Journal of Experimental Botany, Vol. 60, No. 8, pp. 2341–2350, 2009
doi:10.1093/jxb/erp032 Advance Access publication 23 February, 2009

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Internal conductance under different light conditions along the plant profile of Ethiopian mustard (*Brassica carinata* A. Brown.)

Andrea Monti*, Guido Bezzi and Gianpietro Venturi

Department of Agroenvironmental Science and Technology, University of Bologna, Viale Fanin 44, 40127 Bologna, Italy

Received 24 December 2008; Revised 22 January 2009; Accepted 26 January 2009

Abstract

This study focused on the internal conductance (g_i) along the plant profile of Ethiopian mustard under two light conditions: (i) light from the top only (I1); (ii) light from the top integrated by supplementary lateral light along the whole plant profile (I2). Lateral light strongly increased the productivity (e.g. +104% of seed oil) and net photosynthesis (A). The latter appeared more driven by g_i (r=0.78) than by stomatal conductance (g_s) (r=0.51*). Importantly, irradiance also considerably shortened the time from leaf appearance to senescence, which means that corresponding leaves in I1 and I2 had different ages. Therefore, since leaf age and irradiance have counteracting effects on g_i, I1 sometimes showed higher g_i values than I2. With respect to irradiance, leaf age had clearly higher effects on g_i, which radically declined from the top to the basal leaves, even under constant light conditions. The internal conductance caused a significant drawdown of CO_2 from the sub-stomatal cavity (C_i) to the site of carboxylation (C_c) that, in turn, led to a substantial underestimation of V_{cmax} calculated using the A/C_i model. Again, the trends of g_i and g_s were not consistent along the plant profile, and so the ratio between stomatal and internal limitations to A changed from top to bottom leaves, accordingly. This study suggests that g_i may be a valuable trait for increasing photosynthetic capacity and productivity; nonetheless, it suggests caution in selecting leaves for high g_i, as the latter can considerably change along the plant profile due to leaf age and irradiance effects.**

Key words: Internal conductance, irradiance, leaf age, light, mesophyll conductance, photosynthesis, plant profile, stomatal conductance.

Introduction

Ethiopian (E.) mustard (*Brassica carinata*) is an interesting C_3 oil crop evolved through the inter-specific hybridization between *B. nigra* and *B. oleracea* (Hemingway, 1995). Because of the high erucic acid content of its seeds, it has recently attracted a growing interest in a number of non-food applications such as biodiesel, bio-polymers, lubricants, soaps, and surfactants (Becker, 1999). In addition, the residual defatted meal has some precursors of biologically active compounds (glucosinolate) which could be used as soil amendments for plant defence (FAIR-CT96-1946 Project report; Anon., 2000). Due to its higher tolerance of drought (Cardone *et al.*, 2003) and the lower tendency to pod-shattering, E. mustard could be very competitive against rapeseed under unfavourable environmental conditions. Furthermore, it produces a higher biomass than rapeseed that could eventually be processed into electricity and/or heat.

Studying internal conductance (g_i) is of enormous importance in crops like E. mustard which are envisaged to be grown in environments where high water use efficiency is imperative. There is, in fact, evidence that increasing g_i may improve photosynthesis and water use efficiency (Warren and Adams, 2006), while the increase in stomatal conductance (g_s) generally involves significant transpiration costs that crops might be unable to support. This was emphasized in a number of studies (Evans and Vellen, 1996; Lauteri

* To whom correspondence should be addressed. E-mail: a.monti@unibo.it

et al., 1997; Flexas *et al.*, 2008) showing consistent and positive correspondence between water use efficiency and the g_i/g_s ratio.

There is increasing evidence that g_i can significantly decrease the CO_2 concentration at the Rubisco site (for a review, see Ethier and Livingston, 2004) to an extent depending on water shortage, salinity, leaf age, mineral nutrition, etc. (Bernacchi *et al.*, 2002; Long and Bernacchi, 2003). The irradiance can significantly modify the leaf anatomy and mesophyll structure (Nobel, 1991; Parkhurst, 1994) and thus it can be envisaged to affect also g_i. Nonetheless, as far as is known (for a review, see Flexas *et al.*, 2008), only eight studies out of over 130 articles on g_i documented the relationships between irradiance and g_i (Lloyd *et al.*, 1992; Hanba *et al.*, 2002; Piel *et al.*, 2002; Gorton *et al.*, 2003; Warren *et al.*, 2003, 2007; Laisk *et al.*, 2005), and, of these, only three involved herbaceous crops (Laisk *et al.*, 2005; Flexas *et al.*, 2007*a*; Evans *et al.*, 2008). Moreover, most of these experiments used natural light gradients or examined instantaneous responses to irradiance (Evans *et al.*, 2008). By contrast, the present study is a manipulative mid-term acclimation experiment, which should provide greater power to disentangle the various determinants of g_i. Specifically, the experiment focused on the influence of irradiance upon g_i along the plant profile.

Materials and methods

Site description

In 2007, 24 plants of E. mustard, variety CT 204, were grown in 0.21-m-diameter plastic pots (two plants per 6.6 l pot) in a 2×2.5 m (2 m height) controlled-environment room where relative humidity, temperature, and photoperiod could be regulated and monitored. Light was provided by six groups of 12 independently controllable neon tubes (Philips Master TL-D 58W/840). Pots were weighed twice a week and watered immediately after, thus maintaining the soil moisture constantly close to the field water capacity, the latter being determined gravimetrically. The soil substrate was a mixture (2:1, v/v) of sand and neutral peat (46% C; 0.8% N; pH 6). The Ruakura solution, a nutrient solution especially designed for growing plants in sandy soils (Smith *et al.*, 1983), was applied at a dose of 150 ml kg^{-1} of soil once a month or once a week during wintertime and after the spring regrowth, respectively.

At the start of light treatment, all plants had at least 10 visible pods and 80% of open pollinated flowers. Plants were randomly disposed in two separate rows. The distance between two succeeding plants was maintained close to that in the field (about 4 cm). Daylength was 16 h, while light/dark temperatures (°C) and relative humidity (RH%) were 25/15 and 50/80, respectively. The environmental parameters inside the room were constantly monitored by a micrometeorological station (μMetos, Pessl Instr., Weiz, Austria) and, at the same time, the ambient CO_2 was measured (IRGA-WM-4 plus datalogger; PP-Systems, Hertfordshire, UK). This concentration was used to set the $[CO_2]$ in the air entering the cuvette during the gas-exchange measurements.

