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RESEARCH PAPER

# Mesophyll conductance to CO<sub>2</sub>, assessed from online TDL-AS records of <sup>13</sup>CO<sub>2</sub> discrimination, displays small but significant short-term responses to CO<sub>2</sub> and irradiance in *Eucalyptus* seedlings

Cyril Douthe<sup>1,2,3</sup>, Erwin Dreyer<sup>1,2,\*</sup>, Daniel Epron<sup>1,2</sup> and Charles R. Warren<sup>3</sup>

<sup>1</sup> INRA, Unité Mixte de Recherches 1147 'Ecologie et Ecophysiologie Forestières', F-54280 Champenoux, France

<sup>2</sup> Nancy Université, Unité Mixte de Recherches 1147 'Ecologie et Ecophysiologie Forestières', Faculté des Sciences, F-54500 Vandoeuvre, France

<sup>3</sup> University of Sydney, School of Biological Sciences, Heydon-Laurence Building, A08, The University of Sydney, NSW 2006, Australia

\* To whom correspondence should be addressed. E-mail: [dreyer@nancy.inra.fr](mailto:dreyer@nancy.inra.fr)

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## Abstract

Mesophyll conductance ( $g_m$ ) is now recognized as an important limiting process for photosynthesis, as it results in a significant decrease of CO<sub>2</sub> diffusion from substomatal cavities where water evaporation occurs, to chloroplast stroma. Over the past decade, an increasing number of studies proposed that  $g_m$  can vary in the short term (e.g. minutes), but these variations are still controversial, especially those potentially induced by changing CO<sub>2</sub> and irradiance. In this study,  $g_m$  data estimated with online <sup>13</sup>C discrimination recorded with a tunable diode laser absorption spectrometer (TDL-AS) during leaf gas exchange measurements, and based on the single point method, are presented. The data were obtained with three *Eucalyptus* species. A 50% decrease in  $g_m$  was observed when the CO<sub>2</sub> mole fraction was increased from 300  $\mu\text{mol mol}^{-1}$  to 900  $\mu\text{mol mol}^{-1}$ , and a 60% increase when irradiance was increased from 200  $\mu\text{mol mol}^{-1}$  to 1100  $\mu\text{mol mol}^{-1}$  photosynthetic photon flux density (PPFD). The relative contribution of respiration and photorespiration to overall <sup>13</sup>C discrimination was also estimated. Not taking this contribution into account may lead to a 50% underestimation of  $g_m$  but had little effect on the CO<sub>2</sub>- and irradiance-induced changes. In conclusion, (i) the observed responses of  $g_m$  to CO<sub>2</sub> and irradiance were not artefactual; (ii) the respiratory term is important to assess absolute values of  $g_m$  but has no impact on the responses to CO<sub>2</sub> and PPFD; and (iii) increasing irradiance and reducing the CO<sub>2</sub> mole fraction results in rapid increases in  $g_m$  in *Eucalyptus* seedlings.

**Key words:** *Eucalyptus globulus*, *Eucalyptus saligna*, *Eucalyptus sieberii*, photosynthesis, stomatal conductance, tunable diode laser absorption spectrometry.

## Introduction

During photosynthesis, CO<sub>2</sub> diffuses from the atmosphere (at a mole fraction  $C_a$ ) to the sites of carboxylation ( $C_c$ ) inside the chloroplasts (Gaastra, 1959; Farquhar *et al.*, 1980). CO<sub>2</sub> crosses the leaf boundary layer and traverses stomatal pores into the substomatal cavity. CO<sub>2</sub> then diffuses through the gas phase between mesophyll cells before reaching cell walls, where it is solubilized. In the

liquid phase, CO<sub>2</sub> crosses the plasma membrane, the cytosol, and finally the chloroplast membranes to reach the sites of carboxylation in the chloroplast stroma. Most of the carboxylation is by RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), while a small fraction (estimated at 5%) is by phosphoenolpyruvate carboxylase (PEPc) in the cytosol. The mesophyll conductance to CO<sub>2</sub> ( $g_m$ ) represents

the conductance from the substomatal cavities at a mole fraction  $C_i$ , to the sites of carboxylation, and includes gas and liquid phase transfer.

In early leaf photosynthesis models,  $g_m$  was considered to be infinite. This was an explicit assumption in the original formulation of Farquhar's model of  $C_3$  photosynthesis. However, many subsequent studies showed that there was a difference between  $C_i$  and  $C_c$ , suggesting that  $g_m$  might be finite (Evans *et al.*, 1986; Lloyd *et al.*, 1992) and that it affects estimation and interpretation of photosynthetic parameters such as the maximal carboxylation activity of RubisCO ( $V_{cmax}$ ) or the maximal light-driven electron flux ( $J_{max}$ ) (Epron *et al.*, 1995; Niinemets *et al.*, 2009a).  $g_m$  can limit the rate of photosynthesis by 25–30% (Epron *et al.*, 1995) and this limitation may be as large as the one due to stomatal conductance (Warren and Adams, 2006; Flexas *et al.*, 2007c). The range of  $g_m$  variation among species is similar to that of stomatal conductance, from 0.005 mol  $m^{-2} s^{-1}$  up to 0.5 mol  $m^{-2} s^{-1}$  (see the reviews by Evans and Von Caemmerer, 1996; Flexas *et al.*, 2008), with high  $g_m$  occurring in herbaceous annuals and lower values in evergreen gymnosperms.  $g_m$  is apparently influenced by leaf traits such as thickness, tissue density (and therefore leaf dry mass per unit area, LMA; for meta analyses, see Flexas *et al.*, 2008; Niinemets *et al.*, 2009b), cell wall thickness, or the proportion of gaseous versus liquid mesophyll phases (Piel *et al.*, 2002; Terashima *et al.*, 2006; Evans *et al.*, 2009). Such morphological leaf properties would confer stable  $g_m$  in the short term (e.g. a few days).

Since the early 1990s,  $g_m$  was shown to be affected by environmental factors. At first, changes were thought to be rather long-term changes such as, for instance, the decline induced by leaf ageing (Scartazza *et al.*, 1998), by a gradual drought stress (Cornic *et al.*, 1989; Roupsard *et al.*, 1996; Warren, 2008b), or by salinity (Bongi and Loreto, 1989). More recently, short-term changes were shown to occur in response to temperature (Bernacchi *et al.*, 2002; Pons and Welschen, 2003; Warren and Dreyer, 2006; Warren, 2008a). Similarly rapid responses of  $g_m$  (at the minutes scale) were reported to occur under varying  $CO_2$  mole fractions. A review by Flexas *et al.* (2008) reported that  $g_m$  decreases with increasing  $CO_2$  (data from Harley *et al.*, 1992; During, 2003; Flexas *et al.*, 2007b). This pattern was also observed by Hassiotou *et al.* (2009) among seven *Banksia* species, by Vrábl *et al.* (2009) in *Helianthus annuus*, and by Bunce (2010) in *Glycine max* and *Phaseolus vulgaris*. Tazoe *et al.* (2011) recently observed the same decreasing pattern among three species with contrasting photosynthetic capacities. Only a few studies have tested the rapid response of  $g_m$  to irradiance. Flexas *et al.* (2008) reported an increase of  $g_m$  with increasing irradiance (data from Gorton *et al.*, 2003; Flexas *et al.*, 2007b).

Nevertheless, there is still no consensus about the reality of such rapid responses of  $g_m$  to  $CO_2$  and irradiance. Several studies reported  $g_m$  to be stable in response to changes in  $CO_2$  (Von Caemmerer and Evans, 1991; Loreto *et al.*, 1992; Tazoe *et al.*, 2009) and irradiance (Tazoe *et al.*, 2009; Yamori *et al.*, 2010). Some of the discrepancy may be

due to measurement accuracy and/or artefacts. This can be true with the two main methods used to estimate  $g_m$ : the fluorescence/gas exchange technique (Loreto *et al.*, 1992) and the isotopic discrimination method (Pons *et al.*, 2009). One of the complications with the isotopic method, which is used here, is that the contribution of  $^{13}C$  discrimination during respiration and photorespiration needs to be taken into account (see the model description below). Some studies have ignored discrimination during respiration and photorespiration (Flexas *et al.*, 2007b, c; Vrábl *et al.*, 2009), or approximated respiration in the light by that in the dark (Tazoe *et al.*, 2009, 2011). The fractionation factors associated with respiration and photorespiration can be taken into consideration using recent estimates (Lanigan *et al.*, 2008). An alternative approach is to limit the impact of fractionation during photorespiration by using low  $O_2$  in the measurement atmosphere (Tazoe *et al.*, 2009).

