Global Change Biology (2005) **11,** 1207–1219, doi: 10.1111/j.1365-2486.2005.00990.x

The effect of elevated CO_2 on diel leaf growth cycle, leaf carbohydrate content and canopy growth performance of *Populus deltoides*

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Abstract

Image sequence processing methods were applied to study the effect of elevated CO_2 on the diel leaf growth cycle for the first time in a dicot plant. Growing leaves of *Populus deltoides*, in stands maintained under ambient and elevated CO_2 for up to 4 years, showed a high degree of heterogeneity and pronounced diel variations of their relative growth rate (RGR) with maxima at dusk. At the beginning of the season, leaf growth did not differ between treatments. At the end of the season, final individual leaf area and total leaf biomass of the canopy was increased in elevated CO_2. Increased final leaf area at elevated CO_2 was achieved via a prolonged phase of leaf expansion activity and not via larger leaf size upon emergence. The fraction of leaves growing at 30–40% day^{-1} was increased by a factor of two in the elevated CO_2 treatment. A transient minimum of leaf expansion developed during the late afternoon in leaves grown under elevated CO_2 as the growing season progressed. During this minimum, leaves grown under elevated CO_2 decreased their RGR to 50% of the ambient value. The transient growth minimum in the afternoon was correlated with a transient depletion of glucose (less than 50%) in the growing leaf in elevated CO_2, suggesting diversion of glucose to starch or other carbohydrates, making this substrate temporarily unavailable for growth. Increased leaf growth was observed at the end of the night in elevated CO_2. Net CO_2 exchange and starch concentration of growing leaves was higher in elevated CO_2. The extent to which the transient reduction in diel leaf growth might dampen the overall growth response of these trees to elevated CO_2 is discussed.

Keywords: biomass, Biosphere 2 Laboratory, carbohydrates, elevated CO_2, gas exchange, image processing, leaf growth

Received 14 May 2004; revised version received 15 April 2005 and accepted 16 April 2005

Introduction

Although earlier studies showed that leaf growth of poplars under elevated CO_2 in free air CO_2 enrichment treatments was stimulated by both increased cell production and cell expansion, species of *Populus* differed in the relative extent of these two responses (Ferris *et al.*, 2001). In *Populus euramericana* leaf expansion was stimulated by elevated CO_2 in early and late stages of leaf development (Taylor *et al.*, 2003), but relationships between cell initiation and substrate supply for growth and expansion remain uncertain. Masle (2000) examined the importance of carbohydrate supply for leaf development under elevated CO_2 in wheat, but there have been few studies of these relationships in trees.

Photosynthesis of plants is usually enhanced in elevated CO_2; however, interactions with other factors such as temperature (e.g. Turnbull *et al.*, 2002) and

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nutrient availability (e.g. Kruse *et al*., 2003) alter the degree of photosynthetic enhancement. Moreover, acclimation takes place progressively and reduces photosynthetic enhancement (e.g. Stitt, 1991; Ainsworth *et al*., 2003). Assimilation in developing leaves is often more strongly stimulated than in mature leaves under elevated CO_2 (Pearson & Brooks, 1995; Miller *et al*., 1997; Wait *et al*., 1999). The predominant effect of elevated CO_2 on metabolites is an increase in concentrations of nonstructural carbohydrates such as starch, sucrose, glucose and fructose (Poorter *et al*., 1997). The relationship between leaf metabolite pools and leaf growth is complex, but ultimately determines leaf area and plant growth (Seneweera *et al*., 1995). Carbohydrates are utilized as substrates for growth processes such as cell wall extension and for respiration, which provides energy for metabolic processes involved in growth. Glucose is a key metabolite because it is the structural unit of cellulose and starch and is a respiratory substrate. The glucose pool is connected to the pools of fructose and sucrose, which is the central transport form of carbohydrates between sinks and sources. Often, carbohydrates are transiently stored in the chloroplast in the form of starch. Starch builds up during the day when photosynthates are available, and it is depleted during the night providing a buffer reservoir for the carbohydrate demand of the tissue (e.g. Geiger *et al*., 1998; Matt *et al*., 2001).

Taking into account the huge variability among species and experiments, overall plant growth usually shows a smaller response to elevated CO_2 than photosynthesis (Poorter & Navas, 2003). The mechanisms that modulate the relationship between leaf or plant growth and photosynthesis remain enigmatic, possibly because the two processes are often studied at vastly different time scales. While the dynamics of photosynthesis have been analyzed intensely, very few studies have dealt with short-term growth variations.

