

# Genetic Resources of European Beech (*Fagus sylvatica* L.) for Sustainable Forestry

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MINISTERIO DE CIENCIA E INNOVACIÓN  
INSTITUTO NACIONAL DE INVESTIGACIÓN  
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## **Genetic resources of European beech (*Fagus sylvatica* L.) for sustainable forestry**

Proceedings of the COST E52 «Evaluation of beech genetic  
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## ABSTRACT

Beech is a dominant forest tree species of high economic as well as ecological importance with a wide distribution range linking Scandinavia and the Mediterranean. Due to its functional flexibility and large genetic plasticity, beech can be utilized to study wide reaching influences affecting ecosystems, *e.g.* climate factors in different parts of Europe. The COST Action E52 «Evaluation of Beech Genetic Resources for Sustainable Forestry» commenced March 2006. During the final meeting of this COST action (Burgos, Spain, 4<sup>th</sup> to 6<sup>th</sup> of May, 2010) results of numerous research areas were presented, of which a special selection is published here. Among them, the evaluation of data from provenance trials located in most of the regions of beech occurrence show how well populations have adapted to certain site-inherent environmental features, *e.g.* limited water availability, late frost occurrence, acidic or calcareous soil, as well as how non-adapted populations react to such situations, and how successfully they might cope with them. This is of great significance for assessing the value of both, a given beech population and its ecosystem with respect to the conservation of beech ecosystems in a broad sense and particularly the genetic resources of beech.

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

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## ABSTRACT

Seasonal variation and the differential effect of drought on photosynthetic parameters involved in carbon assimilation in forest species have been poorly studied under natural conditions. 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 seedling received periodic watering in addition to natural rainfall to reduce the severity of the summer drought which is typical in the area. Plant water status was evaluated by measuring predawn water potential. Basic biochemical parameters were inferred from photosynthesis-CO<sub>2</sub> curves (A-C<sub>c</sub>) under saturating light and chlorophyll fluorescence. The curves were established on three dates during the summer months. 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 a 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. A 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. Furthermore, non-stomatal limitations were also important limiting net photosynthesis as water stress increased. In addition, it should be highlighted that the 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 understand of the functional mechanisms influencing carbon fixation capacity of beech seedlings. This capacity may influence the total carbon balance of beech seedlings, and feed forward to their growth and survival under drought-prone environments such as those in the south of Europe.

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

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## 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 principle limitations to carbon uptake ope-

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rate at the leaf level, which represent the main control point in the process of carbon fixation by plants. Although great advances have been made since pioneering studies scaling net photosynthesis from cellular to ecosystem levels, there are some uncertainties that remains in our understanding of how the factors limiting CO<sub>2</sub> fixation are modulated (Grassi and Magnani, 2005; Díaz-Espejo *et al.*, 2007). For instance, only recently has the importance of CO<sub>2</sub> diffusion from the leaf inter-cellular spaces to the inside chloroplast has been recognized, and its effect on photosynthesis (see Flexas *et al.*, 2008, for a comprehensive review). Technical advances in the measurement of gas exchange and fluorescence, and isotopic techniques have provided more accurate means to assess the successive resistances across the overall CO<sub>2</sub> diffusion pathway through the leaf, prompting the reconsideration their importance for carbon uptake (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). Both biochemical factors and diffusion resistances act in concert on the potential of seedlings to maintain a positive leaf carbon balance during forest regeneration. This dual limitation should be accounted 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,  $V_{\text{cmax}}$ , or maximum rate of electronic transport,  $J_{\text{max}}$ ) might change from well-watered to water stressed plants (Flexas *et al.*, 2006; Grassi *et al.*, 2009). For an accurate estimation of  $V_{\text{cmax}}$  and  $J_{\text{max}}$ , we need to account for the mesophyll conductance of CO<sub>2</sub>. Indeed, any change in the estimation of  $V_{\text{cmax}}$  and  $J_{\text{max}}$  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 control of water loss incurs as penalty, since the increase of the stomatal limitation to CO<sub>2</sub> diffusion into the leaf, reductions in the carbon uptake potential (Wilson *et al.*, 2000a; Aranda *et al.*, 2000; Medrano *et al.*, 2002). In addition, mesophyll conductance to CO<sub>2</sub> is finite and impairs 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 conductances is unclear, as is the 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 in controlled conditions (Galmés *et al.*, 2007), these responses have been less studied in seedlings of forest tree species under natural environments.

