

# Leaf structural responses to pre-industrial, current and elevated atmospheric [CO<sub>2</sub>] and temperature affect leaf function in *Eucalyptus sideroxylon*

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**Abstract.** Leaf structure and chemistry both play critical roles in regulating photosynthesis. Yet, a key unresolved issue in climate change research is the role of changes in leaf structure in photosynthetic responses to temperature and atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]), ranging from pre-industrial to future levels. We examined the interactive effects of [CO<sub>2</sub>] (290, 400 and 650  $\mu\text{L L}^{-1}$ ) and temperature (ambient, ambient +4°C) on leaf structural and chemical traits that regulate photosynthesis in *Eucalyptus sideroxylon* A.Cunn. ex Woolls. Rising [CO<sub>2</sub>] from pre-industrial to elevated levels increased light-saturated net photosynthetic rates ( $A_{\text{sat}}$ ), but reduced photosynthetic capacity ( $A_{\text{max}}$ ). Changes in leaf N per unit area ( $N_{\text{area}}$ ) and the number of palisade layers accounted for 56 and 14% of the variation in  $A_{\text{max}}$ , respectively, associated with changes in leaf mass per area. Elevated temperature increased stomatal frequency, but did not affect  $A_{\text{max}}$ . Further, rising [CO<sub>2</sub>] and temperature generally did not interactively affect leaf structure or function. These results suggest that leaf  $N_{\text{area}}$  and the number of palisade layers are the key chemical and structural factors regulating photosynthetic capacity of *E. sideroxylon* under rising [CO<sub>2</sub>], whereas the lack of photosynthetic responses to elevated temperature may reflect the limited effect of temperature on leaf structure and chemistry.

**Additional keywords:** leaf anatomy, leaf physiology, nitrogen, photosynthesis, pre-industrial [CO<sub>2</sub>], stomata, temperature.

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## Introduction

Rising atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]) have a significant effect on global climate by increasing temperature. Over the past 200 years, atmospheric [CO<sub>2</sub>] has risen from 280 to 390  $\mu\text{L L}^{-1}$  (Sage and Coleman 2001; Körner 2006). Models project [CO<sub>2</sub>] to reach 600  $\mu\text{L L}^{-1}$  within this century, accompanied by a 0.3–3.4°C rise in mean air temperature for Australia (Hennessy *et al.* 2007). Forests may play a key role in ameliorating rises in [CO<sub>2</sub>] and temperature because trees account for ~70% of terrestrial primary production (Field *et al.* 1998; Atwell *et al.* 2007) and sequester a sizeable fraction of CO<sub>2</sub> released to the atmosphere (Melillo *et al.* 1993; Schimel *et al.* 2001; Norby *et al.* 2005). Consequently, it is crucial that we understand how the capacity of trees to absorb CO<sub>2</sub> is influenced by a changing climate. Although tree responses to elevated [CO<sub>2</sub>] have been well documented (Saxe *et al.* 1998; Norby *et al.* 1999; Ainsworth and Long 2005), less is known about responses to past rises in [CO<sub>2</sub>] (Lewis *et al.* 2010) or the interactive effects of [CO<sub>2</sub>] and temperature (Ghannoum *et al.* 2010a).

A key unresolved issue in the response of carbon sequestration by trees to climate change is the role of leaf anatomy. Leaf structure is closely linked to the function and growth of the whole plant and is sensitive to environmental conditions such as temperature (Klich 2000; Niinemets *et al.* 2009). Growth [CO<sub>2</sub>] may also affect leaf structure, but few general trends have emerged. Leaf thickness has been reported to increase (Pritchard *et al.* 1999) and decrease (Rengifo *et al.* 2002) with rising [CO<sub>2</sub>]. Growth in elevated [CO<sub>2</sub>] is also reported to increase (Thomas and Harvey 1983) and decrease mesophyll cell layer thickness (Oksanen *et al.* 2005). The effect of elevated temperature similarly varies (Saxe *et al.* 2001) and elevated temperature sometimes enhances (Zha *et al.* 2001; Vu 2005) or does not enhance (Bannayan *et al.* 2009) the response to elevated [CO<sub>2</sub>]. There have been few studies of the effects of pre-industrial [CO<sub>2</sub>] on leaf anatomy; however, leaf mass per area (LMA) has been shown to increase with rising [CO<sub>2</sub>] from glacial or pre-industrial levels (Tissue *et al.* 1995; Ghannoum *et al.* 2010a; Tissue and Lewis 2010). Studies involving current and elevated [CO<sub>2</sub>] also report an increase in LMA, which

suggests leaves become more dense or thicker with increasing  $[\text{CO}_2]$  (Pritchard *et al.* 1999).

Changes in LMA in response to climate change may significantly alter leaf function and plant growth, as LMA is closely related to photosynthetic rates (Reich *et al.* 1998; Wright *et al.* 2005) and relative growth rates in many species (Atkin *et al.* 1999; Wilson *et al.* 1999; Wright and Westoby 2000). LMA reflects both leaf thickness and density, but these two traits differ in their relationship to leaf function. Increases in thickness reflect anatomical changes, such as increases in the size and number of layers of palisade cells (Pereira and Kozlowski 1976; James *et al.* 1999), whereas increases in density also reflect changes in leaf chemistry, including increases in soluble protein and starch per unit leaf area and changes in cell wall content (Dijkstra and Lambers 1989). Increased protein per unit leaf area is associated with increased photosynthetic rates (Field and Mooney 1986; Evans 1989), but increased starch accumulation may reduce photosynthetic rates through source:sink limitation (Herold 1980; Lewis *et al.* 2002b). As a result, to understand how the response of LMA to climate change may affect photosynthesis, it is critical to consider the relationship between LMA and both leaf thickness and chemistry.

Changes in leaf anatomy may also affect photosynthetic responses to climate change by altering  $\text{CO}_2$  diffusion from the atmosphere to the site of photosynthesis (Guerfel *et al.* 2009).  $\text{CO}_2$  diffusion is primarily regulated by stomates and reductions in stomatal frequency with rising  $[\text{CO}_2]$  reduce  $\text{CO}_2$  diffusion (Woodward *et al.* 2002). Conversely, growth in pre-industrial  $[\text{CO}_2]$  may increase stomatal frequency, reducing the resistance to  $\text{CO}_2$  diffusion, but increasing water loss by transpiration (Ward and Strain 1997). Mesophyll packing also alters gas diffusion (Evans 1995), by altering the total surface area of mesophyll cells exposed to intercellular air space (James *et al.* 1999; Marchi *et al.* 2008; Evans *et al.* 2009).

In related studies, we have assessed the effects of rising  $[\text{CO}_2]$  and temperature on growth (Ghannoum *et al.* 2010a), photosynthesis (Ghannoum *et al.* 2010b), light energy partitioning (Logan *et al.* 2010) and nocturnal stomatal conductance during drought (Zeppel *et al.* 2011) of *Eucalyptus sideroxylon* A.Cunn. ex Woolls. Ghannoum *et al.* (2010a) observed that elevated temperature increased the stimulatory effect of rising  $[\text{CO}_2]$  on *E. sideroxylon*, but temperature did not affect photosynthetic responses to rising  $[\text{CO}_2]$ . Upregulation of electron transport capacity ( $J_{\text{max}}$ ) was a key factor driving the observed photosynthetic responses (Ghannoum *et al.* 2010b). Upregulation of  $J_{\text{max}}$  in response to climate change is uncommon, but Ghannoum *et al.* (2010b) lacked a mechanistic explanation for the response because they did not determine whether the change was the result of anatomical or chemical changes. Our objective in this study was to extend our previous research by identifying the structural (e.g. number of palisade layers, stomatal frequency) and chemical (e.g. leaf  $[\text{N}]$ ) changes that may have regulated the photosynthetic response of *E. sideroxylon* to rising  $[\text{CO}_2]$  and temperature. This study was conducted on plants grown separately from Ghannoum *et al.* (2010a, 2010b), but under similar environmental conditions. The studies primarily differ

in that plants in this study were grown for ~210 days, compared with ~140 days in Ghannoum *et al.* (2010a, 2010b). We focussed on *Eucalyptus* because few studies have examined structural and functional responses of *Eucalyptus* to climate change (Conroy 1992; Roden and Ball 1996; Roden *et al.* 1999), despite *Eucalyptus* being an iconic genus with ecological importance in Australia and commercial importance worldwide. We selected *E. sideroxylon* because it represents an ecologically important eucalypt with slower growing, more drought tolerant traits than eucalypts which have been more commonly studied.

## Materials and methods

### Growth conditions

Detailed experimental set-up is described by Ghannoum *et al.* (2010a). Briefly, 9 kg of air-dried loamy-sand field soil was added to 10-L PVC pots, which were transferred to six adjacent, naturally-lit and temperature controlled glasshouse compartments. Three compartments were programmed to simulate the local ambient temperature (Richmond, NSW) and the remaining three compartments were maintained at ambient +4°C. Average temperatures for the ambient and elevated temperature treatments were 26/18 and 30/22°C (day/night) respectively. Within each temperature treatment, compartments were maintained at pre-industrial (280  $\mu\text{L L}^{-1}$  target), current (400  $\mu\text{L L}^{-1}$  target) or elevated (640  $\mu\text{L L}^{-1}$  target)  $[\text{CO}_2]$ . Atmospheric  $[\text{CO}_2]$  was controlled and monitored as described by Ghannoum *et al.* (2010a). Mean daytime  $[\text{CO}_2]$  during the experiment for the pre-industrial, current and elevated treatments was 290, 400 and 650  $\mu\text{L L}^{-1}$  respectively. RH, monitored by Tinytag data loggers (TinyView, Gemini Data Loggers LTD, Chichester, UK), averaged 57% during the study and did not differ among  $[\text{CO}_2]$  and temperature treatments. As a result, vapour pressure deficit (VPD) was higher in the elevated compared with the ambient temperature treatment (1.8 vs 1.4 kPa, on average). Maximum mid-day photosynthetically active radiation (PAR), measured at a nearby (1 km away) weather station, was 2360  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Across the study, peak midday PAR averaged 1250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The glasshouse structure attenuated direct sunlight by ~10–15%.