Shoots and leaves show clear diel growth rhythms that differ in phasing between species (e.g. Lecharny *et al*., 1985; Jouve *et al*., 1998; Walter & Schurr, 2005). It is known that most key enzymes and metabolic processes within a plant are governed by diel or circadian rhythms and can be switched on and off within short time frames. The dynamics of some of those regulatory mechanisms are affected by external CO_2 (Geiger *et al*., 1998; Matt *et al*., 2001), and the diel growth cycle is affected by the diel carbohydrate metabolism (Kehr *et al*., 1998; Walter *et al*., 2002). Furthermore, it is likely that additional carbohydrates, produced in elevated CO_2, enhance cell production and cell expansion (Masle, 2000). Thus, a strong effect of elevated CO_2 on amplitude and phasing of the diel leaf growth cycle is conceivable.

In the only study dealing with the effect of elevated CO_2 on phasing and amplitude of the diel leaf growth cycle, Seneweera *et al*. (1995) examined rice leaf growth with a small number of replicates and a relatively coarse method, but did not find growth differences that were attributable to the CO_2 treatments. Yet, they found a correlation between the phasing of diel growth rate, sucrose-phosphate synthase activity and soluble carbohydrate concentrations, further supporting an important role of carbohydrate metabolism for diel leaf growth control. With emerging optical techniques for growth measurements (Schmundt *et al*., 1998) it has now become feasible to investigate diel growth cycles of dicot leaves with relatively high spatial and temporal resolution. Thus, the central question examined here was whether there is a difference in the general phasing and shape of the diel growth cycle in leaves of *P. deltoides* grown at different levels of atmospheric CO_2. The experiments explore the importance of diel patterns of expansion growth in leaves of *P. deltoides* at ambient and elevated CO_2, and relationships to leaf photosynthesis and to carbon availability. The importance of these factors in determining the seasonal progression to a larger canopy leaf area under elevated CO_2 is discussed.

Materials and methods

Plants, cultivation and control of environmental parameters

P. deltoides Bartr. ex. Marsh (clone S7c8) was grown in three different compartments (bays) of the Biosphere 2 Laboratory with an area of 550–650 m^2, volumes of about 12 000 m^3 (the air handling system of each bay provided an air turnover of 4000 $m^3 min^{-1}$) and an average soil depth of approximately 1 m, allowing for tree heights of up to 15 m (Walter & Lambrecht, 2004). Stands were planted in 1999 with cuttings from a single clone (Westvaco, Summerville, SC, USA). In 2002 and 2003, 22 or more trees grew in each of the three bays. Until 2001/2002, trees were coppiced each year in late winter and resprouting was initiated the following April by increasing the bay temperature. Trees were not coppiced in 2002/2003, and leaf growth was initiated a month earlier than usual on established stems and branches, affording an opportunity to compare leaf growth under the two conditions. Temperature and humidity were controlled to simulate climate conditions in the southeastern US (Fig. 1). The soil nutrient conditions were similar to those of commercial forest plantation sites with a soil organic carbon content of 24 g kg^{-1} and a carbon : nitrogen ratio of 12 (Silverstone *et al*., 1999; Torbert & Johnson, 2001).

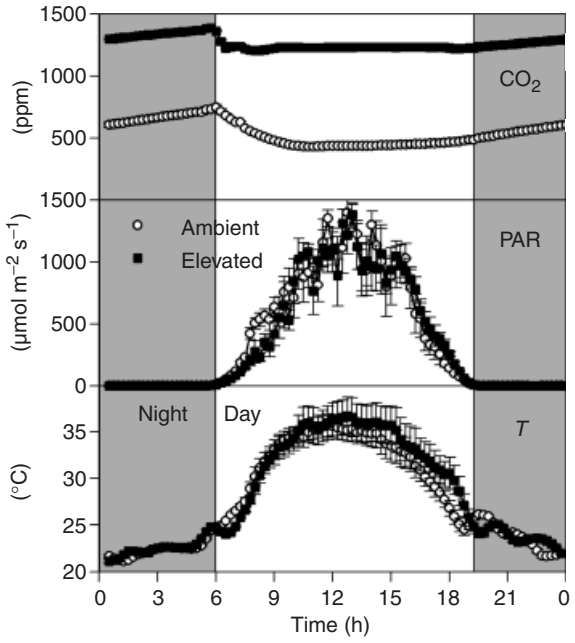

Fig. 1 CO_2 concentration, photosynthetically active radiation (PAR) and temperature conditions within the two experimental bays (August 2002, mean value and SE, $4 < n < 12$).