The growth room was modified in order to provide, from one side, light from the top only (I1), and from the other, light from two sides, i.e. light from the top plus supplementary lateral light through horizontal neon tubes (I2). A plastic wall permeable to air flux was placed between the two treatments in order to protect I1 from the lateral light of I2. For each treatment, photosynthetic photon flux density (PPFD) was measured vertically along the plant profile, and horizontally throughout the plant, by a 0.75-m-long portable light sensor system with 30 PAR sensors covered by a diffuser (EMS 7/L Canopy Analyser; S.W & W.S. Burrage, Kent, UK) (Fig. 1).

Growth and production

Phenological stages were determined every other week on four randomly selected plants in each treatment. To distinguish different growth stages, the CETIOM phenological scale (CETIOM, 2003) was used, which is an alphanumeric code tailored for rapeseed, representing seven main growing stages and a number of sub-stages classifying the plant growth from emergence to seed physiological maturity. In addition, plant height and the number of leaves, siliqua, and seeds, as well as their areas, were measured using allometric models specially created for this experiment (data not shown). Briefly, the allometric models for leaves and siliqua were obtained from the linear relationship between their actual areas as measured by an area meter (Li-3000; LI-COR, Nebraska, USA), and the estimated areas, which were calculated from the rectangles subtending

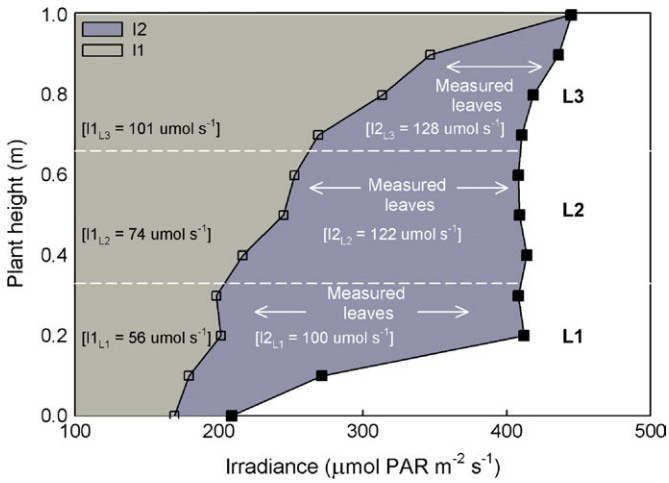

Fig. 1. Light extinction along the plant profile under higher (I2) and lower (I1) light conditions. Light intensity was maintained fairly constant in I2 plants by a supplementary lateral light. The arrows indicate the exact position of leaves taken for measurements. Numbers between brackets indicate the cumulative intercepted PAR (photosynthetically active radiation) within each layer (L1–L3 delimited by horizontal broken lines) of I1 and I2 plants. A trapezoidal rule was used to integrate PAR values over the plant profile.

them. The seed area was calculated assuming the seed shape is a sphere.

In all plants, three arbitrary and equally dimensioned (0.3 m height) plant layers (L1–L3) were characterized on the base of their productivity parameters (biomass, siliqua, seed, and oil production) and physiological traits. The seed oil was analysed according to the Soxhlet's method (Soxhlet, 1879). To extract oil completely, 0.5 g seed flour was mixed with Na-sulphate anhydrous and quartz sand then treated in the Soxhlet device for 6 h in a solution of 200 ml *n*-hexane. Because of the low seed production in each single layer, the amount of flour was slightly lower than indicated in the official Soxhlet method. For this reason, the Soxhlet device was first subjected to a test run in order to correct systematic errors due to the small samples.

Physiological parameters

Leaf gas-exchange measurements were performed by a portable open-path system (CIRAS-2; PP-Systems). During measurements the air flow entering the chamber was 250 cm^3 min^{-1}, RH ~60%, leaf temperature 24±0.5 °C (mean ±SD), the latter calculated using the energy balance method. Leaf-to-air vapour pressure deficit was between 0.8 kPa and 1.0 kPa.

In three layers (L1–L3) of three labelled plants per treatment (n=3), the two main parameters representing photosynthetic capacity, i.e. the maximum carboxylation rate (V_{cmax}) and the electron transport rate (J_{max}), were determined at 7 and 21 d after the start of treatment (DAT) (36 A/C_i curves in total) at a constant PPFD of 1500 µmol photons m^{-2} s^{-1} provided by an LED light unit above the cuvette. V_{cmax} and J_{max} were calculated using the biochemical model proposed by Farquhar *et al.* (1980), which represents the mechanistic relationship between net photosynthesis (A) and the intercellular CO_2 concentration (C_i). Briefly, the model relies on the concept that A is co-limited by three processes: (i) the activity and kinetics of Rubisco (W_c); (ii) the regeneration of ribulose-1,5-bisphosphate (RuBP) that is supported by the electron transport chain (W_j); (iii) the availability of triose phosphates for the Calvin cycle (W_p):

$$A=[1-(\Gamma^*/C_i)]\min(W_c,W_j,W_p)-R_d$$

where Γ* is the CO_2 compensation point in the absence of dark respiration; R_d is the day respiration, i.e. the mitochondrial respiration during illumination. In this experiment, the apparent Γ* (Γ_a*) and R_d were determined in the three plant layers (L1–L3) of the I1 plants using the method described by Laisk (1977), i.e. the determination of the intercept of four A/C_i curves generated at different low PPFDs (from 100 to 500 µmol photons m^{-2} s^{-1}) for C_i values below 15 Pa. To calculate the true Γ*, Γ_a* was corrected according to von Caemmerer and Evans (1991) as: $\Gamma^* = \Gamma_a^* + (R_d/g_i)$; where g_i is the internal conductance. Since Γ* and R_d were not significantly different along the plant profile, their average values were taken for the subsequent calculations: Γ* and R_d were 35.8±3.2 µmol CO_2 mol^{-1} and 0.80±0.11 µmol CO_2 m^{-2} s^{-1} (mean ±SD), respectively. These values were also used for I2 as previous studies showed that both Γ* and Γ_a* were not significantly affected by irradiance level (Piel *et al.*, 2002; Warren *et al.*, 2007).

Each point of the A/C_i curve took about 4 min. Measurements started at C_a, the [CO_2] in the incoming air, of about 400 µmol mol^{-1}. First, C_a was progressively reduced to 200 and 100 µmol mol^{-1} and then gradually increased up to about 2000 µmol mol^{-1} across the series of 400, 500, 600, 800, 1000, and 1600 µmol mol^{-1} using a CO_2 mass flow controller (generally 10 data points for each A/C_i curve). Each data point was recorded after equilibration to a steady state (CV <2% was considered tolerable). The software Photosyn Assistant (Dandee Sci., Scotland, UK) was used to fit the model and calculate the photosynthetic parameters. Before running the model, it was assumed that the threshold between W_c and W_j was 20–25 Pa (Wullschleger, 1993; Manter and Kerrigan, 2004).

