además, las limitaciones no-estomáticas, definidas por  $V_{cmax}$  and  $J_{max}$ , fueron también importantes como restricciones fisiológicas a la fotosíntesis neta. Sin embargo, debe subrayarse que las limitaciones bioquímicas marcadas por modificaciones en  $V_{cmax}$  y  $J_{max}$  estuvieron marcadas fundamentalmente por factores ontogénicos asociados a la fecha de medición, sin una clara relación con la sequía bajo el estrés hídrico moderado soportado por los brinzales a lo largo del estudio.

Los resultados presentados pueden ayudar a comprender mejor los mecanismos funcionales que condicionan la capacidad de fijación de carbono en brinzales de haya bajo condiciones naturales.

**Palabras clave:** limitaciones a la difusión; conductancia estomática; conductancia del mesófilo; fotosíntesis; sequía; regeneración.

## Introduction

Water scarcity is recognized as one of the main environmental factors limiting leaf CO<sub>2</sub> fixation, and in turn growth and yield in plants (Chaves, 1991). The principal limitations to carbon uptake operate at the leaf level, which represents the main control point in the process of carbon fixation by plants. Although great advances have been made since pioneering studies there are some uncertainties that remain in our understanding of how the factors limiting CO<sub>2</sub> fixation are modulated (Grassi and Magnani, 2005; Diaz-Espejo *et al.*, 2007). For instance, the importance of CO<sub>2</sub> diffusion from the leaf inter-cellular spaces into the chloroplast and its effect on photosynthesis (see Flexas *et al.*, 2008 for a comprehensive review) has only recently been recognized. Technical advances in the measurement of gas exchange and fluorescence, and isotopic techniques, have provided more-accurate means to assess successive resistances across the entire CO<sub>2</sub> diffusion pathway through the leaf, prompting their importance for carbon uptake to be reconsidered (Flexas *et al.*, 2002; Ennahli and Earl, 2005; Warren, 2006). In this context, it is important to elucidate the changes in biochemical factors and diffusion resistances during photosynthesis when plants are submitted to naturally stressful conditions, such as drought (Niinemets *et al.*, 2005; Galmés *et al.*, 2007; Flexas *et al.*, 2009). This dual limitation influences the potential of seedlings to maintain a positive leaf carbon balance, and should be accounted for when assessing the ultimate consequences of water stress on ecological succession and niche partitioning under sub-Mediterranean environments (Kunstler *et al.*, 2005; Robson *et al.*, 2009). Moreover, the basic physiological parameters that drive the process of carbon uptake (i.e. maximum velocity of carboxylation,  $V_{cmax}$ ,

or maximum rate of electronic transport,  $J_{max}$ ) might differ between well-watered and water-stressed plants (Flexas *et al.*, 2006; Grassi *et al.*, 2009). For an accurate estimation of  $V_{cmax}$  and  $J_{max}$ , we need to account for the mesophyll conductance of CO<sub>2</sub>. Indeed, any change in the estimation of  $V_{cmax}$  and  $J_{max}$  could modify the way that models, such as that from Farquhar *et al.* (1980), are applied, and their outcomes in process-based modelling from leaves to ecosystems (Bernacchi *et al.*, 2002; Ethier and Livingston, 2004; Keenan *et al.*, 2010).

Under conditions of low water availability in the soil or atmosphere, plants first trigger mechanisms aimed to minimize water loss. Of these, stomatal closure is one of the most extensively studied and widely recognized (Chaves *et al.*, 2002; Brodribb and Jordan, 2008). However, the stomatal control of water loss incurs a penalty, since CO<sub>2</sub> diffusion into the leaf is concomitantly limited, leading to reduced carbon uptake potential (Wilson *et al.*, 2000a; Aranda *et al.*, 2000; Medrano *et al.*, 2002.). In addition, mesophyll conductance of CO<sub>2</sub> can become non-negligible and impair carbon fixation during drought periods (Flexas and Medrano, 2002; Niinemets *et al.*, 2004; Warren, 2006). Though it has been postulated that  $g_m$  and  $g_s$  respond to the same environmental variables and in a similar manner (Flexas *et al.*, 2008), the mechanistic linkage between both types of diffusive conductance is unclear, as is their impact on other functional processes such as the water use efficiency (Hanba *et al.*, 2003). While a decrease of  $g_s$  and  $g_m$  under water stress has been reported under controlled conditions (Galmés *et al.*, 2007; Galle *et al.*, 2009), these responses have been less studied in seedlings of forest tree species in natural environments.

The effect of drought on the physiological response of beech has been an important topic of research in eco-

physiology since the 1990's (Madsen, 1994; Tognetti *et al.*, 1994; Fotelli *et al.*, 2001; Leuschner *et al.*, 2001). However, only recently has the importance of an increased risk of drought across large areas of the species range started to be considered (Leuzinger *et al.*, 2005; Geßler *et al.*, 2007; Granier *et al.*, 2007), as extreme weather events have become more common at sites which were historically unperturbed by drought. Beech is known for its high sensitivity to water stress (Bréda *et al.*, 2006 and references therein), but previous studies were focused mainly on stomatal closure as the main limitation to carbon uptake capacity at different scales; from the leaf (Backes and Leuschner, 2000, Aranda *et al.*, 2002) to the ecosystem (Granier *et al.*, 2000). However, the contribution of other non-stomatal factors on carbon balance in beech leaves is poorly understood (Epron *et al.*, 1995; Warren *et al.*, 2007; Montpied *et al.*, 2009).

The main aim of this paper is to quantify the biochemical and diffusional limitations on leaf carbon assimilation by beech seedlings growing in natural gaps and exposed to two contrasting soil moisture regimes. We tested three hypotheses: that i)  $g_m$  co-limits carbon uptake to a similar degree as  $g_s$  under non-water-stressed conditions; ii)  $g_s$  and  $g_m$  decrease in response to moderate water-stress but at a different pace; iii) moderate drought involves a higher penalty on leaf carbon uptake incurred via an increase in CO<sub>2</sub> diffusion limitations through  $g_s$  and  $g_m$  rather than via biochemical limitations (e.g. decrease of  $V_{cmax}$  and  $J_{max}$ ).

## Material and methods

### Site Characteristics

The study was carried out in the beech-oak forest of *Montejo de la Sierra* (41°1'N 3°5'W 1,400 masl), composed of a mixture of temperate and sub-Mediterranean broadleaved tree species. The forest is at the southwestern limit of European beech (*Fagus sylvatica* L.) distribution in Europe, and it is subjected to moderate drought. The site has previously been described in detail (Aranda *et al.*, 2000, 2002, 2005; Rodríguez-Calcerrada *et al.* 2008a,b, 2010; Robson *et al.*, 2009).

