

CO₂ enrichment predisposes foliage of a eucalypt to freezing injury and reduces spring growth

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ABSTRACT

Seedlings of *Eucalyptus pauciflora*, were grown in open-top chambers fumigated with ambient and elevated [CO₂], and were divided into two populations using 10% light transmittance screens. The aim was to separate the effects of timing of light interception, temperature and [CO₂] on plant growth. The orientation of the screens exposed plants to a similar total irradiance, but incident during either cold mornings (east-facing) or warm afternoons (west-facing). Following the first autumn freezing event elevated CO₂-grown plants had 10 times more necrotic leaf area than ambient CO₂ plants. West-facing plants had significantly greater (25% more) leaf damage and lower photochemical efficiency (F_v/F_m) in comparison with east-facing plants. Following a late spring freezing event east-facing elevated CO₂ plants suffered a greater sustained loss in F_v/F_m than west-facing elevated CO₂- and ambient CO₂-grown plants. Stomatal conductance was lower under elevated CO₂ than ambient CO₂ except during late spring, with the highest leaf temperatures occurring in west-facing plants under elevated CO₂. These higher leaf temperatures apparently interfered with cold acclimation thereby enhancing frost damage and reducing the ability to take advantage of optimal growing conditions under elevated CO₂.

Key-words: *Eucalyptus pauciflora*; chlorophyll fluorescence; cold acclimation; elevated CO₂; frost damage; leaf temperature; photosynthesis.

INTRODUCTION

It is well established that plants grown under elevated CO₂ exhibit enhanced photosynthesis and biomass production at least in the short to medium term (see Drake, Gonzalez-Meler & Long 1997). However the impact of the complex interaction between timing of light interception, tempera-

ture and elevated CO₂ on plant photosynthesis and growth is less well documented. With the predicted changes in climate, specifically, doubling [CO₂] and increasing temperature combined with more frequent temperature extremes that are likely to occur over the next century (Houghton *et al.* 2001) it is important to better understand how these environmental parameters will affect plant growth, survival and distribution. To this end we aimed to address the question: does exposure to elevated CO₂ alter physiological and growth responses to light and temperature?

Plant responses to elevated CO₂ are altered by temperature (Long 1991). The temperature dependence of both the rubisco-limited and electron transport-limited components of photosynthetic assimilation rates (A) are well known (Farquhar, von Caemmerer & Berry 1980; Medlyn *et al.* 2002). At lower temperatures A is limited by rubisco activity whereas at higher temperatures the rate of electron transport is limiting, although other components may also be affected at temperatures above 30 °C (e.g. increasing RuBP pool and decreasing activation of rubisco) (von Caemmerer 2000). The temperature response curve of A also changes with light intensity; over a 10-fold range in PPFD there is very little difference in A at temperatures under 10 °C, whereas A increases significantly with increasing PPFD at higher temperatures, up to 30 °C (von Caemmerer 2000). These components will also be differentially affected by [CO₂]. As [CO₂] rises carboxylation rates increase as CO₂ competitively inhibits the oxygenase reaction of rubisco. Therefore, photorespiratory CO₂ production will be reduced, thus increasing net CO₂ uptake (Farquhar *et al.* 1980). Long (1991) showed that light-saturated assimilation rates will be much more responsive to [CO₂] at warmer leaf temperatures (35% increase under elevated CO₂ at 30 °C) than at low leaf temperatures (4% increases under elevated CO₂ at 10 °C).

Previous studies have shown that plants may suffer the detrimental effects of cold and heat stress to a greater extent when grown under elevated CO₂ (Roden & Ball 1996; Lutze *et al.* 1998; Beerling *et al.* 2002). During warm periods that are optimal for loblolly pine growth, the efficiency of electron transport through photosystem II (PSII) was higher in elevated CO₂ than ambient CO₂-grown plants, but during the winter the situation was reversed as elevated CO₂ plants had lower electron transport rates

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(Hymus *et al.* 1999). Elevated CO₂ may amplify the interactive effects of low temperature and high light accelerating the rate of de-acclimation in freeze tolerance during spring thereby leaving plants more susceptible to frost damage events (Lutze *et al.* 1998; Terry, Quick & Beerling 2000). Studies of electrolyte leakage and ice-nucleation on evergreen and deciduous species have demonstrated significant increases in the minimum temperature at which leaf damage occurs when plants grown under elevated CO₂ are subjected to freezing temperatures (Lutze *et al.* 1998; Terry *et al.* 2000; Beerling *et al.* 2001). Evaluation of plant performance under elevated CO₂ must take into account temperature-dependent effects.

To separate the interactive effects of elevated CO₂ at high light and variable temperature conditions throughout the day and season, seedlings of the broad-leaf evergreen *Eucalyptus pauciflora* Sieb. Ex Spreng (snow gum) were grown within open-top chambers. The chambers were fumigated with air containing ambient and 2× ambient [CO₂], and seedlings were divided into east- and west-facing populations using shade screens. It was hypothesized that elevated CO₂ would amplify the effects, beneficial or detrimental, of east- or west-facing plants. It was predicted that: (1) east-facing plants would be less able to take advantage of the high light in the early morning due to the low temperatures, and would be at greater risk of frost damage; and (2) west-facing plants would be more likely to benefit from high light when temperatures are higher and would be protected from frost damage to a greater extent than east-facing populations. A third hypothesis was developed following a serendipitous finding that an early autumn freezing event resulted in significantly greater leaf damage under elevated than ambient CO₂ particularly in west-facing plants. Therefore it was hypothesized that: (3) growth under elevated CO₂ resulted in less cold acclimation and greater susceptibility to freezing damage because of higher leaf temperatures due to reduced stomatal conductance.