The affect of drought on the physiological response of beech has been an important topic of research in ecophysiology 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 the increased risk of droughts 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 (Breda *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, analysis of 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 diffusion 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-limit 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  $CO_2$  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 semi-natural 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 south-western 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.*, 2008, 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-x-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 un-watered counterparts (D) by a 0.4 m un-watered buffer zone. Irrigation started on June 27<sup>th</sup>, and consisted on adding 40 L water per m<sup>2</sup> of ground area every 7-10 days. For detailed explanations of the original design see Robson *et al.* (2009). 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  $0.43 \pm 0.06$  ( $10.3 \pm 1.5 \text{ mol m}^{-2} \text{ day}^{-1}$ ). 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, July and August, gas exchange and chlorophyll fluorescence were measured in four to six seedlings per treatment. One attached, first-flush leaf per plant receiving direct sunlight, was selected for measurements. In June, a failure in the chlorophyll fluorescence system precluded chlorophyll fluorescence measurement.

Net  $CO_2$  assimilation rate ( $A_n$ ) was measured using portable photosynthesis system equipped with a blue-red light source (LI-6400; Li-Cor Inc., NE; USA) under different  $CO_2$  concentrations. Measure-

ments were carried out at constant light of  $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . 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.*, 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 on the chamber. This constrained measurements to a temporal window between 9:00 a.m. and 13:00 p.m. Afterwards it was not possible to maintain temperature into the chamber close to the target temperature and, in addition, midday stomatal closure was observed in some of the drought stressed plants even though water stress was not very intense. Measurements were carried out during three consecutive days. After 15 minutes of stabilizing gas exchange rates to a 400 ppm  $\text{CO}_2$  concentration ( $C_a$ ), gas exchange was 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. First, the supply of  $\text{CO}_2$  was reduced step-wise to minimum values; 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 net 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 of 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 Kramer *et al.* (2004) as:

$$\Phi_{PSII} = \frac{F_m' - F_s}{F_m'} \quad [9]$$

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

$$ETR = 0.5 \Phi_{PSII} PPF 0.85 \quad [13]$$

A value of 0.85 for total leaf absorbance 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  $A_n$ - $C_c$  curves to estimate the maximum rate of carboxylation ( $V_{cmax}$ ) and the light saturating maximum rate of RUBP regeneration limited electron transport rate ( $J_{max}$ ). Regression models were constructed according to equations of 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 the limitation triose phosphate regeneration (TPU) which takes place only under very high  $C_i$ .

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

$$A_c = V_{cmax}^a \frac{C_c - \Gamma}{C_c + K_c(1 + O/K_o)} \quad [2]$$

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

$R_d$  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  $\text{CO}_2$ ) and  $K_o$  (Michaelis-Menten coefficient of Rubisco for  $\text{O}_2$ ) and the  $\text{CO}_2$  compensation point in the absence of mito-

chondrial 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 that allowed estimating  $g_m$  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).

Taking into account mean values of variables involved in the Farquhar *et al.* (1980) leaf photosynthetic model after modification included mesophyll conductance in Harley *et al.* (1992), Grassi and Magnani (2005) developed a method to evaluate limitations to photosynthesis through vegetative period when it was limited by amount, activity and kinetics of Rubisco (eq [1]). They partition the decline of optimum photosynthesis by three main absolute limitations. These were stomatal limitation ( $S_L$ ), mesophyll conductance limitation ( $MC_L$ ) and biochemical limitation ( $B_L$ ). In turn, these parameters can be subdivided into the product of the relative limitations on the actual difference with the reference value. The relative limitations were named as: stomatal limitations ( $l_s$ ), mesophyll limitations ( $l_{mc}$ ) and biochemical limitations ( $l_b$ ). Complete mathematical development can be found in the original work (for the full theoretical development see Grassi and Magnani (2005). We compared drought treatment (D) with well watered ones (WW) in each measured date to avoid the effect of the seasonal pattern on the different parameters, and assess better the role played by water treatments without ontogenic influences.