### Plant growth

Seeds of red ironbark (*Eucalyptus sideroxylon* A.Cunn. ex. Woolls) were obtained from Ensis (Australian Tree Seed Centre, ACT) and germinated at ambient  $[\text{CO}_2]$  in plastic greenhouses. Four weeks after germination, seedlings were transplanted by planting one seedling into the middle of each prepared pot. Pots were irrigated every 2–3 days as needed. Pots were irrigated on three occasions (30, 120 and 135 days after planting (DAP)) with a commercial fertiliser (General Purpose, Thrive Professional, Yates, Sydney, NSW) at a concentration of 0.2 g  $\text{N L}^{-1}$  (N:P:K:S:Fe:Mn:B; 25:4.1:17.3:1.6:0.06:0.003:0.022%). Pots were routinely moved within the glasshouse compartments during the experimental period. Five pots from each treatment were randomly selected for this study.

### Internal leaf anatomy

Duplicate  $1.0 \times 0.5$  cm sections were collected, after 7 months of growth, on opposite sides of the midvein in the mid region of the lamina; one leaf was used per pot. The leaf tissue was immediately fixed in double aldehyde fixative containing 0.01 g of Triton-X, washed in three changes of 0.1 M phosphate buffer (pH 6.9) and then dehydrated in an ethanol series before being embedded in LR White Resin (ProSci Tech, Townsville, Qld). Transverse sections  $2\mu\text{m}$  thick were stained with 0.1% toluidine blue. Sections were photographed using a JenOptik C14 digital camera attached to an Olympus compound light microscope (Olympus BX60, Center Valley, PA). Five images were recorded at various points along the leaf section. Image-Pro Plus (ver. 5.1; Media Cybernetics Inc., Bethesda, MD) was used to analyse leaf and epidermal thickness, palisade cell size, number of palisade layers and the contributions of cell layers and intercellular air space to leaf thickness and density. Percent palisade and percent intercellular airspace were calculated as the fraction of leaf structure between the epidermal layers occupied by palisade cells and intercellular air space respectively.

Leaf mass per area (LMA) was measured in conjunction with gas-exchange measurements. A minimum of 40 leaf disks from 8–10 recently fully expanded leaves per plant were collected using a  $0.2\text{ cm}^2$  leaf corer. The disks were oven-dried at  $70^\circ\text{C}$  for 48 h and weighed for dry mass. LMA was calculated as leaf dry mass/area ( $\text{g m}^{-2}$ ).

### Surface leaf anatomy

Two  $1\text{-cm}^2$  sections per plant were taken from the mid-lamina region of recently fully expanded mature leaves after 7 months of growth. Epidermal peels were made using a method adapted from Jain (1976). The epidermal peels were stained in 0.5% aqueous safranin overnight at room temperature, washed with distilled water and mounted in 40% glycerol. Three images were taken on the upper and lower surface of each epidermal peel using a JenOptik C14 digital camera attached to a compound light microscope (Olympus BX60). The counting function in Image-Pro Plus was used to determine the number of stomata and epidermal cells per field of view. Stomatal index (SI) was calculated as:  $\text{SI (\%)} = (\text{SF}/(\text{SF} + \text{EF})) \times 100$ , where SF is stomatal frequency  $\text{mm}^{-2}$  and EF is epidermal cell frequency  $\text{mm}^{-2}$ .

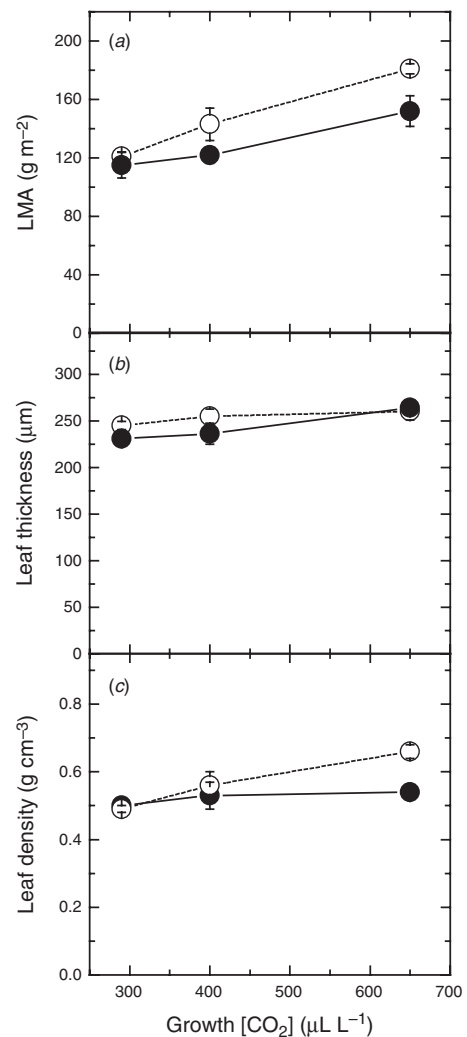
### Leaf gas-exchange measurements

Net photosynthesis at saturating light ( $A_{\text{sat}}$ ) and near-saturating  $\text{CO}_2$  ( $A_{\text{max}}$ ), stomatal conductance ( $g_s$ ), the ratio of intercellular to ambient  $[\text{CO}_2]$  ( $C_i/C_a$ ) and water-use efficiency (WUE) were measured, after 7 months of growth, on one attached, recently fully expanded leaf per plant in the top-third of the plant, using a portable open gas-exchange system (Li-6400XT, Li-Cor, Lincoln, NE).  $A_{\text{sat}}$  and  $A_{\text{max}}$  measurements were made on the same leaf at saturating light ( $1800\mu\text{mol m}^{-2}\text{s}^{-1}$ ) with a target VPD of 1.4 kPa.  $A_{\text{sat}}$  was measured at target growth  $[\text{CO}_2]$  (280, 400 or  $640\mu\text{L L}^{-1}$ ) and midday growth temperature (26 or  $30^\circ\text{C}$ ).  $A_{\text{max}}$  was measured at  $1200\mu\text{L L}^{-1}$   $[\text{CO}_2]$  and  $26^\circ\text{C}$ . Each leaf was allowed to stabilise before measurements were taken.

### Leaf chemistry

The leaf used for gas-exchange measurements was immediately harvested from the plant and snap frozen in liquid nitrogen for analysis of leaf [N]. Leaves were freeze-dried for 24 h, ground to a fine dust, then analysed for N concentration using a CHN analyser (LECO TruSpec, LECO Corporation, St Joseph, MI).

Three leaves per plant were harvested on a sunny day during the gas-exchange campaign, between 1100 and 1300 hours, for carbohydrate and cell wall content analysis. Leaf samples were snap frozen in liquid nitrogen and stored in a  $-85^\circ\text{C}$  freezer. Before analysis, subsamples were freeze-dried for 24 h, then ground to a fine dust. Soluble sugars were assayed using a modified anthrone method as described in Ebell (1969) and total starch was enzymatically assayed on the residual pellet using a Megazyme total starch kit (Megazyme



**Fig. 1.** Leaf structural characteristics of *Eucalyptus sideroxylon* grown at three atmospheric  $[\text{CO}_2]$  (280, 400 or  $640\mu\text{L L}^{-1}$ ) and two air temperatures (ambient (○) or ambient +  $4^\circ\text{C}$  (●)). Values represent means  $\pm$  s.e.

International Ireland Ltd, Wicklow, Ireland); total non-structural carbohydrate (TNC) was calculated as the sum of soluble sugar and starch.

Cell wall content was determined as described by Harrison *et al.* (2009). About 10 mg of ground, freeze-dried leaf material was vortexed in 1.5 mL of buffer (50 mM tricine, pH 8.1) containing 1% PVP40 (Sigma-Aldrich, St Louis, MO), then centrifuged at 12 000g for 5 min. The supernatant was removed and the pellet re-suspended in 1.5 mL of buffer without PVP containing 1% sodium dodecyl sulfate (Sigma-Aldrich). The tube was vortexed and incubated in a 90°C water bath for 5 min, then centrifuged at 12 000g for 5 min. This step was repeated. The pellet was then washed twice with 0.2 M KOH, twice with distilled water and twice with ethanol, mixing well and centrifuging at 12 000g for 5 min at each step. The pellet was oven-dried at 70°C and the dry mass was assumed to represent the leaf structural biomass.

Four leaf disks from recently fully expanded leaves were collected from each plant, in conjunction with samples collected for carbohydrate analyses, for chlorophyll and

soluble protein content. Leaf disks were snap frozen in liquid nitrogen and stored in a -85°C freezer until extraction. Disks were extracted in 1.49 mL of buffer (0.077 g 5 mM DTT, 1 g 10% glycerol, 100 µL 1 mM MgCl<sub>2</sub>, 40 µL 0.5 M EDTA, 20 µL 0.5 M EGTA, 120 µL aminocaproic acid, 80 µL benzamidine, made up to 10 mL with 50 mM HEPES (pH 8.0)), 0.1 g 1% PVP and 10 µL 10 mM PMSF in a pre-cooled mortar. Total chlorophyll content was determined on subsamples of the extract using an acetone-extract colourimetric method, as described by Porra *et al.* (1989). The remaining extract was centrifuged for 2 min and soluble protein content was determined on aliquots of the supernatant using a Coomassie Plus kit (VWR International, Brisbane, Qld).