The bays were kept at daytime CO_2 levels of 400 ppm (east bay, 'ambient CO_2 treatment') and 1200 ppm (west bay, 'elevated CO_2 treatment'), respectively (Fig. 1), by controlled CO_2 injection via Sierra mass flow controllers (60 L min^{-1}) using Li-Cor 'Gashound' infrared gas analyzers (Li-Cor, Lincoln, NE, USA). These bays were separated by a buffer center bay kept at 800 ppm, but we did not examine this stand in the experiments reported here. The air-mass exchange and the exchange of water vapor and CO_2 with the outside atmosphere were monitored and quantified continuously (Rosenthal *et al*., 1999). Water, purified via reverse osmosis, was used for irrigation via rotary sprinkler heads mounted high in the roof-support system (space frame) of each bay. Atmospheric vapor pressure deficit was lowered using high pressure fogging nozzles and raised by removing water vapor as condensate in the air handling system. Incident light levels in the bays reached maximal values of up to 1500 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (Fig. 1). The space frame structure and the laminated glass reduced the irradiation in the biomes to about 50% of the incident irradiation and blocked out UV radiation completely. Because of the N–S orientation of the three adjacent bays, irradiation and temperature was slightly higher in the elevated CO_2 treatment in the late afternoon. Averaging over all available 12 sensors per bay, the temperature difference at 16:00 hours was usually 1 °C and rarely exceeded 2 °C throughout the afternoon (Fig. 1). Statistical analyses revealed that the temperature difference between the bays at 16:00 hours was not significant (P>0.05). The variation between different locations within each bay was generally larger than the variation between bays. Because of the arrangement of the leaf growth measurement setup, it was not possible to measure leaf temperature. Hence, leaves for growth measurements were chosen at random positions in each bay and from relatively open sites to ensure maximal air circulation. Average daytime temperatures were practically identical for all bays throughout the entire growth period (Barron-Gafford *et al*., 2005). Throughout each bay, CO_2 concentration, temperature, vapor pressure deficit and irradiation were measured at several locations and at different heights. Temperature and vapor pressure deficit (vpd)-control were maintained at identical values in both treatments (Murthy *et al*., 2005). Environmental data were collected by Campbell Scientific CR 10X data loggers and managed by Campbell Scientific Loggernet software (Campbell Scientific, Logan, UT, USA).

Measurement of leaf growth with digital image sequence processing

The diel cycle of leaf growth was monitored in newly emerged leaves of the lower canopy by a digital image sequence processing method (Schmundt *et al*., 1998; Walter & Schurr, 2005). In this procedure, a leaf was fixed to the focal plane of a CCD-camera (640 × 480 pixels; equipped with an 880 nm interference filter; Schott, Mainz, Germany) and illuminated with infrared diodes (880 nm) throughout day and night. The attached leaf was stretched perpendicularly to the optical axis of the camera with weights that were attached to the leaf border via threads. Weights were chosen carefully so as not to enhance or diminish leaf growth (Walter *et al*., 2002). The leaf was covered with an infrared cut off filter (Edmund Scientific, Tucson, AZ, USA), which eliminated the infrared part of the sunlight (>800 nm) and ensured constant image brightness. This filter reduced the light intensity on the investigated leaf to about 70% of the incident PFD in the bay. Near-infrared images of the adaxial side of the leaf lamina were captured every 3 min and were evaluated with algorithms written in a digital image processing macrolanguage (Heurisko, Aeon, Hanau, Germany), rendering maps of relative growth rate (RGR) distribution on the leaf for each image. Growth rate distributions were calculated using a structure-tensor approach (Haußecker & Spies, 1999). In short, the movement of all distinguishable gray value structures within an image sequence (such as vein intersections, internal leaf structures, hairs) led to the calculation of a dense velocity field. Then, velocities of

all individual pixels comprising the image were assigned. RGRs were calculated by taking the divergence of this velocity field (differences of x and y velocities across the borderline of each selected region on the leaf, normalized to the area of the selected region).

Time series of the average RGR (measured in $\%\,h^{-1}$) were extracted as required from the image sequences. Since two setups were used, growth of one leaf in ambient CO_2 and one leaf in elevated CO_2 was continually monitored from May to September 2002. The setups were transferred to a newly emerged leaf every 2–7 days. For the purpose of this study, full emergence was defined as the time when a developing leaf was fully unrolled and all of its lamina was visible. At this time, leaves were typically between 30 and 50 mm long.