### Water potential and soil moisture

A pressure chamber (PMS Instrument Co. 7000, Corvallis, USA) was used to take measurements of leaf water potential. Leaf water potentials were carried out on the same leaves previously used to A-C<sub>i</sub> 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 Kjeldahl 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).

### Statistical analysis

The effect of drought and time during the season on the different physiological parameters 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 F post-hoc test (LSD test). Linear regression models and Pearson correlation were used to analyse relationships between variables.

## RESULTS

### Climatic conditions and water status of seedlings

Temperature and relative humidity were moderate during the course of the experimental period. Temperature was seldom beyond 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 reported in previous studies at the same place (*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 sub-plots 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 (Fig. 1). However, the  $\Psi_{pd}$  remained similar between seedlings on the two treatments, and the water stress endured should be considered only moderate for the three dates ( $\Psi_{pd}$  over  $-0.5$  MPa on average). Only on the last sampling date, did seedlings receiving additional water attain a statistically significantly higher  $\Psi_{pd}$  (Table 1).

### Gas exchange

There were not clear differences in  $A_n$  and  $g_s$  (measured at ambient 400 ppm) between treatments during June and July. Only on the last date, there was a tendency in both parameters towards a decrease for those seedlings enduring the natural rainfall regime, compared with those receiving additional water (Table 2). However, overall differences were not statistically significant.

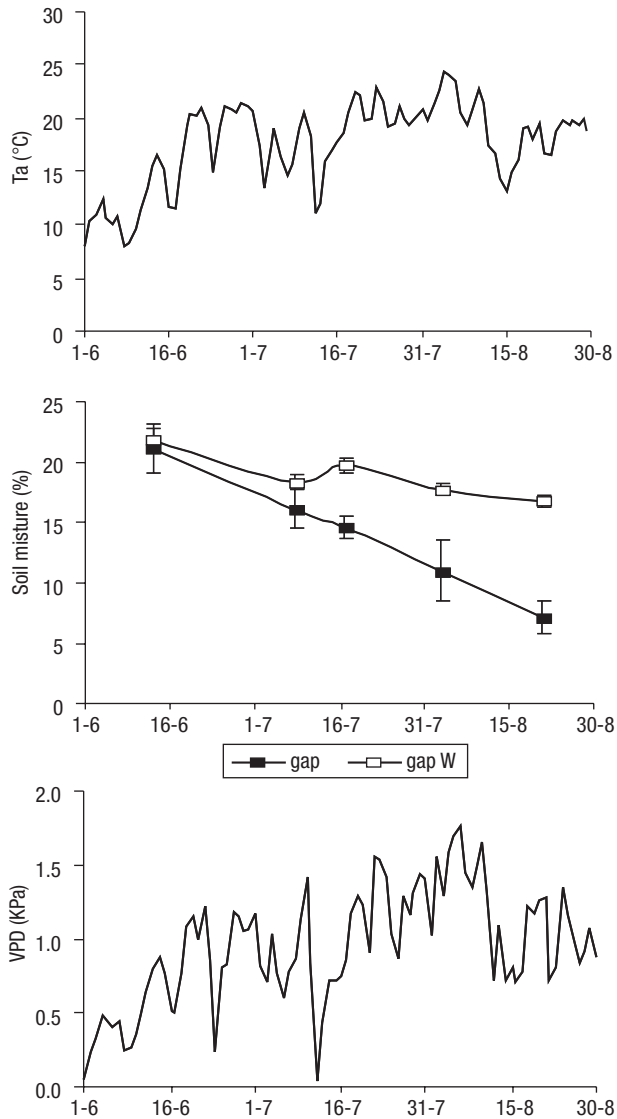
Both  $g_s$  and  $g_m$  were related to the draw down in  $CO_2$  from the air ( $C_a$ ) to the interior of leaf ( $C_a-C_i$ ) or chloroplast ( $C_i-C_c$ ) respectively (Table 2). The largest drop from ambient  $CO_2$  ( $C_a = 400$  ppm) to that in the intercellular spaces of leaves ( $C_i$ ) was caused by  $g_s$  (range  $154 \pm 14 - 123 \pm 11$ ). The resistance to diffusion from the inter-cellular spaces into the chloroplasts ( $g_m$ ) also promoted a decrease in  $CO_2$  levels, albeit lower than  $g_s$  ( $C_i-C_c$ : range  $51 \pm 7 - 105 \pm 17$ ). 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  $g_s$  to  $CO_2$  (Table 2).