### Data analyses

The main and interactive effects of growth [CO<sub>2</sub>] and temperature on leaf anatomy, chemistry and gas exchange were tested using two-way analysis of variance (Statistica, StatSoft Inc., Tulsa, OK). Data were tested for normality and

**Table 1. Summary of the two-way ANOVA results for the effects of temperature and [CO<sub>2</sub>] on leaf structural and functional parameters of *Eucalyptus sideroxylon* grown at two temperatures and three [CO<sub>2</sub>] (see ‘Materials and methods’)**

Significance levels are: n.s., not significant ( $P > 0.05$ ); \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . TNC, total non-structural carbohydrate

Variable	Significance level		
	Temperature	CO <sub>2</sub>	Temperature × CO <sub>2</sub>
<i>Leaf anatomy</i>			
LMA (g m <sup>-2</sup> )	*	***	n.s.
Density (g cm <sup>-3</sup> )	*	**	n.s.
Leaf thickness (µm)	n.s.	*	n.s.
Mesophyll thickness (µm)	n.s.	**	n.s.
Epidermal thickness (µm)	n.s.	**	**
No. of palisade layers	n.s.	***	n.s.
Palisade cell length (µm)	***	n.s.	n.s.
Palisade cell width (µm)	n.s.	*	n.s.
Intercellular air space (%)	*	**	n.s.
Palisade (%)	*	**	n.s.
Cell wall content (%)	n.s.	n.s.	n.s.
<i>Leaf surface anatomy</i>			
Epidermal cell frequency (no. per mm <sup>-2</sup> )	n.s.	***	n.s.
Stomatal frequency (no. per mm <sup>-2</sup> )	*	***	n.s.
Stomatal index (%)	*	**	*
<i>Leaf gas exchange</i>			
$A_{\max}$ (µmol m <sup>-2</sup> s <sup>-1</sup> )	n.s.	***	n.s.
$A_{\text{sat}}$ (µmol m <sup>-2</sup> s <sup>-1</sup> )	**	***	n.s.
$g_s$ (mol m <sup>-2</sup> s <sup>-1</sup> )	n.s.	**	n.s.
$C_i/C_a$	n.s.	n.s.	n.s.
<i>Leaf biochemistry</i>			
Leaf N <sub>mass</sub> (mg g <sup>-1</sup> )	n.s.	***	n.s.
Leaf N <sub>area</sub> (g m <sup>-2</sup> )	n.s.	**	n.s.
Soluble protein (g m <sup>-2</sup> )	*	**	*
Chl <i>a</i> + <i>b</i> (g m <sup>-2</sup> )	*	**	n.s.
Chl <i>a/b</i>	n.s.	n.s.	n.s.
Total soluble sugars (g glucose equivalents m <sup>-2</sup> )	n.s.	***	n.s.
Starch (g glucose equivalents m <sup>-2</sup> )	***	***	**
TNC (g glucose equivalents m <sup>-2</sup> )	**	***	*

homogeneity of variances; extreme outliers were removed from the datasets before proceeding with the statistical analysis. Where  $[\text{CO}_2]$  and temperature interactions were significant, pair-wise comparison of treatment means was done using a Newman-Kewls post-hoc test (Statistica, StatSoft Inc.). Relationships between leaf gas exchange and leaf structure and chemistry were analysed using linear regression analysis (Microcol Origin ver. 6.0, Microcol Software, Inc., Northampton, MA). In all analyses, test results were considered significant if  $P \leq 0.05$ .

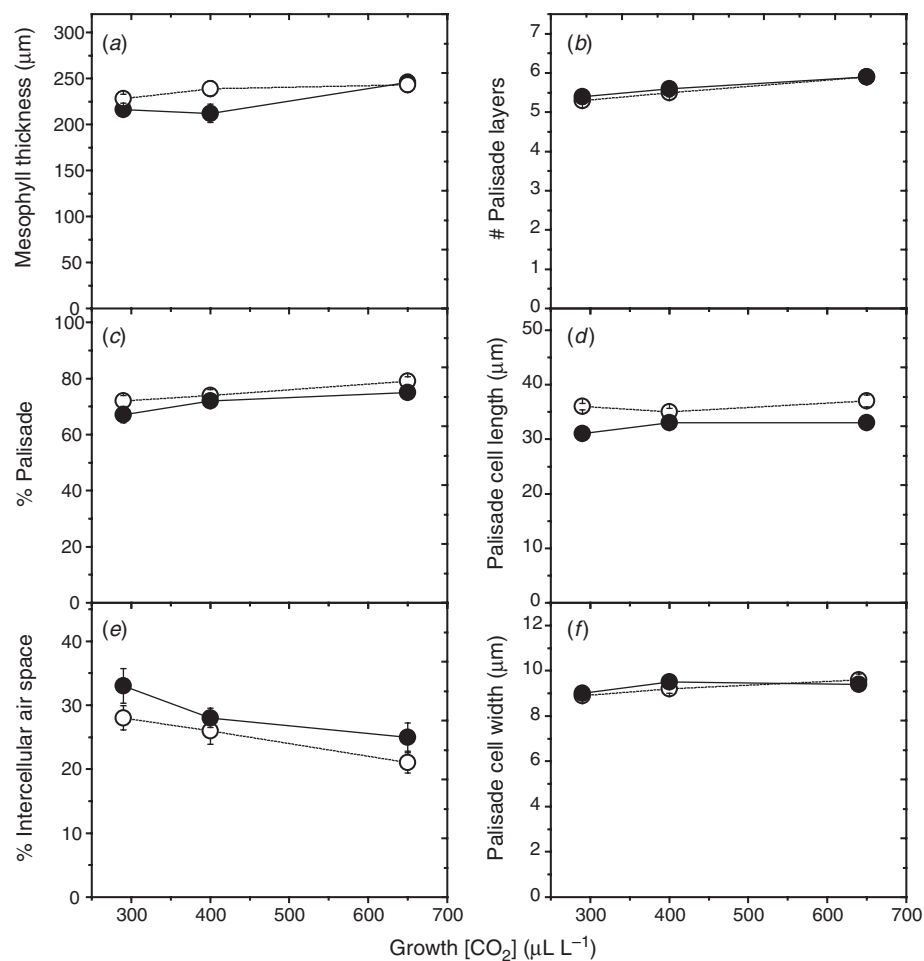
## Results

### Leaf internal anatomy

LMA increased ~35% between pre-industrial and elevated  $[\text{CO}_2]$  (Fig. 1; Table 1). Growth in elevated temperature decreased LMA by 10% compared with ambient temperature. Although the effect of rising  $[\text{CO}_2]$  appeared to vary between temperature treatments, the interaction was not significant (Table 1). Effects of elevated  $[\text{CO}_2]$  on LMA were associated with increases in leaf thickness and density, whereas elevated

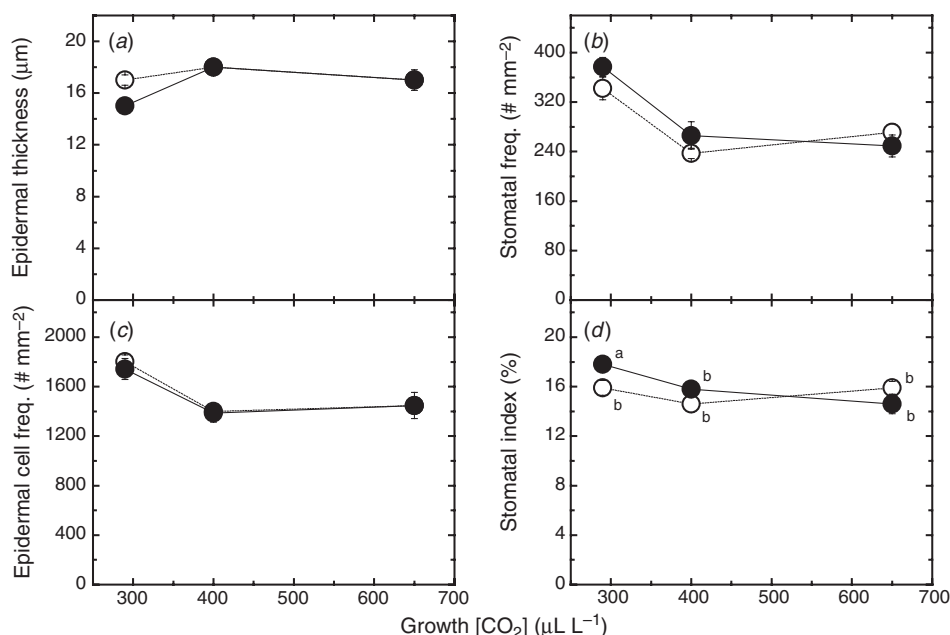
temperature increased leaf density, but did not affect leaf thickness (Fig. 1; Table 1). As with LMA, although the effect of rising  $[\text{CO}_2]$  appeared to differ between temperature treatments, the interactions were not significant (Table 1).