### Experimental design

Two-year old beech seedlings were randomly selected in the spring of 2009 from a plantation of beech

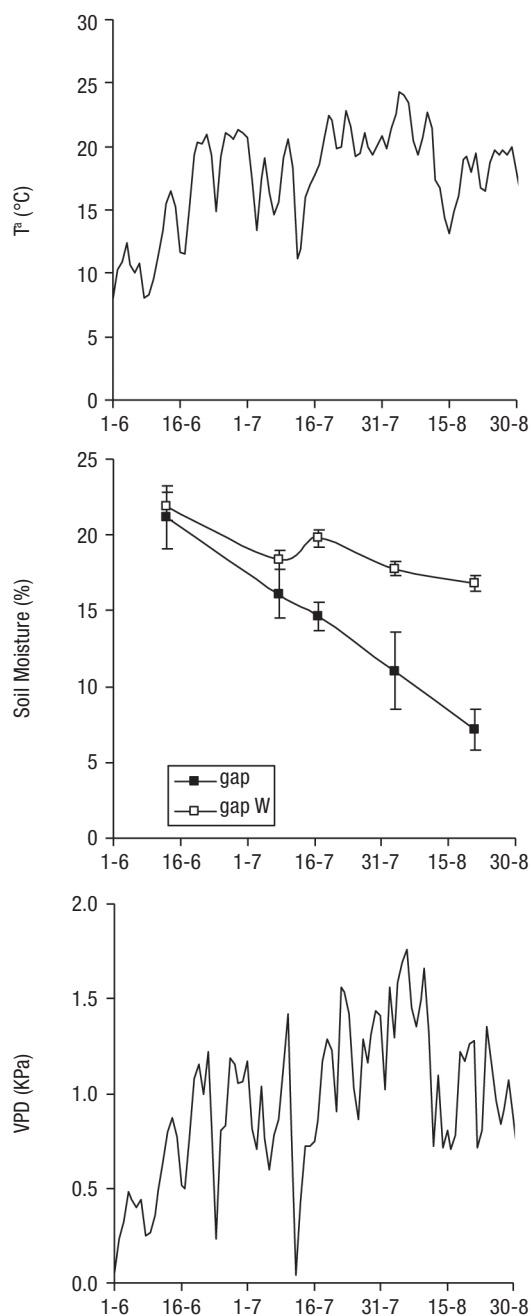
nuts carried out in the winter of 2007, in three plots in natural gaps created by fallen canopy trees. Each plot was split into two 1.3 × 2 m sub-plots, and each sub-plot was randomly assigned to either natural rainfall (D) or natural rainfall plus periodic watering (WW). Watered plants (WW) were separated from their unwatered counterparts (D) by a 0.4 m un-watered buffer zone. Irrigation started on June 27<sup>th</sup>. It consisted on adding 40 L water per m<sup>2</sup> of ground area every 7-10 days, and it finished two days before the last sampling date at the middle of August. Additional rainfall events were recorded during the summer months until the beginning of July, afterwards rainfall was almost absent (see Robson *et al.*, 2009, and Figure 1a in Rodríguez-Calcerrada *et al.*, 2010 for more details on the design and watering regime). Hemispherical photographs were taken during late summer, when the overstorey trees were in full leaf, to characterise the light environment for seedlings at two points in each sub-plot. A Global Site Factor (GSF%) for each plot was calculated, using an atmospheric transmittivity to solar radiation of 0.8 and 0.1 diffuse:direct radiation (canopy analysis software Hemiview 2.1, Delta-T devices Ltd, USA). GSF is an indicator of light availability that ranges between 0 (full canopy closure) and 1 (full sun light). GSF was 0.43 ± 0.06 (10.3 ± 1.5 mol m<sup>-2</sup> day<sup>-1</sup>). There was no difference in radiation received between dry and watered sub-plots ( $F_{1,6} = 0.02$ ,  $P = 0.890$ ).

## Gas Exchange and Chlorophyll fluorescence

### Gas exchange measurements

On three dates during the summer in June (18-20), July (16-18), and August (20-22), gas exchange and chlorophyll fluorescence were measured in four to six seedlings per treatment. One attached, first-flush and fully expanded leaf per plant receiving direct sunlight, was selected for measurements. In June, a failure in the chlorophyll fluorescence system precluded chlorophyll fluorescence measurement.

Light-saturated CO<sub>2</sub> assimilation rate ( $A$ ) was measured using portable photosynthesis system equipped with a blue-red light source (LI-6400; Li-Cor Inc., NE; USA) under different CO<sub>2</sub> concentrations. Measurements were carried out at constant light of 1,200 μmol m<sup>-2</sup> s<sup>-1</sup>. This level of irradiance has been shown to be enough to saturate photosynthesis in leaves of beech seedlings in the field without eliciting photoinhibition (Aranda *et al.*



**Figure 1.** Seasonal changes in air temperature ( $^{\circ}\text{C}$ , top panel), volumetric soil moisture content measured as average value between 10 and 30 cm depth (% , middle panel); white points (gap W) depict well-watered seedlings, black points (gap) refer to unwatered seedlings receiving only natural precipitation, and air vapour pressure deficit (kPa, bottom panel) during the summer months in a gap at the Montejo de la Sierra beechwood.

2002). Leaf temperature was maintained close to  $25^{\circ}\text{C}$  (actual leaf temperature:  $25.8 \pm 0.1^{\circ}\text{C}$ ) by controlling the temperature of the chamber. This constrained measure-

ment time to a temporal window between 9:00 a.m. and 13:00 p.m. Afterwards it was not possible to reasonably maintain the target temperature in the chamber and, in addition, midday stomatal closure was observed in some of non-watered plants even though water stress was not very intense. Measurements were carried out during three consecutive days. After, allowing 15 minutes at 400 ppm  $\text{CO}_2$  concentration ( $C_a$ ) for gas exchange rates to stabilize, gas exchange were recorded over a range of intercellular  $\text{CO}_2$  ( $C_i$ ) resulting from changing the  $\text{CO}_2$  supply in twelve steps from 50 to 1,800 ppm. The supply of  $\text{CO}_2$  was reduced step-wise to the minimum value; then returned to 400 ppm again, and increased step-wise from that concentration to complete the  $A-C_i$  curve at the high  $C_a$  end. Five records were taken at each target  $\text{CO}_2$  concentration when photosynthesis and transpiration showed a CV lower than 5%. This was usually reached after three to four minutes.

Chlorophyll fluorescence was measured simultaneously with gas exchange at each target  $\text{CO}_2$  concentration for  $A-C_c$  curves. Steady-state fluorescence ( $F_s$ ) and maximum fluorescence ( $F_m'$ ) were measured, in the case of  $F_m'$  after applying a saturating pulse of actinic light. The photochemical efficiency of PSII ( $\Phi_{PSII}$ ) was then calculated according to Genty *et al.*, (1989) and Kramer *et al.*, (2004) as:

$$\Phi_{PSII} = \frac{F_m' - F_s}{F_m'} \quad (9)$$

The rate of electron transport through PSII ( $ETR$ ) was calculated following Rosenqvist and van Kooten (2003) as:

$$ETR = 0.5 \Phi_{PSII} PPFD 0.85 \quad (13)$$

A value of 0.85 for total leaf absorptance was assumed (Evans and Loreto, 2000), and a factor of 0.5 for the partitioning of light between the two photosystems (Laisk and Loreto, 1996).

A non-linear least squares fitting procedure was applied to measured  $A - C_c$  curves, to estimate the maximum rate of carboxylation ( $V_{cmax}$ ) and the light-saturated maximum rate of RuBP-regeneration-limited electron transport rate ( $J_{max}$ ). Regression models were constructed according to equations from Farquhar *et al.*, (1980), including mesophyll conductance and some other modifications (see von Caemmerer, 2000) in which  $A_n$  was modelled as the minimum value of Rubisco-limited ( $A_c$ ) and RuBP-limited ( $A_j$ ) rate of photosynthesis according to (1), (2), (3), and without

considering limitation by triose phosphate regeneration (TPU) which takes place only under very high  $C_i$ .

$$A_n = \min(A_c, A_j) - R_d \quad (1)$$

$$A_c = V^a_{cmax} \frac{C_c - \Gamma^*}{C_c + K_c(1 + O/K_o)} \quad (2)$$

$$A_j = J_{max} \frac{C_c - \Gamma^*}{4(C_c + 2\Gamma^*)} \quad (3)$$

$R_d$  is the mitochondrial respiration in light. The concentration of oxygen ( $O$ ) was considered 20 kPa. Temperature-dependent parameters  $K_c$  (Michaelis-Menten coefficient of Rubisco for  $CO_2$ ) and  $K_o$  (Michaelis-Menten coefficient of Rubisco for  $O_2$ ) and the  $CO_2$  compensation point in the absence of mitochondrial respiration in light ( $\Gamma^*$ ) were calculated following the equations derived by Bernacchi *et al.*, (2002). All the parameters estimated were recalculated to a standard temperature of 25°C (Sharkey *et al.*, 2007).