METHODS AND MATERIALS

Plant material and growth conditions

Seeds of *Eucalyptus pauciflora* Sieb. Ex Spreng. were collected from trees in the Gudgenby Valley, Australian Capital Territory (ACT), elev. 1000 m. Seeds were cold-stratified under moist conditions at 4 °C for 4 weeks before germinating on sand in a glasshouse. Seedling were grown for 2 weeks and then selected for uniformity of size and transferred to seedling tubes and grown outside under natural conditions in Canberra (elev. 600 m) for 3 months. After this time the plants were again selected for uniformity of size and leaf number, and transferred into larger (15 cm × 40 cm) open-bottom pots which were buried in the ground within open-top chambers (OTCs) and the soil surface around the pots was covered with a layer of straw. The pots were filled with soil from the surrounding area. The experiment began on 22 March 2001 and the final harvest was performed on 27 November 2001. However, a subsam-

ple of plants was left in the chambers until January 2002 to continue non-destructive measurements.

Open-top chambers

In a pasture near Bungendore in south-eastern Australia (35°15' S, 149°27' E; elev. 700 m), 10 OTCs were installed as five replicate pairs, flushed with air containing either ambient or 2× ambient [CO₂]. The [CO₂] in the chambers was monitored periodically using a portable gas exchange system (LI 6400; Li-Cor Inc., Lincoln, NE, USA) and averaged 652 µmol mol⁻¹ (95% confidence interval, 25.5 µmol mol⁻¹). The flow through the chambers was 1.61 m³ min⁻¹. Chambers were as described by Ashenden, Baxter & Rafarel (1992) and modified by Lutze *et al.* (1998) and Roden, Egerton & Ball (1999). Chambers were further modified for the current experiment by the placement of 10% transmittance shade screens in the middle of the chambers at a height of 0.5 m. These screens separated the plants (5–7 per side) into east- and west-facing populations that received direct light in either the morning or afternoon, respectively. The leaves of all plants were constrained using small wooden skewers to face either the east, for east facing plants or west, for west facing plants. This ensured that there was as little shading as possible.

Assessment of leaf frost damage

Necrotic leaf area was assessed using visual criteria. The leaf area of every leaf was determined using allometric data and the percentage of the necrotic area was estimated. Frost damage was assessed on 25 April following the first significant frost on 13 April and again on 26 September to determine how the freezing damage had effected the plants during the winter.

Chlorophyll fluorescence

The chlorophyll *a* fluorescence parameter F_v/F_m was measured weekly on exposed leaves from each side of the divided chambers, using a portable plant efficiency analyser (PEA; Hansatech, King's Lynn, UK). Following 30 min dark adaptation five replicate plants in each orientation and gas treatment were measured at 1030 h and then again at 1530 h.

Thermocouple measurements of leaf and air temperature

Temperature was measured on one leaf and of the adjacent air (less than 5 cm from the measurement leaf) in each treatment combination in three of the chamber pairs. Temperatures were measured with copper-constantan thermocouples (64 µm diameter) referenced against a PT-100 platinum resistance thermometer. Thermocouples were attached to the leaf lamina on the inward facing surface using a small piece of surgical tape, so were not exposed to any direct sunlight. Thermocouples were scanned every 10 s

and a 15-min average was recorded on a DT 100 data-logger (Data Electronics, Rowville, Victoria, Australia). Reference measurements were made in a weather screen 1.2 m above the ground located on the western side of the study site. Precipitation was recorded from weekly readings of a rain gauge near the weather screen.

Gas exchange measurements

Photosynthetic CO_2 assimilation rates at saturating irradiance (A_{sat}) $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ were measured in the field with a portable gas exchange system (LI 6400; Li-Cor Inc.), fitted with a red/blue LED light source (6400-02B). Light saturation had been previously determined by Egerton *et al.* (2000). Measurements were made on one leaf from east- and west-facing plants in every chamber pair on 26 June, 11 September and 22 November 2001. East-facing leaves were measured between 0830 and 1100 h, whereas west-facing leaves were measured between 1300 and 1530 h. Times were chosen such that the integrated light dose experienced for east- and west-facing plants was near equal. The same leaves were used for CO_2 assimilation measurements as had been used to measure chlorophyll *a* fluorescence. To estimate plant carbon gain measurements were made at ambient temperature and at growth [CO_2] (either 350 or $700 \mu\text{mol mol}^{-1}$) in the cuvette.

Leaf spectral properties

Leaf transmittance (*T*) and reflectance (*R*) was measured and absorbance calculated ($A: A = 100 - \%T - \%R$) every 5 nm between 350 and 1100 nm using a portable spectroradiometer with an external integrating sphere connected by a quartz fibre-optic cable (LI-1800 with LI-1800-12S; Li-Cor Inc.). In spring (late October and November), measurements were made on young leaves and also on mature leaves from the previous year's growth under the two CO_2 treatments. In autumn (February to April) young plants that had developed outside the chambers at ambient CO_2 were transplanted into the chambers, and leaf spectra measured on mature leaves subsequently developed within the chambers 6 weeks later.

Stomatal density measurements

From plants growing in treatment for 11 months one youngest fully expanded leaf from each treatment combination in each block was selected in January 2002. Clear acrylic varnish was painted in a thin layer on both abaxial and adaxial surfaces of the attached leaves, creating a mirror image of the leaf surface. Once dry the layer of varnish was removed using clear adhesive tape, both varnish and tape were placed on a glass microscope slide and viewed under a binocular microscope at 40 \times objective magnification. Stomata were easily distinguishable from oil glands. Stomatal numbers in three distinct regions on each leaf surface were counted ($n = 15$).

Statistics

The randomised split plot design was utilized for the purpose of statistical analysis. Data were analysed by two-way analysis of variance (ANOVA) and appropriate post-hoc tests using Genstat (v. 6) (VSN, Hemel Hempstead, UK).

RESULTS

Environmental conditions

The daily minimum and maximum temperatures at the field site were recorded throughout the duration of the experiment, March to November 2001 (i.e. beginning of autumn to the end of spring) (Fig. 1a). On 13 April the first freezing event of the year occurred with a low of -5.8°C , temperatures rose to an afternoon high of 20.4°C giving a diurnal temperature range for that day of 26.2°C . The average daily maximum temperature was 16°C and the average minimum temperature was 2.1°C , with the last freezing event of the year taking place on 22 October. During the experimental period 330 mm of rain fell, slightly below the long-term average (Fig. 1b). East- and west-facing leaves intercepted similar integrated daily irradiance (Fig. 1c).