Mesophyll thickness increased with rising  $[\text{CO}_2]$ , but did not vary with growth temperature (Fig. 2; Table 1). Further, although both rising  $[\text{CO}_2]$  and elevated temperature affected the size and distribution of palisade cells, the effects differed between these factors (Fig. 2). Rising  $[\text{CO}_2]$  enhanced the number of palisade layers; on average, one extra layer of palisade cells was observed at elevated compared with pre-industrial  $[\text{CO}_2]$ . Palisade cell width increased with rising  $[\text{CO}_2]$ , whereas palisade cell length was not affected by growth  $[\text{CO}_2]$ . In contrast, increasing temperature reduced palisade cell length by an average of 12%, but did not affect the number of layers or the width of palisade cells. Rising  $[\text{CO}_2]$  also increased the fraction of internal leaf space occupied by palisade cells and reduced that occupied by intercellular air space (Fig. 2). In contrast, elevated temperature increased the fraction of intercellular air space and reduced the fraction of internal leaf space occupied by palisade cells (Fig. 2).



**Fig. 2.** Mesophyll characteristics of *Eucalyptus sideroxylon* grown at three atmospheric  $[\text{CO}_2]$  (290, 400 or  $650 \mu\text{L L}^{-1}$ ) and two air temperatures (ambient (○) or ambient +  $4^\circ\text{C}$  (●)). Values represent means  $\pm$  s.e.





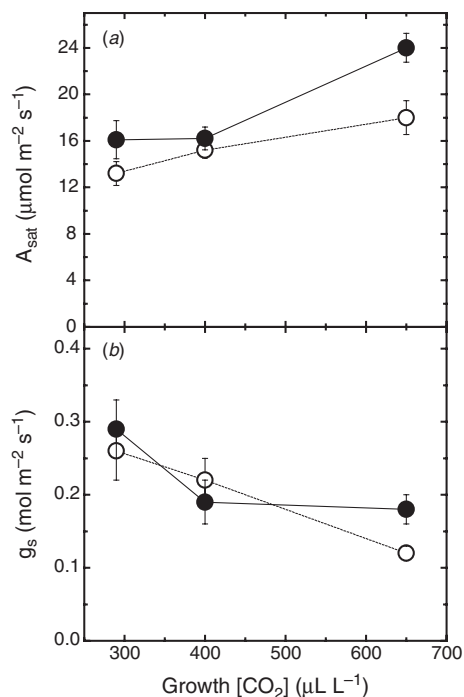
**Fig. 3.** (a) Epidermal thickness, (b) stomatal frequency, (c) cell frequency and (d) stomatal index of *Eucalyptus sideroxylon* grown at three atmospheric  $[\text{CO}_2]$  (290, 400 or 650  $\mu\text{L L}^{-1}$ ) and two air temperatures (ambient  $\circ$ ) or ambient +4°C ( $\bullet$ ). Values represent means  $\pm$  s.e. There was a significant interaction between  $[\text{CO}_2]$  and temperature treatments on stomatal index only; superscripts in (d) indicate significant differences between treatments.

#### Leaf surface anatomy

Epidermal thickness increased and epidermal cell frequency decreased with rising  $[\text{CO}_2]$ , but temperature did not affect epidermal thickness or cell frequency (Fig. 3; Table 1). The effects of elevated  $[\text{CO}_2]$  on stomatal frequency and stomatal index differed between temperature treatments. Stomatal frequency and stomatal index decreased with increasing  $[\text{CO}_2]$  between pre-industrial and current  $[\text{CO}_2]$  in both temperature treatments. However, stomatal frequency and stomatal index also decreased with rising  $[\text{CO}_2]$  between current and elevated  $[\text{CO}_2]$  in the elevated temperature treatment, but both increased with increasing  $[\text{CO}_2]$  between current and elevated  $[\text{CO}_2]$  in the ambient temperature treatment. As a result, although elevated temperature increased stomatal frequency and stomatal index at pre-industrial and current  $[\text{CO}_2]$ , elevated temperature decreased stomatal frequency and stomatal index in elevated  $[\text{CO}_2]$ .

#### Leaf gas exchange

$A_{\text{sat}}$  increased with rising  $[\text{CO}_2]$  and elevated temperature (Fig. 4; Table 1), but the increase was larger between current and elevated  $[\text{CO}_2]$  (33% increase) than between pre-industrial and current  $[\text{CO}_2]$  (7% increase). The comparatively smaller effect in the transition from pre-industrial and current  $[\text{CO}_2]$  reflected a 27% decrease in  $A_{\text{max}}$  in the transition from pre-industrial to current  $[\text{CO}_2]$ ; no change was observed from current to elevated  $[\text{CO}_2]$  (Table 2).  $A_{\text{max}}$  did not differ between temperature treatments (Table 1).



**Fig. 4.** (a) Light-saturated photosynthetic rates measured at growth  $[\text{CO}_2]$  and temperature ( $A_{\text{sat}}$ ) and (b) stomatal conductance ( $g_s$ ) of *Eucalyptus sideroxylon* grown at three atmospheric  $[\text{CO}_2]$  (290, 400 or 650  $\mu\text{L L}^{-1}$ ) and two air temperatures (ambient  $\circ$ ) or ambient +4°C ( $\bullet$ ). Values represent means  $\pm$  s.e.

**Table 2.** Leaf biochemistry traits of *Eucalyptus sideroxylon* grown at two temperatures and three [CO<sub>2</sub>] (see 'Materials and methods')

Values represent means  $\pm$  s.e. For those leaf traits where there was a significant interaction between [CO<sub>2</sub>] and temperature treatments, different letters indicate significance between treatments. In all cases  $n=5$ . TNC, total non-structural carbohydrate

Variable	Temperature	[CO <sub>2</sub> ] (μL L <sup>-1</sup> )		
		290	400	650
Leaf anatomy				
Cell wall content (%)	Ambient	30 ± 1	30 ± 1	32 ± 1
	High	30 ± 1	30 ± 1	28 ± 1
Leaf gas exchange				
A <sub>max</sub> (μmol m <sup>-2</sup> s <sup>-1</sup> )	Ambient	32.0 ± 0.1	24.4 ± 1.7	23.2 ± 1.9
	High	32.9 ± 1.8	23.5 ± 1.1	26.2 ± 1.1
C <sub>i</sub> /C <sub>a</sub>	Ambient	0.64 ± 0.04	0.66 ± 0.03	0.59 ± 0.03
	High	0.68 ± 0.04	0.59 ± 0.04	0.60 ± 0.03
Leaf biochemistry				
Leaf N <sub>mass</sub> (mg g <sup>-1</sup> )	Ambient	20.9 ± 0.8	14.1 ± 1.8	11.7 ± 1.2
	High	24.5 ± 2.8	15.9 ± 2.8	13.5 ± 1.1
Leaf N <sub>area</sub> (g m <sup>-2</sup> )	Ambient	2.5 ± 0.1	1.9 ± 0.1	2.1 ± 0.2
	High	2.9 ± 0.3	1.9 ± 0.3	2.0 ± 0.1
Soluble protein (g m <sup>-2</sup> )	Ambient	8.8 ± 0.4b	6.3 ± 1.0a	4.7 ± 0.6a
	High	5.5 ± 0.8a	5.6 ± 0.5a	4.8 ± 0.2a
Chl <i>a</i> + <i>b</i> (g m <sup>-2</sup> )	Ambient	0.55 ± 0.06	0.37 ± 0.02	0.37 ± 0.05
	High	0.62 ± 0.04	0.45 ± 0.07	0.51 ± 0.01
Chl <i>a</i> / <i>b</i>	Ambient	2.5 ± 0.0	2.4 ± 0.1	2.4 ± 0.2
	High	2.4 ± 0.0	2.2 ± 0.1	2.4 ± 0.4
Total soluble sugars (g glucose equivalents m <sup>-2</sup> )	Ambient	9.6 ± 1.2	13.8 ± 2.5	19.9 ± 2.2
	High	10.2 ± 1.7	15.5 ± 1.6	17.9 ± 1.8
Starch (g glucose equivalents m <sup>-2</sup> )	Ambient	6.0 ± 0.9a	7.2 ± 0.3a	14.3 ± 1.1b
	High	3.1 ± 0.6a	4.6 ± 0.5a	6.4 ± 0.5a
TNC (g glucose equivalents m <sup>-2</sup> )	Ambient	15.5 ± 1.3a	21.9 ± 3.2b	35.6 ± 2.5c
	High	13.8 ± 1.6a	20.1 ± 1.8ab	24.4 ± 2.0b

Results showed that  $g_s$  decreased with rising [CO<sub>2</sub>] but did not vary with growth temperature (Fig. 4; Table 1).  $C_i/C_a$  was not affected by either growth [CO<sub>2</sub>] or temperature (Table 2). Overall, leaf gas exchange was affected more by growth [CO<sub>2</sub>] than temperature (Table 1). Temperature and [CO<sub>2</sub>] had no interactive effects on leaf gas-exchange parameters (Table 1).

#### Leaf chemistry

Leaf N per unit dry mass ( $N_{\text{mass}}$ ) decreased 45% with rising [CO<sub>2</sub>], but did not vary with growth temperature (Tables 1, 2). Similar responses to rising [CO<sub>2</sub>] and temperature were observed when leaf N was expressed on an area or structural dry mass basis. Although growth temperature did not affect the response of leaf N per unit area ( $N_{\text{area}}$ ) to rising [CO<sub>2</sub>], the response of soluble protein content to rising [CO<sub>2</sub>] differed between growth temperatures. At ambient temperature, soluble protein decreased by 28% in the transition from pre-industrial to current [CO<sub>2</sub>], but did not differ between current and elevated [CO<sub>2</sub>]. At elevated temperature, soluble protein did not differ among [CO<sub>2</sub>] treatments. Elevated temperature reduced soluble protein in pre-industrial [CO<sub>2</sub>], but did not affect soluble protein in current or elevated [CO<sub>2</sub>]. Total chlorophyll decreased with

rising [CO<sub>2</sub>] and increased with temperature. Neither growth [CO<sub>2</sub>] nor temperature had an effect on the chlorophyll  $a/b$  ratio or on cell wall content.