The decrease in  $g_s$  had a greater effect on IWUE on the three sampling dates than the concomitant changes of  $g_m$ . This suggests a larger effect of stomatal regulation on water use efficiency into the range of water stress endured by plants. This expectation was confirmed by the negative relationship between  $g_s$  and IWUE for the three dates, without a clear relationship with the  $g_m$  (Fig. 2) or the biochemical variables  $V_{Cmax}$  or  $J_{max}$  influencing the carbon uptake potential (data not shown).

TABLE 1

Water status ( $\Psi_{pd}$ , leaf predawn water potential) and leaf morphological traits (LMA, leaf mass per area;  $N_m$ , nitrogen content per leaf dry mass) 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). Mean values  $\pm$  SE. for each parameter. Significant differences are indicated by different letters (LSD-test after ANOVA)

	$\Psi_{pd}$ (MPa)		LMA (g m <sup>-2</sup> )		$N_m$ (mg g <sup>-1</sup> )	
	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	$-0.13 \pm 0.02^d$	$-0.43 \pm 0.14^{abc}$	$49.97 \pm 3.33^{ab}$	$49.31 \pm 2.15^{ab}$	$2.36 \pm 0.08^{ab}$	$1.95 \pm 0.10^a$



**Figure 1.** Seasonal changes in air temperature ( $^{\circ}\text{C}$ , top panel), volumetric soil moisture content (% 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.

There were small seasonal changes in those parameters driving the uptake of carbon within chloroplast ( $V_{\text{cmax}}$  and  $J_{\text{max}}$ ), with the lowest values on the last sampling date in August, even for plants receiving additional watering and with good water status ( $\Psi_{\text{pd}} \sim -0.2\text{MPa}$ ). Water stress prompted a seasonal decrease in  $V_{\text{cmax}}$  and  $J_{\text{max}}$  ( $P < 0.05$  for sampling date). In this sense, a weak negative relationship was observed between  $g_s$  and  $g_m$ , and  $\Psi_{\text{pd}}$  (Fig. 3), but not for  $V_{\text{cmax}}$  and  $J_{\text{max}}$ , where leaf-age had a gre-