To date, there is no consensus regarding whether  $g_m$  responds rapidly to irradiance and the  $CO_2$  mole fraction, despite the importance of this for interpreting responses of photosynthesis to environmental variables. The aim of this research was to assess whether short-term variations of  $g_m$  occur in response to changes in irradiance and the  $CO_2$  mole fraction. Rapid response of  $g_m$  was assessed by recording online  $^{13}CO_2$  discrimination during photosynthesis with a custom-built photosynthesis chamber adapted to a LI-COR 6400, and coupled to a TDL-AS (or tunable diode laser absorption spectrometer). To examine the ubiquity of responses, three species of *Eucalyptus* with differing rates of photosynthesis were used, and the respiratory term of online  $^{13}C$  discrimination was taken into consideration to reinforce the findings. Measurements were carried out on species with contrasting photosynthetic capacity to check whether  $g_m$  variability is species dependent and how  $g_m$  is related to  $A$  and  $g_s$  within and among species.

## Materials and methods

### Plant material

Three species in the genus *Eucalyptus* were used: *E. globulus* Labill., *E. saligna* Sm., and *E. sieberi* L. A. S. Johnson. Plants were grown for 4 months in a naturally illuminated greenhouse at an average daily temperature of 25 °C in 8.0 l pots filled with compost-based substrate. They were watered with automatic drip irrigation. At the time of the experiment they were ~60 cm high.

### Gas exchange measurements

The response of the net  $CO_2$  assimilation rate ( $A$ ,  $\mu mol m^{-2} s^{-1}$ ) to variations in substomatal  $CO_2$  mole fraction ( $C_i$ ) and photosynthetic photon flux density (PPFD,  $\mu mol m^{-2} s^{-1}$ ) was measured in each of four replicate plants of the three species, for each treatment (e.g. 2 treatments  $\times$  3 species  $\times$  4 replicates = 24 plants). All measurements were made on the youngest fully expanded leaves. Plants were transferred from the greenhouse to the laboratory at 25 °C. To assess photosynthesis and discrimination against  $^{13}CO_2$  simultaneously, a LI-6400 portable gas exchange system (LI-COR, Lincoln, NE, USA) equipped with a custom-built chamber of 18  $cm^2$  and coupled to a TDL-AS (TGA100A, Campbell Scientific, Logan, UT, USA) was used.

Before measurements, leaves were exposed to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and 400  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  for 30 min to induce photosynthesis and stomatal opening. CO<sub>2</sub> responses of photosynthesis were measured under a constant PPFD of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  while the CO<sub>2</sub> mole fraction for the reference gas ( $C_e$ ) was sequentially set at 300, 500, 700, and 900  $\mu\text{mol mol}^{-1}$ . The PPFD responses at 200, 500, 800, and 1100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  were measured under a constant CO<sub>2</sub> mole fraction of 400  $\mu\text{mol mol}^{-1}$ . During each step of the CO<sub>2</sub> or PPFD responses, at least 20 min was allowed for  $A$  and  $g_s$  to reach a steady state, and three individual points separated by 180 s were recorded. Each set of three individual data points was averaged to characterize the response at one step in the response curve. The standard error of the three individual points was used to assess the analytical variability.

Air flow into the chamber was set at 400  $\mu\text{mol s}^{-1}$ , and leaf temperature at 25 °C. Irradiance was provided by a LI-COR RGB light source (6400-18, LI-COR-0128), which covered the entire chamber surface. A subsample of air from the sample and reference gas lines of the LI-6400 was diverted to the TDL-AS. The TDL-AS measured sequentially gas from two calibration tanks, the LI-6400 reference gas, and finally the LI-6400 sample gas. Each intake was measured for 45 s, with the first 15 s ignored to minimize carryover and enable stabilization between intakes. The total time for a sequence of measurements was therefore 180 s.

The TDL-AS gas was connected through a 'T' tubing to the reference tube of the LI-6400 between the console and the IRGA. In the same way, the TDL-AS intake of sample gas was connected in between the LI-6400 chamber exhaust. The TDL-AS was set to continuously withdraw 150  $\text{ml min}^{-1}$  ( $\sim 102 \mu\text{mol s}^{-1}$ ) from each of the sample and reference fluxes of the LI-6400. This withdrawal of air was much smaller than the flow through the LI-6400 chamber, which means that the TDL-AS could sample air from the LI-6400 while maintaining a positive pressure in the LI-6400 chamber. The existence of a positive pressure inside the LI-6400 chamber was checked through the curvature of the propafilm covering the top of the chamber. TDL-AS data were matched with LI-6400 data by taking into account the time lag of 37 s that was recorded between the chamber and the TDL-AS. The TDL-AS data were only used to record the isotopic composition of the reference and sample gas, while all photosynthesis parameters were estimated from the LI-6400 data.

#### Isotopic measurements and system testing

Discrimination by a leaf was assessed by measuring the isotopic composition in reference ( $\delta^{13}\text{C}_e$ , ‰) and sample gas ( $\delta^{13}\text{C}_o$ , ‰). The isotopic composition ( $\delta^{13}\text{C}$ ) was expressed as:

$$\delta = \left( \frac{R_s}{R_{\text{VPDB}}} - 1 \right) \times 1000$$

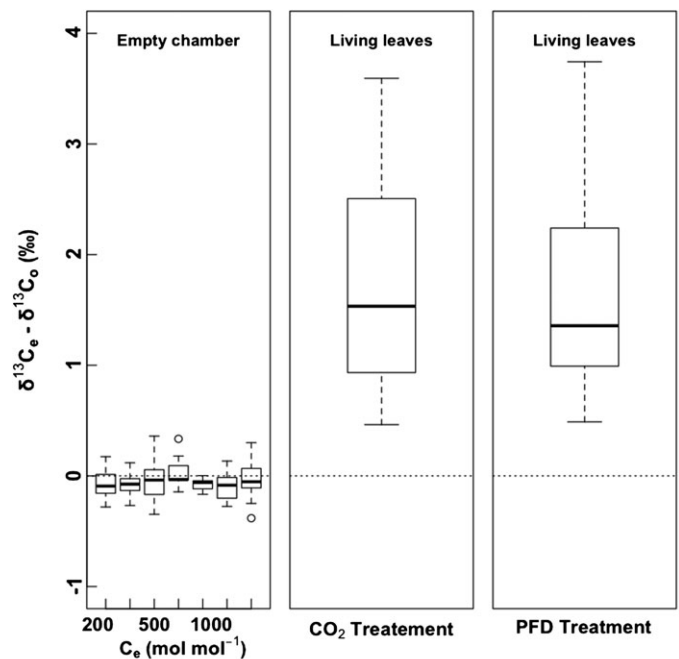
where  $R_s$  is the isotopic ratio ( $R = {}^{13}\text{CO}_2/{}^{12}\text{CO}_2$ ) of the sample and  $R_{\text{VPDB}}$  is the isotopic ratio of Vienna Pee Dee Belemnite (VPDB, 0.0112372).