Soluble sugars nearly doubled between pre-industrial and elevated [CO<sub>2</sub>], but the effect of rising [CO<sub>2</sub>] on starch differed between temperature treatments (Tables 1, 2). At ambient temperature, starch nearly doubled between current and elevated [CO<sub>2</sub>], but there was no effect between pre-industrial and current [CO<sub>2</sub>] (Tables 1, 2). At elevated temperature, starch increased by 48% between pre-industrial and current [CO<sub>2</sub>] and 40% between current and elevated [CO<sub>2</sub>]. Elevated temperature decreased starch ~50% across [CO<sub>2</sub>] treatments. As a result of the combined treatment effects on soluble sugars and starch, TNC doubled with rising [CO<sub>2</sub>] and declined ~20% with elevated temperature (Tables 1, 2). Overall, growth [CO<sub>2</sub>] had a greater effect on leaf chemical properties than high temperature (Table 1).

#### Relationships between leaf structure and function

To assess those factors that may have regulated  $A_{\text{max}}$ , we examined relationships between  $A_{\text{max}}$  and structural and chemical variables (LMA, leaf and mesophyll thickness, number of palisade layers, fraction of intercellular air space, and leaf  $N_{\text{area}}$ , starch, soluble sugars, TNC and soluble

proteins).  $A_{\max}$  increased with increasing fraction of intercellular air space and leaf  $N_{\text{area}}$ , whereas  $A_{\max}$  decreased with increasing LMA, leaf and mesophyll thickness, number of palisade layers and starch, soluble sugar and TNC per unit area ( $P < 0.05$  in all cases). The strongest relationships were between  $A_{\max}$  and LMA, number of palisade layers, leaf  $N_{\text{area}}$  and TNC per unit area (Fig. 5).  $A_{\max}$  did not vary with leaf soluble proteins per unit area ( $P = 0.186$ ).

To assess those factors that may have regulated leaf density, we examined the relationships between leaf density and structural (fraction of palisade cells) and chemical (leaf starch, soluble sugars, TNC) variables that may have influenced leaf density. Leaf density increased with leaf starch and the fraction of palisade cells (Fig. 6). Leaf starch per unit volume accounted for 41% of the variation in leaf density, while fraction of palisade cells accounted for 29% of the variation in leaf density.

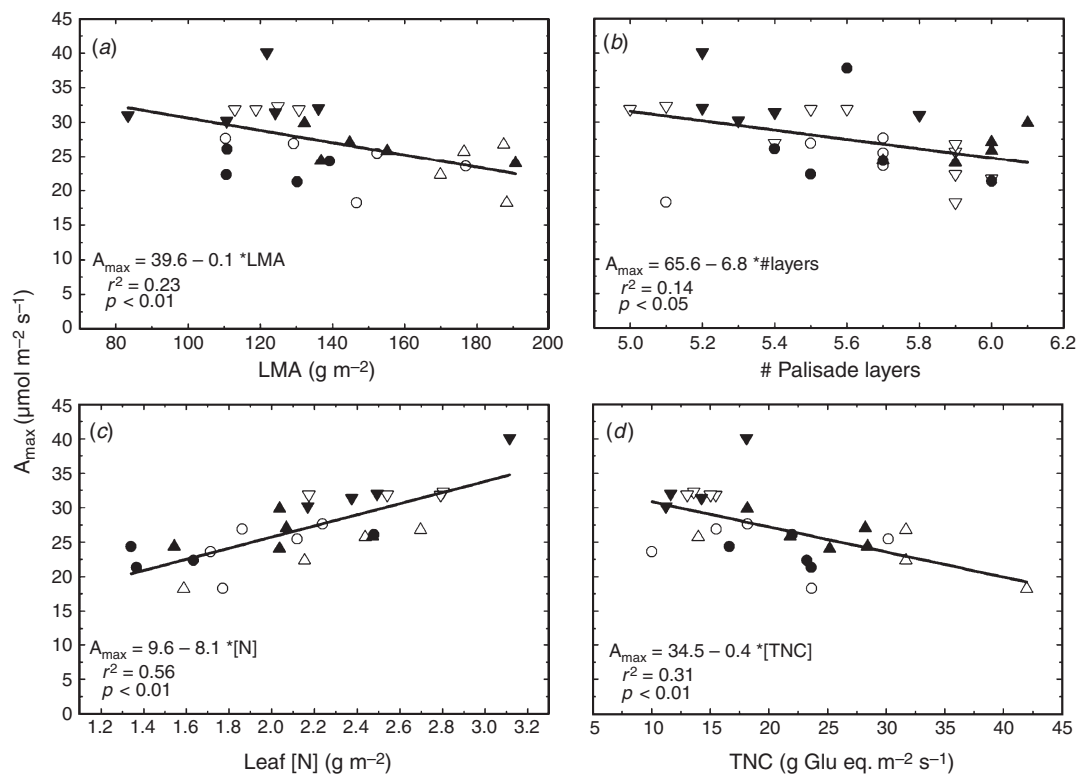
## Discussion

Rising  $[\text{CO}_2]$  affected  $A_{\text{sat}}$  directly and through effects on photosynthetic capacity ( $A_{\max}$ ). Rising  $[\text{CO}_2]$  reduced  $A_{\max}$  by reducing leaf  $N_{\text{area}}$  but increased the number of palisade layers. Leaf  $N_{\text{area}}$  accounted for 56% of the variation in  $A_{\max}$  and the number of palisade layers accounted for 14% of the variation, respectively. Reduction in  $N_{\text{area}}$  was associated with increased leaf mass per area (LMA), which, in turn, was associated with

increased starch and number of palisade layers. Changes in these three factors were associated with linear reductions in  $A_{\max}$  between pre-industrial and elevated  $[\text{CO}_2]$ , suggesting consistent patterns in the effects of these factors on  $A_{\max}$  across  $\text{CO}_2$  concentrations. In contrast, although elevated temperature increased stomatal frequency, it did not affect  $A_{\max}$ , indicating acclimation to growth temperature. Further, there generally were no interactions between rising  $[\text{CO}_2]$  and temperature on leaf structure or function. These results indicate leaf  $N_{\text{area}}$  and the number of palisade layers, associated with changes in LMA, were the key chemical and anatomical factors regulating photosynthetic responses of *E. sideroxylon* to rising  $[\text{CO}_2]$ , whereas the lack of photosynthetic responses to elevated temperature may reflect both acclimation of photosynthesis and the limited effect of elevated temperature on leaf anatomy.

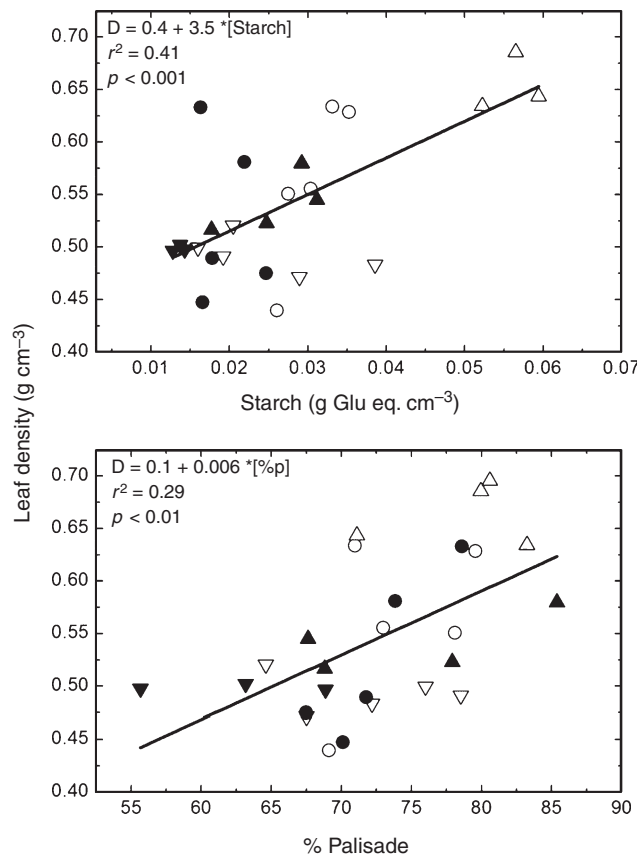
## Relationships between leaf structure and function

In  $\text{C}_3$  plants, long-term exposure to rising  $[\text{CO}_2]$  often is associated with reductions in  $A_{\max}$ , reducing the relative  $A_{\text{sat}}$  response to  $[\text{CO}_2]$  (Tissue and Oechel 1987; Ainsworth and Rogers 2007), as we observed in this study. These reductions may result from a wide range of structural and physiological changes, including changes in leaf thickness and reductions in total Rubisco activity. Further, these changes may be tied to changes in leaf chemistry, including reductions in leaf  $N_{\text{area}}$



**Fig. 5.** The relationships of *Eucalyptus sideroxylon* photosynthetic capacity ( $A_{\max}$ ) with (a) LMA, (b) number of palisade layers, (c) leaf  $N_{\text{area}}$  and (d) total non-structural carbohydrates. Symbols represent growth  $[\text{CO}_2]$ ; 290  $\mu\text{L L}^{-1}$  (▼), 400  $\mu\text{L L}^{-1}$  (●) and 650  $\mu\text{L L}^{-1}$  (▲), with open symbols representing ambient temperature and closed symbols representing ambient +4°C. There were five replicates per treatment and each data point represents a single observation. Data were fitted using a linear regression (solid line). The equation of the linear fit, adjusted  $R^2$  value and its significance are shown.