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 CO<sub>2</sub> 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°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 <sup>bc</sup>	7.25 $\pm$ 0.72 <sup>bc</sup>	10.19 $\pm$ 0.79 <sup>a</sup>	8.62 $\pm$ 0.68 <sup>ab</sup>	8.23 $\pm$ 0.94 <sup>abc</sup>	5.98 $\pm$ 0.19 <sup>c</sup>
$g_s$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	0.056 $\pm$ 0.013 <sup>b</sup>	0.064 $\pm$ 0.006 <sup>b</sup>	0.098 $\pm$ 0.010 <sup>a</sup>	0.075 $\pm$ 0.013 <sup>ab</sup>	0.073 $\pm$ 0.011 <sup>ab</sup>	0.044 $\pm$ 0.005 <sup>b</sup>
$V_{c\text{max}}$ ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ )	44.4 $\pm$ 6.1 <sup>a</sup>	49.4 $\pm$ 7.7 <sup>a</sup>	67.7 $\pm$ 7.1 <sup>a</sup>	57.4 $\pm$ 5.5 <sup>a</sup>	50.01 $\pm$ 4.1 <sup>a</sup>	40.9 $\pm$ 6.1 <sup>a</sup>
$J_{\text{max}}$ ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ )	79.3 $\pm$ 8.8 <sup>a</sup>	78.2 $\pm$ 7.4 <sup>a</sup>	93.0 $\pm$ 8.5 <sup>a</sup>	79.0 $\pm$ 2.2 <sup>a</sup>	75.3 $\pm$ 6.4 <sup>a</sup>	69.2 $\pm$ 11.2 <sup>a</sup>
$g_m$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	0.124 $\pm$ 0.019 <sup>a</sup>	0.097 $\pm$ 0.011 <sup>a</sup>	0.107 $\pm$ 0.016 <sup>a</sup>	0.104 $\pm$ 0.015 <sup>a</sup>	0.142 $\pm$ 0.02 <sup>a</sup>	0.116 $\pm$ 0.013 <sup>a</sup>
$g_m^*$ ( $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	—	—	0.098 $\pm$ 0.019 <sup>a</sup>	0.066 $\pm$ 0.012 <sup>a</sup>	0.075 $\pm$ 0.010 <sup>a</sup>	0.052 $\pm$ 0.010 <sup>a</sup>
IWUE (mmol mol <sup>-1</sup> )	86 $\pm$ 8 <sup>ab</sup>	71 $\pm$ 2 <sup>ab</sup>	68 $\pm$ 7 <sup>b</sup>	77 $\pm$ 9 <sup>ab</sup>	73 $\pm$ 4 <sup>ab</sup>	88 $\pm$ 9 <sup>a</sup>
$C_i$ ( $\mu\text{mol mol}^{-1}$ )	247 $\pm$ 12 <sup>a</sup>	270 $\pm$ 3 <sup>a</sup>	277 $\pm$ 10 <sup>a</sup>	262 $\pm$ 14 <sup>a</sup>	268 $\pm$ 7 <sup>a</sup>	246 $\pm$ 14 <sup>a</sup>
$C_c$ ( $\mu\text{mol mol}^{-1}$ )	186 $\pm$ 18 <sup>a</sup>	192 $\pm$ 11 <sup>a</sup>	172 $\pm$ 10 <sup>a</sup>	173 $\pm$ 18 <sup>a</sup>	205 $\pm$ 11 <sup>a</sup>	195 $\pm$ 10 <sup>a</sup>
$C_a - C_i$ ( $\mu\text{mol mol}^{-1}$ )	153 $\pm$ 12 <sup>b</sup>	130 $\pm$ 3 <sup>ab</sup>	123 $\pm$ 11 <sup>b</sup>	138 $\pm$ 14 <sup>ab</sup>	154 $\pm$ 14 <sup>ab</sup>	131 $\pm$ 7 <sup>ab</sup>
$C_i - C_c$ ( $\mu\text{mol mol}^{-1}$ )	61 $\pm$ 13 <sup>bc</sup>	79 $\pm$ 10 <sup>abc</sup>	105 $\pm$ 17 <sup>a</sup>	88 $\pm$ 10 <sup>ab</sup>	63 $\pm$ 7 <sup>bc</sup>	51 $\pm$ 7 <sup>c</sup>