Finally, chlorophyll index (SPAD-502 chlorophyll meter; Minolta), which is widely known to be related to leaf nitrogen content (Debaeke *et al.*, 2006), was measured in the leaves used for gas-exchange analysis.

Soluble sugars extraction, photosynthetic pigments, and carbon isotope composition

Two leaf discs (15 mm diameter) were collected about 4 h after each A/C_i curve: one disc was used to determine the relative water content, i.e. the ratio of tissue fresh weight minus dry weight to tissue turgid weight minus dry weight, the specific leaf area (SLA, leaf area/dry mass) and photosynthetic pigments [chlorophylls *a* and *b* (Chl*a* and *b*) and carotenoids (Car)]; the other disc was used for the extraction of leaf soluble sugars and for the determination of the carbon isotope composition ($\delta^{13}C$). Briefly, the soluble sugars were first dried at 40 °C in a ventilated stove, then re-diluted with 8 ml water, and finally centrifuged for 20 min at 3000 *g*. The solution was sequentially filtered with two ionic-exchange resins, DOWEX-50 (H^+) and DOWEX-1 (Cl^-), in order to separate amino acids and organic acids from soluble sugars (Brugnoli *et al.*, 1988). Successively, 2 ml sugar solution was treated with a colouring DNS solution (3.5-dinitrosalicylic acid) in order to quantify the extracted sugars through the absorbance at λ 530 nm (Summer, 1921). The rest of the solution was maintained at –80 °C and then analysed for $\delta^{13}C$. The latter was determined through the combustion of 1 g sample in an elemental analyser (model NA 1500; Carlo Erba, Milan, Italy), coupled to a dual-inlet mass spectrometer (model SIRA II; GV-Instruments, Middlewich, UK). $\delta^{13}C$ of the sample was compared with that of a working standard CO_2 calibrated against the international standard Vienna-Pee Dee Belemnite. The possible fractionation during combustion was controlled by testing a standard sucrose (Sigma Chemicals, USA) with a known $\delta^{13}C$ (–25.09±0.06‰). The carbon isotope discrimination (Δ‰) was then calculated as:

$$\Delta(‰)=(\delta_a-\delta_p)/(1+\delta_p)$$

where δ_a and δ_p are the carbon isotope composition of source air and plant material, respectively, relative to Vienna-Pee Dee Belemnite.

Chl*a* and Chl*b*, and Car were extracted by 10 ml ethanol mix (95%, v/v) into a cold shaded mortar. The extract was centrifuged at 3000 *g* for 10 min, then 1 ml of supernatant was diluted in 4 ml of ethanol 95% (v/v), and finally, the absorbance was determined at λ of 750, 665, 649, and 470 nm through a spectrophotometer (Perkin-Elmer Lambda 1 UV/VIS) (Aranjuelo *et al.*, 2007). Lichtenthaler's equation (Lichtenthaler, 1987) was used to calculate Chl*a*, Chl*b*, and Car.

Internal conductance and C_c

As given by Brugnoli *et al.* (1988) and further developed by Lauteri *et al.* (1997), g_i was calculated through the discrepancy between Δ determined in soluble sugars (Δ_s) and the expected Δ (Δ_i) calculated using the Farquhar model (Farquhar *et al.*, 1982):

$$g_i=[(b-e_s-a_1)(A/C_a)]/[(\Delta_i-\Delta_s)-(f\Gamma^*)/C_a)]$$

where, *b* is the discrimination associated with carboxylation reactions; e_s, the fractionation occurring during the dissolution of CO_2; a_1, the discrimination caused by the diffusion of CO_2 in the liquid phase; *f*, the discrimination during photorespiration. Details on values and references were reported elsewhere (Monti *et al.*, 2006). The concentration of CO_2 at the site of carboxylation (C_c) was then obtained as $C_c=C_i-A/g_i$. Once C_c was calculated, V_{cmax} and J_{cmax} were recalculated using the A/C_c model (V_{cmax_Cc}; J_{cmax_Cc}).

Stomatal and internal limitations to photosynthesis were calculated as proposed by Jones (1985). In short, the drawdown of CO_2 from the leaf surface to the site of carboxylation is a function of stomatal and internal conductance, i.e.

$$C_c=C_a-(A/g_s)-(A/g_i)$$

Assuming that mitochondrial respiration is not significantly changing during the experimental period, the relationship between *A* and V_{cmax} can be expressed (Farquhar *et al.*, 1980) as:

$$\delta A/\delta A_{max}=A/V_{cmax}$$

where A_{max} is the light-saturated net photosynthesis. As δC_c can also be expressed as:

$$\delta C_c=[(A/g_s)(\delta g_s/g_s)]+[(A/g_i)(\delta g_i/g_i)]-[(1/g_s)+(1/g_i)\times\delta A]$$

Combining the last three equations gives the following:

$$(\delta A/A)=[l_s(\delta g_s/g_s)+l_m(A/g_i)+l_b(\delta/V_{cmax}/V_{cmax})]$$

Therefore, the stomatal (l_s) and internal (l_i) limitations can be calculated as:

$$l_s=[(g_{tot}/g_s)(\delta A/\delta/C_c)]/[g_{tot}(\delta A/\delta/C_c)];$$
$$l_i=[(g_{tot}/g_i)(\delta A/\delta/C_c)]/[g_{tot}(\delta A/\delta/C_c)]$$

where g_{tot} is the total conductance to CO_2 ($1/g_{tot}=1/g_s+1/g_i$).

Statistical analysis

All data were subjected to statistical analysis according to the general linear model for repeated measurements performed by SYSTAT 10.2 (Systat Software, Inc., Chicago, IL, USA). The Bartlett's test ($P \leq 0.05$) was used to test the homogeneity of the variance. The LSD Fisher's test ($P \leq 0.05$) was applied to separate the significantly different means. The statistical significance of the correlation coefficients was tested according to Pearson's correlation test ($P \leq 0.05$). To solve the area under a curve (see Fig. 1), the trapezoidal rule was used.