**Fig. 6.** The relationships of *Eucalyptus sideroxylon* leaf density with leaf starch and fraction palisade cells. Symbols represent growth  $[\text{CO}_2]$ ; 290  $\mu\text{L L}^{-1}$  (▼), 400  $\mu\text{L L}^{-1}$  (●) and 650  $\mu\text{L L}^{-1}$  (▲), with open symbols representing ambient temperature and closed symbols representing ambient +4°C. There were five replicates per treatment and each data point represents a single observation. Data were fitted using a linear regression (solid line). The equation of the linear fit, adjusted  $R^2$  value and its significance are shown.

and increased carbohydrate concentrations (Griffin *et al.* 2001; Lewis *et al.* 2004; Ainsworth and Rogers 2007). Our results suggest that variation in  $A_{\text{max}}$  was primarily driven by changes in LMA and associated changes in leaf chemistry and the number of palisade layers. These changes, in turn, partially reflected changes in allocation of resources within the leaf.

Changes in leaf  $N_{\text{area}}$  had the largest effect on  $A_{\text{max}}$ , accounting for 56% of the variation in  $A_{\text{max}}$ . The effect of leaf  $N_{\text{area}}$  on  $A_{\text{max}}$  may have been in part through effects on soluble protein content per unit area, which exhibited similar patterns of response to rising  $[\text{CO}_2]$  and temperature as leaf  $N_{\text{area}}$ . In  $C_3$  leaves, Rubisco constitutes ~25% of leaf N content and up to 50% of the soluble protein fraction (Evans 1983; Conroy 1992). As a result, soluble protein content and  $A_{\text{max}}$  closely vary with leaf  $N_{\text{area}}$  (Field and Mooney 1986; Evans 1989); reductions in leaf  $N_{\text{area}}$  and soluble protein content with rising  $[\text{CO}_2]$  were an indication that Rubisco content was reduced (Tissue *et al.* 1993). Reductions in  $A_{\text{max}}$  also occurred in conjunction with increased TNC, suggesting that photosynthetic downregulation may have occurred at least in part due to a

source:sink imbalance. Downregulation of  $A_{\text{max}}$  in response to carbohydrate accumulation has been observed in a wide range of studies (Thomas and Strain 1991; Lewis *et al.* 2002b; Ainsworth and Long 2005).

The close relationships between  $A_{\text{max}}$  and leaf  $N_{\text{area}}$ , soluble proteins and TNC per unit area at least partially account for the differential responses of  $A_{\text{max}}$  to pre-industrial versus elevated  $[\text{CO}_2]$ .  $A_{\text{max}}$ , leaf  $N_{\text{area}}$ , and soluble proteins decreased while carbohydrates increased with rising  $[\text{CO}_2]$ . The differential effects on leaf chemistry of pre-industrial  $[\text{CO}_2]$  compared with elevated  $[\text{CO}_2]$  were partially a function of changes in leaf structure, as has been observed in previous studies (e.g. Poorter and Evans 1998; Reich *et al.* 1998). Leaf structure, which is often correlated with LMA, affects processes such as N allocation and concentration (Field and Mooney 1986). Accordingly, there often is a strong correlation between LMA and photosynthesis–nitrogen relationships (Reich *et al.* 1998; Wright *et al.* 2005). For instance, species with high LMA generally have higher  $A_{\text{max}}$  and  $N_{\text{area}}$  than species with low LMA (Poorter and Evans 1998; Sefton *et al.* 2002). In this study, *E. sideroxylon* with high-LMA leaves had lower  $A_{\text{max}}$  than that observed in the low-LMA leaves; low leaf  $N_{\text{area}}$  and high LMA together generated low  $A_{\text{max}}$ . This result highlights the role of N in determining  $A_{\text{max}}$  and in regulating responses to rising  $[\text{CO}_2]$  from pre-industrial to elevated  $[\text{CO}_2]$ .

Higher TNC at elevated  $[\text{CO}_2]$  was associated with the production of an additional palisade cell layer, as has been observed in other studies (Thomas and Harvey 1983; Pritchard *et al.* 1999). Increased numbers of palisade cell layers generally increase  $A_{\text{max}}$  (Lin *et al.* 2001). However, increases in the number of palisade cell layers may reduce  $A_{\text{max}}$  by reducing leaf  $N_{\text{area}}$  through effects on LMA. Increases in palisade layer number may also affect processes such as  $\text{CO}_2$  diffusion (James *et al.* 1999; Marchi *et al.* 2008; Evans *et al.* 2009). Here, we observed with rising  $[\text{CO}_2]$  an increase in the fraction of internal leaf surface occupied by palisade cells and a reduction in the fraction occupied by intercellular air space. Reductions in the intercellular air space have been associated with reduced mesophyll conductance of  $\text{CO}_2$ , which would offset the stimulatory effect of an additional palisade layer on  $A_{\text{max}}$ . Likewise, increasing the fraction of intercellular air space at pre-industrial  $[\text{CO}_2]$  facilitates plant compensation for low  $[\text{CO}_2]$  by reducing the resistance to  $[\text{CO}_2]$  diffusion.

Accordingly, rising  $[\text{CO}_2]$  may have reduced  $A_{\text{sat}}$  through effects on both mesophyll and stomatal conductance. Stomates are the primary factor regulating  $\text{CO}_2$  diffusion into the leaf and reductions in stomatal frequency with rising  $[\text{CO}_2]$  reduce  $\text{CO}_2$  diffusion into the leaf (Woodward *et al.* 2002). Consistent with this expectation as well as empirical data (Medlyn *et al.* 2001; Lewis *et al.* 2002a; Ainsworth and Long 2005),  $g_s$  decreased with rising  $[\text{CO}_2]$ . Growth in pre-industrial  $[\text{CO}_2]$  often is associated with comparatively high stomatal frequency, reducing the resistance to  $\text{CO}_2$  diffusion and offsetting the reduction in the driving gradient for  $\text{CO}_2$  diffusion into the leaf (Ward and Strain 1997). The observed changes in stomatal frequency likely reflected effects of rising  $[\text{CO}_2]$  on epidermal cell production rather than on stomatal initiation. Rising  $[\text{CO}_2]$  had a small negative effect on stomatal index, which suggests that changes in stomatal frequency were largely proportional to changes in

epidermal cell frequency (stomatal index is the ratio of the number of epidermal cells to stomata). Hence, rising  $[\text{CO}_2]$  is unlikely to have affected stomatal initiation but instead stimulated epidermal cell production and expansion.

We noted that the response of  $A_{\text{sat}}$  to rising  $[\text{CO}_2]$  was independent of temperature. During short-term exposure, the response of  $A_{\text{sat}}$  to rising  $[\text{CO}_2]$  is predicted to increase with increasing temperature because of differences in the temperature responses of photosynthesis and photorespiration (Berry and Björkman 1980; Sage and Kubien 2007). However, responses to long-term exposure to elevated  $[\text{CO}_2]$  and temperature depend on the extent to which photosynthesis acclimates to the new conditions. In our study, the strong thermal acclimation of  $A_{\text{max}}$  precluded temperature  $\times$   $[\text{CO}_2]$  interactions. The lack of a temperature effect on  $A_{\text{max}}$  indicates that photosynthesis underwent partial thermal acclimation in response to growth temperature (Ghannoum *et al.* 2010b). Photosynthetic acclimation to high temperature is commonly observed in a wide variety of plants (Berry and Björkman 1980; Cowling and Sage 1998), although not always (Lewis *et al.* 2001). The lack of a temperature effect on  $A_{\text{max}}$  further indicates that the anatomical changes associated with elevated temperature, including the decrease in leaf density, decreased palisade cell length and percent palisade cells, were not sufficient to alter photosynthetic processes.

## Conclusions

Rising  $[\text{CO}_2]$  increased  $A_{\text{sat}}$  through direct effects, but indirectly affected  $A_{\text{sat}}$  through reductions in  $A_{\text{max}}$ . Reductions in  $A_{\text{max}}$  with rising  $[\text{CO}_2]$  were driven by reductions in leaf  $N_{\text{area}}$  and an increase in the number of palisade layers, which accounted for 56 and 14%, respectively, of the variation in  $A_{\text{max}}$ . Reductions in  $N_{\text{area}}$  were associated with increased LMA, which, in turn, was associated with increased starch and numbers of palisade layers.  $A_{\text{max}}$  responded linearly to changes in these traits, suggesting consistent effects of these traits on  $A_{\text{max}}$  across  $[\text{CO}_2]$  levels. Elevated temperature was associated with increases in stomatal frequency, but did not significantly affect  $A_{\text{max}}$ . Further, as has been found in a wide range of tree species (Lewis *et al.* 1999; Wang *et al.* 2003; Allen and Vu 2009; Ghannoum *et al.* 2010b), the effects of rising  $[\text{CO}_2]$  and elevated temperature generally were additive, rather than interactive. Our results suggest rising  $[\text{CO}_2]$  affects photosynthesis in *E. sideroxylon* primarily through effects on leaf  $N_{\text{area}}$ , stomatal frequency and the number of palisade layers, while the lack of effect of elevated temperature on  $A_{\text{max}}$  indicated photosynthetic acclimation to elevated temperature and that structural changes associated with rising temperature were not extensive enough to alter  $A_{\text{max}}$ .