We used an application for Microsoft Excel developed by Sharkey *et al.*, (2007) for calculation of photosynthetic parameters. This application implements the curve-fitting method to iteratively calculate mesophyll conductance ( $g_m$ ; see Warren 2006 and Flexas *et al.*, 2008 for a comprehensive review on the methodologies to estimate  $g_m$ ). The reliability of the method was checked by comparing the values of  $g_m$  in July and August according to the curvature method, with those estimated from chlorophyll fluorescence measurements in parallel to gas exchange, which allowed  $g_m$  to be estimated by the J constant method (Harley *et al.*, 1992; Warren, 2006; Flexas *et al.*, 2008). Because the estimation of  $g_m$  is sensitive to errors in both  $R_d$  and  $\Gamma^*$  (Harley *et al.*, 1992), we used the  $R_d$  at the leaf temperature given from empirical relationships between  $R_d$  and temperature obtained in a parallel experiment on the same plants (Rodríguez-Calcerrada *et al.*, 2010).

By taking into account the mean values of those variables involved in the Farquhar *et al.*, (1980) leaf photosynthetic model, which after modification include mesophyll conductance (Harley *et al.*, 1992), Grassi and Magnani (2005) developed a method to evaluate the limitations to photosynthesis during a plant's vegetative period by the amount, activity and kinetics of Rubisco (eq 1). They partitioned the decline of optimum photosynthesis by three main limitations: these are stomatal limitation ( $S_L$ ), mesophyll-conductance limitation ( $MC_L$ ) and biochemical limitation ( $B_L$ ). In turn, these parameters can be subdivided into the contribution of each relative limitation

to the recorded difference from the reference value. The relative limitations are identified as: stomatal limitations ( $I_s$ ), mesophyll limitations ( $I_{mc}$ ) and biochemical limitations ( $I_b$ ). The complete mathematical formulae and full theoretical development of the model are given by Grassi and Magnani, (2005). We compared drought-treatment plants (D) with watered ones (WW) on each measurement date to circumvent any seasonal effect on the different parameters, and to better assess the role played by our water treatments irrespective of ontogenic influences.

## Water potential and soil moisture

A pressure chamber (PMS Instrument Co. 1000, Corvallis, USA) was used to take measurements of leaf water potential. These were carried out on the same leaves previously used for  $A-C_i$  curves. The same leaves were kept hydrated for twelve hours, and used to estimate specific leaf mass per area (LMA), and nitrogen content on a per mass basis ( $N_m$ ) by the Kjeldhal method after oven drying. Nitrogen content on a leaf area basis ( $N_a$ ) was estimated from LMA and  $N_m$ .

Volumetric soil moisture was measured at 10 and 30 cm depths several times during the summer months using a Time Domain Reflectometer (TDR, Trase System I, Soil Moisture Equipment Corp., Santa Barbara, USA). Soil moisture was recorded in two well-separated points in each sub-plot within the three main plots ( $n = 6$ ).

## Statistical Analysis

The effect of drought and time during the season on each physiological parameter was tested using a two-way analysis of variance. All computations were performed in Statistica 6.0. The pair-wise comparison between drought treatments on each date was tested by a post-hoc F test (LSD test). Linear regression models and Pearson correlation were used to analyse the relationships between variables.

## Results

### Climatic conditions and water status of seedlings

Temperature and relative humidity were moderate during the course of the experimental period. Tem-

**Table 1.** Mean values  $\pm$  SE for water status ( $\Psi_{pd}$ , leaf predawn water potential) and leaf morphological traits (LMA, leaf mass per area;  $N_m$ , nitrogen content on a leaf dry mass basis) measured on beech seedlings growing in three canopy gaps. Half of the plants received natural rainfall during the summer months (D) and the other half had supplementary watering several times throughout the summer (WW). Significant differences are indicated by different letters (LSD-test after ANOVA)

	$\Psi_{pd}$ (MPa)		LMA ( $\text{g m}^{-2}$ )		$N_m$ (%)	
	WW	D	WW	D	WW	D
June	$-0.27 \pm 0.02$ cd	$-0.32 \pm 0.03$ bc	$49.19 \pm 2.82$ ab	$47.95 \pm 1.54$ ab	$2.21 \pm 0.13$ ab	$2.26 \pm 0.15$ ab
July	$-0.42 \pm 0.05$ ab	$-0.54 \pm 0.05$ a	$54.11 \pm 1.34$ b	$45.72 \pm 5.71$ a	$2.23 \pm 0.10$ ab	$2.59 \pm 0.04$ b
August	$-0.13 \pm 0.02$ d	$-0.43 \pm 0.14$ abc	$49.97 \pm 3.33$ ab	$49.31 \pm 2.15$ ab	$2.36 \pm 0.08$ ab	$1.95 \pm 0.10$ a

perature seldom reached above 25°C and the maximum VPD was never higher than 1.5 kPa. These represent moderate climatic conditions during summer months in central Spain, since much higher temperatures and evaporative demands have been encountered in previous years at the same site (e.g. Aranda *et al.*, 2002, Aranda *et al.*, 2004).

Soil moisture measured at 20 cm depth followed a very different pattern between treatments. In the subplots receiving additional water the soil moisture was within the range 15-20%, whereas in the subplots receiving just natural rainfall, soil moisture decreased to around 7.5% by the middle of August (Figure 1). However, the  $\Psi_{pd}$  remained similar between seedlings in the two treatments, and the water stress endured should only be considered moderate for the three dates ( $\Psi_{pd}$  over  $-0.5$  MPa on average). Seedlings receiving additional water only attained a significantly higher  $\Psi_{pd}$  than in the un-watered plots on the last sampling date (T). The slightly higher  $\Psi_{pd}$  in WW treatment in August than on previous dates could be explained by the lowest VPD during the night immediately prior to that predawn water potential measurement. Otherwise, the absence of larger drops in the  $\Psi_{pd}$  at the end of summer for D seedlings, even though soil moisture reached the minimum value at this time, may be explained by deeper rooting of seedlings to below the depths where soil moisture was recorded. Overall, the dry period of the summer was not sufficiently intense to elicit decreases in predawn water potential as large as those reported in previous studies at the same stand in other years (Aranda *et al.*, 2001, Aranda *et al.*, 2002, Robson *et al.*, 2009).

### Gas exchange

There were no clear differences between treatments in  $A_n$  and  $g_s$  (measured at ambient 400 ppm) during June and July. In July as much  $A_n$  or  $g_s$  were higher

than in June, despite water status at dawn in both treatments was slightly worse. Only on the last date (August), there was a tendency for both parameters to decrease in those seedlings enduring the natural rainfall regime (D), compared with those receiving additional water (WW: Table 2). However, differences were not statistically significant when considering all dates and treatments.