Shading experiments were performed to estimate the effect of the partial shading exerted by the infrared cut off filter that was technically necessary for image acquisition (constant image brightness at 880 nm throughout day and night). It was possible to study the effect of reduced photosynthesis on the expansion process of the growing leaf. Shaded leaves were covered with a tent-like construction using black plastic foil that was open at the bottom to ensure air ventilation, reducing PFD to less than 10% of incident light in the bay. Growth of shaded leaves in elevated CO_2 was recorded with a third setup in July and August 2002.

Thirty diurnal courses were acquired each month in each treatment, but only three to 12 were reported in the monthly averages. Leaves with growth rates smaller than $10\%\,day^{-1}$ were not used for data evaluation, as the signal-to-noise ratio was too low. Many image sequences were rejected because of technical problems (e.g. loss of power, unscheduled rain events, insect damage). The raw data of the imaging growth results were normalized to compare leaves from different developmental stages, as described below. The coefficient of variation of the diel growth cycle was calculated as a measure for the amplitude or variance of growth throughout 24 h by computing the standard deviation of all 1 h average values within a 24 h day and relating this to the average RGR during this day.

Manual measurement of leaf growth

Leaf growth was followed manually in June 2002 (6 weeks after resprouting, $n = 100$ leaves per treatment) and September 2002 (4 months after resprouting, $n = 120$ leaves per treatment), by measuring length and width of postemergent leaves with a ruler every second or third day over a period of 3 weeks. Leaf area was calculated as

$$\text{Leaf area} = \text{length} \times \text{width} \times 0.61,$$

where 0.61 was a geometric factor determined via calibration measurements ($n = 30$) of differently sized leaves. RGRs were calculated from area measurements at consecutive days as

$$\text{RGR} = \ln(A2/A1)/\mathrm{d}t,$$

where $A2$ is area at measurement time 2, $A1$ area at measurement time 1, and $\mathrm{d}t$ time between measurements.

In July, the diel course of leaf growth was followed by measuring leaf length every 4 h ($n = 100$ per treatment). The diel leaf growth cycles determined in these measurements matched those obtained with the imaging system (data not shown), confirming that the forces chosen for leaf mounting were appropriate (Walter *et al*., 2002).

Biomass measurements

At the end of the season, trees were coppiced and measured for branch, stem and leaf biomass. Fresh and dry weight was obtained for $n = 9$ trees per bay, while fresh weight was measured on all trees. Regressions from the subset of nine trees were used to calculate total aboveground biomass for each stand. Specific leaf area on a subset of trees coupled with dry weight of all leaves was used to determine total leaf area in each bay (for more details see Barron-Gafford *et al*., 2005).

Leaf gas exchange and carbohydrate analysis

These analyses were done on 'growing' (first fully unrolled leaf, RGR: $30 \pm 10\%\,day^{-1}$) and 'mature' (fully expanded, six to nine positions below 'growing' leaf) leaves in the growing season in 2003. Leaf gas exchange was measured with a portable open-flow gas exchange system (Li-Cor 6400, Li-Cor, Lincoln, NE, USA) using either a standard $2 \times 3\,cm^2$ leaf chamber with ambient light or a closed leaf chamber with an internal light source (6400–02B LED Light Source, Li-Cor, Lincoln, NE, USA). CO_2 concentration, humidity and temperature within the leaf chamber were maintained at ambient conditions of the bay. Identical light intensities were used for measurements in each bay using the mean value at each time point (1200, 1100 and 675 μmol photons $m^{-2}\,s^{-1}$ PAR at 11:00, 13:00, and 15:00 hours, respectively). In diel measurements, the standard leaf chamber was clamped to a single leaf for 24 h.

Two sets of samples were taken for carbohydrate analyses. One set (three leaves per population) was harvested between 15:00 and 18:00 hours. Five disks of interveinal tissue (diameter = 7 mm), distributed along the lamina from base to tip, were sampled from each leaf, weighed, frozen in liquid nitrogen, stored at

−80 °C until further extraction and analyzed separately. Since no base-tip-gradient in carbohydrate composition was observed, data from the five disks were pooled after analysis. The second set of discs ($n = 3$ per population) was sampled every 4 h throughout 24 h; each sample contained one disk from the base and one from the tip.