Photosynthetic efficiency and canopy loss following early season frost

Photosynthetic efficiency, F_v/F_m was depressed following the 13 April frost (Fig. 2a). F_v/F_m was 27% lower ($P = 0.05$) for west-facing elevated CO_2 plants than the average of the other treatments when measured 4 d after the frost event. There was no difference in F_v/F_m between east-facing ambient CO_2 and elevated CO_2 plants.

Leaf damage was estimated 12 d after the frost event that caused foliar necrosis (Fig. 2b). Median leaf damage was significantly higher ($P < 0.001$) for plants grown under elevated CO_2 in comparison with ambient CO_2 -grown plants (52 and 4% canopy loss, respectively). West-facing plants experienced, significantly greater damage (67% leaf damage) in comparison with east-facing plants (37% leaf damage), $P = 0.03$. The same pattern was revealed in the ambient CO_2 -grown plants yet the loss of leaf tissue was considerably lower (5% loss for west-facing and 3% loss for east-facing plants).

At the end of winter, surviving leaf tissue was estimated non-destructively to compare the net effect of frost damage on elevated CO_2 , vis-à-vis ambient CO_2 -grown plants (Fig. 2c). Elevated CO_2 -grown plants had significantly less surviving leaf area than plants grown under ambient CO_2 ($P = 0.02$). Further, the west-facing elevated CO_2 plants had significantly less leaf area than the east-facing plants ($P = 0.03$).

Leaf temperature

The surprising result described above, namely that frost damage was greater in west- than east-facing plants at elevated CO_2 , implies that difference in leaf temperature

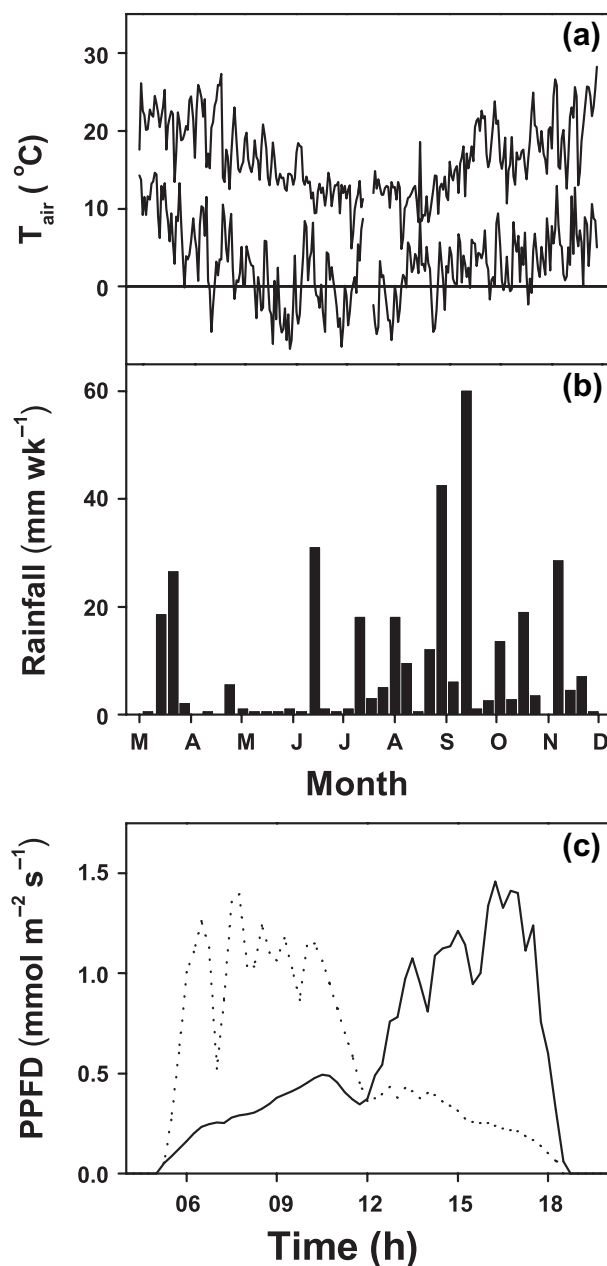


Figure 1. (a) Daily screen air temperature and (b) weekly rainfall from February to December 2001 at Bungendore, NSW and (c) an example of a daily time course of photosynthetic photon flux density (PPFD) measured on the east- (dotted line) and west-sides (solid line) of one OTC on 29 October 2001. The integrated light received on that day on the east-side was 29.28 mol m⁻² d⁻¹ and on the west-side was 30.59 mol m⁻² d⁻¹.

could affect acclimation to freezing. Thermocouples were placed on leaves to determine if plants growing under elevated CO₂ were warmer than ambient CO₂-grown plants. Figure 3a and b show leaf temperature for a single leaf in each of the treatments over the course of a single day (5 July 2001). East-facing plants had higher leaf temperature under elevated than ambient CO₂ during exposure to direct morning sunlight. Similarly, west-facing plants had higher

leaf temperature under elevated than ambient CO₂ during exposure to direct afternoon sunlight.

The difference in leaf minus air temperature (ΔT) (between 1100 and 1300 h on the east and 1300 and 1500 h on the west) was calculated and the observation divided into temperature classes. This made it possible to establish the most commonly observed difference in maximum leaf and air temperature (ΔT_m) (Fig. 4a & b) and to compare the distribution of differences between leaf and air temperature for all measurements made during each day (ΔT_a) for each of the treatments (Fig. 4c & d). ΔT_m was calculated from the absolute maximum leaf temperature minus the corresponding air temperature at that time. For east-facing