Both  $g_s$  and  $g_m$  were related to the draw-down of  $\text{CO}_2$  from the air ( $C_a$ ) to the interior of leaf ( $C_a-C_i$ ) or chloroplast ( $C_i-C_c$ ) (Table 2). The largest drop from ambient  $\text{CO}_2$  ( $C_a = 400$  ppm) to that in the intercellular spaces of leaves ( $C_i$ ) was caused by  $g_s$  (range  $154 \pm 14$  to  $123 \pm 11$ ). The resistance to diffusion from the intercellular spaces into the chloroplasts ( $g_m$ ) also promoted a decrease in  $\text{CO}_2$  concentrations, albeit lower ( $C_i-C_c$ : range  $51 \pm 7$  to  $105 \pm 17$ ). The  $g_m$  estimated by the fitting-curve method always gave higher values than the  $g_m^*$  estimated by the method of the J constant. In both cases, the trend was to maintain higher values of  $g_m$  than of  $g_s$  of  $\text{CO}_2$  (Table 2).

The decrease in  $g_s$  from July to August had a greater effect on IWUE than the concomitant changes in  $g_m$ . This suggests a large effect of stomatal regulation on water use efficiency, reaching into the range of water stress endured by plants. This expectation was consistent with the negative relationship between  $g_s$  and IWUE, whereas there was not a clear relationship between IWUE and  $g_m$  (Figure 2) nor with the biochemical variables influencing the carbon uptake potential,  $V_{cmax}$  and  $J_{max}$  (data not shown). However, this relationship should be viewed cautiously as estimated IWUE and  $g_s$  are not independent.

There were small seasonal changes in those parameters driving the uptake of carbon within chloroplasts ( $V_{cmax}$  and  $J_{max}$ ), with the lowest values tending to occur on the last sampling date in August, even for plants receiving additional water and with good water status ( $\Psi_{pd} \sim -0.2$  MPa). However, non statistical differences

**Table 2.** Gas exchange variables estimated from *A-Cc* curves in leaves of seedlings growing in three canopy gaps. Half of the plants received natural rainfall during the summer months (D) and the other half had supplementary watering several times throughout the summer (WW). Mean values  $\pm$  SE. are displayed ( $n = 3-6$ ). Mesophyll conductance to  $\text{CO}_2$  was estimated by the fitting curve method ( $g_{m^*}$ ) after Ethier *et al.* (2004) and the  $J$  constant method ( $g_m$ ) following to Harley *et al.* (1992). Values were normalized to  $25^\circ\text{C}$  following the equations of Sharkey *et al.* (2007). Failure of the fluorescence system in June meant that  $g_m$  could not be calculated. Significant differences are indicated by different letters (LSD-test after ANOVA)

	June		July		August	
	WW	D	WW	D	WW	D
$A_n$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	7.03 $\pm$ 0.96 bc	7.25 $\pm$ 0.72 bc	10.19 $\pm$ 0.79 a	8.62 $\pm$ 0.68 ab	8.23 $\pm$ 0.94 abc	5.98 $\pm$ 0.19 c
$g_s$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	0.056 $\pm$ 0.013 b	0.064 $\pm$ 0.006 b	0.098 $\pm$ 0.006 b	0.075 $\pm$ 0.013 ab	0.073 $\pm$ 0.011 ab	0.044 $\pm$ 0.005 b
$V_{\text{cmax}}$ ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ )	44.4 $\pm$ 6.1 a	49.4 $\pm$ 7.7 a	67.7 $\pm$ 7.1 b	57.4 $\pm$ 5.5 b	50.01 $\pm$ 5.5 b	40.9 $\pm$ 6.1 a
$J_{\text{max}}$ ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ )	79.3 $\pm$ 8.8 a	78.2 $\pm$ 7.4 a	93.0 $\pm$ 8.5 a	79.0 $\pm$ 2.2 a	75.3 $\pm$ 4.1 a	69.2 $\pm$ 6.1 a
$g_m$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	0.124 $\pm$ 0.019 a	0.097 $\pm$ 0.011 a	0.107 $\pm$ 0.016 a	0.104 $\pm$ 0.015 a	0.142 $\pm$ 0.02 a	0.116 $\pm$ 0.013 a
$g_{m^*}$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	–	–	0.098 $\pm$ 0.019 a	0.066 $\pm$ 0.012 a	0.075 $\pm$ 0.010 a	0.052 $\pm$ 0.010 a
IWUE ( $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ )	86 $\pm$ 8 ab	71 $\pm$ 2 ab	68 $\pm$ 7 ab	77 $\pm$ 9 ab	73 $\pm$ 4 ab	88 $\pm$ 9 ab
$C_i$ ( $\mu\text{mol mol}^{-1}$ )	247 $\pm$ 12 a	270 $\pm$ 3 a	277 $\pm$ 10 a	262 $\pm$ 14 a	268 $\pm$ 7 a	246 $\pm$ 14 a
$C_c$ ( $\mu\text{mol mol}^{-1}$ )	186 $\pm$ 18 a	192 $\pm$ 11 a	172 $\pm$ 10 a	173 $\pm$ 18 a	205 $\pm$ 11 a	195 $\pm$ 10 a
$C_a - C_i$ ( $\mu\text{mol mol}^{-1}$ )	153 $\pm$ 12 b	130 $\pm$ 3 b	123 $\pm$ 11 b	138 $\pm$ 14 ab	154 $\pm$ 14 ab	131 $\pm$ 7 ab
$C_r - C_c$ ( $\mu\text{mol mol}^{-1}$ )	61 $\pm$ 13 bc	79 $\pm$ 10 bc	105 $\pm$ 10 bc	88 $\pm$ 10 ab	63 $\pm$ 7 ab	51 $\pm$ 7 c

were observed for treatments, being only significant for date when grouping data of both treatments within each date (after ANOVA).