Soluble sugars were exhaustively extracted from frozen leaf material in 400 µL ethanol/water (80 : 20 v/v, 2 mM HEPES) for 20 min at 80 °C. The supernatant was stored at 4 °C and the extraction was repeated first with 400 µL ethanol/water (50 : 50 v/v, 2 mM HEPES) and then with 200 µL ethanol/water (80 : 20 v/v, 2 mM HEPES) until the leaf disks were colorless. Glucose, fructose and sucrose were analyzed with a coupled enzyme assay (Jones *et al.*, 1977) using a multiplate photometer (ht II, Anthos Mikrosysteme GmbH, Krefeld, Germany).

The extracted leaf material was prepared for starch analysis by grinding it in 300 µL of distilled water with a stainless-steel ball (diameter 7 mm) in a mixer mill (MM200, Retsch GmbH & Co. KG, Haan, Germany), then washing the ball with additional 200 µL distilled water. The samples were autoclaved for 2 h at 120 °C, 1 bar. One hundred microliters of the autoclaved sample were incubated in 400 µL Na-acetate buffer (50 mM, pH 4.9 with 1.4 U amyloglucosidase and 1 U α-amylase) for 16 h at 37 °C. The starch content was then determined enzymatically as glucose concentration using the same procedure as described above for soluble sugars.

Statistical analysis

Comparisons between CO_2 treatments for air temperature, carbohydrate concentrations, RGR and final leaf area were performed using two-tailed Student's *t*-tests (software: Microsoft Excel).

Results

Leaf and canopy growth

Leaf and canopy growth were monitored throughout the entire growing season of 2002. In June 2002, manual growth measurements were made on newly emerged leaves on stems that had attained a height of about 2 m in the different treatments and were just beginning to branch (Fig. 2). Leaf RGR declined monotonically, independent of treatment, and these leaves did not differ significantly with respect to final leaf area ($P > 0.05$) or kinetics of growth, although a slightly higher RGR was found in early stages of leaf development in the elevated CO_2 treatment.

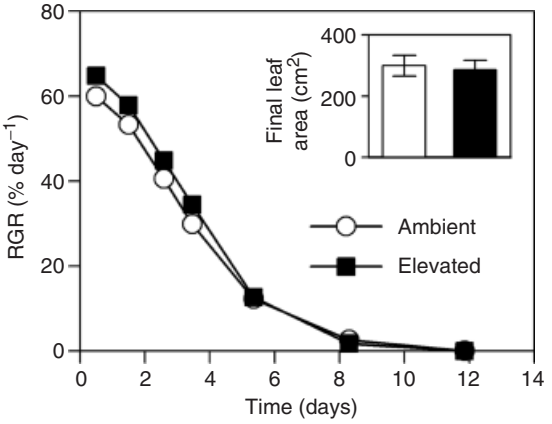

Fig. 2 Leaf growth at the beginning of the growth season in June 2002. Final leaf area and temporal development of the relative growth rate (RGR) in leaves of *Populus deltoides* from the main axis. Day 0 corresponds to leaf emergence (mean value and SE, $n = 10$).

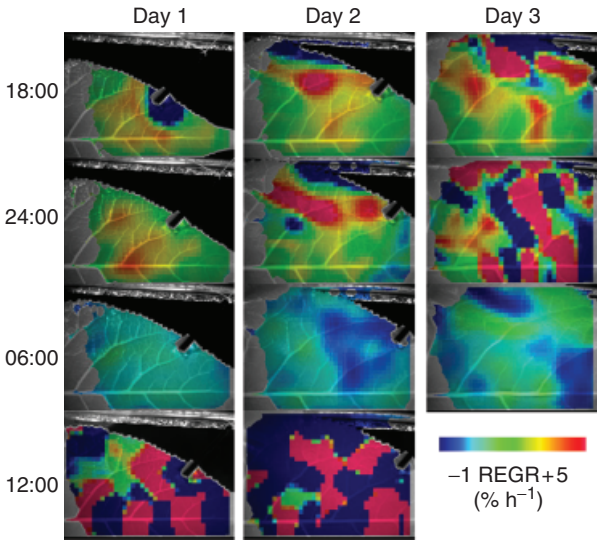

Fig. 3 Color-coded distribution of relative elemental growth rates (REGR) on the leaf blade of *Populus deltoides*. Data from a typical leaf at ambient CO_2 measured over 3 consecutive days in August 2002.