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## References

- Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air  $\text{CO}_2$  enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising  $\text{CO}_2$ . *New Phytologist* **165**, 351–372. doi:10.1111/j.1469-8137.2004.01224.x
- Ainsworth EA, Rogers AL (2007) The response of photosynthesis and stomatal conductance to rising  $\text{CO}_2$ : mechanisms and environmental interactions. *Plant, Cell & Environment* **30**, 258–270. doi:10.1111/j.1365-3040.2007.01641.x
- Allen LH, Vu JCV (2009) Carbon dioxide and high temperature effects on growth of young orange trees in a humid, subtropical environment. *Agricultural and Forest Meteorology* **149**, 820–830. doi:10.1016/j.agrformet.2008.11.002
- Atkin OK, Schortemeyer M, McFarlane N, Evans JR (1999) The response of fast- and slow-growing *Acacia* species to elevated atmospheric  $\text{CO}_2$ : an analysis underlying components of relative growth rate. *Oecologia* **120**, 544–554. doi:10.1007/s004420050889
- Atwell BJ, Henery ML, Rogers GS, Seneweera SP, Treadwell M, Conroy JP (2007) Canopy development and hydraulic function in *Eucalyptus tereticornis* grown in drought in  $\text{CO}_2$ -enriched atmospheres. *Functional Plant Biology* **34**, 1137–1149. doi:10.1071/FP06338
- Bannayan M, Tojo Soler CM, Garcia A, Guerra LC, Hoogenboom G (2009) Interactive effects of elevated  $[\text{CO}_2]$  and temperature on growth and development of a short- and long-season peanut cultivar. *Climatic Change* **93**, 389–406. doi:10.1007/s10584-008-9510-1
- Berry J, Björkman O (1980) Photosynthetic response and adaptation to temperature in higher plants. *Annual Review of Plant Physiology* **31**, 491–543. doi:10.1146/annurev.pp.31.060180.002423
- Conroy JP (1992) Influence of elevated atmospheric  $\text{CO}_2$  concentrations on plant nutrition. *Australian Journal of Botany* **40**, 445–456.
- Cowling SA, Sage RF (1998) Interactive effects of low atmospheric  $\text{CO}_2$  and elevated temperature on growth, photosynthesis and respiration in *Phaseolus vulgaris*. *Plant, Cell & Environment* **21**, 427–435. doi:10.1046/j.1365-3040.1998.00290.x
- Dijkstra P, Lambers H (1989) Analysis of specific leaf area and photosynthesis of two inbred lines of *Plantago major* differing in relative growth rate. *New Phytologist* **113**, 283–290. doi:10.1111/j.1469-8137.1989.tb02405.x
- Ebell LF (1969) Variation in total soluble sugars of conifer tissues with method of analysis. *Phytochemistry* **8**, 227–233. doi:10.1016/S0031-9422(00)85818-5
- Evans JR (1983) Nitrogen and photosynthesis in flag leaf of wheat (*Triticum aestivum* L.). *Plant Physiology* **72**, 297–302. doi:10.1104/pp.72.2.297
- Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of  $\text{CO}_2$  plants. *Oecologia* **78**, 9–19. doi:10.1007/BF00377192
- Evans JR (1995) Carbon fixation profiles do reflect light absorption profiles in leaves. *Australian Journal of Plant Physiology* **22**, 865–873. doi:10.1071/PP950865
- Evans JR, Kaldenhoff R, Benty B, Terashima I (2009) Resistances along the  $\text{CO}_2$  diffusion pathway inside leaves. *Journal of Experimental Botany* **60**, 2235–2248. doi:10.1093/jxb/erp117
- Field C, Mooney HA (1986) The photosynthesis-nitrogen relationship in wild plants. In 'On the economy of plant form and function'. (Ed. TJ Givnish) pp. 25–55. (Cambridge University Press: Cambridge)
- Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* **281**, 237–240. doi:10.1126/science.281.5374.237
- Ghannoum O, Phillips NG, Conroy JP, Smith RA, Attard RD, Woodfield R, Logan BA, Lewis JD, Tissue DT (2010a) Exposure to preindustrial, current and future atmospheric  $\text{CO}_2$  and temperature differentially affects growth and photosynthesis in *Eucalyptus*. *Global Change Biology* **16**, 303–319. doi:10.1111/j.1365-2486.2009.02003.x

- Ghannoum O, Phillips NG, Sears MA, Logan BA, Lewis JD, Conroy JP, Tissue DT (2010b) Photosynthetic responses of two eucalypts to industrial-age changes in atmospheric [CO<sub>2</sub>] and temperature. *Plant, Cell & Environment* **33**, 1671–1681. doi:10.1111/j.1365-3040.2010.02172.x
- Griffin KL, Anderson OR, Gastrich MD, Lewis JD, Lin GH, Schuster W, Seeman JR, Tissue DT, Turnbull MH, Whitehead D (2001) Plant growth in elevated CO<sub>2</sub> alters mitochondrial number and chloroplast fine structure. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2473–2478. doi:10.1073/pnas.041620898
- Guerfel M, Baccouri O, Boujnah D, Chaibi W, Zarrouk M (2009) Impacts of water stress on gas exchange, water relations, chlorophyll content and leaf structure in the two main Tunisian olive (*Olea europaea* L.) cultivars. *Scientia Horticulturae* **119**, 257–263. doi:10.1016/j.scienta.2008.08.006
- Harrison MT, Edwards EJ, Farquhar GD, Nicotra AB, Evans JR (2009) Nitrogen in cell walls of sclerophyllous leaves accounts for little variation in photosynthetic nitrogen-use efficiency. *Plant, Cell & Environment* **32**, 259–270. doi:10.1111/j.1365-3040.2008.01918.x
- Hennessey K, Fitzharris B, Bates BC, Harvey N, Howden SM, Hughes L, Salinger J, Warrick R (2007) Australia and New Zealand. In 'Climate change 2007: impacts, adaptation and vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change'. (Eds ML Parry, OF Canziani, JP Palutikof, PJ van der Linden, CE Hanson) pp. 507–540. (Cambridge University Press: Cambridge, UK)
- Herold A (1980) Regulation of photosynthesis by sink activity: the missing link. *New Phytologist* **86**, 131–144. doi:10.1111/j.1469-8137.1980.tb03184.x
- Jain KK (1976) Hydrogen peroxide and acetic acid for preparing epidermal peels from conifer leaves. *Biotechnic & Histochemistry* **51**, 202–204. doi:10.3109/10520297609116701
- James SA, Smith WK, Vogelmann TC (1999) Ontogenetic difference in mesophyll structure and chlorophyll distribution in *Eucalyptus globulus* ssp. *Globulus* (Myrtaceae). *American Journal of Botany* **86**, 198–207. doi:10.2307/2656937
- Klich MG (2000) Leaf variations in *Elaeagnus angustifolia* related to environmental heterogeneity. *Environmental and Experimental Botany* **44**, 171–183. doi:10.1016/S0098-8472(00)00056-3
- Körner C (2006) Plant CO<sub>2</sub> responses: an issue of definition, time and resource supply. *New Phytologist* **172**, 393–411. doi:10.1111/j.1469-8137.2006.01886.x
- Lewis JD, Olszyk D, Tingey DT (1999) Seasonal patterns of photosynthetic light response in Douglas fir seedlings subjected to elevated atmospheric CO<sub>2</sub> and temperature. *Tree Physiology* **19**, 243–252.
- Lewis JD, Lucash M, Olszyk D, Tingey DT (2001) Seasonal patterns of photosynthesis in Douglas fir seedlings during the third and fourth year of exposure to elevated CO<sub>2</sub> and temperature. *Plant, Cell & Environment* **24**, 539–548. doi:10.1046/j.1365-3040.2001.00700.x
- Lewis JD, Lucash M, Olszyk DM, Tingey DT (2002a) Stomatal responses of Douglas fir seedlings to elevated carbon dioxide and temperature during the third and fourth years of exposure. *Plant, Cell & Environment* **25**, 1411–1421. doi:10.1046/j.1365-3040.2002.00923.x
- Lewis JD, Wang XZ, Griffin KL, Tissue DT (2002b) Effects of age and ontogeny on photosynthetic responses of a determinate annual plant to elevated CO<sub>2</sub> concentrations. *Plant, Cell & Environment* **25**, 359–368. doi:10.1046/j.0016-8025.2001.00815.x
- Lewis JD, Lucash M, Olszyk DM, Tingey DT (2004) Relationships between needle nitrogen concentration and photosynthetic responses of Douglas fir seedlings to elevated CO<sub>2</sub> and temperature. *New Phytologist* **162**, 355–364. doi:10.1111/j.1469-8137.2004.01036.x
- Lewis JD, Ward JK, Tissue DT (2010) Phosphorus supply drives nonlinear responses of cottonwood (*Populus deltoides*) to increases in CO<sub>2</sub> concentration from glacial to future concentrations. *New Phytologist* **187**, 438–448. doi:10.1111/j.1469-8137.2010.03307.x
- Lin J, Jach ME, Ceulemans R (2001) Stomatal density and needle anatomy of Scots pine (*Pinus sylvestris*) are affected by elevated CO<sub>2</sub>. *New Phytologist* **150**, 665–674. doi:10.1046/j.1469-8137.2001.00124.x
- Logan BA, Hricko CR, Lewis JD, Ghannoum O, Phillips NG, Smith R, Conroy JP, Tissue DT (2010) Examination of pre-industrial and future [CO<sub>2</sub>] reveals the temperature-dependent CO<sub>2</sub> sensitivity of light energy partitioning at PSII in eucalypts. *Functional Plant Biology* **37**, 1041–1049. doi:10.1071/FP101113
- Marchi S, Tognetti R, Minnoccini A, Borghi M, Sebastiani L (2008) Variation in mesophyll and photosynthetic capacity during leaf development in a deciduous mesophyte fruit tree (*Prunus perisca*) and an evergreen sclerophyllous Mediterranean shrub (*Olea europaea*). *Trees – Structure & Function* **22**, 559–571. doi:10.1007/s00468-008-0216-9
- Medlyn BE, Barton CVM, Broadmeadow MSJ, Ceulemans R, De Angelis P, Forstreuter M, Freeman M, Jackson SB, Kellomaki S, Laitat E, Rey A, Robertz P, Sigurdsson BD, Strassmeyer J, Wang K, Curtis PS, Jarvis PG (2001) Stomatal conductance of forest species after long-term exposure to elevated CO<sub>2</sub> concentration: a synthesis. *New Phytologist* **149**, 247–264. doi:10.1046/j.1469-8137.2001.00028.x
- Melillo JM, McGuire AD, Kicklighter DW, Moore B, Vorosmarty CJ, Schloss AL (1993) Global climate change and terrestrial net primary production. *Nature* **363**, 234–240. doi:10.1038/363234a0
- Niinemetts U, Díaz-Espejo A, Flexas J, Galmés J, Warren CR (2009) Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. *Journal of Experimental Botany* **60**, 2249–2270. doi:10.1093/jxb/erp036
- Norby RJ, Wullschlegel SA, Gunderson CA, Johnson DW, Ceulemans R (1999) Tree responses to rising CO<sub>2</sub> in field experiments: implications for the future forest. *Plant, Cell & Environment* **22**, 683–714. doi:10.1046/j.1365-3040.1999.00391.x
- Norby RJ, DeLucia EH, Gielen B, Calfapietra C, Giardina CP, Kings JS, Ledford J, McCarthy HR, Moore DJP, Ceulemans R, De Angelis P, Finzi AC, Karnosky DF, Kubiske ME, Lukac M, Pregitzer KS, Scarascia-Mugnozza GE, Schlesinger WH, Oren R (2005) Forest response to elevated CO<sub>2</sub> is conserved across a broad range of productivity. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18 052–18 056. doi:10.1073/pnas.0509478102
- Oksanen E, Riikonen A, Kaakinen S, Holopainen T, Vapaavuori E (2005) Structural characteristics and chemical composition of birch (*Betula pendula*) leaves are modified by increasing CO<sub>2</sub> and ozone. *Global Change Biology* **11**, 732–748. doi:10.1111/j.1365-2486.2005.00938.x
- Pereira JS, Kozłowski TT (1976) Leaf anatomy and water relations of *Eucalyptus camaldulensis* and *E. globulus* seedlings. *Canadian Journal of Botany* **54**, 2868–2880. doi:10.1139/b76-308
- Poorter H, Evans JR (1998) Photosynthetic-nitrogen use efficiency of species that differ inherently in specific leaf area. *Oecologia* **116**, 26–37. doi:10.1007/s004420050560
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *BBA – Bioenergetics* **975**, 384–394. doi:10.1016/S0005-2728(89)80347-0
- Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO<sub>2</sub> and plant structure: a review. *Global Change Biology* **5**, 807–837. doi:10.1046/j.1365-2486.1999.00268.x