The TDL-AS was calibrated with two tanks (T1 and T2) with CO<sub>2</sub> concentrations of  $419 \pm 10 \mu\text{mol mol}^{-1}$  and  $290 \pm 7 \mu\text{mol mol}^{-1}$  [mean  $\pm$  the confidence interval (CI)], respectively, given by the provider and checked with a recently factory-calibrated LI-8100 IRGA (LI-COR). The isotopic composition of the each tank was measured by sampling air into 12 ml exetainers (Labco Limited, Buckinghamshire, UK) and analysed via the gas-bench inlet of an IRMS (Delta S, Finningan, Bremen, Germany) at INRA Nancy ( $n=10$  exetainers per tank). For T1 and T2,  $\delta^{13}\text{C}$  was  $-36.6 \pm 0.08\text{‰}$  and  $-36.9 \pm 0.2\text{‰}$  [mean  $\pm$  standard deviation (Sd),  $n=10$ ], respectively. Absolute values of  ${}^{12}\text{CO}_2$  and  ${}^{13}\text{CO}_2$  were, respectively, 414.51  $\mu\text{mol mol}^{-1}$  and 4.487  $\mu\text{mol mol}^{-1}$  for T1 and 286.89  $\mu\text{mol mol}^{-1}$  and 3.104  $\mu\text{mol mol}^{-1}$  for T2, considering the CO<sub>2</sub> mole fraction indicated by the provider took into consideration both isotopologues. A linear interpolation was

used between these two points for each isotopologue. For further calibration, a range of CO<sub>2</sub> mole fractions of 200, 300, 500, 700, 1000, 1500, and 2000  $\mu\text{mol mol}^{-1}$  was generated using the CO<sub>2</sub> injector of the LI-6400 fed with the same CO<sub>2</sub> cartridge during the whole test. CO<sub>2</sub> mole fractions above the calibration range led to a small deviation of +2.5‰. A second order polynomial was fitted to describe the deviation of apparent  $\delta^{13}\text{C}$  from reference values measured at  $C_e=300 \mu\text{mol mol}^{-1}$  (because this is within the calibration range) along the extended CO<sub>2</sub> range ( $\delta^{13}\text{C}=0.000007[\text{CO}_2]^2-0.0035[\text{CO}_2]-5.2922$ ;  $R^2=0.92$ ,  $n=140$ ). All TDL-AS values were corrected and a stable  $\delta^{13}\text{C}$  signal was obtained along the extended CO<sub>2</sub> mole fraction range.

The noise of the system was assessed by observing the Sd of  $\delta^{13}\text{C}$  values within each CO<sub>2</sub> step. An average Sd of 0.16 ‰ was observed for  $\delta^{13}\text{C}$  ( $n=21$ ). These values were used for the computation of the standard deviation of the observed discrimination by the leaf ( $\text{Sd}_{\Delta\text{obs}}$ , see below).

Finally the empty photosynthesis chamber was used to test the absence of a  $\delta^{13}\text{C}$  difference between the inlet and outlet that might be caused by leaks and/or CO<sub>2</sub> adsorption/desorption processes. It was observed that the  $\delta^{13}\text{C}_e - \delta^{13}\text{C}_o$  difference was stable along the CO<sub>2</sub> mole fraction range used during the test, and it was confirmed that the empty chamber did not affect the isotopic composition of the air (i.e. leaks and CO<sub>2</sub> adsorption/desorption processes were negligible). The observed  $\delta^{13}\text{C}_e - \delta^{13}\text{C}_o$  difference was much smaller than the  $\delta^{13}\text{C}$  difference recorded during actual measurements (Fig. 1), although under specific conditions (low photosynthesis) the two could overlap. A data filtering procedure was therefore implemented (see below).



**Fig. 1.** Boxplots of the difference in  $\delta^{13}\text{C}$  between the outlet and inlet of the 18  $\text{cm}^2$  chamber for (i) an empty chamber with inlet air CO<sub>2</sub> varying from 200  $\mu\text{mol mol}^{-1}$  to 1000  $\mu\text{mol mol}^{-1}$  ( $n=10$ ); (ii) the whole set of measurements under varying CO<sub>2</sub> concentrations (300  $\mu\text{mol mol}^{-1}$  to 900  $\mu\text{mol mol}^{-1}$ ,  $n=40$ ); and (iii) the whole set of measurements under varying PPFD (from 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  up to 1100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $n=40$ ). The middle line represents the median, the upper and lower box limit the 75% and 25% quartiles, respectively, and whiskers represent the extreme value.

## Model description

The observed discrimination ( $\Delta_{\text{obs}}$ ) is usually calculated following Evans *et al.* (1986):

$$\Delta_{\text{obs}} = \frac{\xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)}{1000 + \delta^{13}\text{C}_o - \xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)} \quad (1)$$

where:

$$\xi = \frac{C_e}{C_e - C_o} \quad (2)$$

$\xi$  is the ratio of  $\text{CO}_2$  entering the chamber over the  $\text{CO}_2$  drawdown induced by the leaf.

$\Delta$  is the result of discrimination by diffusion processes during  $\text{CO}_2$  movement from the atmosphere to the chloroplast, and biochemical fractionation during carboxylation processes. Each fractionation step is characterized by a fractionation factor (due to diffusion or biochemistry) weighted by the gradient of concentration. In the complete form,  $\Delta$  is predicted by (Evans *et al.*, 1986):

$$\Delta = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (e_s + a_i) \frac{C_i - C_c}{C_a} + b \frac{C_c}{C_a} - \frac{eR_d + f\Gamma^*}{C_a} \quad (3)$$

where:

- $a_b$  is the fractionation during  $\text{CO}_2$  diffusion in the boundary layer (2.9‰, Evans *et al.*, 1986);
- $a$  is the fractionation during  $\text{CO}_2$  diffusion in air through stomata into the leaf (4.4‰, O'Leary, 1981);
- $e_s$  is the fractionation occurring when  $\text{CO}_2$  is dissolved in the cell solution (1.1‰ at 25 °C, O'Leary, 1981);
- $a_i$  is the fractionation during  $\text{CO}_2$  diffusion in the liquid phase (0.7‰, O'Leary, 1981);
- $b$  is the discrimination during carboxylation, and is dependent on fractionation by both RubisCO ( $b_3=30\%$ ) and PEPc ( $b_4=-5.7\%$ ),  $b$  is computed as:

$$b = (1 - \beta)b_3 - \beta b_4 \quad (4)$$

where  $\beta$  (between 0.05 and 0.1) is the relative amount of carbon fixed by PEPc (Farquhar and Richards, 1984). In the present experiment a value of  $b=28\%$  was used; that is, a  $\beta$  value of 0.055.

- $f$  denotes overall discrimination during photorespiration. The value of  $f$  was set at 11‰, according to the theoretical approach of Tcherkez (2006), which was confirmed later by Lanigan *et al.* (2008);
- $e$  denotes overall fractionation during day respiration relative to photosynthetic products ( $R_d$ ), and can vary between -10‰ and +10‰ (Ghashghaie *et al.*, 2003).  $e$  was set at 1‰ before correction following Wingate *et al.* (2007);
- $k$  is the carboxylation efficiency computed as (Farquhar *et al.*, 1982);

$$k = (A + R_d)/(C_i - \Gamma^*) \quad (5)$$

- $\Gamma^*$  is the  $\text{CO}_2$  compensation point in the absence of day respiration (Brooks and Farquhar, 1985).

The estimation of  $g_m$  is based on the difference between the observed discrimination by the leaf ( $\Delta_{\text{obs}}$ ) and the discrimination predicted from the simplified form of the model ( $\Delta_i$ ) in which

decarboxylation terms are ignored and  $g_m$  is considered to be infinite:

$$\Delta_i = a + \left( b - a \right) \frac{C_i}{C_a} \quad (6)$$

With this 'single point method' first developed by Lloyd *et al.* (1992) and recently described by Pons *et al.* (2009),  $g_m$  can be estimated from a single value of  $\Delta_{\text{obs}}$ :

$$g_m = \frac{(b - e_s - a_i)A/C_a}{\left( \Delta_i - \Delta_{\text{obs}} \right) - \frac{eR_d/k + f\Gamma^*}{C_a}} \quad (7)$$