Imaging growth measurements were conducted between May and September 2002. The two most prominent features in sequences from both treatments were (a) a distinctive diel variation of growth activity (Fig. 3), with a maximum in the evening and a minimum in the morning, and (b) a high degree of small-scale growth heterogeneity (patchiness). Throughout the entire day, there were regions on the leaf lamina growing with high intensity and neighboring regions that were not growing at all (depicted in different colors in Fig. 3). These regions or patches

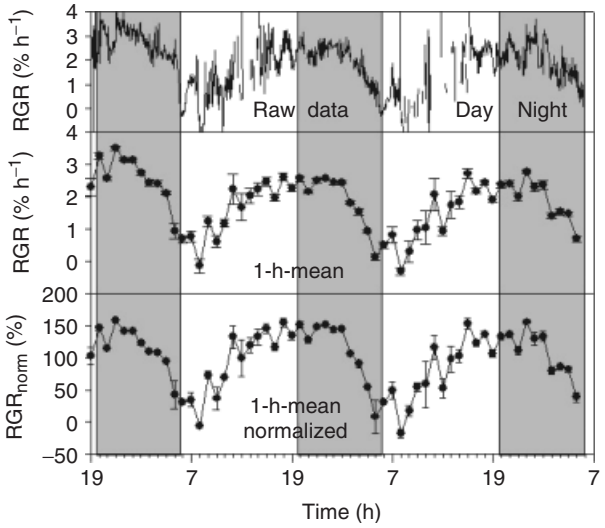

Fig. 4 Typical diel *Populus deltoides* leaf growth cycles over 3 consecutive days in August 2002 in ambient CO_2. Top panel: raw data (values calculated every 3 min), lower two panels: absolute values of 1 h average and normalized 1 h average data ($n = 20$ for each point; SE given as a measure of confidence).

did not maintain stable expansion rates for long periods of time and patchiness did not seem to differ between leaves from the two treatments. No strong base-tip growth gradient was present in leaves of either treatment. Detailed analysis of the patchiness of growth exceeds the scope of this study.

As the focus of this study was to examine diel variation of growth activity of entire leaves, the entire imaged leaf area was used to extract the averaged RGR of the leaf lamina every 3 min. Figure 4 shows the steps that are needed to transform the automated measurements of leaf growth to normalized diel patterns. Normalization was performed by calculating 1 h average values for the growth data and then relating those 1 –h averages to the average of a 24 h period. Normalization was necessary for comparisons between treatments because it was impossible to select leaves with exactly the same average RGR on a given day of the experiment from the two treatments because of the continuously declining growth rate of each individual leaf (Fig. 2). Minimum RGR was observed at the beginning of the day. RGR increased during the day, reached a maximum around dusk and decreased during the night (Figs 3–5). No prominent change in