Results

Light effect on growth and production

Increasing the light distribution along the plant profile visibly modified the cycle length and productivity: I2 took 14 d less than I1 to complete the cycle (Fig. 2) and it produced 47%, 140%, and 61% more biomass, siliqua, and seeds, respectively (Fig. 3). In addition, I2 had a higher oil content (%) than I1, with a consequent double oil yield (Fig. 3). Similarly, the size of siliqua and seed was significantly affected by the irradiance: the total siliqua and seed areas were 71 and 31 cm^2 higher in I2 than I1. The leaf area (LA) and SLA were conversely not influenced by the irradiance decreasing rapidly with seed maturation. Nonetheless, LA greatly changed along the plant profile: in L1, it was 2- and 5-fold as high as in L2 and L3, respectively. SLA was lower in L3 (25 $m^2\ kg^{-1}$) and similar in L1 and L2 (37 $m^2\ kg^{-1}$,

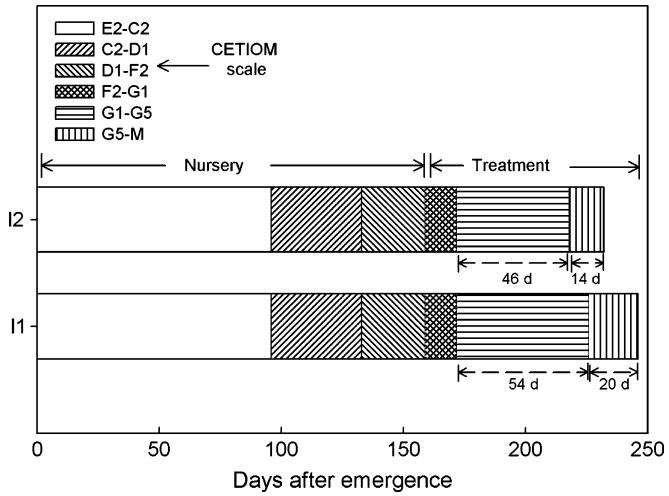

Fig. 2. Length of the different phenological stages (CETIOM scale) under the two light treatments. The higher irradiance (I2) clearly reduced the total cycle length.

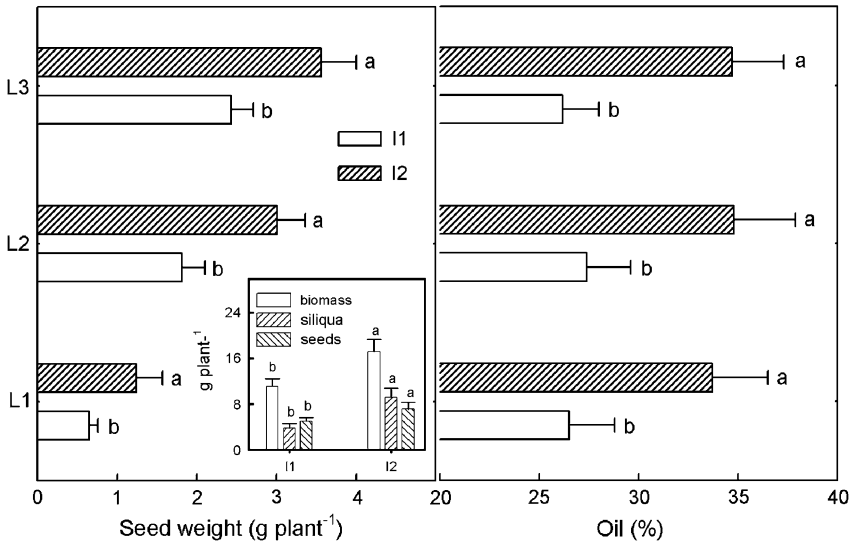

Fig. 3. Productivity and seed oil content in the three plant layers (L1–L3). The inset figure shows the average biomass and siliqua and seed yields under high (I2) and low (I1) irradiance conditions. Different letters indicate significantly different means (LSD Fisher's test for $P \leq 0.05$, $n=12$) within each layer.

on average). The relative water content averaged 0.85±0.09 and was not influenced by irradiance or by leaf position along the plant profile. The oil content (%) was also modified by light intensity: 25% higher oil content was found in I2 compared with I1 (Fig. 3).

Light effects on g_i

Since the light regime substantially modified the cycle length, the corresponding leaves in I1 and I2 had different ages. Therefore, the differences in g_i between I1 and I2, within each layer, probably reflected cumulative effects of leaf age and irradiance. Nonetheless, a reliable estimate of the leaf age effects on g_i could be obtained in I2, since these plants maintained a constant light level along the profile (Fig. 1). Similarly, the effects of the leaf age on g_i were quantified through the comparison of the same leaf at 7 and 21 DAT.

The internal conductance drastically declined from top to basal leaves irrespective of irradiance level (Fig. 4). Therefore, leaf age effects are likely to have prevailed over irradiance effects in changing g_i. Nevertheless, the influence of irradiance upon g_i was not negligible, as testified by the significant interaction between irradiance and plant layer. The reason behind this interaction was that g_i was significantly higher in L3 of I2 with respect to I1, while the opposite occurred in L1. By contrast, there were no significant differences in g_i between I1 and I2, in L2 (Fig. 4). The interaction between irradiance and plant layer was also significant 14 d later when, however, it was no longer possible to measure L1 due to the advanced senescence of the basal leaves. Besides, in L2, g_i was higher in I1, while in L3 it not longer differed between I1 and I2 (Fig. 4).

Photosynthesis and g_i

Internal conductance had a significant impact on A, which appeared more driven by g_i than by g_s (Fig. 5). Overall, g_i

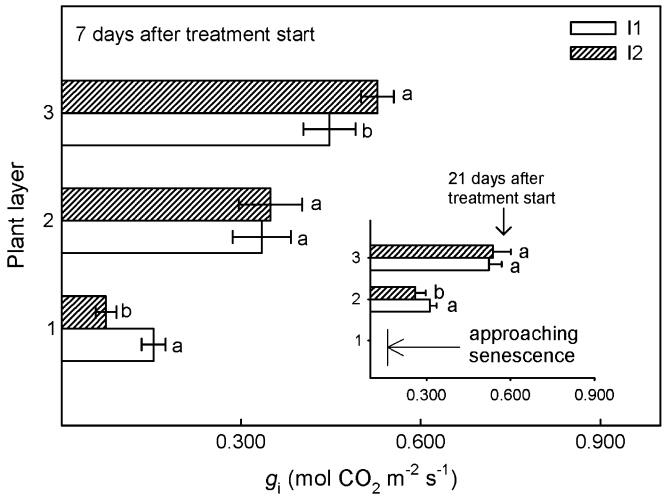

Fig. 4. Internal conductance (g_i) measured in the three leaf layers (L1–L3) at 7 and 21 d (inset figure) after the start of treatment. On DAT 21 it was no longer possible to determine g_i in L1 leaves because of advanced leaf senescence. Different letters indicate significantly different means (LSD Fisher's test for $P \leq 0.05$; n=3).

was fairly consistent with A, being higher in L3 and then gradually decreasing downward (Table 1). Moreover, since A declined downward more rapidly in I2 than in I1, the leaf-age effect on A was likely to be more prevalent than that of irradiance. A result which is consistent with the effects of leaf age on g_i; however, unlike for g_i, I2 showed higher A values than I1 also in L2 (Table 1).