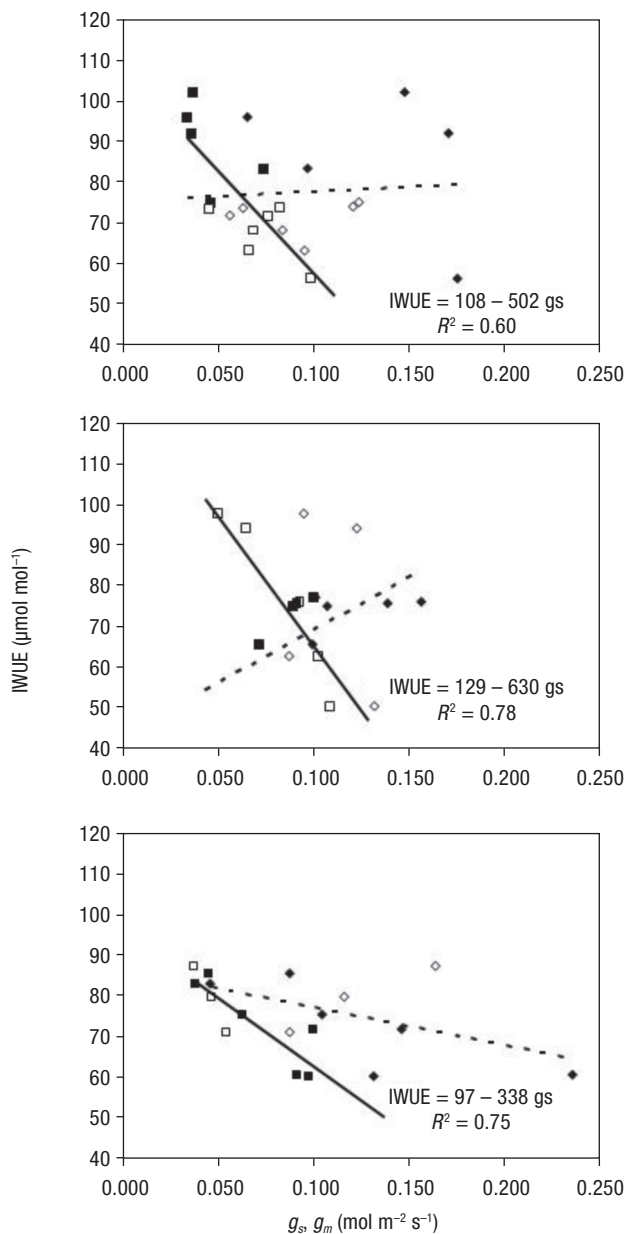
A weak negative relationship was observed between  $g_s$  and  $g_m$ , and  $\Psi_{pd}$  ( $g_s = 0.10 + 0.06 \Psi_{pd}$ ,  $R^2 = 0.23$ ,  $P = 0.07$ ;  $g_m = 0.18 + 0.13 \Psi_{pd}$ ,  $R^2 = 0.27$ ,  $P = 0.08$ ). However, neither  $V_{\text{cmax}}$  nor  $J_{\text{max}}$  showed any relationship with  $\Psi_{pd}$ , suggesting that leaf-age could have had a greater effect in driving changes in biochemical regarding diffusional parameters (Table 2). In this respect, as much  $V_{\text{cmax}}$  as  $J_{\text{max}}$  were positively correlated with the nitrogen content on a leaf area basis ( $N_a$ ) (Figure 3), and not with  $\Psi_{pd}$ .

Following the approach of Grassi and Magnani (2005), by comparing the relative effect of dry conditions (D plants) as a proportion of normal WW plant traits, we observed that stomatal limitations increased significantly in July and even more so in August. Values of  $S_L$  accounted for 51 and 59% and  $MC_L$  for 6 and 11% of photosynthetic down-regulation, in July and August respectively; while the all rest was due to biochemical limitations  $B_L$  of 43 and 30%. So in August, the reduction of 27% in net photosynthetic rate was mainly due to diffusional limitations ( $S_L + MC_L$  contributed 70% of this). The relative contribution of each single limitation to net photosynthesis was 33% by the stomatal limitation ( $I_s$ ), for all treatments with the exception of water stress treatment on the last date; 22% from mesophyll related limitations ( $I_{mc}$ ); and 45% from biochemical related limitation ( $I_b$ ).

## Discussion

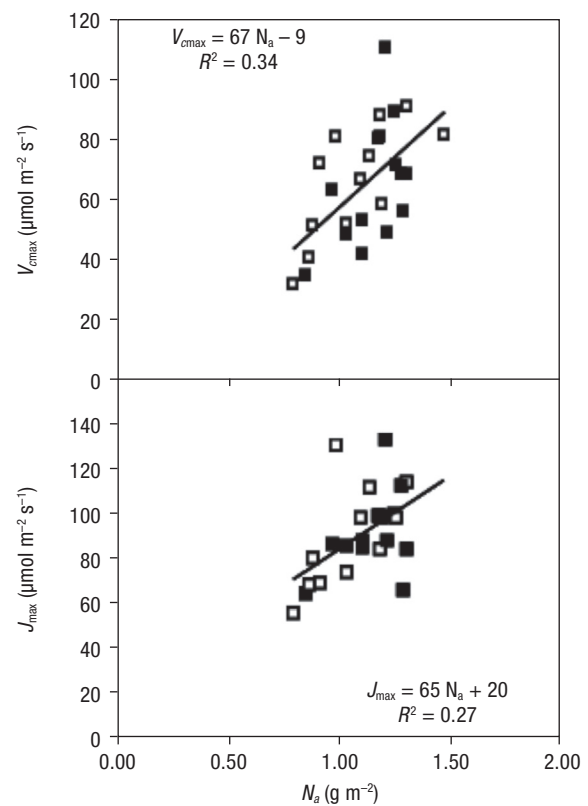
### Diffusional limitations to photosynthesis under moderate water stress

The range of water stress beech seedlings had to cope with in 2009 was lower than during previous studies carried out in the recent past at the same site (minimum  $\Psi_{pd}$  around  $-0.5$  MPa), but nevertheless enough to prompt changes in the stomatal conductance to water vapour at the end of summer (Aranda *et al.*, 2002). In this respect, the present study furthers our understanding of the role played by different diffusional limitations and biochemical variables during  $\text{CO}_2$  assimilation by leaves of beech seedlings growing under natural environments. We provide new information on how these processes operate under natural forest conditions, which compliments and builds upon previous reports on the same species under semi-controlled conditions and environmental manipulations (e.g. Epron *et al.*, 1995; Dreyer *et al.*, 2001; Warren *et al.*, 2007). The main factor constraining photosynthesis under moderate water stress was diffusional limitation through stomata, as previously reported (Aranda *et al.*, 2002, 2004, Gallé and Feller, 2007; Robson *et al.*, 2009). In the range of water stress endured by beech seedlings, stomatal closure comprised one of the main limiting factors to carbon uptake (Chaves *et al.*, 2002; Medrano *et al.*, 2002). Water stress of  $\Psi_{pd} = -0.5$  MPa was enough to prompt significant partial stomatal clo-



**Figure 2.** Relationship between intrinsic water use efficiency (IWUE  $\mu\text{mol mol}^{-1}$ ) and stomatal ( $g_s$ ,  $\text{mol m}^{-2} \text{s}^{-1}$ —squares and continuous line) or mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ,  $\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ —diamonds and dashed line) in June (top), July (middle) and August (down). Black points represent watered seedlings, and white ones those submitted to the natural rainfall pattern through the summer months at the beech-wood of Montejo de la Sierra.

sures in beech-seedling leaves, but only at the end of summer (Aranda *et al.*, 2002), that otherwise are able to endure a minimum  $\Psi_{pd}$  in the range  $-1.5$  to  $-2.0$  MPa as previously reported (Aranda *et al.* 2001). Accordingly, in this study  $g_s$  of  $\text{CO}_2$  decreased from 0.073 to



**Figure 3.** Positive relationship between basic leaf biochemical parameters: maximum rate of carboxylation,  $V_{c\text{max}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ) and light saturating maximum rate of RUBP regeneration limited electron transport,  $J_{\text{max}}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ), and nitrogen content on a leaf area basis ( $N_a$ — $\text{g m}^{-2}$ ). It's depicted a unique relationship for well watered (black points) and un-watered seedlings (white points). No clear trends were observed when considering watering treatments, thus a unique linear relationship was fitted to the pooled data ( $P < 0.05$ ).