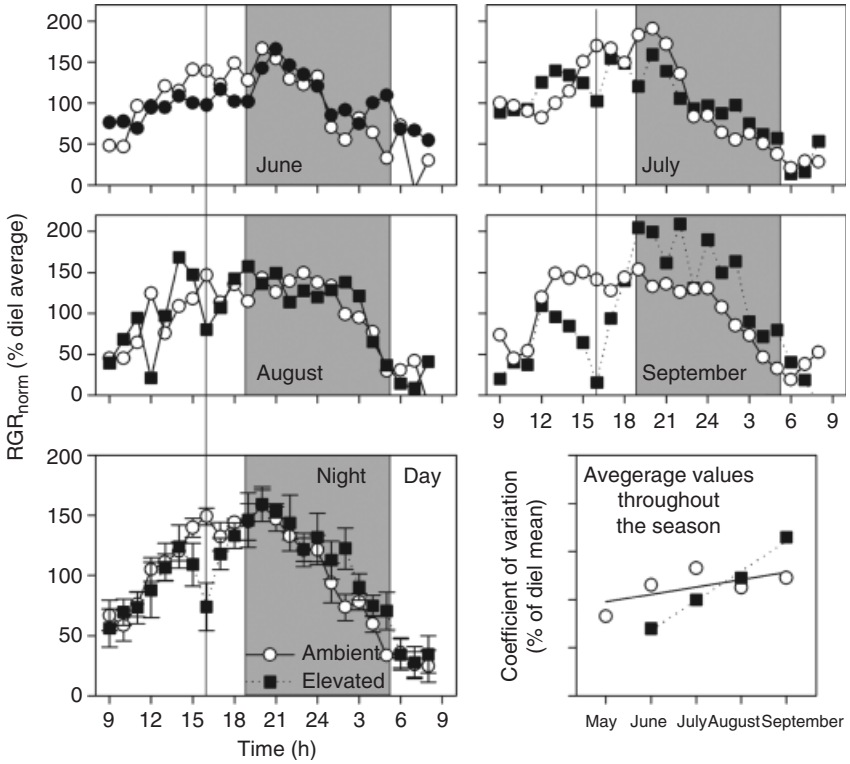

Fig. 5 Development of diel leaf growth cycle in *Populus deltoides* in ambient and elevated CO_2 throughout the growing season. Data show mean values for each treatment and each month (upper panels) as well as the seasonal average (lower left panel) for the normalized data (June: $n = 6$, 4 for ambient and elevated treatment, respectively; July: $n = 12$, 7; August: $n = 3$, 3; September: $n = 4$, 3; season average: $n = 25$, 17; $\pm$ SE). The lower right panel shows the coefficient of variation for each month and treatment as a measure of the amplitude and variance of the diel leaf growth cycle.

shape and phasing of the diel growth cycle was observed during the development of an individual leaf. Comparison of leaves from ambient and elevated CO_2 showed that in both treatments the diel cycle became more pronounced throughout the season, showing deeper troughs and higher peaks (increased coefficient of variation) towards the end of the season (Fig. 5). A pronounced, transient afternoon trough in leaf growth was observed in the elevated CO_2 treatment. It was compensated for by a higher growth rate in the second half of the night at elevated CO_2. The most pronounced difference between the growth curves of leaves from both treatments occurred at 16:00 hours, when growth rates of leaves from ambient CO_2 exceeded those of leaves from elevated CO_2 significantly ($P<0.001$) by a factor of two.

At the end of the growth season in September and October 2002, another growth analysis was performed using manual leaf area measurements on more than hundred leaves per treatment from different positions in the canopy (Fig. 6). A paired *t*-test for the mean leaf areas of each investigated position within the canopy revealed a significant difference between leaves from both treatments at this time of the season ($P<0.01$). Trees were then about 5 m tall and extensively branched. The overall area per leaf was 22% higher in elevated CO_2. There was a slight gradient in leaf area distribution from low to high branches within the tree. Leaves from high branches were about 20% larger compared with those from low branches. In ambient CO_2, leaves on the south side of the trees reached higher final areas than leaves from the slightly more shaded northern side; in elevated CO_2 it was vice versa. Leaves emerging directly from the main shoot of the tree were 27% larger in the high CO_2 treatment compared with the ambient treatment and reached a final leaf area twice that of leaves from the branches.

Higher final leaf area at elevated CO_2 was achieved via a prolonged phase of leaf expansion activity and not via larger sizes of the leaves upon emergence. Emergent