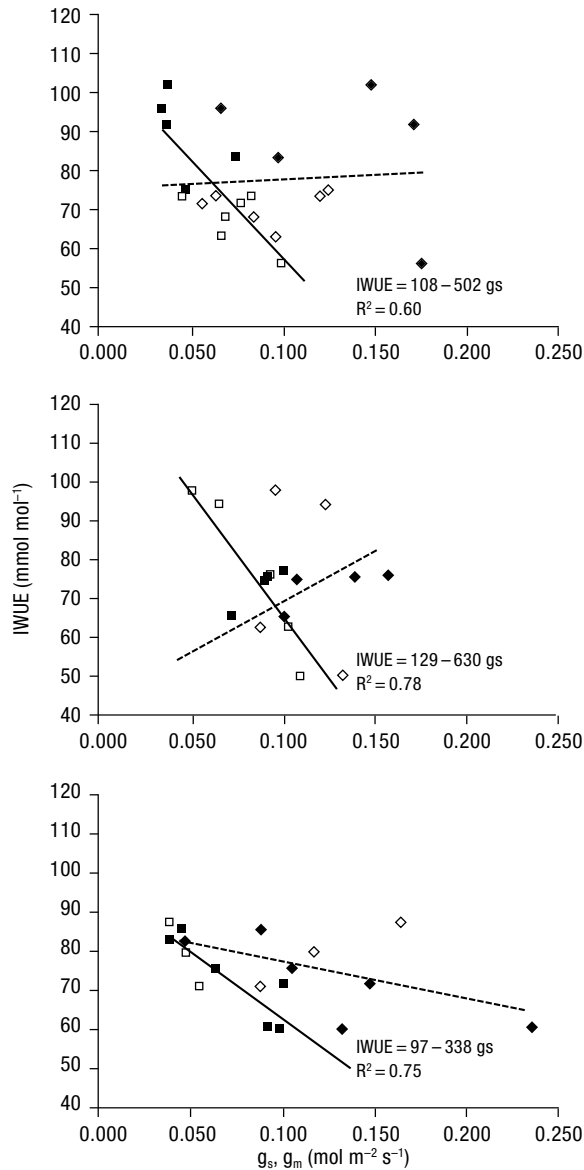
ater effect in driving the biochemical changes (Table 2). In this case, both parameters showed a positive correlation with the nitrogen content on a leaf area basis ( $N_a$ ) (Fig. 4), and none with  $\Psi_{pd}$  (Fig. 3).

Following the approach of Grassi and Magnani (2005), and comparing D plants with WW ones taken as reference, we observed stomatal and mesophyll limitations increased in July and even more in August. Values of  $S_L$  accounted for 51 and 59% and  $MC_L$  for 6 and 11% of the down photosynthetic regulation in July and August respectively, while the rest until 100% was restricted by 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 to 70%). Relative contributions of each single limitation to net photosynthesis were a mean of 33% by the stomatal limitation ( $l_s$ ) for all treatments with the exception of water stress treatment in the last date; mesophyll relative limitations ( $l_{m0}$ ) of 22% and biochemical relative limitation ( $l_b$ ) of 45%.

## DISCUSSION

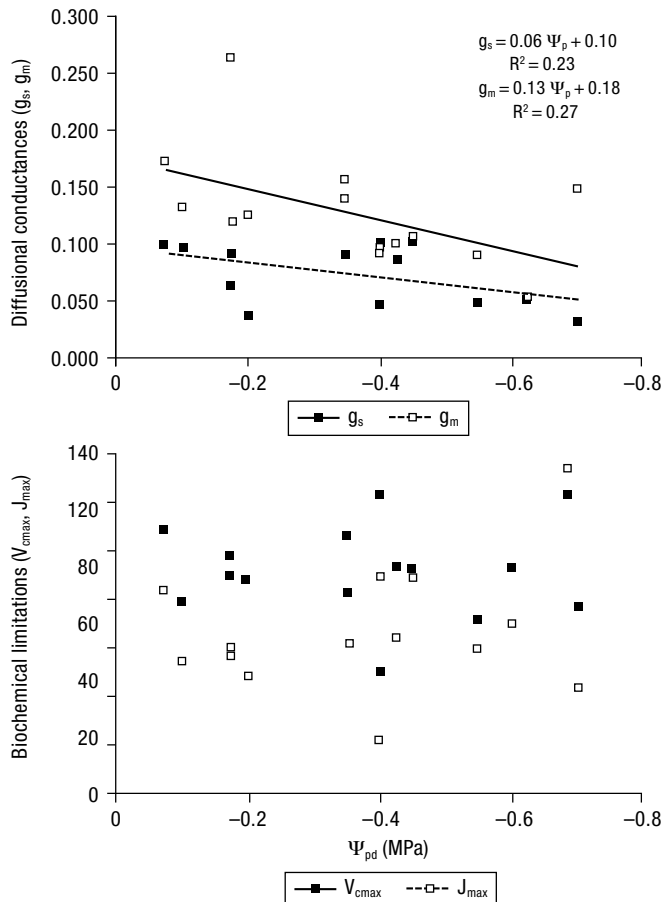
### Diffusional limitations to photosynthesis under moderate water stress