## Model parameters

$R_d$  and  $\Gamma^*$  were estimated with the 'Laisk method' (Viil *et al.*, 1977) for the three species (i.e. from the intersection of three  $A-C_i$  curves recorded at PPFDs of 100, 50, and 25  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  and  $C_e$  of 125, 100, and 50  $\mu\text{mol mol}^{-1}$ ). The 'Laisk method' provides  $C_i^*$  or the 'apparent'  $\text{CO}_2$  compensation point in the absence of day respiration (Von Caemmerer *et al.*, 1994), and was used as a proxy of  $\Gamma^*$ . Because these measurements are sensitive to errors due to  $\text{CO}_2$  leak diffusion (low  $A$  and low  $C_a$  compared with the atmosphere), the potential  $\text{CO}_2$  leaks due to diffusion through chamber gaskets were estimated (Flexas *et al.*, 2007a; Rodeghiero *et al.*, 2007). A diffusion coefficient of the gaskets was computed with the procedure provided in the user manual of LI-COR, and a value of 0.938  $\mu\text{mol s}^{-1}$  was found (while it usually is 0.46 for smaller 6  $\text{cm}^2$  chambers). This correction was incorporated into all gas exchange computations used for  $\Gamma^*$  and  $R_d$  estimations. The computed values of  $\Gamma^*$  did not differ between species, so the mean ( $\Gamma^*=38.7 \pm 0.51 \mu\text{mol mol}^{-1}$ ,  $n=13$ ) was used as a common value for the three species. For *E. globulus*, *E. saligna*, and *E. sieberi*,  $R_d$  was  $0.41 \pm 0.09$ ,  $0.31 \pm 0.09$ , and  $0.68 \pm 0.07 \mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively.

As the isotopic signature of the reference gas provided by cartridges differed from that used by the leaves for earlier photosynthesis,  $e$  was replaced by  $e' = e + \delta^{13}\text{C}_{\text{tank}} - \delta^{13}\text{C}_{\text{atmosphere}}$  (Wingate *et al.*, 2007).  $\delta^{13}\text{C}$  in the cartridge was measured with the TDL-AS at the chamber inlet. In the present case, the LI-6400 was fed with compressed  $\text{CO}_2$  cartridges with  $\delta^{13}\text{C}_{\text{tank}}$  varying between -1‰ and -4‰ except for two cartridges with  $\delta^{13}\text{C}_{\text{tank}} = -19\%$ .  $e'$  therefore varied between +4‰ and +6‰, except for two plants where it was -11‰.

$C_c$ , the  $\text{CO}_2$  concentration at the site of carboxylation, was calculated from Fick's Law as:

$$C_c = C_i - \frac{A}{g_m} \quad (8)$$

Finally, the total leaf conductance to  $\text{CO}_2$  was calculated following Ball (1988), assuming resistances in series as:

$$g_t = \frac{1}{\frac{1}{g_{sc}} + \frac{1}{g_m}} \quad (9)$$

where the stomatal conductance to  $\text{CO}_2$  is  $g_{sc} = g_{sw}/1.6$ .

Propagation of uncertainty from measurement to  $\Delta$  calculation and data filtering

The uncertainty (standard deviation) of  $\Delta_{\text{obs}}$  due to the finite precision of  $\delta^{13}\text{C}$  measurements was estimated. This was achieved by propagating uncertainty (standard deviations) of  $\delta^{13}\text{C}_e$  and  $\delta^{13}\text{C}_o$  through the equations estimating  $\Delta_{\text{obs}}$  (see Appendix for details):

$$Sd_{\Delta_{obs}} = \left( \frac{\xi \sqrt{Sd_{\delta^{13}C_e}^2 + Sd_{\delta^{13}C_o}^2}}{\xi(\delta^{13}C_o - \delta^{13}C_e)} + \frac{1 + \sqrt{Sd_{\delta^{13}C_o}^2 - \xi \sqrt{Sd_{\delta^{13}C_e}^2 + Sd_{\delta^{13}C_o}^2}}}{1 + \delta^{13}C_o - \xi(\delta^{13}C_o - \delta^{13}C_e)} \right) \Delta_{obs} \quad (10)$$

The propagated uncertainty in  $\Delta_{obs}$  (i.e.  $Sd_{\Delta_{obs}}$ ) was used as the basis for a filter to remove unreliable estimates of  $\Delta_{obs}$ . Computation of  $g_m$  is based on the difference between  $\Delta_i$  and  $\Delta_{obs}$  (i.e.  $\Delta_i - \Delta_{obs}$ ), thus it was reasoned that  $g_m$  estimates would be unreliable if the difference  $\Delta_i - \Delta_{obs}$  was smaller than  $Sd_{\Delta_{obs}}$ . Consequently all values where  $\Delta_{obs} + Sd_{\Delta_{obs}} > \Delta_i$  were rejected. This filter was applied to the individual points in the data set, rejecting 33 among 238 points.

### Statistical analyses

All statistical analyses were performed with R (R Development Core Team 2010, <http://www.R-project.org>). Mixed-effect linear models were run to assess species and treatment effects on  $A$ ,  $g_s$ ,  $g_m$ ,  $\Delta_i - \Delta$ ,  $C_c$ , and the  $C_i - C_c$  drawdown, as shown in Table 1. For the CO<sub>2</sub> treatment, 'species' (as a factor) and ' $C_i$ ' (as a covariate) were incorporated into the model as fixed effects, and 'individual within species' as a random effect. For variations of  $C_c$  and the  $C_i - C_c$  drawdown,  $C_a$  was used as covariate. For PPFD treatment, species and PPFD were set as factors. Normality and heteroscedasticity were graphically checked with QQ-plots. In the case of heteroscedastic data, the mean was weighted as a function of the variance. In the case of non-normal distribution, variables were log-transformed. The species  $\times$  treatment interaction was tested for each procedure, and was removed from the model when not significant. In the absence of interaction, comparison of the intercepts was performed to assess differences between species. In the case of interaction, slope comparisons were performed to test if species responses differed from each other. Significance was accepted at  $P < 0.05$ . Mean least squares regression was used to assess the correlation between variables ( $R^2$  and  $P$ -value).

## Results

### Variation of $g_m$ under changing $C_e$

The CO<sub>2</sub> mole fraction was changed in the air entering the chamber in three steps from 300  $\mu\text{mol mol}^{-1}$  to 900  $\mu\text{mol mol}^{-1}$ , inducing a range of  $C_i$  from 185  $\mu\text{mol mol}^{-1}$  to 745  $\mu\text{mol mol}^{-1}$ . Net CO<sub>2</sub> assimilation rate ( $A$ ) was positively related to  $C_i$  and varied between 3  $\mu\text{mol mol}^{-2} \text{s}^{-1}$  and 18  $\mu\text{mol mol}^{-2} \text{s}^{-1}$ , while stomatal conductance to water vapour ( $g_s$ ) was negatively related to  $C_i$  and varied

between 0.02  $\text{mol m}^{-2} \text{s}^{-1}$  and 0.8  $\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 2). *Eucalyptus sieberi* had significantly higher  $A$  and  $g_s$  than *E. globulus* and *E. saligna* ( $t$ -test  $P < 0.05$ , Fig. 2).

The difference between  $\delta^{13}C_e$  and  $\delta^{13}C_o$  decreased with increasing  $C_i$  (data not shown). Among all species,  $\Delta_{obs}$  varied between 12‰ and 22‰ and was positively correlated to  $C_i/C_a$  ( $R^2=0.79$ ,  $P < 0.001$ , data not shown). The difference between  $\Delta$  calculated with infinite  $g_m$  and no respiratory term (simple model) and observed  $\Delta$  ( $\Delta_i - \Delta_{obs}$ ) varied between 3‰ and 7‰ but showed no clear trend with  $C_i$  (Fig. 2, Table 1).

$g_m$  computed by taking into account the respiratory component of discrimination varied from 0.025  $\text{mol m}^{-2} \text{s}^{-1}$  to 0.55  $\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 2).  $g_m$  was larger in *E. sieberi* than in the other two species ( $t$ -test,  $P < 0.05$ ).  $g_m$  was affected by  $C_i$  (Table 1), and decreased when  $C_i$  increased. Post-hoc tests revealed that *E. globulus* and *E. saligna* displayed  $g_m - C_i$  slopes significantly different from zero. In *E. sieberi*, three individuals out of four showed a clear negative pattern when  $C_i$  increased, but not the fourth. The relationship between  $g_m$  and  $g_s$  was significant among all species ( $R^2=0.54$ ,  $P < 0.001$ ), and within *E. globulus* and *E. saligna* when treated separately (data not shown).