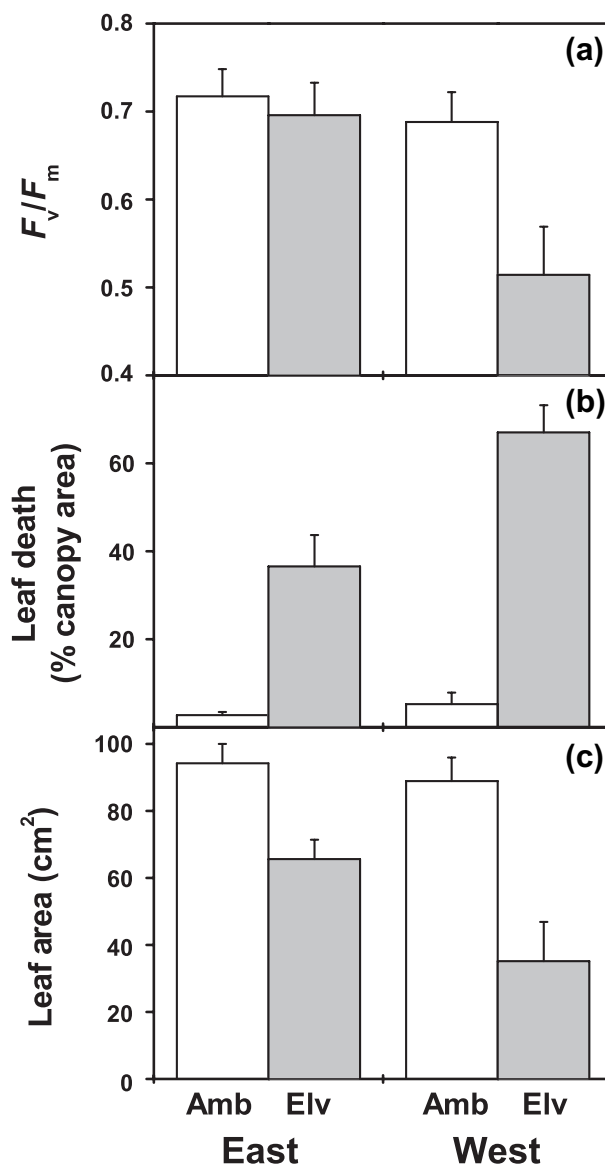


Figure 2. (a) Mean chlorophyll *a* fluorescence (dark-adapted F_v/F_m) measured in the morning for east-facing and in the afternoon for west-facing plants; (b) median leaf death as a percentage of total canopy area; and (c) median living leaf area of plants following the 13 April frost of east- and west-facing plants grown under elevated CO₂ and ambient CO₂. $n = 5$, bar = SEM.

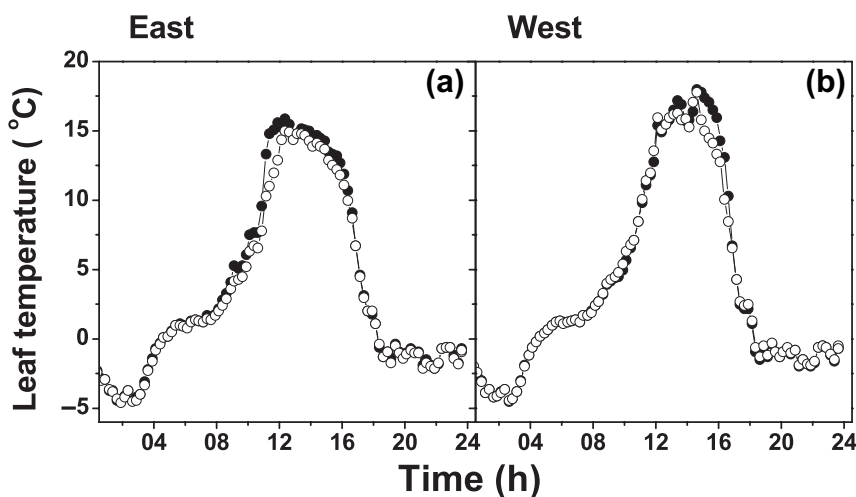


Figure 3. Diurnal temperature measurements of a single leaf growing under elevated CO_2 (solid circles) or ambient CO_2 (clear circles) of (a) east-facing or (b) west-facing plants. Data are from 5 July 2001.

plants the majority of observations of ΔT_m for both elevated CO_2 and ambient CO_2 treatments fell into the 1°C class (Fig. 4a). For west-facing plants the greatest number of observations for the ambient CO_2 -grown plants fell into the 2°C temperature class whereas for elevated CO_2 -grown plants most of the observation were in the 3°C class (Fig. 4b). When all data (ΔT_a) were considered, leaf temperature was most frequently 1°C above ambient air temperature (Fig. 4c & d). However, there was more frequent

occurrence of leaf temperatures ranging from 2 to 5°C above ambient air temperature under elevated CO_2 , with the effect being most pronounced in west-facing plants.

Stomatal density and leaf spectral characteristics

As leaf temperature was affected by our treatments we examined two sets of leaf attributes that may influence leaf