The leaf Chl and Car contents were also rather consistent with A. Specifically, Chl*a*, Chl*b*, and Car did not change between L3 and L2, while it declined drastically in L1 (Table 2). Conversely, no significant effects of the irradiance were observed on Chl*a*, Chl*b*, and Car. Similarly, SPAD values were only slightly influenced by the irradiance; however, unlike photosynthetic pigments, SPAD values

radically decreased downward, thus revealing a likely decline in leaf N-content parallel to leaf ageing (Table 2).

Stomatal conductance (g_s) was weakly influenced by the irradiance, and it appeared significantly related to g_i (Fig. 5). Nonetheless, unlike g_i, g_s remained rather constant between L3 and L2 in I2. More than that, g_i decreased more markedly than g_s from the apex to the basal leaves, with the result that the ratio between g_i and g_s significantly changed with the position of the leaf and, to a lesser extent, with the irradiance (Fig. 6). Specifically, in L3, g_i was about three times larger than g_s both in I1 and I2, while in L2 g_i was much larger than g_s only in I2. In the basal leaves, g_i and g_s were nearly equivalent (Fig. 6). The diffusive limitations to A through stomata (l_s) averaged 21% and they were significantly higher than internal limitation (l_m) (Fig. 6). Overall, l_s values were higher in the upper layer and then decreased downward, contrary to that observed for l_m. The latter, however, was not negligible, ranging from 9% to 15% in L3 and L1, respectively. The irradiance effects on diffusive limitations to CO_2 were generally negligible; the only significant effect was in L2 where l_m was significantly higher in I2 than I1, both on DAT 7 and DAT 21 (Fig. 6).

The drawdown of CO_2 from the atmosphere to the carboxylation site was significantly influenced by g_i. C_c resulted in being 7–9% lower than C_i in L2 and L3, and 12–14% lower in L1 (Table 1). This led to a significant underestimation of V_{cmax} as calculated by the conventional A/C_i model, and, to a lesser extent, of J_{max} as well (Table 1). Importantly, the correlation between V_{cmax} and J_{max} significantly improved (r=0.90**) once the two parameters were recalculated using the A/C_c model.

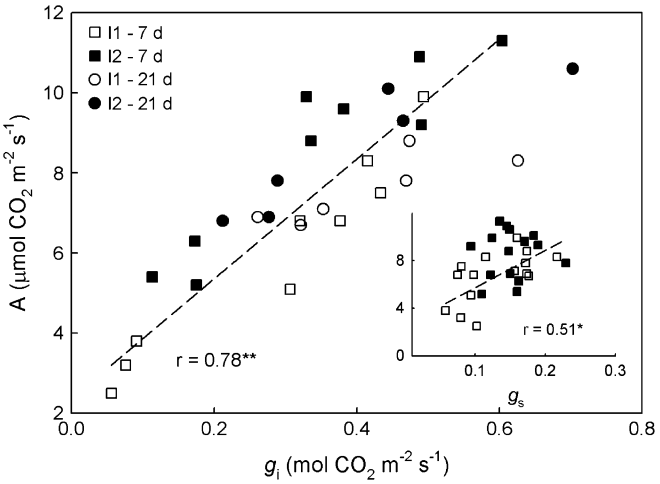

Fig. 5. Relationship between net assimilation rate (A) and stomatal (g_s, inset figure) or internal (g_i) conductance. r, Correlation coefficient; * and **, statistical significance of r for $P \leqslant 0.05$ and $\leqslant 0.01$, respectively (Pearson's correlation test). Each point represents a single leaf.

Discussion

This study revealed that light distribution along the plant profile can enormously affect biomass, seed and oil yields, and, to a lesser extent, the fatty acid composition. Therefore, plant density and its architecture should be taken into account by agronomists and breeders in establishing the optimal growing conditions towards maximizing the productivity of this crop.

A bottom-up comparison of leaves under two light conditions was the original goal of this study. Nonetheless, irradiance had an unexpected strong influence upon the plant growth that caused an early maturation of I2 plants. Consequently, the difference between corresponding leaves of I1 and I2 were probably the consequence of the cumulative effects of the irradiance and leaf age. Anyway, since I2 plants were subjected to constant light intensities along the whole profile, the ageing effects on g_i could be reasonably estimated along the profile of I2 plants or, possibly, by measuring the same leaf at 7 d and 21 d after the treatment started.

Generally, g_i increases until the leaf is fully expanded and then decreases parallel to the leaf senescing (Hanba *et al.*, 2002; Ethier *et al.*, 2006). Similarly, the present results show

Table 1. Net assimilation rate (A), CO_2 concentration at the substomatal cavity (C_i) and at the chloroplasts (C_c,) and maximum carboxylation (V_{cmax_Cc}), and electron transport (J_{max_Cc}) rates

The two latter parameters were calculated on C_c basis (A/C_c curve). The underestimation (%) of V_{cmax} and J_{max} if calculated using the A/C_i curve is given in parenthesis. Different letters indicate vertical significant differences ($P \leqslant 0.05$, LSD Fishers's test) within each DAT (days after the start of treatment; n=3).

DAT	Light	Layer	A		C_i		C_c		V_{cmax_Cc}		J_{max_Cc}		V_{cmax}/J_{max}	
7	I1	L3	8.6	b	319	a	285	ab	108 (22%)	a	222 (7%)	a	1.5	b
		L2	6.2	c	328	a	289	ab	93 (19%)	b	166 (4%)	b	1.8	ab
		L1	5.6	c	316	a	302	a	70 (41%)	c	108 (9%)	c	2.3	a
	I2	L3	10.5	a	320	a	274	b	113 (21%)	a	224 (4%)	a	1.9	ab
		L2	8.7	b	327	a	287	ab	97 (22%)	b	178 (6%)	b	1.9	ab
		L1	3.2	d	327	a	293	a	65 (35%)	c	116 (9%)	c	2.2	a
21	I1	L3	8.3	b	335	a	319	a	91 (23%)	a	196 (5%)	a	2.2	a
		L2	6.9	c	339	a	316	a	85 (28%)	a	173 (6%)	b	2.0	a
	I2	L3	10.0	a	321	a	302	a	93 (26%)	a	182 (4%)	ab	2.0	a
		L2	7.1	c	334	a	306	a	69 (31%)	b	129 (7%)	c	1.9	a

Table 2. Chlorophyll (Chl*a* and *b*), carotenoid (Car) contents, and SPAD values determined at 7 d and 21 d after the start of treatment (DAT)

L1, L2, and L3 indicate the plant layers from bottom to top, respectively; I1 and I2, less and more illuminated plants, respectively. Different letters indicate vertical significant differences ($P \leq 0.05$, LSD Fishers's test; $n=3$) within each DAT.