$C_c$  and the  $C_i - C_c$  drawdown were, as expected, severely affected by  $C_a$  (Table 1). The  $C_i - C_c$  drawdown was  $\sim 50 \mu\text{mol mol}^{-1}$  at  $C_a=200 \mu\text{mol mol}^{-1}$  and increased up to 200  $\mu\text{mol mol}^{-1}$  at  $C_a=900 \mu\text{mol mol}^{-1}$  (Fig. 3). When tested individually, all slopes of the responses of  $C_c$  and  $C_i - C_c$  to  $C_i$  were different from zero.

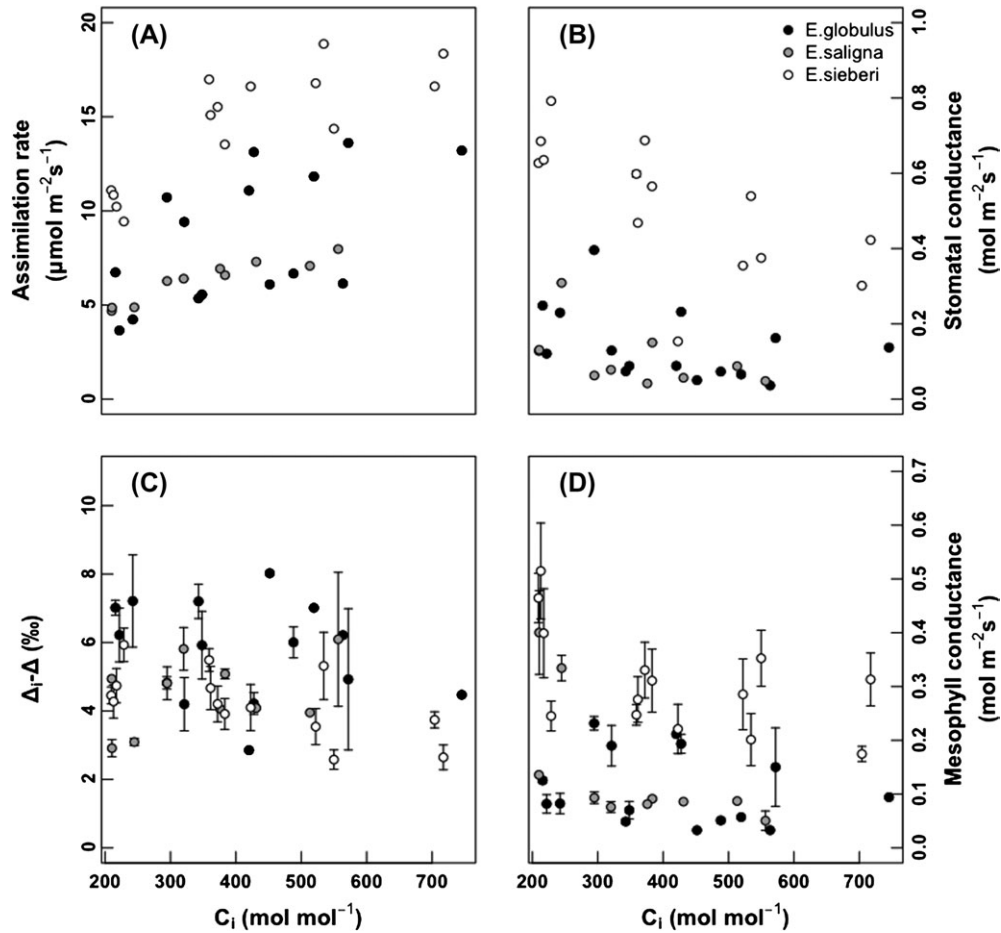
### Variation of $g_m$ with changing irradiance

$A$  and  $g_s$  increased significantly with irradiance (Table 1).  $A$  and  $g_s$  were larger in *E. sieberi* than in the two other species (see Fig. 4,  $t$ -test  $P < 0.05$ ). In each species,  $g_s$  increased significantly from 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, and then stabilized (Fig. 4).  $A$  and  $g_s$  were positively correlated, for all species taken together (Fig. 5C,  $R^2=0.76$ ,  $P < 0.001$ ). Across the range of irradiance,  $\Delta_{obs}$  varied between 11‰ and 20‰ and was positively correlated with  $C_i/C_a$  ( $R^2=0.74$ ,  $P < 0.001$ , data not shown).  $\Delta_i - \Delta_{obs}$  varied between 3‰ and 9‰ but was not affected by irradiance or by species (Table 1, Fig. 4).

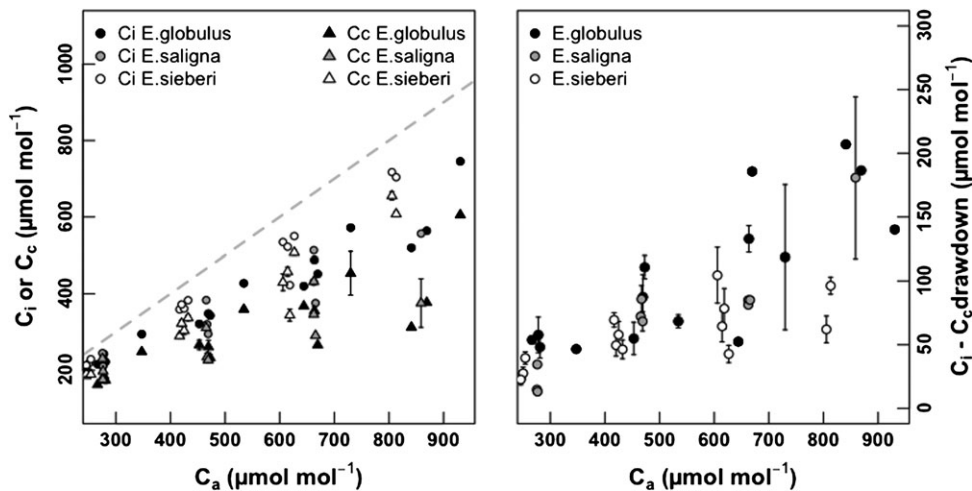
**Table 1.** Mixed effects model for  $A$ ,  $g_s$ ,  $g_m$ , and  $\Delta_i - \Delta$

Species,  $C_a$  and  $C_i$ , or PPFD effects were incorporated into the model as fixed effects, and individual plant as a random effect. In the case of heteroscedastic data the mean was weighted as a function of the variance. For  $C_i$  and  $C_a$ , the degree of freedom (df) was 1, for PPFD df=3, and for species df=2. Significant values ( $P < 0.05$ ) are shown in bold.

		$C_i$				$C_a$		PPFD					
		$A$	$g_s$	$g_m$	$\Delta_i - \Delta$	$C_c$	$C_i - C_c$	$A$	$g_s$	$g_m$	$\Delta_i - \Delta$	$C_c$	$C_i - C_c$
Variable	$F$	<b>168.11</b>	<b>38.82</b>	<b>18.67</b>	3.70	<b>165.01</b>	<b>102.90</b>	<b>86.01</b>	<b>34.23</b>	<b>6.41</b>	NS	NS	NS
	$P$	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	(0.06)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.001</b>	<b>&lt;0.001</b>	<b>0.002</b>	NS	NS	NS
Species	$F$	<b>13.31</b>	<b>26.33</b>	<b>10.11</b>	<b>6.36</b>	<b>4.64</b>	<b>7.65</b>	<b>14.65</b>	(2.91)	NS	NS	NS	NS
	$P$	<b>0.002</b>	<b>&lt;0.001</b>	<b>0.006</b>	<b>0.02</b>	<b>0.045</b>	<b>0.013</b>	<b>&lt;0.01</b>	(0.10)	NS	NS	NS	NS
Interaction	$F$	3.05	NS	<b>3.44</b>	NS	<b>5.84</b>	<b>3.72</b>	<b>14.88</b>	<b>4.55</b>	NS	NS	NS	NS
	$P$	(0.06)	NS	<b>0.047</b>	NS	<b>0.008</b>	<b>0.038</b>	<b>&lt;0.001</b>	<b>0.002</b>	NS	NS	NS	NS