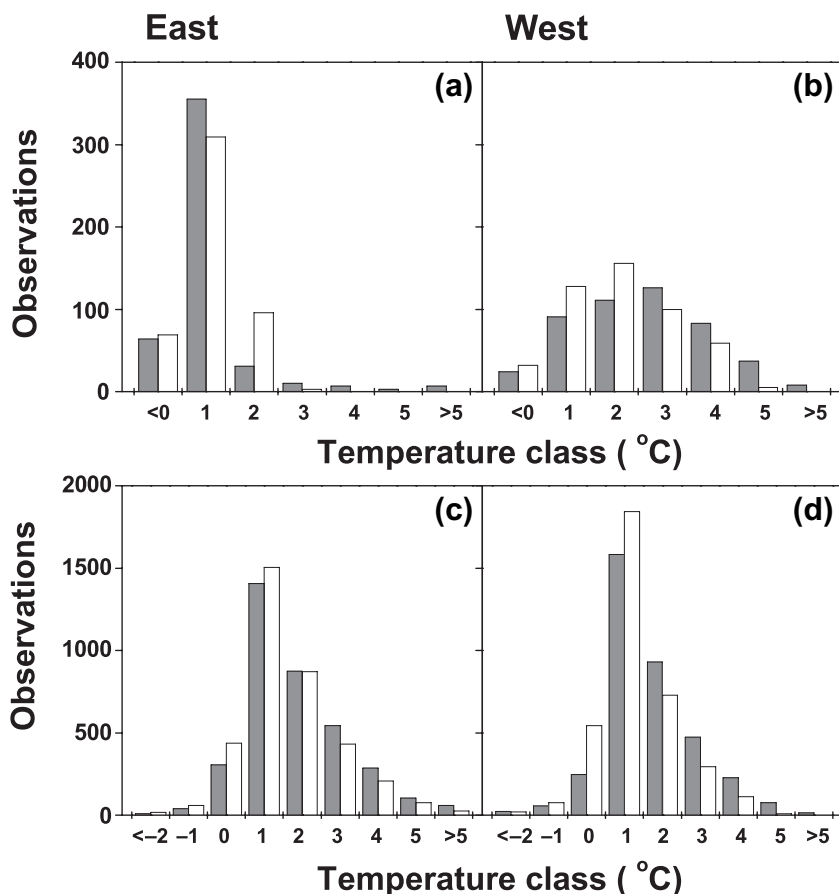


Figure 4. Observations of maximum leaf minus corresponding air temperature at a given time (ΔT_m) for all chambers ($n = 5$) were classified into temperature classes for elevated CO_2 (solid bars) and ambient CO_2 (clear bars)-grown plants, east-facing (a) and west-facing (b). The difference between all corresponding leaf and air temperature measurements (ΔT_a) was also analysed this way for elevated and ambient plants east-facing (c) and west-facing (d). As with Fig. 3 east data includes 1100–1300 h records while west data includes 1300–1500 h.

temperature: stomatal density and leaf spectral characteristics. It has been observed that stomatal density can change when plants are grown in elevated CO₂. Initial observations suggested that stomatal density declined in a CO₂-enriched atmosphere (Woodward 1987); however, accumulated observations show that plant responses are likely to be species specific. If stomatal densities were different between our treatments this may affect leaf temperature. In our study stomatal densities were unchanged by either growth [CO₂] or aspect: west elevated CO₂, $218.9 \mu\text{m}^{-2} \pm 22.3$, west ambient CO₂, $237.5 \mu\text{m}^{-2} \pm 25.5$, east elevated CO₂, $235.3 \mu\text{m}^{-2} \pm 21.0$, east ambient CO₂, $228.1 \mu\text{m}^{-2} \pm 11.7$.

There were no differences in the optical properties of leaves that might contribute to temperature variation between treatments. The only spectral difference between leaves developed under the two CO₂ treatments was a very small and transient decrease in transmittance at 555 nm for young leaves on the elevated CO₂ plants; this difference was evident in spring (October) but disappeared when the same leaves were re-measured 2 weeks later. By that stage, spectra taken for leaves developed under the two regimes were identical, as they were for mature leaves developed the previous year. For both young and mature leaves, and for both CO₂ treatments, the abaxial surface reflected slightly more radiation than the adaxial surface; this difference was most pronounced in the visible portion of the spectrum, but never more than a few percent.

Stem elongation and bud break

West-facing plants under elevated CO₂ were the slowest to respond with stem elongation and bud-break to increasing temperature in spring; this was probably a consequence of the severe frost damage suffered at the beginning of winter (Fig. 5a & b). Bud break of west-facing elevated CO₂ plants lagged behind ambient CO₂-grown plants throughout the measured period. However, by mid-November west-facing plants were able to take advantage of the benefits of growing under elevated CO₂ and stem elongation matched that of the ambient CO₂-grown plants. Stem elongation and bud break (Fig. 5a & b) of east-facing plants under elevated CO₂ was adversely affected by a late spring frost on 3 October (Fig. 1a) after which time plant growth of east-side plants greatly decreased.

Seasonal changes in photochemical efficiency

The values of F_v/F_m decreased with lower temperature and the approach of winter, and increased with rising temperature during the spring (c.f. Figs 6 & 1a). However, during autumn F_v/F_m was consistently lower for elevated CO₂ than ambient CO₂-grown plants of either orientation. As temperatures increased from winter into spring the F_v/F_m of east-facing plants under elevated CO₂ failed to recover following a late frost in September (Figs 1a, 6a & c). In contrast the F_v/F_m of west-facing elevated CO₂-grown plants recovered and by mid-September was not different than ambient CO₂-grown plants (Fig. 6b & d).

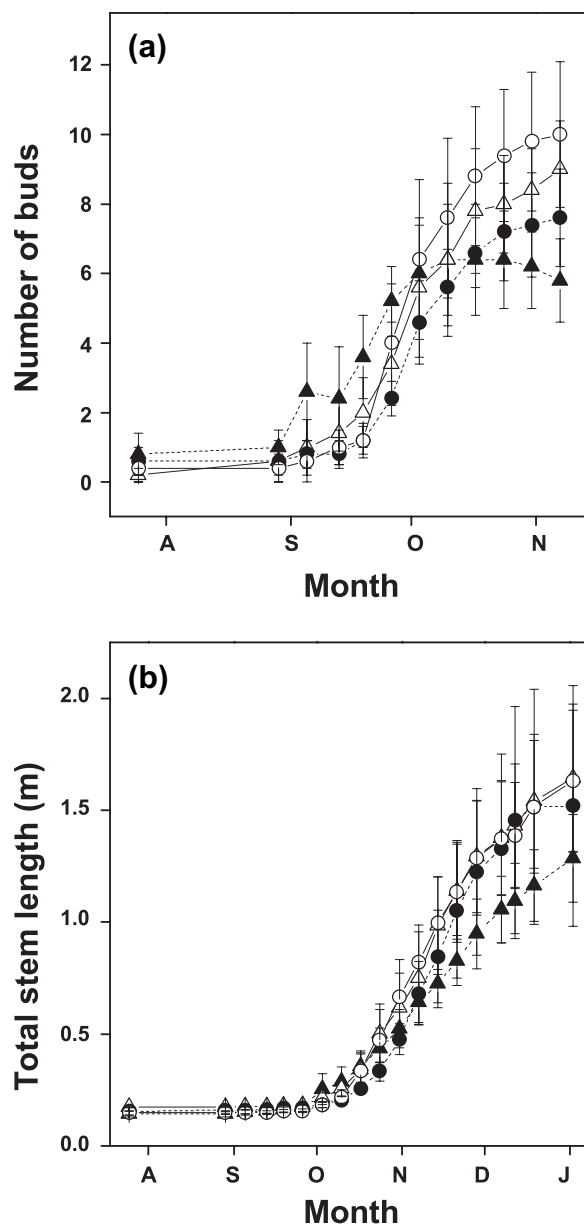


Figure 5. Number of buds (a) and total stem length (b) of plants growing under all treatments; elevated CO₂ east-facing (solid triangles), ambient CO₂ east-facing (clear triangles), elevated CO₂ west-facing (solid circles) and ambient CO₂ west-facing (open circles) $n = 5$, bar = SEM.