The present study provides information on the role played by different diffusional limitations and biochemical variables to CO<sub>2</sub> assimilation by leaves of beech seedlings growing under natural environments. New information is shown on how these processes operate under natural forest conditions, which compliment and build 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, 2007). This assessment of the different factors that contribute to limit carbon uptake under natural environments at the leaf level, is of prime importance to further our understanding of the basic physiological limitations imposed on carbon uptake under more realistic field conditions. The main constraint to photosynthesis under moderate water stress was related to diffusional limitation through stomata, as previously reported (Aranda *et al.*, 2002, 2004; Gallé and Feller, 2007; Robson *et al.*, 2009). Thus, in the range of water



**Figure 2.** Relationship between intrinsic water use efficiency-(IWUE mmol mol<sup>-1</sup>) and stomatal ( $g_s$ , mol m<sup>-2</sup> s<sup>-1</sup>—squares and continuous line) or mesophyll conductance to CO<sub>2</sub> ( $g_m \cdot CO_2$ , mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>— diamonds and dashed line) depict what it's only a non-significant trend) 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.

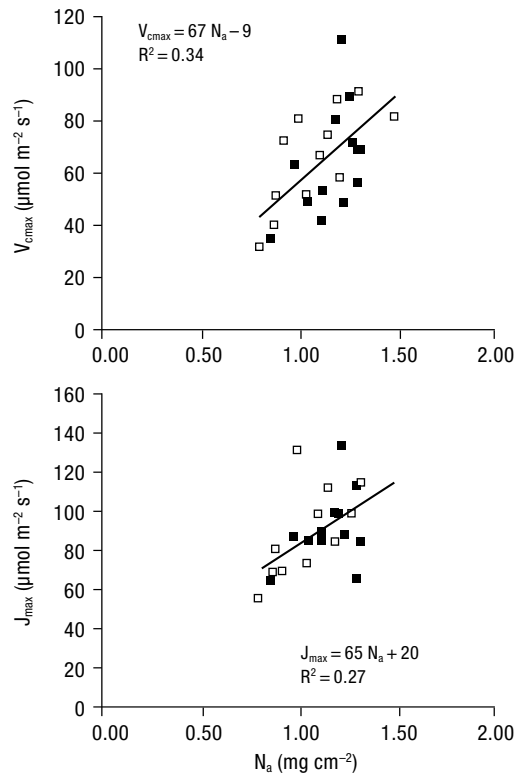
stress endured by seedlings, stomatal closure comprised one of the main limiting factors to carbon uptake (Chaves *et al.*, 2002; Medrano *et al.*, 2002). Previously, water stress of  $\Psi_{pd} = -0.5$  MPa was enough to prompt significant partial stomatal closures in beech seedling leaves (Aranda *et al.*, 2002). Accor-



**Figure 3.** There was a weak relationship between the water stress experienced by seedlings in terms of  $\Psi_{pd}$  and diffusional conductances, for both the  $g_s$ - $\Psi_{pd}$  (black squares,  $P = 0.07$ ) and the  $g_m$ - $\Psi_{pd}$  relationship (white squares  $P = 0.08$ ). There was no relation between the biochemical limitations imposed by  $V_{cmax}$  and  $J_{max}$  on carbon uptake, and the water stress endured by seedlings in the range of  $\Psi_{pd}$  analyzed.

dingly, in this study  $g_s$  to  $CO_2$  decreased from  $0.073$  to  $0.044 \mu mol m^{-2} s^{-1}$  on the last date when largest were the differences between treatments in the water stress experienced. However, the responsiveness of stomata to water stress could only be partly confirmed 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; Lendzion and Leuzinger, 2008). This could combine with less sensibility to drought of carbon losses by respiration regarding carbon uptake (Flexas *et al.*, 2005; Flexas *et al.*, 2006; Rodríguez-Calcerrada *et al.*, 2010). If maintain 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 to the overall leaf carbon balance of seedlings could be exacerbated when occurring in combination with low soil moisture and deep shade in the understory of mature stands (Aranda *et al.*, 2002; Aranda *et al.*, 2004; Rodríguez-Calcerrada *et al.*, 2008; Robson *et al.*, 2009).