DAT	Light	Layer	Chl*a* (mg g^{-1})		Chl*b* (mg g^{-1})		Chl*a*/*b* (mg g^{-1})		Car (mg g^{-1})		SPAD (adim.)	
7	I1	L3	9.9	a	3.1	a	3.2	a	2.6	a	58	a
		L2	9.2	a	3.0	a	3.2	a	3.0	a	43	b
		L1	6.8	b	2.0	b	3.4	a	1.9	b	32	c
	I2	L3	10.3	a	3.3	a	3.3	a	2.7	a	62	a
		L2	10.0	a	3.1	a	3.2	a	2.6	a	46	b
		L1	4.0	c	1.4	c	3.4	a	2.1	b	39	c
21	I1	L3	10.5	a	3.8	a	2.7	b	2.7	a	56	a
		L2	10.1	a	3.4	a	3.0	b	2.7	a	45	b
	I2	L3	11.2	a	3.7	a	3.0	b	2.6	a	59	a
		L2	8.7	b	2.4	b	3.6	a	1.9	b	40	b

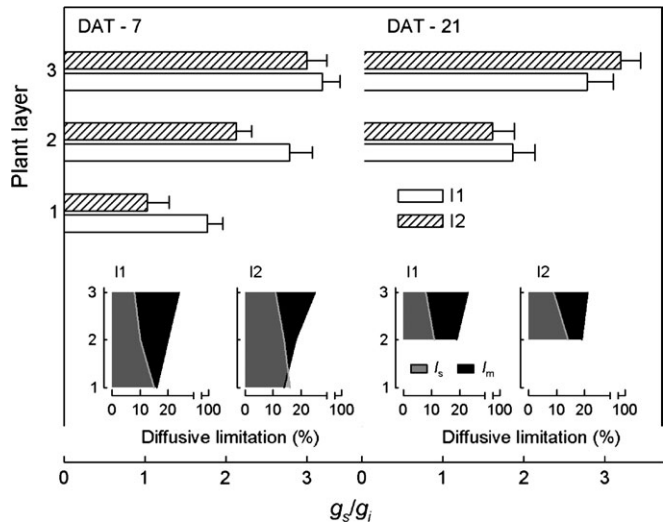

Fig. 6. Ratios (horizontal bars) between stomatal (g_s) and internal (g_i) conductance in the three plant layers (L1–L3, bottom-up) under high (I2) and low (I1) light conditions on 7 d and 21 d after the start of treatment (DAT). The four inset figures represent the stomatal (l_s, black) and internal (l_m, grey) limitations to photosynthesis (A) along the plant profile (L1–3).

that under I2 conditions, g_i strongly decreased (from 0.527 to 0.074 mol CO_2 m^{-2} s^{-1} in L3 and L1, respectively), thus supporting the evidence of likely important effects of leaf age on g_i (Warren, 2008*a*, and references therein). Moreover, since the downward decrease in g_i occurred to a similar extent in I1 and I2, leaf age clearly seemed to have more prevalent effects on g_i than irradiance. Nevertheless, the effect of the irradiance on g_i should not be ignored since in L3, where the leaves were young and likely to be more active, g_i changed significantly between I1 and I2, and this caused a diverse overestimation of the CO_2 concentration at the carboxylation site between I1 and I2. Similar to g_i, A declined from top to bottom leaves; however, unlike g_i, I2 showed higher A values than I1 also in L2, and therefore, irradiance can be supposed to have a higher influence on g_i than A.

In spite of a considerable change in g_i and A, C_i and C_c remained fairly constant along the plant profile (C_i/C_c ratio of 1.08±0.14 SD), irrespective of the light treatment. It derives that C_c was never significantly related to A; when A changed, g_i and g_s scaled accordingly and so C_c remained constant. A fact that would indicate, presumably, a feedforward mechanism on the diffusive stomatal limitation to CO_2 through the internal CO_2 concentration. The drawdown from C_i to C_c (on average 30 μmol mol^{-1}) was pretty low if compared with the average value of 226 species calculated by Warren (2008*a*). Nonetheless, it was not negligible as it determined a substantial underestimation of V_{cmax} and, to a lesser extent, of J_{max} as calculated by the conventional A/C_i model. Specifically, V_{cmax} and J_{max} calculated on a C_c basis were on average 1.24 (r=0.91**) and 1.08 (r=0.67**) higher than V_{cmax} and J_{max} calculated on the C_i basis, respectively. These two coefficients were slightly lower (V_{cmax}) than or very similar (J_{max}) to those calculated (1.62 and 1.08, respectively) in a recent review (Warren, 2008*a*). Moreover, the correlation between V_{cmax} and J_{max} increased when the A/C_c model was used instead of the A/C_i model. As also ascertained in other herbaceous crops (Monti *et al.*, 2006), not g_i taking into account may lead to the decrease in A to be attributed to biochemical constraints, while diffusive limitations could be the major cause.

The sum of stomatal and non-stomatal limitations was about 30% with little variation along the plant profile. Nevertheless, the diffusive limitations to CO_2 were modified by the significant interaction between leaf age and irradiance. As a result, the relative importance of stomatal and non-stomatal limitations changed accordingly. Specifically, in the two upper layers, the diffusive limitations to CO_2 in mesophyll were about half those in stomata, while in the bottom leaves (L1) they were nearly equal or even higher in the mesophyll (Fig. 6).