Carbon assimilation and stomatal conductance

When measured at growth [CO₂], light-saturated rates of assimilation (A_{sat}) for the east-facing plants were not affected by [CO₂] suggesting that down-regulation of photosynthesis under elevated CO₂ had occurred (Fig. 7). The A_{sat} of west-facing plants grown at elevated CO₂ was higher than the A_{sat} of plants growing at ambient CO₂ for all measurement periods. Stomatal conductance (g_s) was generally lower in plants grown at elevated CO₂ in comparison with ambient CO₂ plants. For the most part west-facing plants had lower g_s than east-facing plants. Stomatal conductance

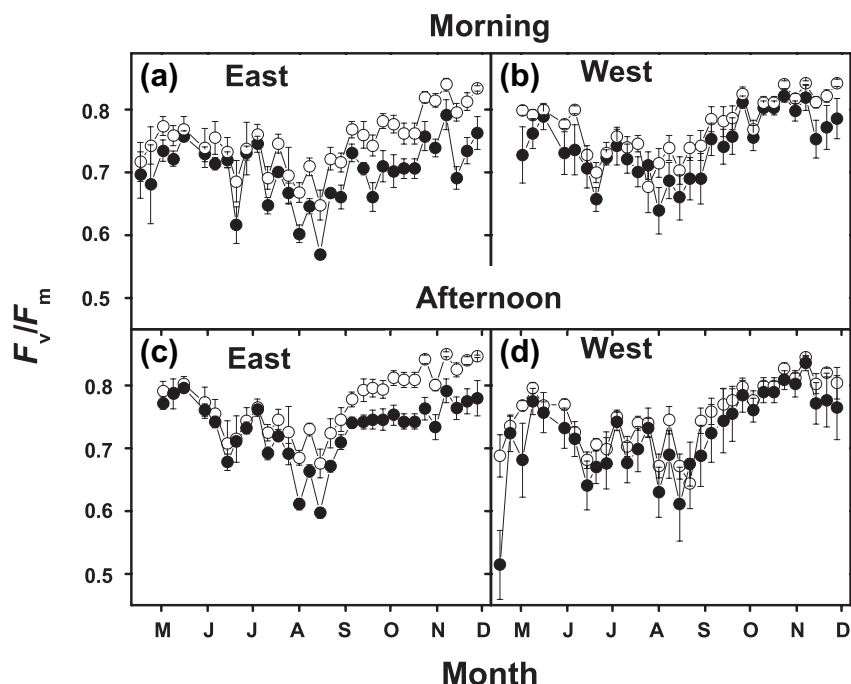


Figure 6. Seasonal patterns of intrinsic efficiency of photosystem II (dark-adapted F_v/F_m) for elevated CO_2 (solid circles) and ambient CO_2 -grown plants. For the first 2 weeks of measurement at the end of April F_v/F_m was only recorded on west-facing plants in the afternoon and on east-facing plants in the morning; by May measurements were taken in the morning and afternoon for both the east-facing (a and c) and the west-facing plants (b and d). $n = 5$, bar = SEM.

was significantly lower in winter and early spring ($P < 0.05$) for elevated CO_2 -grown plants but not in late spring when g_s was considerably higher, probably due to increased water availability and temperature (Fig. 7).

Biomass allocation and growth

By the final harvest at the end of spring (November) biomass accumulation indicated that the west-facing plants growing under elevated CO_2 had not completely recovered from the damage suffered from the initial freezing event in April (Fig. 8). Although leaf area was somewhat lower for elevated CO_2 compared to ambient CO_2 -grown plants, root dry mass was significantly lower ($P = 0.03$), there was also a moderately significant CO_2 by aspect interaction ($P = 0.06$). The significant reduction in root biomass accounted for half of the reduction in total biomass that was apparent in the west-facing elevated CO_2 plants. For east-facing plants there was no difference in biomass accumulation by late spring between elevated CO_2 and ambient CO_2 -grown plants.

DISCUSSION

This study shows that the enhanced growth that is often observed under elevated CO_2 in controlled environment studies may not always be exhibited when plants are exposed to other environmental factors that can constrain growth. The interaction between changes in physiology that occur as a result of exposure to elevated CO_2 and other environmental parameters is likely to be complex. Our first two hypotheses that east-facing populations would be less able to take advantage of high light in the early morning due to low temperatures and would also be at greater risk of frost damage than protected west-facing populations was supported, at least during the spring. Elevated CO_2 reduced

the ability of leaves to resist frost-damage particularly for west-facing plants in autumn (Fig. 2) and resulted in a decline in F_v/F_m for the east-facing plants in spring (Fig. 6). Frost damage lowered photochemical efficiency and reduced leaf area thereby diminishing the ability to take advantage of optimal growing conditions during spring, ultimately decreasing biomass production. The greater rate of carbon assimilation throughout the year of west-facing plants under elevated CO_2 (Fig. 7) did not compensate for tissue loss and the subsequent constraint on growth rate.

The increased freezing damage of west-facing elevated CO_2 leaves was unexpected. Our original prediction, based on the well-documented response of the biochemistry of photosynthesis to temperature, light and $[\text{CO}_2]$ was that west-facing leaves would be less likely to suffer freezing damage. Protected from the cold, high light conditions that are experienced by east-facing plants, west-facing plants would be expected to take greater advantage of elevated CO_2 during the warmer afternoons. Although CO_2 assimilation was enhanced for west-facing plants under elevated CO_2 , they were less prepared for a frost event in autumn and were therefore more susceptible to frost damage. We have previously shown that late spring frost damage occurs due to an acceleration in de-acclimation when snow gum was grown at elevated CO_2 (Lutze *et al.* 1998), and here we show that an apparent lag in acclimation as winter approached may have led to significant foliar damage during autumn frosts.