**Figure 4.** Positive relationship between basic leaf biochemical parameters (maximum rate of carboxylation,  $V_{c\max}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and light saturating maximum rate of RUBP regeneration limited electron transport,  $J_{\max}$  ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and nitrogen content on a leaf area basis ( $N_a$ ). It's depicted a unique relationship for well watered and unwatered seedlings. No clear trends were observed when considering watering treatments.

The stomatal limitation to carbon uptake, even under moderate water stress, encompassed with low mesophyll diffusion conductance to  $\text{CO}_2$ . The low  $g_m$ , otherwise 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 a 22% of the relative photosynthesis limitations, a bit lower to that around 30% evaluated by Epron *et al.* (1995) with a different approach. On the other hand, stomatal resistance was a bit higher (33-40% in our case vs. 30%). 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  $g_m$ , being qualitatively similar whether the J constant or the curvature method was used to estimate  $g_m$ . In conclusion, both diffusional limitations 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  $\text{CO}_2$  diffusing to carbon fixing sites (Medrano *et al.*, 2002; Chaves *et al.*, 2003; Flexas *et al.*, 2008). This increases the overall diffusional limitations imposed on a first step by stomata (Medrano *et al.*, 2002).

In the present study, where moderate water stress was suffered by beech seedlings, only a seasonal reduction in  $g_s$  in response to water stress was evident. In this respect, the  $g_m$  did not show any consistent pattern in response to the moderate 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 was clear that main reduction was due to diffusional resistences, mainly stomatal one, accounting over 50% of the photosynthesis decrease. Under moderate water stress stomatal limitation on 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). The increase in IWUE with decreasing  $g_s$  on the three dates seems to reinforce the prevailing role of stomatal limitations under mild water stress, supporting this view. Only on the last date, was there a similar trend in the IWUE- $g_m$  relationship, but this was not statistically significance (see Fig. 2).

### 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 their photosynthetic capacity in terms of decreases in  $V_{cmax}$  and  $J_{max}$  (Wilson *et al.*, 2000a; Balandier *et al.*, 2007). Thus,  $V_{cmax}$  and  $J_{max}$  decreased slightly between July to August irrespective of watering, and caused a down-regulation of photosynthesis because of reduced biochemical capacity to fix carbon. Seasonally-induced decreases in photosynthetic capacity by the end of summer in beech have been reported before (Balandier *et al.*, 2008), and they add to the increase in the stomatal limitation in contributing to the impairment of carbon uptake under natural conditions of moderate water stress. 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 watering, reinforcing the ontogenic effect upon the changes in the basic biochemical parameters influencing net photosynthesis. Accordingly, both variables showed a stronger relationship with the leaf nitrogen content (Balandier *et al.*, 2008), than with the water stress experienced. Nevertheless, and as observed in other studies, there was also a slight direct down-regulation of  $V_{cmax}$  and  $J_{max}$  as consequence of the moderate drought (Wilson *et al.*, 2000b; Xu and Baldocchi, 2003; Damour *et al.*, 2009). In this respect, the differences between D and WW plants, though not significant were consistently lower in D than in WW on the last two measurement dates.

### CONCLUSIONS

The combination of shade tolerance at juvenile stages and a positive reaction to higher light levels makes beech very competitive in both wet and mesic sites, conditioning the ecology and silviculture of the species (see Wagner *et al.*, 2010, for a compressive review). However, even a moderate drought may change the competitiveness of the species when water is not limiting. 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 (Cornic, 1994). However, internal conductance to  $CO_2$  was also an important limitation to carbon uptake comprising a 22% of the total limitation to carbon fixing. This diffusional limitation could continue to increase, like those related with biochemical parameters, in years extremely dry.

What seems clear is the high sensitivity of beech to moderate water stress, confirming previous studies (Madsen, 1994; Aranda *et al.*, 2004; Robson *et al.*, 2009). Ultimately, this could jeopardize the

future of the species in marginal areas according to the expectations of a worsening in the climatic conditions for some beech stands, and where an increase in the temperature together with a decrease in the seasonal rainfall are expected (Peñuelas and Boada, 2003).

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