Contrasting effects occurred on g_i along the plant profile because g_i is known to decrease with leaf ageing (Bernacchi *et al.*, 2005; Grassi and Magnani, 2005; Flexas *et al.*, 2007*b*), while it increases with irradiance (Terashima *et al.*,

2006). Since the leaves aged faster in I2 than in I1, it was not surprising that g_i was sometimes higher in I1, as the older I2 leaves were probably less capable of responding promptly to light. Therefore, on the basis of these results, three scenarios along the plant profile could be identified. (i) At the top (L3), the leaves were still young both in I1 and I2. In this layer, g_i was mostly influenced by light and, to a much lesser extent, by leaf age. Thus it follows that irradiance effects on g_i could be reliably estimated in this layer, since the counteracting effects of leaf age on g_i can be assumed to be negligible. In this layer, g_i and irradiance were found to be positively related in agreement with other studies (Boardman, 1977; Warren *et al.*, 2003; Evans *et al.*, 2008; Flexas *et al.*, 2008). (ii) In the intermediate layer (L2), I2 had significantly older leaves than I1. The negative age effects offset or prevailed over the positive effects of irradiance on g_i, with the result that 21 DAT the internal conductance was higher in I1. (iii) In the basal leaves (L1), the age effects were further evident both in I1 and I2.

There is evidence that g_i and g_s are positively related (Loreto *et al.*, 1992; Centritto *et al.*, 2003; Warren, 2008*b*). Nonetheless, Warren (2008*b*) demonstrated that this relationship is not ubiquitous as no relationship was found between g_s and g_i when g_s was modulated by the atmospheric water deficit. The present study, though it showed g_s and g_i being generally related ($r = 0.51$*), seems somehow to support the results of Warren (2008*b*) since g_s and g_i were sometimes independent in responding to irradiance. Under I2 conditions, g_s remained fairly stable from L3 to L2 to then decreased appreciably in L1; conversely, g_i declined by 25% from L3 to L2, and then it decreased drastically from L2 to L1 (from 0.335 to 0.074 mol m^{-2} s^{-1}). Consequently, the responsiveness of stomata and mesophyll to light changed along the plant profile and so the g_i/g_s ratio changed accordingly. The reason behind the different responses of g_s and g_i to light was not clear. Whether or not the mesophyll can drive g_s is still an open matter. Recent studies suggest that stomatal response to light is mostly driven by a signal generated by the mesophyll (Mott *et al.*, 2008). Nonetheless, stomata also responded to red-light, probably through a mechanism regulated by the chloroplasts of the guard cells (Tominaga *et al.*, 2001). As a result, mesophyll should have little or no effect on g_s. Furthermore, Messinger *et al.* (2006) showed that stomatal response to red-light was evident even when C_i was maintained constant through regulating the ambient CO_2. Of course, the fact remains that other mechanisms might influence the relationship between stomata and mesophyll. Therefore, since the light source used in the present experiment (neon tubes) is characterized by a prevailing far-red-to-orange light spectrum, an hypothesis could be that this specific light spectrum, or the still uncertain responsiveness to blue-light of the mesophyll and stomata (Marten *et al.*, 2007; Loreto *et al.*, 2008), was the cause of the differing behaviour of stomata and mesophyll, as guard cells could be more sensitive to far-red light than mesophyll receptors.

In summary, four main findings could be underlined: (i) productivity was strongly increased by the lateral light through the rise in photosynthetic rates of basal and intermediate leaves as well as other green organs (Ruuska *et al.*, 2004); (ii) irrespective of irradiance level, g_i declined drastically from the top to the bottom leaves thus suggesting a likely prevalent effect of leaf age compared with irradiance—for this reason, the positive effects of irradiance on g_i are visible only in the top leaves; (iii) once again, g_i led to significant underestimation of V_{cmax} which was higher related to J_{max} once it was recalculated using the A/C_c curve; (iv) g_i and g_s were significantly related and they scaled with the photosynthetic capacity, but the ratio between stomatal and non-stomatal limitations was not constant along the profile.

References

Anon. 2000. FAIR-CT96–1946 Project. *Brassica carinata* the outset of a new crop for biomass and industrial non-food oil. EC Final Report.

Aranjuelo I, Irigoyen JJ, Sanchez-Diaz M. 2007. Effect of elevated temperature and water availability on CO_2 exchange and nitrogen fixation of nodulated alfalfa plants. *Environmental and Experimental Botany* **59,** 99–108.

Becker HC, Löptien H, Röbbelen G. 1999. Breeding: an overview. In: Gómez-Campo C, ed. *Biology of* Brassica *cenospecies*. Amsterdam: Elsevier Science BV, 413–460.

Bernacchi CJ, Morgan PB, Ort DR, Long SP. 2005. The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing *in vivo* Rubisco capacity. *Planta* **220,** 434–446.

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.

Boardman NK. 1977. Comparative photosynthesis of sun and shade plants. *Annual Review of Plant Physiology* **28,** 355–377.

Brugnoli E, Hubick TH, von Caemmerer S, Wong SC, Farquhar GD. 1988. Correlation between the carbon isotope discrimination in leaf starch and sugars of C_3 plants and the ratio of intercellular and atmospheric partial pressure of carbon dioxide. *Plant Physiology* **88,** 1418–1424.

Cardone M, Mazzoncini M, Menini S, *et al.* 2003. *Brassica carinata* as an alternative oil crop for the production of biodiesel in Italy: agronomic evaluation, fuel production by transesterification and characterization. *Biomass and Bioenergy* **25,** 623–636.

Centritto M, Loreto F, Chartzoulakis K. 2003. The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. *Plant, Cell & Environment* **26,** 585–594.

CETIOM. 2003. Stades repères du colza. Cetiom, Colza atouts rendez-vous. http://www.cetiom.fr/index.php?id=10408.

Debaeke P, Rouet P, Justes E. 2006. Relationship between the normalized SPAD index and the nitrogen nutrition index: application to durum wheat. *Journal of Plant Nutrition* **29,** 75–92.

Ethier GH, Livingston NJ. 2004. On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant, Cell & Environment* **27,** 137–153.

Ethier GJ, Livingston NJ, Harrison DL, Black TA, Moran JA. 2006. Low stomatal and internal conductance to CO_2 versus Rubisco deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. *Plant, Cell & Environment* **29,** 2168–2184.

Evans JR, Tazoe Y, Hassiotou F, von Caemmerer S. 2008. Does internal conductance vary with irradiance? In: Flexas J, ed. *ESF Exploratory Workshop on Mesophyll conductance to CO_2*. Spain: Universitat del les Illes Balears, 35–36.

Evans JR, Vellen L. 1996. Wheat cultivars differ in transpiration efficiency and CO_2 diffusion inside their leaves. In: Ishii R, Horie T, eds. *Crop research in Asia: achievements and perspective*. Tokyo: ACSA, 326–329.

Farquhar GD, O'Leary MH, Berry JA. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9,** 121–137.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149,** 78–90.

Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007*a*. Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell & Environment* **30,** 1284–1298.