Our third hypothesis that growth under elevated CO_2 reduced stomatal conductance and thus increased leaf temperature, particularly of west-facing plants, leading to changes in timing of acclimation was also supported. Our data suggest that the reduction in g_s commonly observed in plants grown at elevated CO_2 (Drake *et al.* 1997; Curtis & Wang 1998; Nowak *et al.* 2000) was likely to result in higher

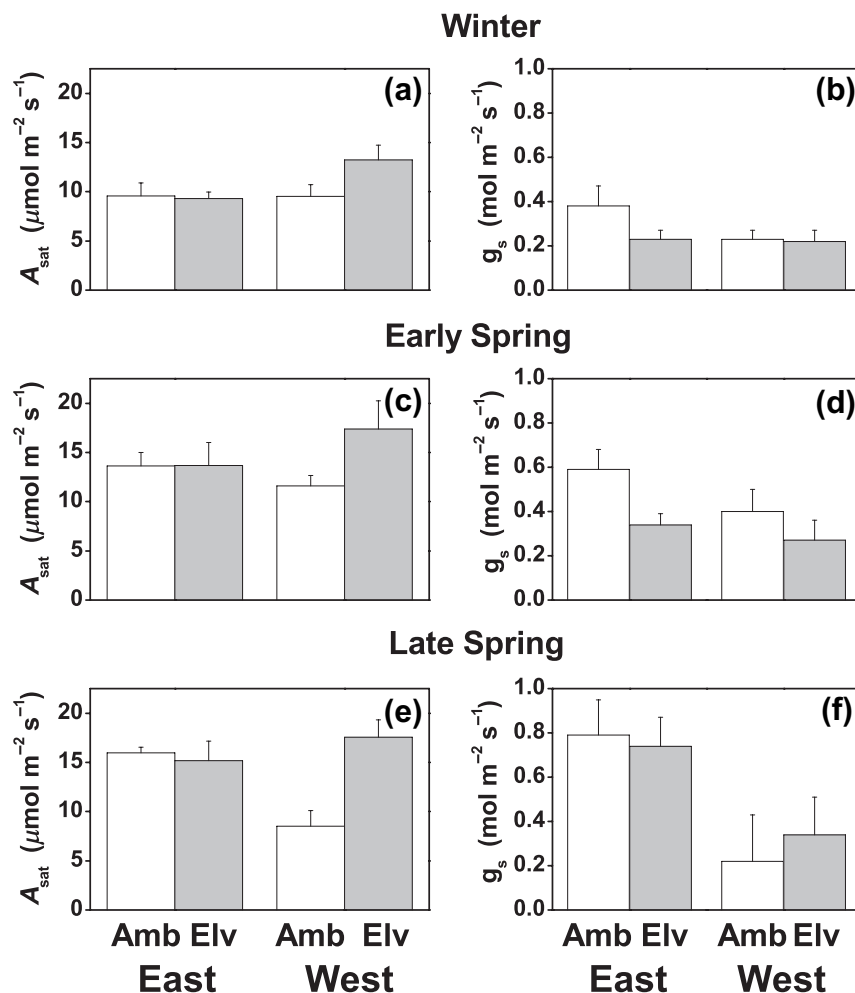


Figure 7. Light-saturated photosynthesis (A_{sat}) and stomatal conductance (g_s) measured in winter (a and b), early spring (c and d) and late spring (e and f) for plants growing under elevated CO₂ (solid bars) and ambient CO₂ (clear bars) east-facing and west-facing. $n = 5$, bar = SEM.

leaf temperature (Figs 3 & 4) (Siebke *et al.* 2002) possibly delaying the necessary signal transduction required to cold acclimate evergreen leaves. Acclimation to higher leaf temperature comes at the cost of low temperature acclimation and increased susceptibility to low temperature membrane damage. Data presented here revealed that reduced conductance and greater leaf temperature in west-facing, elevated CO₂-grown plants, may have increased their susceptibility to frost damage during autumn, suggesting that the signal for cold acclimation may have been delayed or altered. However, during the spring, it was predicted that warmer leaves may falsely signal the rapid advance of summer to elevated CO₂-grown plants, increasing the rate of premature de-acclimation and increasing the chance of frost-induced damage (Lutze *et al.* 1998). Similarly, seasonal F_v/F_m data (Fig. 6), bud count and stem elongation data (Fig. 5) suggested that east-facing plants growing under elevated CO₂ were more susceptible to early spring frosts due to premature de-acclimation.

Spectral and stomatal density data revealed no changes with respect to either growth [CO₂] or aspect. It is therefore likely that changes in leaf temperature were due primarily to changes in g_s . Phenology is considered one of the most sensitive plant traits to climate change (Sparks & Menzel 2002) and must be considered when assessing the impact of

our treatments on freeze tolerance and growth. However leaf turnover for *Eucalyptus* species is slow and occurs every 1–3 years, with the average leaf age of 18 months for evergreen eucalypts (Chapin 1980; Boland *et al.* 1984). As our experiment lasted only 8 months it is unlikely that there was any significant leaf turnover or change to leaf phenology that would have influenced our results.

In a natural environment there are additional factors relating to temporal separation of resources that must be taken into account when predicting plant growth. Wayne & Bazzaz (1993) proposed a hypothesis, the resource congruency hypothesis which states that plants often experience high light levels when other resources are limiting, particularly in forest gaps, therefore reducing potential photosynthetic carbon gain. This has important implications for the success of tree seedlings growing on east- or west-facing sides of forest fragments and is likely to result in an asymmetric growth response. The inevitability of the solar track of the sun across the sky results in cold, bright conditions prevailing for east-facing plants that are unlikely to be as favourable for photosynthesis and growth as the warmer high-light conditions characteristic of west-facing populations. In an ecological framework it therefore appears that the process of delayed acclimation is likely to have a greater impact for west-facing plants of forest fragments whereas