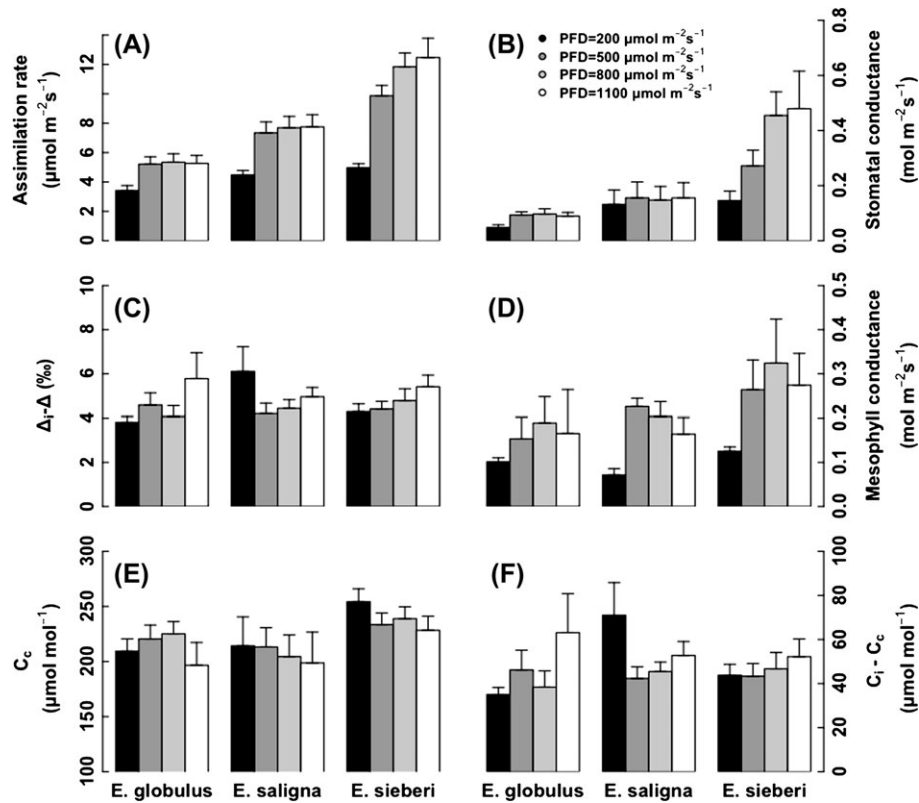


**Fig. 2.** Relationships between the  $\text{CO}_2$  mole fraction in the substomatal cavities ( $C_i$ ) and (A) net  $\text{CO}_2$  assimilation rate ( $A$ ). (B) Stomatal conductance to water vapour ( $g_s$ ). (C) Difference between predicted and measured isotopic discrimination ( $\Delta_i - \Delta$ ). (D) Mesophyll conductance ( $g_m$ ). *Eucalyptus globulus* is in black, *E. saligna* in grey, and *E. sieberi* in white. The SE is provided by the average of three measurements taken at 180 s intervals. Measurements were made at four levels of  $C_e$  on four plants per species, and were filtered against noisy values of  $\delta^{13}\text{C}_e - \delta^{13}\text{C}_o$ .

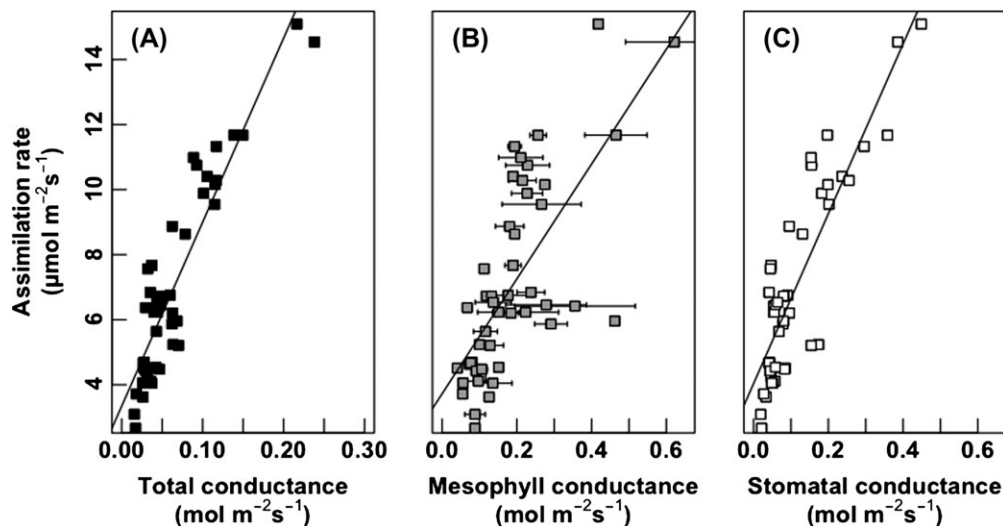


**Fig. 3.** Left:  $\text{CO}_2$  mole fraction in the substomatal cavities ( $C_i$ , disks) and at carboxylation sites ( $C_c$ , triangles) as a function of  $C_a$ . The dashed grey line is the 1:1 relationship. Right:  $C_i - C_c$  drawdown as a function of  $C_a$ . Each point represents the mean of 1–3 analytical measurements  $\pm$  SE, with *E. globulus* in black, *E. saligna* in grey, and *E. sieberi* in white.

$g_m$  varied between  $0.04 \text{ mol mol}^{-2} \text{ s}^{-1}$  and  $0.6 \text{ mol mol}^{-2} \text{ s}^{-1}$ , and was positively related to PPFD. As for  $g_s$ , the response consisted of a significant increase between  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$  and  $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPFD with



**Fig. 4.** (A) Net CO<sub>2</sub> assimilation rate (A). (B) Stomatal conductance to water vapour ( $g_s$ ). (C) Difference between predicted and measured isotopic discrimination ( $\Delta_i - \Delta$ ). (D) Mesophyll conductance ( $g_m$ ). (E) CO<sub>2</sub> mole fraction at carboxylation sites ( $C_c$ ). (F) The  $C_i - C_c$  drawdown at four different levels of PPFD. Means  $\pm$  SE ( $n=2-4$  replicate plants), with *E. globulus* in black, *E. saligna* in grey, and *E. sieberi* in white.



**Fig. 5.** Relationships between net CO<sub>2</sub> assimilation rate (A) and (A) total leaf conductance to CO<sub>2</sub> ( $g_t$ , black squares), (B) mesophyll conductance to CO<sub>2</sub> ( $g_m$ , grey squares), and (C) stomatal conductance to CO<sub>2</sub> ( $g_{sc}$ , white squares) under varying PPFD.

a stabilization above this threshold.  $g_m$  was positively correlated with  $g_s$  ( $R^2=0.36$ ,  $P < 0.001$ , data not shown) and  $A$  ( $R^2=0.49$ ,  $P < 0.001$ , Fig. 5B), among all species. Significant  $A-g_m$  and  $g_m-g_s$  relationships within each species were also detected (except for  $g_s-g_m$  in *E. saligna*).

Total leaf conductance to CO<sub>2</sub> ( $g_t$ ) was strongly correlated to  $A$  ( $R^2=0.83$ ,  $P < 0.001$ ) as shown in Fig. 5A. Over the full set of irradiance values,  $C_c$  varied between 200  $\mu\text{mol mol}^{-1}$  and 260  $\mu\text{mol mol}^{-1}$ , and the  $C_i - C_c$  drawdown between 40  $\mu\text{mol mol}^{-1}$  and 60  $\mu\text{mol mol}^{-1}$ . None of these



parameters displayed any variation with irradiance (Table 1), or with species.

## Discussion

This study provides support to recent evidence that  $g_m$  varies rapidly (within minutes) in response to environmental conditions. The rapid responses were observed under two different sources of variation: CO<sub>2</sub> mole fraction and irradiance. In seedlings of three *Eucalyptus* species a modest but significant decrease of  $g_m$  with increasing CO<sub>2</sub> mole fraction, and a significant increase with irradiance, was found. The effect was visible in the three species irrespective of the photosynthetic capacity.