Flexas J, Ortuño MF, Ribas-Carbó M, Díaz-Espejo A, Florez-Sarasa ID, Medrano H. 2007*b*. Mesophyll conductance to CO_2 in *Arabidopsis thaliana*. *New Phytologist* **175,** 501–511.

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H. 2008. Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell & Environment* **31,** 602–621.

Gorton HL, Herbert SK, Vogelmann TC. 2003. Photoacoustic analysis indicates that chloroplast movement does not alter liquid-phase CO_2 diffusion in leaves of *Alocasia brisbanensis*. *Plant Physiology* **132,** 1529–1539.

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 & Environment* **28,** 834–849.

Hanba YT, Kogami H, Terashima I. 2002. The effect of growth irradiance on leaf anatomy and photosynthesis in *Acer* species differing in light demand. *Plant, Cell & Environment* **25,** 1021–1030.

Hemingway JS. 1995. Mustards: *Brassica* spp. and *Sinapis alba* (Cruciferae). In: Smartt J, Simmonds NW, eds. *Evolution of crop plants*. Singapore: Longman Scientific and Technical, 82–88.

Jones HG. 1985. Partitioning stomatal and non-stomatal limitations to photosynthesis. *Plant, Cell & Environment* **8,** 95–104.

Laisk AK. 1977. *Kinetics of photosynthesis and photorespiration in C_3 plants*. Moscow: Nauka [in Russian].

Laisk A, Eichelmann H, Oja V, *et al*. 2005. Adjustment of leaf photosynthesis to shade in a natural canopy: rate parameters. *Plant, Cell & Environment* **28,** 375–388.

Lauteri M, Scartazza A, Guido MC, Brugnoli E. 1997. Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll conductance in provenances of *Castanea sativa* adapted to different environments. *Functional Ecology* **11,** 675–683.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: Douce R, Packer L, eds. *Methods in enzymology*. New York, NY: Academic Press, 350–382.

Lloyd J, Syversten JP, Kriedemann PE, Farquhar GD. 1992. Low conductance for CO_2 diffusion from stomata to the sites of carboxylation in leaves of woody species. *Plant, Cell & Environment* **15,** 873–899.

Long SP, Bernacchi CJ. 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany* **54,** 2393–2401.

Loreto F, Harley PC, Di Marco G, Sharkey TD. 1992. Estimation of mesophyll conductance to CO_2 flux by three different methods. *Plant Physiology* **98,** 1437–1443.

Loreto F, Tsonev T, Centritto M. 2008. The impact of blue-light on mesophyll conductance. In: Flexas J, ed. *ESF Exploratory Workshop on Mesophyll Conductance to CO2*. Spain: Universitat del les Illes Balears, 37–38.

Manter DK, Kerrigan J. 2004. A/C_i curve analysis across a range of woody plant species: influence of regression analysis parameters and mesophyll conductance. *Journal of Experimental Botany* **55,** 2581–2588.

Marten H, Hedrich R, Roelfsema MRG. 2007. Blue light inhibits guard cell plasma membrane anion channels in a phototropin-dependent manner. *The Plant Journal* **50,** 29–39.

Messinger S, Buckley TN, Mott KA. 2006. Evidence for involvement of photosynthetic processes in the stomatal response to CO_2. *Plant Physiology* **140,** 771–778.

Monti A, Brugnoli E, Scartazza A, Amaducci MT. 2006. The effect of transient and continuous drought on yield, photosynthesis and carbon isotope discrimination in sugar beet (*Beta vulgaris* L.). *Journal of Experimental Botany* **57,** 1253–1262.

Mott KA, Sibbernsen ED, Shope JC. 2008. The role of the mesophyll in stomatal responses to light and CO_2. *Plant Cell & Environment* **9,** 1299–1306.

Nobel PS. 1991. *Physicochemical and environmental plant physiology*. San Diego, CA: Academic Press.

Parkhurst DF. 1994. Diffusion of CO_2 and other gases inside leaves. *New Phytologist* **126,** 449–479.

Piel C, Frak E, Le Roux X, Genty B. 2002. Effect of local irradiance on CO_2 transfer conductance of mesophyll in walnut. *Journal of Experimental Botany* **53,** 2423–2430.

Ruuska SA, Schwender J, Ohlrogge JB. 2004. The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes. *Plant Physiology* **136,** 2700–2709.

Smith GS, Johnston CM, Conforth IS. 1983. Comparison of nutrient solutions for growth of plants in sand culture. *New Phytologist* **94,** 537–548.

Soxhlet F. 1879. Die gewichtsanalytische Bestimmung des Milchfettes. *Dingler's Polytechnisches Journal* **232,** 461.

Summer JB. 1921. Dinitro-salicylic acid, a regent for estimation of sugar in normal and diabetic urine. *Journal of Biological Chemistry* **47,** 5–7.

Terashima I, Hanba YT, Tazoe Y, Vyas P, Yano S. 2006. Irradiance and phenotype: comparative eco-development of sun and

shade leaves in relation to photosynthetic CO_2 diffusion. *Journal of Experimental Botany* **57,** 343–354.

Tominaga M, Kinoshita T, Shimazaki K. 2001. Guard-cell chloroplasts provide ATP required for H+ pumping in the plasma membrane and stomatal opening. *Plant and Cell Physiology* **42,** 795–802.

Von Caemmerer S, Evans JR. 1991. Determination of the average partial pressure of CO_2 in chloroplasts from leaves of several C_3 plants. *Australian Journal of Plant Physiology* **18,** 287–305.

Warren CR. 2008*a*. Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany* **59,** 1475–1487.

Warren CR. 2008*b*. Soil water deficits decrease the internal conductance to CO_2 transfer but atmospheric water deficits do not. *Journal of Experimental Botany* **59,** 327–334.

Warren CR, Adams MA. 2006. Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and nitrogen use in photosynthesis. *Plant, Cell & Environment* **29,** 192–201.

Warren CR, Löw M, Matyssek R, Tausz M. 2007. Internal conductance to CO_2 transfer of adult *Fagus sylvatica*: variation between sun and shade leaves and due to free-air ozone fumigation. *Environmental and Experimental Botany* **59,** 130–138.

Warren CR, Ethier GJ, Livingston NJ, Grant NJ, Turpin DH, Harrison DL, Black TA. 2003. Transfer conductance in second growth Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) canopies. *Plant, Cell & Environment* **26,** 1215–1227.

Wullschleger SD. 1993. Biochemical limitations to carbon assimilation in C_3 plants: a retrospective analysis of the A/C_i curves from 109 species. *Journal of Experimental Botany* **44,** 907–920.