$0.044 \mu\text{mol m}^{-2} \text{s}^{-1}$  on the last date when differences between watering treatments were largest (Table 1). However, the responsiveness of stomata to water stress could only be partly validated from the weak  $g_s$ - $\Psi_{pd}$  relationship. The high sensitivity of carbon uptake to water stress is mediated by a sensitive stomatal response to low soil moisture or high evaporative demand (Aranda *et al.*, 2000; Lenzion and Leuzinger, 2008). This stomatal limitation to carbon uptake may combine with any response of respiration to drought when considering the balance of carbon uptake and loss (Flexas *et al.*, 2005; Flexas *et al.*, 2006; Rodríguez-Calcerrada *et al.*, 2010). If maintained for an extended time period this disequilibrium could precipitate a negative carbon balance, making growth unsustainable in long lasting stressful conditions, such as those typical for sub-Mediterranean beechwoods. This impairment of the



overall leaf carbon balance of seedlings could be exacerbated when occurring in combination with low soil moisture and deep shade in the understorey of mature stands (Aranda *et al.*, 2002; Aranda *et al.*, 2004; Rodríguez-Calcerrada *et al.*, 2008a; Robson *et al.*, 2009). On other hand, it's difficult to explain the lower values of gas exchange recorded in June compared with July. Predawn water potential was slightly higher in June, though a slight effect of water stress at the late spring could not be ruled out as a possible cause of the low gas exchange maintained in WW and D plants. Other possible explanation could be leaves of seedlings in both treatments had not achieved the full physiological competence in the first measurement date. In fact, a similar result has been observed previously in beech where a seasonal lag in maximum gas exchange rates was observed despite leaf unfolding have been completed by the middle-end of June (Aranda *et al.*, 2000).

The stomatal limitation to carbon uptake, even under moderate water stress, sums to a low mesophyll diffusion conductance to CO<sub>2</sub>. The low  $g_m$ , common to woody plant species (Wilson *et al.*, 2000a; Grassi and Magnani, 2005), may be responsible for the low photosynthetic capacity of beech seedlings (Valladares *et al.*, 2002; Aranda *et al.*, 2004; Balandier *et al.*, 2007). This finding agrees with the typically low photosynthetic capacity of shade-tolerant tree species. Accordingly, relative mesophyll limitation ( $l_{mc}$ ) accounted for 22% of the relative photosynthesis limitation, a little lower than the 30% value proposed by Epron *et al.*, (1995) using a different approach. On the other hand, stomatal resistance was a bit higher (33-40% in our case vs. 30% from Epron *et al.*, 1995). In our case,  $g_m$  was slightly higher than the  $g_s$ , with values close to those previously reported by Epron *et al.*, (1995), and following the same pattern as that observed by Warren *et al.*, (2007) when comparing sun and shade leaves in mature trees. These results were consistent for changes in  $g_m$ , and qualitatively similar whether the J constant or the curvature method was used to estimate  $g_m$ . In conclusion, both components of diffusional limitation comprised a high percent of the overall limitation to carbon uptake.

It has been noted that as stress intensifies there is a reduction in the mesophyll conductance of CO<sub>2</sub> (Medrano *et al.*, 2002; Chaves *et al.*, 2003; Flexas *et al.*, 2008). This increases the overall diffusional limitations imposed at the first step by stomata (Medrano *et al.*, 2002). In the present study, where moderate water stress was suffered by beech seedlings, only a sea-

sonal reduction in  $g_s$  in response to water stress was evident. There was no consistent pattern in  $g_m$  in response to the water stress imposed, except on the last date when the differences in  $\Psi_{pd}$  between treatments were greatest. Taking into account the absolute limitations to photosynthesis, it is clear that the main reduction was due to diffusional resistances, mainly through the stomata, accounting for over 50% of the photosynthetic decrease. Under moderate water stress, stomatal limitation of photosynthesis is thought to be the main restriction on carbon uptake (Lawlor and Cornic, 2002; Medrano *et al.*, 2002; Grassi and Magnani, 2005; Díaz-Espejo *et al.*, 2007; Grassi *et al.*, 2009).

### Limitations to carbon uptake imposed by biochemical factors and time of year

Beech seedlings had a low biochemical capacity for photosynthesis, as previously reported in a comparative study with other co-occurring species (Dreyer *et al.*, 2001). In addition to the increase in diffusional limitations to carbon uptake imposed by water stress, there was also a seasonal reduction in seedlings' photosynthetic capacity (Wilson *et al.*, 2000a; Balandier *et al.*, 2007).  $V_{cmax}$  and  $J_{max}$  decreased slightly, though only significantly for  $V_{cmax}$ , between July and August irrespective of watering and caused a reduction in the capacity to fix carbon. Seasonally-induced decreases in photosynthetic capacity by the end of summer in beech have been reported before (Balandier *et al.*, 2007), and they add to the impairment of carbon uptake caused by increased stomatal limitation under natural conditions of moderate water stress. The trend in the degree of down regulation of  $V_{cmax}$  and  $J_{max}$  was similar between July and August in plants enduring the natural rainfall pattern and those receiving supplementary water, reinforcing the idea that this was an ontogenic effect. Accordingly, both variables showed a stronger relationship with the leaf nitrogen content (Balandier *et al.* 2007), than with the water stress experienced. The direct down-regulation of  $V_{cmax}$  as consequence of the moderate drought has been reported elsewhere (Wilson *et al.*, 2000b, Xu and Baldocchi, 2003, Damour *et al.*, 2009), and although not statistically significant, there was also a tendency towards consistently lower values in D than in WW on the last two measurement dates in the present study. However, the ontogenic effect should be recognized as the most plausible reason for the decrease of both biochemical parameters in the present study.

## Conclusions

The combination of shade tolerance at juvenile stages and a positive reaction to higher light levels makes beech very resilient in both wet and mesic sites, conditioning the ecology and silviculture of the species (see Wagner *et al.*, 2010 for a comprehensive review). However, even moderate drought may change the competitiveness of the species when water is not limiting (Cornic, 1994). Thus, carbon uptake at the leaf level was compromised in seedlings by moderate soil moisture causing stomatal closure which prevailed as the main limitation to net photosynthesis under moderate water stress, what is a well-known fact. Nevertheless, internal conductance of CO<sub>2</sub> was also an important limitation to carbon uptake comprising a 22% of the total limitation to carbon assimilation. This diffusional limitation could continue to increase, like those related with biochemical parameters, in extremely dry years.

The high sensitivity of beech to just moderate water stress is clearly apparent from this and previous studies (Madsen, 1994; Aranda *et al.*, 2004; Robson *et al.*, 2009), but also underscores the importance that the relatively low  $g_m$  has on the carbon potential uptake of beech leaves whichever water stress endured by seedlings. Ultimately this sensitivity could jeopardize the future of the species in currently marginal beech stands, where future climatic conditions are expected to worsen, and where an increase in the temperature together with a decrease in the seasonal rainfall could compromise much more the low carbon uptake capacity of young beech seedlings.

## Acknowledgements

This research was supported by the project of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria SUM2008-00004-C03-01s and CGL2007-66066-C04-03/BOS of the Ministry of Science and Innovation of Spain (MSI). T.M.R. & D.S.G were funded through the programme “Juan de la Cierva” of the MSI, and associated to the projects [AGL2006-03242 to D.S.G., CGL2007-66066-C04-03/BOS to T.M.R.]. FJ Cano was funded by a PhD fellowship from Regional Government of Madrid (CM). The authors are grateful to the Autonomous Community of Madrid (CM) for permission to work at *el Hayedo de Montejo*.