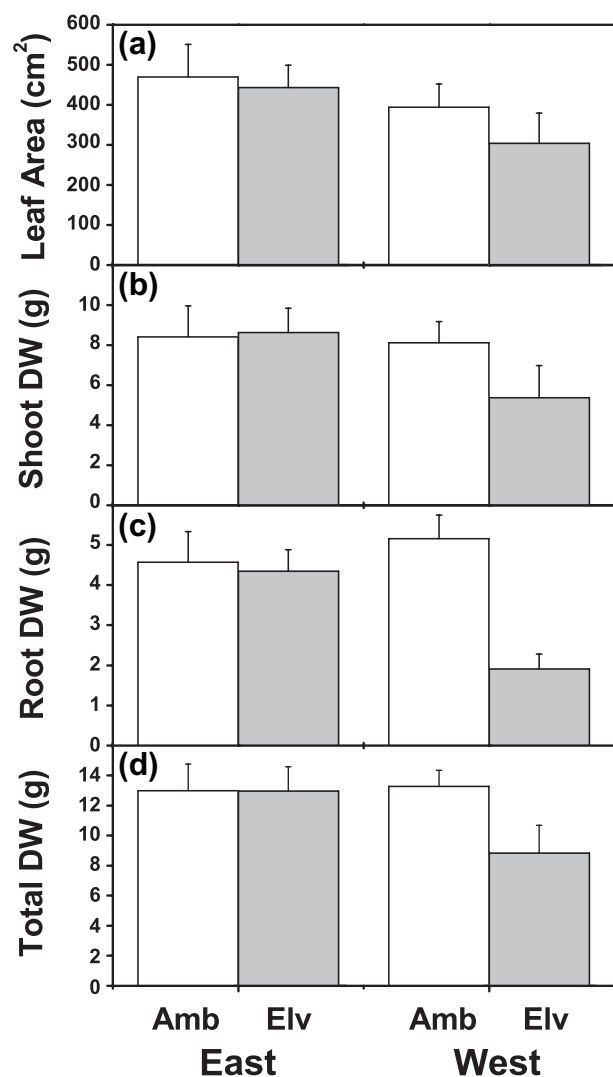


Figure 8. Final harvest in late spring (November 2001), leaf area (a), shoot dry weight (b), root dry weight (c) and total plant dry weight (d) for elevated CO₂ (solid bars) and ambient CO₂ (clear bars) east- and west-facing plants. $n = 5$, bar = SEM.

premature de-acclimation is more likely to affect the east-facing plants in forest fragments.

The literature documenting the interaction between elevated CO₂ and exposure to freezing temperatures shows contrasting results. Tolerance to freezing temperatures has been shown to increase in *Betula alleghaniensis* (Wayne, Reekie & Bazzaz 1998) and *Yucca brevifolia*, *Y. scidigera* and *Y. whipplei* (Loik *et al.* 2000). Conversely, freezing tolerance declines in *Pinus sylvestris* (Repo, Hanninen & Kellomaki 1996) *Ginkgo biloba* (Terry *et al.* 2000), *Eucalyptus pauciflora* (Lutze *et al.* 1998), *Vaccinium myrtillus* (Taulavuori *et al.* 1997), *Picea mariana* (Margolis & Vezina 1990), *Bromus erectus*, *Cirsium acaule*, *Sangisorba minor*, *Salvia pratensis* and *Trifolium medium* (Obrist, Arnone & Korner 2001). Finally there are also several reports of elevated CO₂ having no effect on freeze tolerance in evergreen species (Wiemken, Kossatz & Ineichen 1996; Dalen, Johnsen & Ogner 2001; Naumburg, Loik & Smith 2004).

Obviously there are species-specific changes in freeze tolerance at elevated CO₂ which may be related to the signalling mechanisms that lead to acclimation and de-acclimation of plants to freezing temperatures. For example, some species rely only on the gradual decline of non-freezing temperatures (e.g. *E. pauciflora*) to initiate cold acclimation (Tibbitts & Reid 1987), whereas other species respond to a combination of minimum temperatures and shortening day length (e.g. *P. abies*) to begin the acclimation process (Dalen & Johnsen 2004). Differences between studies may also be due to different experimental systems and designs as the list above includes results from field studies, open-top chambers and glasshouse experiments. As the mechanisms for acclimation are likely to be a complex combination of physiological and biochemical processes (Browse & Xin 2001), it is not surprising that contrasting responses to multiple factors are observed. If perception of gradually declining temperature is the most important signal to induce freeze tolerance, then the increased leaf temperature that we have reported for west-facing plants growing at elevated CO₂ will certainly delay the signal transduction for cold acclimation. However, for species that utilize a combination of environmental signals to initiate cold acclimation, their signal transduction pathways may be less influenced by increases in atmospheric [CO₂].

CONCLUSION

Effects of climate change on phenology for both evergreen and deciduous species through either early onset of bud break in spring, lengthening of growing season or delay in leaf senescence in autumn (see Walther 2003) have been widely interpreted as positive in relation to net CO₂ uptake. Specifically, there is likely to be an increase in the duration of time for plants to harness and utilize incident light, therefore potentially increasing global carbon sinks. However, for over-wintering plants, growth under elevated CO₂ and the resulting increase in leaf temperatures may cause a delay in the detection of environmental signals to initiate the cold-acclimation process resulting in plants being more susceptible to the inevitable frost events that precede winter in temperate and boreal forests. Therefore increases in carbon gain caused by changes in the timing of phenological events resulting from climate change are likely to be offset by potentially significant loss of photosynthetic tissue due to the enhanced susceptibility of elevated CO₂-grown plants to frost damage. This observation has broad-reaching consequences for forest growth dynamics and revegetation practices for disturbed lands.

ACKNOWLEDGMENTS

The authors would like to thank Mr Tim Hobbs and Mr Paul Sillis for permission to conduct experiments on their property, Mr Wayne Pippen for technical assistance and Drs John Evans, Dan Bruhn, Paul Kriedemann and Michael Robinson for comments on earlier versions of this manuscript. Dr John Evans is also thanked for the loan of

the spectroradiometer. Many thanks to Dr Dan Bruhn for providing OTC [CO₂] data.

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Received 14 March 2005; received in revised form 9 May 2005; accepted for publication 10 May 2005