- Reich PB, Ellsworth DS, Walters MB (1998) Leaf structure (specific leaf area) modulates photosynthesis-nitrogen relations: evidence from within and across species and functional groups. *Functional Ecology* **12**, 948–958. doi:10.1046/j.1365-2435.1998.00274.x
- Rengifo E, Urich R, Herrera A (2002) Water relations and leaf anatomy of the tropical species, *Jatropha gossypifolia* and *Alternanthera crucis*, grown under an elevated CO<sub>2</sub> concentration. *Photosynthetica* **40**, 397–403. doi:10.1023/A:1022679109425
- Roden JS, Ball MC (1996) Growth and photosynthesis of two eucalypt species during high temperature stress under ambient and elevated [CO<sub>2</sub>]. *Global Change Biology* **2**, 115–128. doi:10.1111/j.1365-2486.1996.tb00056.x
- Roden JS, Egerton JJG, Ball MC (1999) Effect of elevated [CO<sub>2</sub>] on photosynthesis and growth of snow gum (*Eucalyptus pauciflora*) seedlings during winter and spring. *Australian Journal of Plant Physiology* **26**, 37–46. doi:10.1071/PP98134
- Sage RF, Coleman JR (2001) Effects of low atmospheric CO<sub>2</sub> on plants: more than a thing of the past. *Trends in Plant Science* **6**, 18–24. doi:10.1016/S1360-1385(00)01813-6
- Sage RF, Kubien DS (2007) The temperature response of C<sub>3</sub> and C<sub>4</sub> photosynthesis. *Plant, Cell & Environment* **30**, 1086–1106. doi:10.1111/j.1365-3040.2007.01682.x
- Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO<sub>2</sub> atmosphere. *New Phytologist* **139**, 395–436. doi:10.1046/j.1469-8137.1998.00221.x
- Saxe H, Cannell MGR, Johnsen B, Ryan MG, Vourlitis G (2001) Tree and forest functioning in response to global warming. *New Phytologist* **149**, 369–399. doi:10.1046/j.1469-8137.2001.00057.x
- Schimel DS, House JI, Hibbard KA, Bousquet P, Ciais P, *et al.* (2001) Recent patterns and mechanisms of carbon exchange by terrestrial ecosystems. *Nature* **414**, 169–172. doi:10.1038/35102500
- Sefton CA, Montagu KD, Atwell BJ, Conroy JP (2002) Anatomical variation in juvenile eucalypt leaves accounts for differences in specific leaf area and CO<sub>2</sub> assimilation rates. *Australian Journal of Botany* **50**, 301–310. doi:10.1071/BT01059
- Thomas JF, Harvey CN (1983) Leaf anatomy of four species grown under continuous CO<sub>2</sub> enrichment. *Botanical Gazette* **144**, 303–309. doi:10.1086/337377
- Thomas RB, Strain BR (1991) Root restriction as a factor in photosynthetic acclimation of cotton seedlings grown in elevated carbon dioxide. *Plant Physiology* **96**, 627–634. doi:10.1104/pp.96.2.627
- Tissue DT, Lewis JD (2010) Photosynthetic responses of cottonwood seedlings grown in glacial through future atmospheric [CO<sub>2</sub>] vary with phosphorus supply. *Tree Physiology* **30**, 1361–1372. doi:10.1093/treephys/tpq077
- Tissue DT, Oechel WC (1987) Response of *Eriophorum vaginatum* to elevated CO<sub>2</sub> and temperature in the Alaskan tussock tundra. *Ecology* **68**, 401–410. doi:10.2307/1939271
- Tissue DT, Thomas RB, Strain BR (1993) Long-term effects of elevated CO<sub>2</sub> and nutrients on photosynthesis and rubisco in loblolly pine seedlings. *Plant, Cell & Environment* **16**, 859–865. doi:10.1111/j.1365-3040.1993.tb00508.x
- Tissue DT, Griffin KL, Thomas RB, Strain BR (1995) Effects of low and elevated CO<sub>2</sub> on C<sub>3</sub> and C<sub>4</sub> annuals. 2. Photosynthesis and leaf biochemistry. *Oecologia* **101**, 21–28. doi:10.1007/BF00328895
- Vu JCV (2005) Acclimation of peanut (*Arachis hypogaea* L.) leaf photosynthesis to elevated growth CO<sub>2</sub> and temperature. *Environmental and Experimental Botany* **53**, 85–95. doi:10.1016/j.envexpbot.2004.03.006
- Wang KY, Kellomäki S, Zha T (2003) Modifications in photosynthetic pigments and chlorophyll fluorescence in 20-year-old pine trees after a four-year exposure to carbon dioxide and temperature elevation. *Photosynthetica* **41**, 167–175. doi:10.1023/B:PHOT.0000011948.00870.db
- Ward JK, Strain BR (1997) Effects of low and elevated CO<sub>2</sub> partial pressure on growth and reproduction of *Arabidopsis thaliana* from different elevations. *Plant, Cell & Environment* **20**, 254–260. doi:10.1046/j.1365-3040.1997.d01-59.x
- Wilson PJ, Thompson K, Hodgson JG (1999) Specific leaf area and leaf dry matter content as alternative predictors of plant strategies. *New Phytologist* **143**, 155–162. doi:10.1046/j.1469-8137.1999.00427.x
- Woodward FI, Lake JA, Quick WP (2002) Stomatal development and CO<sub>2</sub>: ecological consequences. *New Phytologist* **153**, 477–484. doi:10.1046/j.0028-646X.2001.00338.x
- Wright IJ, Westoby M (2000) Cross-species relationships between seedlings relative growth rate, nitrogen productivity and root vs leaf function in 28 Australian woody species. *Functional Ecology* **14**, 97–107. doi:10.1046/j.1365-2435.2000.00393.x
- Wright IJ, Reich PB, Cornelissen JHC, Falster DS, Garnier E, Hikosaka K, Lamont BB, Lee W, Oleksyn J, Osada N, Poorter H, Villar R, Warton DI, Westoby M (2005) Assessing the generality of global leaf trait relationships. *New Phytologist* **166**, 485–496. doi:10.1111/j.1469-8137.2005.01349.x
- Zeppel MJB, Lewis JD, Chaszar B, Smith RA, Medlyn BE, Huxman TE, Tissue DT (2011) Nocturnal stomatal conductance responses to rising [CO<sub>2</sub>], temperature and drought. *New Phytologist* doi:10.1111/j.1469-8137.2011.03993.x
- Zha T, Ryppö A, Wang K, Kellomäki S (2001) Effects of elevated carbon dioxide concentration and temperature on needle growth, respiration and carbohydrate status in field-grown Scots pines during the needle expansion period. *Tree Physiology* **21**, 1279–1287.