## References

- Aranda I, Gil L, Pardos JA. 2000. Water relations and gas exchange in *Fagus sylvatica* L. and *Quercus petraea* (Matuschka) Liebl. in a mixed stand at their southern limit of distribution in Europe. *Trees* 14, 344-352.
- Aranda I, Gil L, Pardos JA. 2001. Effects of thinning in a *Pinus sylvestris* L. stand on foliar water relations of *Fagus sylvatica* L. seedlings planted within the pinewood. *Trees, Structure and Function* 15: 358- 364.
- Aranda I, Gil L, Pardos JA. 2002. Physiological responses of *Fagus sylvatica* L. seedlings under *Pinus sylvestris* L. and *Quercus pyrenaica* Willd. overstories. *Forest Ecology and Management* 162, 153-164.
- Aranda I, Gil L, Pardos JA. 2004. Improvement of growth conditions and gas exchange of *Fagus sylvatica* L. seedlings planted below a recently thinned *Pinus sylvestris* L. stand. *Trees* 18, 211-220.
- Aranda I, Gil L, Pardos JA. 2005. Seasonal changes in apparent hydraulic conductance and their implications for water use of European beech (*Fagus sylvatica* L.) and sessile oak [*Quercus petraea* (Matt.) Liebl ] in South Europe. *Plant Ecology*. 179, 155-167.
- Backes K, Leuschner C. 2000. Leaf water relations of competitive *Fagus sylvatica* and *Quercus petraea* trees during 4 years differing in soil drought. *Canadian Journal of Forest Research* 30, 335-346.
- Balandier P, Sinoquet H, Frak E, Giuliani R, Vandame M, Descamps S, Coll L, Adam B, Prévosto B, Curt T. 2007. Six-year evolution of light use efficiency, carbon gain and growth of beech saplings (*Fagus sylvatica* L.) planted under Scots pine (*Pinus sylvestris* L.) shelterwood. *Tree Physiology* 27, 1073-1082.
- Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP. 2002. Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. *Plant Physiology* 130, 1992-1998.
- Breda N, Huc R, Granier A, Dreyer E. 2006. Temperate forest trees and stands under severe drought: a review of ecophysiological responses, adaptation processes and long-term consequences. *Annals of Forest Science* 63, 625-644.
- Brodribb TJ, Jordan GJ. 2008. Internal coordination between hydraulics and stomatal control in leaves. *Plant Cell and Environment* 31, 1557-1564.
- Von Caemmerer S. 2000. *Biochemical Models of Leaf Photosynthesis*. CSIRO, Collingwood, Australia.
- Cornic G. 1994. Drought stress and high light effects on leaf photosynthesis. In: Baker NR, Bowyer JR, eds. *Photoinhibition of photosynthesis*. Oxford: Bios Scientific Publishers, 297-313.
- Chaves MM. 1991. Effects of water deficits on carbon assimilation. *Journal of Experimental Botany* 42, 1-16.

- Chaves MM, Pereira JS, Maroco JP, Rodrigues ML, Ricardo CPP, Osorio ML, Carvalho I, Faria T, Pinheiro C. 2002. How plants cope with water stress in the field. Photosynthesis and growth. *Annals of Botany* 89, 907-916.
- Chaves MM, Maroco JP, Pereira JS. 2003. Understanding plant responses to drought - from genes to the whole plant. *Functional Plant Biology* 30, 239-264.
- Damour Ga, Vandame Mb, Urban Lc. 2009. Long-term drought results in a reversible decline in photosynthetic capacity in mango leaves, not just a decrease in stomatal conductance. *Tree Physiology* 29, 675-684.
- Díaz-Espejo A, Nicolás E, Enrique Fernández J. 2007. Seasonal evolution of diffusional limitations and photosynthetic capacity in olive under drought. *Plant Cell and Environment* 30, 922-933.
- Dreyer E, Le Roux X, Montpied P, Daudet FA, Masson F. 2001. Temperature response of leaf photosynthetic capacity in seedlings from seven temperate tree species. *Tree Physiology* 21, 223-232.
- Ennahli S, Earl HJ. 2005. Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. *Crop Science* 45, 2374-2382.
- Epron D, Godard D, Cornic G, Genty B. 1995. Limitation of net CO<sub>2</sub> assimilation rate by internal resistances to CO<sub>2</sub> transfer in the leaves of two tree species (*Fagus sylvatica* L. and *Castanea sativa* Mill.). *Plant Cell and Environment* 18, 43-51.
- Ethier GJ, Livingston NJ. 2004. On the need to incorporate sensitivity to CO<sub>2</sub> transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell and Environment* 27, 137-153.
- Evans JR, Loreto F. 2000. Acquisition and diffusion of CO<sub>2</sub> in higher plant leaves. In: Leegood RC, Sharkey TD, von Caemmerer S (eds) *Photosynthesis, physiology and metabolism*. Kluwer, Dordrecht, pp. 321-351.
- Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO<sub>2</sub> assimilation in leaves of C3 species. *Planta* 149, 78-90.
- Flexas J, Bota J, Escalona JM, Sampol B & Medrano H. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Functional Plant Biology* 29, 461-471.
- Flexas J, Medrano H. 2002. Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of Botany* 89, 183-189.
- Flexas J, Galmés J, Ribas-Carbó M, Medrano H. 2005. The effects of drought in plant respiration. In: Lambers H, Ribas-Carbó M (eds) *Advances in Photosynthesis and Respiration* 18. *Plant Respiration: from Cell to Ecosystem*. Kluwer Academic Publishers, Dordrecht, 85-94.
- Flexas J, Bota J, Galmés J, Medrano H, Ribas-Carbo M. 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiologia Plantarum* 127, 343-352.
- Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H. 2008. Mesophyll conductance to CO<sub>2</sub>: current knowledge and future prospects. *Plant, Cell and Environment* 31, 602-621.
- Flexas J, Barón M, Bota J, Ducruet J.-M, Gallé A, Galmés J, Jiménez M, Pou A, Ribas-Carbó M, Sajani C, Tomás M and Medrano H. 2009. Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandieri*·*V. rupestris*). *Journal of Experimental Botany* 60, 2361-2377.
- Fotelli MN, Geßler A, Peuke AD, Rennenberg H. 2001. Drought affects the competition between *Fagus sylvatica* L. seedlings and an early successional species (*Rubus fruticosus*): growth, water status and δ13C composition. *New Phytologist* 151, 427-435.
- Galmés J, Medrano H, Flexas J. 2007. Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist* 175, 81-93.
- Gallé A, Feller U. 2007. Changes of photosynthetic traits in beech saplings (*Fagus sylvatica*) under severe drought stress and during recovery. *Physiologia Plantarum* 131, 412-421.
- Galle A, Florez-Sarasa I, Tomas M, Pou A, Medrano H, Ribas-Carbo M, Flexas J. 2009. The role of mesophyll conductance during water stress and recovery in tobacco (*Nicotiana sylvestris*): acclimation or limitation? *Journal of Experimental Botany* 60, 2379-2390.
- Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta* 990: 87-92.
- Grassi G, Magnani F. 2005. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant Cell and Environment* 28, 834-849.
- Grassi G, Ripullone F, Borghetti M, Raddi S, Magnani F. 2009. Contribution of diffusional and non-diffusional limitations to midday depression of photosynthesis in *Arbutus unedo* L. *Trees* 23, 1149-1161.
- Geßler A, Keitel C, Kreuzwieser J, Matyssek R, Seiler W, Rennenberg H. 2007. Potential risks for European beech (*Fagus sylvatica* L.) in a changing climate. *Trees* 21, 1-11.
- Granier A, Reichstein M, Bréda N, Janssens IA, Falge E, Ciais P, Grunwald T, Aubinet M, Berbigier P, Bernhofer C, Buchmann N, Facini O, Grassi G, Heinesch B, Ilvesniemi H, Keronen P, Knohl A, Köstner B, Lagergren F, Lindroth A, Longdoz B, Loustau D, Mateus J, Montagnani L, Nys C, Moors E, Papale D, Peiffer M, Pilegaard K, Pita G, Pumpanen J, Rambal S, Rebmann C, Rodrigues A, Seufert G, Tenhunen J, Vesala I, Wang Q. 2007. Evidence for soil water control on carbon and water dynam-