### Importance of the respiratory and photorespiratory terms in the estimation of $g_m$

The isotopic method estimates  $g_m$  from the <sup>13</sup>CO<sub>2</sub> discrimination during photosynthesis by comparing observed values with those derived from a model-based prediction of discrimination under infinite mesophyll conductance. This approach requires a high precision in discrimination records, which is now achieved by combining precise leaf gas exchange measurements with online TDL-AS records of changes in <sup>13</sup>CO<sub>2</sub>/<sup>12</sup>CO<sub>2</sub> in the atmosphere around the leaf (for a discussion of the technique, see Pons *et al.*, 2009; Tazoe *et al.*, 2011). One of the important problems with this method is the fact that several discrimination steps during photosynthesis, respiration, and photorespiration need be taken into account. In particular, a number of earlier studies omitted the respiratory and photorespiratory terms (Flexas *et al.*, 2007b; Vrabl *et al.*, 2009). Moreover, the response of  $g_m$  to CO<sub>2</sub> was affected by the O<sub>2</sub> mole fraction in the air; that is, by the occurrence of photorespiration during measurements: it was visible only under low O<sub>2</sub> (Tazoe *et al.*, 2011).

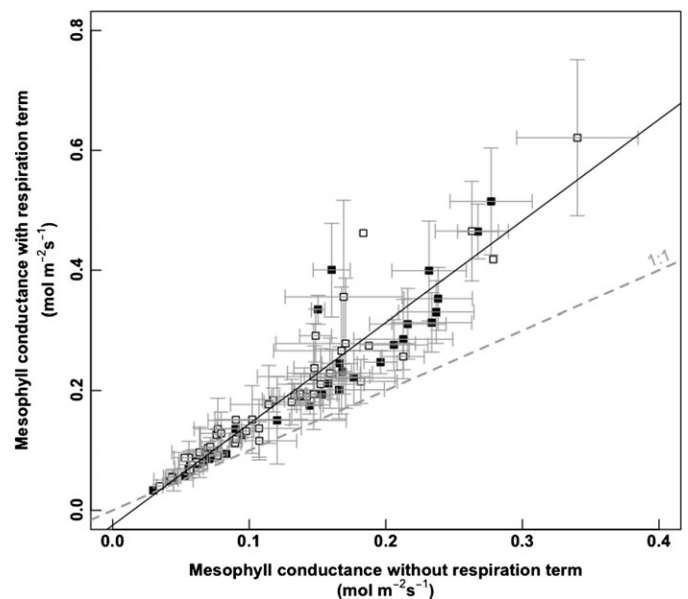
In the present study, these two terms were incorporated into the  $g_m$  estimates displayed in the results. Absolute values of  $g_m$  were up to 50% larger when these terms were incorporated, and this enhancement was independent of the treatments applied (Fig. 6). Whether this potentially large change had an impact on the observed effects of changing the CO<sub>2</sub> mole fraction in the air or irradiance was tested: the response of  $g_m$  to CO<sub>2</sub> mole fraction and irradiance remained significant even when the respiratory term was omitted (see Table 2). The effects of substituting  $e$  (fractionation during day respiration) and  $f$  (fractionation during photorespiration) with extreme values ( $f=0\text{‰}$ ,  $e=-10\text{‰}$ ,  $e=+10\text{‰}$ ) were similarly tested. Despite the fact that these changes resulted in significant differences of computed  $g_m$ , they did not result in any loss of significance of the observed effects of CO<sub>2</sub> or irradiance (data not shown). Vrabl *et al.* (2009) compared  $g_m$  estimated with the fluorescence method (which takes the respiratory terms implicitly into account) and with the isotopic method (without taking them into account) and found the same

range of  $g_m$  values and the same negative response to C<sub>i</sub>. The present computations suggest that the respiratory terms can be important in the estimation of the absolute values of  $g_m$ , but have only little influence on the observed CO<sub>2</sub> or irradiance responses of  $g_m$ . The respiratory terms of isotopic discrimination are unlikely to be responsible for the discrepancies among studies. Addressing the question of changing  $e$  and  $f$  during the CO<sub>2</sub> and irradiance treatments was omitted in this discussion: up to now there is no reason to assume that these values are not stable across the whole range of environmental conditions.

**Table 2.** Impact of omitting the respiratory term in the discrimination model on the assessment of the impact of the CO<sub>2</sub> mole fraction (C<sub>i</sub>) or of irradiance on  $g_m$

Mixed effect model for mesophyll conductance computed including (as shown in Table 1) or omitting the respiration and photorespiration terms.

Variable		C <sub>i</sub>		PPFD	
		$g_m$	$g_m$ (omitting the respiration terms)	$g_m$	$g_m$ (omitting the respiration terms)
C <sub>i</sub>	F	18.67	13.14	6.41	12.09
	P	<0.001	0.001	0.002	<0.001
Species	F	10.11	10.28	NS	4.89
	P	0.006	0.006	NS	0.03
Interaction	F	3.44	3.64	NS	NS
	P	0.047	0.04	NS	NS



**Fig. 6.** Mesophyll conductance  $g_m$  as computed by taking into account the contribution of respiration and photorespiration to <sup>13</sup>CO<sub>2</sub> discrimination versus without taking them into account (i.e. fractionation factors  $e$  and  $f$  set to 0 in Equation 7). Results obtained under changing CO<sub>2</sub> (filled squares) or irradiance (open squares). The dashed line represents the 1:1 relationship. Each point represents the mean  $\pm$ SE of a given measurement ( $n=3$ ).

### Response of $g_m$ to CO<sub>2</sub> mole fraction

There was at maximum a 50% decrease in  $g_m$  with increasing CO<sub>2</sub> mole fraction (from 300  $\mu\text{mol mol}^{-1}$  to 900  $\mu\text{mol mol}^{-1}$ ). This response was general, as all three species displayed a similar response to CO<sub>2</sub>, with the exception of a single individual of *E. sieberi*. The results contrast with earlier studies reporting no response of  $g_m$  to CO<sub>2</sub>. This was the case in *Quercus ilex* and *Citrus aurantium* (chlorophyll fluorescence method; Loreto *et al.*, 1992), *Raphanus sativus* (isotopic discrimination; Von Caemmerer and Evans, 1991), and *Triticum aestivum* (isotopic discrimination; Tazoe *et al.*, 2009). The present results confirm several studies that reported a negative relationship between  $g_m$  and CO<sub>2</sub> (Flexas *et al.*, 2007b; Hassiotou *et al.*, 2009; Vrabl *et al.*, 2009; Bunce, 2010). The same magnitude of decrease of  $g_m$  with  $C_i$  as in Flexas *et al.* (2007b) and Vrabl *et al.* (2009) was found. Tazoe *et al.* (2011) found an ~30% decrease of  $g_m$  when  $C_i$  increased under 1% O<sub>2</sub> but no effect under 21% O<sub>2</sub> in two *Arabidopsis thaliana* genotypes or in *Nicotinia tabacum*, while in the present study a significant effect was found even under 21% O<sub>2</sub>. The absence of a clear pattern of  $g_m$  responses among studies could be interpreted as a species-dependent response of  $g_m$  to  $C_i$ , but no common trait seems to be shared by the 'non-responsive' versus the 'responsive' species.

There are a suite of methodological reasons and artefacts (e.g. choice or calculation of  $b$ ,  $e$ ,  $f$ ,  $R_d$ , or  $\Gamma^*$ ) that might explain the discrepancy among studies. The case for  $e$  and  $f$  was discussed above. Measured values of  $R_d$  and  $\Gamma^*$  (i.e. of its proxy  $C_i^*$ ) were used for each species rather than arbitrary values taken from the literature. Despite some uncertainties regarding the choice of  $b$  or  $f$  (Lanigan *et al.*, 2008; Pons *et al.*, 2009), it is concluded that the response of  $g_m$  that was recorded here is unlikely to be an artefact.