- ics in European forests during the extremely dry year: 2003. *Agricultural and Forest Meteorology* 143, 123-145.
- Granier A, Ceschia E, Damesin C, Dufrêne E, Epron D, Gross P, Lebaube S, Le Dantec V, Le Goff N, Lemoine D, Lucot E, Ottorini JM, Pontailler JY, Saugier B. 2000. The carbon balance of a young Beech forest. *Functional Ecology* 14, 312-325.
- Hanba YT, Kogami H, Terashima I. 2003. The effect of internal CO<sub>2</sub> conductance on leaf carbon isotope ratio. *Isotopes in Environmental and Health Studies* 39, 5-13.
- Harley PC, Loreto F, Di Marco G, Sharkey TD. 1992. Theoretical considerations when estimating the mesophyll conductance to CO<sub>2</sub> flux by analysis of the response of photosynthesis to CO<sub>2</sub>. *Plant Physiology* 98, 1429-1436.
- Keenan T, Sabate S, Gracia C. 2010. The importance of mesophyll conductance in regulating forest ecosystem productivity during drought periods. *Global Change Biology* 16, 1019-1034.
- Kramer DM, Johnson G, Kiirats O, Edwards GE. 2004. New fluorescence parameters for the determination of QA redox state and excitation energy fluxes. *Photosynthesis Research* 79, 209-218.
- Kunstler G, Curt T, Bouchaud M, Lepart J. 2005. Growth, mortality, and morphological response of European beech and downy oak along a light gradient in sub-Mediterranean forest. *Canadian Journal of Forest Research* 35, 1657-1668.
- Laisk A, Loreto F. 1996. Determining photosynthetic parameters from leaf CO<sub>2</sub> exchange and chlorophyll fluorescence: rubisco specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate and mesophyll diffusion resistance. *Plant Physiology* 110, 903-912.
- Lawlor DW, Cornic G. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell and Environment* 25, 275-294.
- Lendzion J, Leuschner Ch. 2008. Growth of European beech (*Fagus sylvatica* L.) saplings is limited by elevated atmospheric vapour pressure deficits. *Forest Ecology and Management* 256, 648-655.
- Leuschner Ch, Backes K, Hertel D, Schipka F, Schmitt U, Terborg O, Runge M. 2001. Drought responses at leaf, stem and fine root levels of competitive *Fagus sylvatica* L. and *Quercus petraea* (Matt.) Liebl. trees in dry and wet years. *Forest Ecology and Management* 149, 33-46.
- Leuzinger S, Zotz G, Asshoff R, Körner C. 2005. Responses of deciduous forest trees to severe drought in Central Europe. *Tree Physiology* 25, 641-650.
- Madsen P. 1994. Growth and survival of *Fagus sylvatica* seedlings in relation to light intensity and soil water content. *Scandinavian Journal of Forest Research* 9, 316-322.
- Medrano H, Escalona JM, Boto J, Gulias J, Flexas J. 2002. Regulation of photosynthesis of C3 plants in response to progressive drought: Stomatal conductance as a reference parameter. *Annals of Botany* 89, 895-905.
- Montpied P, Granier A, Dreyer E. 2009. Seasonal time-course of gradients of photosynthetic capacity and mesophyll conductance to CO<sub>2</sub> across a beech (*Fagus sylvatica* L.) canopy. *Journal of Experimental Botany* 60, 2407-2418.
- Niinemets Ü, Sonninen E, Tobias M. 2004. Canopy gradients in leaf intercellular CO<sub>2</sub> mole fractions revisited: interactions between leaf irradiance and water stress need consideration. *Plant, Cell and Environment* 27, 569-583.
- Niinemets U, Cescatti A, Rodeghiero M, Tosens T. 2005. Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. *Plant Cell and Environment* 28, 1552-1566.
- Robson MT, Rodríguez-Calcerrada J, Sánchez-Gómez D, Aranda I. 2009. Summer drought impedes beech seedlings performance more in a sub-Mediterranean forest understory than in small gaps. *Tree Physiology* 29, 249-259.
- Rodríguez-Calcerrada J, Mutke S, Alonso J, Gil L, Pardos JA, Aranda I. 2008a. Influence of overstory density on understory light, soil moisture, and survival of two underplanted oak species in a Mediterranean montane Scots pine forest. *Investigación Agraria: Sistemas y Recursos Forestales*. 17, 31-38.
- Rodríguez-Calcerrada J, Pardos JA, Gil L, Aranda I. 2008b. Ability to avoid water stress in seedlings of two oak species is lower in a dense forest understory than in a medium canopy gap. *Forest Ecology and Management* 255, 421-430.
- Rodríguez-Calcerrada J, Atkin OK, Robson MT, Zaragoza-Castells J, Gil L, Aranda I. 2010. Acclimation of leaf dark respiration to shifts in summer temperature was independent of degree of canopy closure and water stress in a manipulative field experiment with beech seedlings. *Tree Physiology* 30, 214-224.
- Rosenqvist E, van Kooten O. 2003. Chlorophyll fluorescence: a general description and nomenclature. In: De Ell, J.R. and Toivonen, P.M.A. (eds.) *Practical applications of chlorophyll fluorescence in plant biology*, pp: 31-77. Kluwer Academic Publishers, The Netherlands.
- Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. 2007. Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant Cell and Environment* 30, 1035-1040.
- Tognetti R, Michelozzi M, Borghetti M. 1994. Response to light of shade-grown beech seedlings subjected to different watering regimes. *Tree Physiology* 14, 751-758.
- Valladares F, Chico JM, Aranda I, Balaguer L, Dizengremel P., Manrique E, Dreyer E. 2002. The greater seedling high-light tolerance of *Quercus robur* over *Fagus sylvatica* is

- linked to a greater physiological plasticity. *Trees* 16, 395-403.
- Wagner S, Collet C, Madsen P, Nakashizuka T, Nylandf, Khosro Sagheb-Talebi. 2010. Beech regeneration research: from ecological to silvicultural aspects. *Forest Ecology and Management* 259, 2172-2182.
- Warren CR. 2006. Estimating the internal conductance to CO<sub>2</sub> movement. *Functional Plant Biology* 33, 431-442.
- Warren CR, Löw M, Matyssek R, Tausz M. 2007. Internal conductance to CO<sub>2</sub> transfer of adult *Fagus sylvatica*: variation between sun and shade leaves and due to free-air ozone fumigation. *Environmental Experimental Botany* 59, 130-138.
- Wilson KB, Baldocchi DD & Hanson PJ. 2000a. Quantifying stomatal and non-stomatal limitations to carbon assimilation resulting from leaf ageing and drought in mature deciduous tree species. *Tree Physiology* 20, 787-797.
- Wilson KB, Baldocchi DD, Hanson PJ. 2000b. Spatial and seasonal variability of photosynthetic parameters and their relationship to leaf nitrogen in a deciduous forest. *Tree Physiology* 20, 565-578.
- Xu L, Baldocchi DD. 2003. Seasonal trends in photosynthetic parameters and stomatal conductance of blue oak (*Quercus douglasii*) under prolonged summer drought and high temperature. *Tree Physiology* 23, 865-877.