Rapid responses of  $g_m$  to CO<sub>2</sub> could be mediated by aquaporins that might impact the permeability of plasma and chloroplast membranes to CO<sub>2</sub> (Terashima and Ono, 2002), enhancing CO<sub>2</sub> diffusion in the liquid phase. Flexas *et al.* (2006) stated that the expression of NtAQPI aquaporins can change  $g_m$  values by 20–50% in *N. tabacum*. These variations of  $g_m$  are of the same magnitude as those in the present study, but only direct measurements of aquaporin expression/activity could confirm the role of these proteins in the diffusion of CO<sub>2</sub> through membranes. Establishing a parallel between short-term responses of  $g_m$  and of the expression and activities of aquaporins is still an open area for research. Tholen *et al.* (2008) also found that chloroplast movements can induce a variation of  $g_m$  by 50% in *A. thaliana*. However, it is not known yet whether CO<sub>2</sub> variations can directly mediate a displacement of chloroplasts.

A positive relationship was observed between  $g_m$  and  $g_s$ , as was also observed by Flexas *et al.* (2007c). Interestingly, such a relationship was also found by Flexas *et al.* (2006) when they compared plants overexpressing NtAQPI aquaporin and controls. They suggested that

the variation of  $g_m$  primarily induced by manipulating NtAQPI expression probably also led to an adjustment of  $g_s$  and subsequently of  $A$ . This potentially indicates a physiological link between these two parameters (Flexas *et al.*, 2007c; Vrabl *et al.*, 2009). Nevertheless, the precise signalling cascade that could cause a coordinated response of stomatal and of mesophyll conductance remains to be elucidated.

### Response of $g_m$ to irradiance

Several studies observed an increase in  $g_m$  with increasing irradiance. Flexas *et al.* (2007b) reported that  $g_m$  increased in tobacco by ~40% when irradiance increased from 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Data from Gorton *et al.* (2003), reanalysed by Flexas *et al.* (2008), also showed a positive effect of irradiance on  $g_m$ . Hassiotou *et al.* (2009) detected an ~22% increase in six *Banksia* species as irradiance was switched from 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The present study corroborates this positive effect of irradiance, with an increase in  $g_m$  by 60%, up to a plateau in irradiance reached between 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 1100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , depending on the species. A slightly larger sensitivity of  $g_m$  to irradiance than earlier studies was found. Sensitivity to irradiance could be (i) species dependent or (ii) due to different parameterization of the model enabling  $g_m$  estimation. For instance, in the present case, removing the respiratory and photorespiratory terms in the estimation of  $g_m$  led to a slightly smaller response of  $g_m$  to irradiance (Table 2). A systematic analysis of the reported responses, with a standardized parameterization, would be very helpful.

As during the response to CO<sub>2</sub>,  $g_m$  was positively correlated to  $g_s$ . This relationship seems to be independent of the method used to vary net CO<sub>2</sub> assimilation rates. A review by Flexas *et al.* (2008) insisted that  $g_s$  and  $g_m$  responded in parallel to irradiance, CO<sub>2</sub>, temperature, and drought stress (Warren, 2008b). On the other hand, Warren (2008b) reported that  $g_m$  was unaffected by increases in vapour pressure deficit (VPD) while  $g_s$  decreased strongly, and Vrabl *et al.* (2009) reported that  $g_m$  was unaffected by feeding leaves with abscisic acid (ABA), whereas there was a clear decrease in  $g_s$ . In the two latter cases the net CO<sub>2</sub> assimilation rate remained unaffected by VPD and ABA despite the severe reduction of  $g_s$ . The  $g_s$ – $g_m$  relationship therefore may reflect a tight coordination between  $A$  and  $g_m$ . Thus it seems that  $g_m$  contributes to adjust the CO<sub>2</sub> supply to the sites of carboxylation in response to photosynthetic limitations such as light availability, hydraulic constraints, or biochemical limitations (Warren *et al.*, 2007). In the present study, the coordinated variations of  $g_s$  and  $g_m$  apparently led to a very stable  $C_c$  (and  $C_i$ – $C_c$  drawdown) across irradiance levels, despite large variation in  $A$ . Such a homeostasis of  $C_c$  was already observed across a range of leaf morphologies (Hassiotou *et al.*, 2009), during leaf ageing (Ethier *et al.*, 2006; Warren, 2006), and such an adjustment seems also to occur during short-term fluctuations of irradiance.

## Conclusion

This study with three *Eucalyptus* species confirmed that  $g_m$  estimated with the online  $^{13}\text{C}$  discrimination method declines in response to short-term increases of the  $\text{CO}_2$  mole fraction and increases with irradiance. The response to irradiance is saturated above  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. The respiratory term in the  $^{13}\text{C}$  discrimination equation was found to be important to estimate absolute values of  $g_m$  but had little impact on the  $\text{CO}_2$  and irradiance responses. During the responses to  $\text{CO}_2$  and PPFD,  $g_s$  and  $g_m$  were tightly correlated and varied in parallel independently of the source of variation. Moreover, it was observed that coordinated adjustments of  $\text{CO}_2$  demand ( $A$ ) and supply ( $g_s$  and  $g_m$ ) led to a stability of  $C_c$  across irradiance variations.  $C_c$  homeostasis could be an advantage for the leaf to prevent large variation of the oxygenation/carboxylation ratio of the RubisCO, when the  $\text{CO}_2$  demand increases.

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## Appendix I

Here we describe the procedure to calculate the standard deviation of  $\Delta_{\text{obs}}$  ( $Sd_{\Delta_{\text{obs}}}$ ) based on the standard deviation of the isotopic composition of reference and measured air ( $Sd_{\delta^{13}\text{C}_e}$  and  $Sd_{\delta^{13}\text{C}_o}$ , respectively). In the equation of Evans *et al.* (1986) to calculate  $\Delta_{\text{obs}}$ ,  $\delta^{13}\text{C}_e$  and  $\delta^{13}\text{C}_o$  were substituted by their respective standard errors  $Sd_{\delta^{13}\text{C}_e}$  and  $Sd_{\delta^{13}\text{C}_o}$  to obtain:

$$Sd_{\Delta_{\text{obs}}} \approx \frac{\xi(Sd_{\delta^{13}\text{C}_o} - Sd_{\delta^{13}\text{C}_e})}{1 + Sd_{\delta^{13}\text{C}_o} - \xi(Sd_{\delta^{13}\text{C}_o} - Sd_{\delta^{13}\text{C}_e})}$$

$\frac{Sd_{\delta^{13}\text{C}_o} - Sd_{\delta^{13}\text{C}_e}}{\sqrt{Sd_{\delta^{13}\text{C}_o} + Sd_{\delta^{13}\text{C}_e}}}$  and  $\frac{Sd_{\delta^{13}\text{C}_o}}{\sqrt{Sd_{\delta^{13}\text{C}_o}}}$  were replaced by  $\xi$  and  $1 + \delta^{13}\text{C}_o - \xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)$ , respectively, as is described in Harris (1991) to obtain:

$$Sd_{\Delta_{\text{obs}}} \approx \frac{\xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}{1 + \sqrt{Sd_{\delta^{13}\text{C}_o}^2} - \xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}$$

Then, to calculate the cumulative effect of the upper term and the lower term, their relative Sds were summed to obtain the relative standard error of  $\Delta_{\text{obs}}$ :

$$\frac{Sd_{\Delta_{\text{obs}}}}{\Delta_{\text{obs}}} = \left( \frac{\xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}{\xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)} \right) + \left( \frac{1 + \sqrt{Sd_{\delta^{13}\text{C}_o}^2} - \xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}{1 + \delta^{13}\text{C}_o - \xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)} \right)$$

$Sd_{\Delta_{\text{obs}}}$  is then equal to:

$$Sd_{\Delta_{\text{obs}}} = \left( \frac{\xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}{\xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)} + \frac{1 + \sqrt{Sd_{\delta^{13}\text{C}_o}^2} - \xi \sqrt{Sd_{\delta^{13}\text{C}_o}^2 + Sd_{\delta^{13}\text{C}_e}^2}}{1 + \delta^{13}\text{C}_o - \xi(\delta^{13}\text{C}_o - \delta^{13}\text{C}_e)} \right) \Delta_{\text{obs}}$$

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