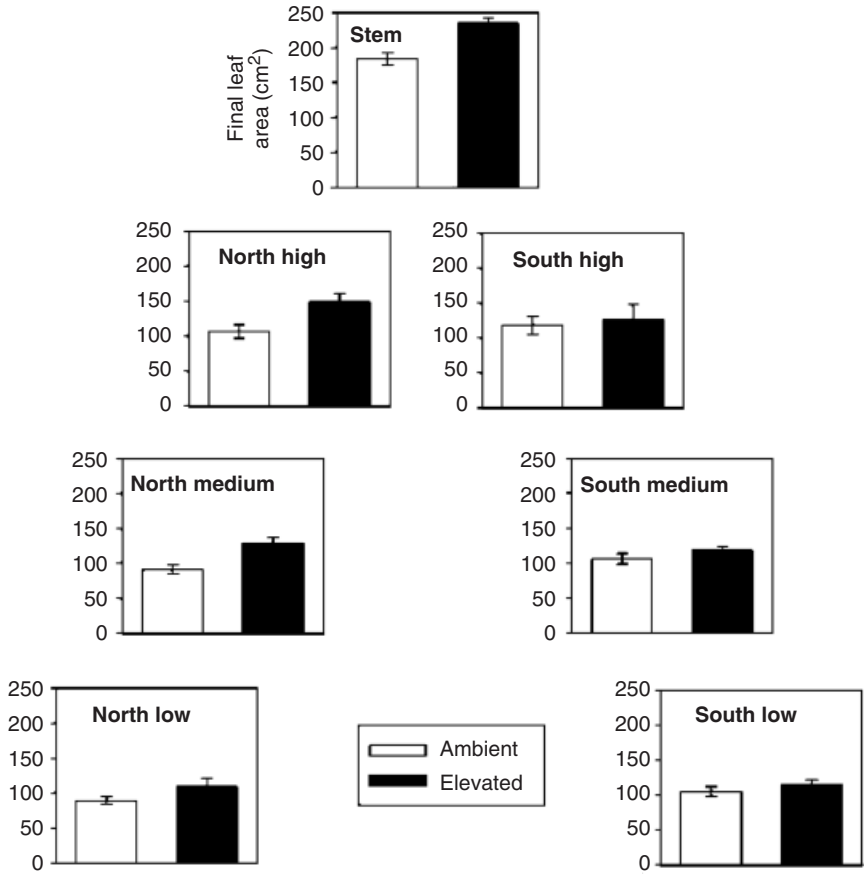

Fig. 6 Leaf growth at the end of the growth season in September 2002. Final leaf area of *Populus deltoides* at seven different positions on the tree (mean value and SE, $n \geq 10$). Leaves were positioned at branches inserting at 1 m (low), 2 m (medium), or 3 m (high) height along the stem (facing south or north) or directly at the tip of the stem (three trees per treatment). Growth of 10 leaves per branch was measured manually two times per week throughout a period of 3 weeks. Final leaf area was recorded for each of those leaves that reached the full grown state within the measurement period.

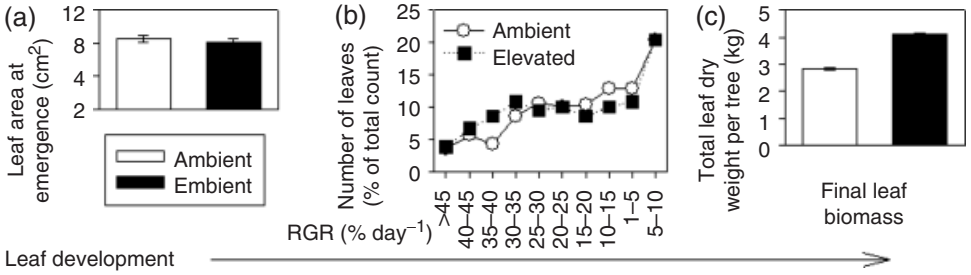

Fig. 7 Leaf growth at the end of the growth season in September 2002. (a) Leaf area at emergence in *Populus deltoides* (mean value and SE, $n = 10, 15$ for ambient and elevated treatment, respectively). (b) Histogram of leaf relative growth rate (RGR) distribution within the entire population of leaves measured as described in Fig. 6 ($n = 299, 385$). (c) Total leaf dry weight per tree at the end of 2002 (mean value and SE, $n = 25, 25$).

leaves had areas of 8.5 cm^2 on average, irrespective of treatment (Fig. 7a). Differences were found in the kinetics with which leaves reached the full-grown state. The kinetics were quantified by grouping all measured leaves, irrespective of their position within the canopy, according to their RGRs (Fig. 7b). Whereas leaves in ambient CO_2 showed a 'normal' spectrum of RGR distribution with monotonically increasing numbers towards the full-grown state, leaves from the high CO_2 treatment showed high frequencies in the upper part of the RGR scale, indicating a prolonged growth activity in an early stage of postemergent leaf development. There were about twice as many leaves growing at 30–40% day^{-1} in the elevated CO_2 treatment compared with the ambient CO_2 treatment.

The individual leaf area increase of 22% in elevated CO_2 (Fig. 6) matched the observed increase in leaf biomass per tree of 46% (Fig. 7c), as specific leaf area declined in the middle and upper part of the canopy in elevated CO_2 (Barron-Gafford *et al.*, 2005; in large part because of the accumulation of starch in leaves). Yet, specific leaf area did not differ between treatments in the lower part of the leaf canopy, which was used in our study to investigate diel growth cycles and carbohydrate concentrations. Over the 4 year experiment with *P. deltoides*, total leaf biomass production increased by 36% at elevated CO_2, and total aboveground biomass increased by 27.5% (Barron-Gafford *et al.*, 2005). Although we used an unusually high CO_2 treatment comparison, the stimulation of leaf and total aboveground biomass in plantations grown at 800 ppm was only slightly less than that at 1200 ppm (Barron-Gafford *et al.*, 2005).

Relationship to leaf photosynthesis and carbon availability

Experiments were performed with shaded leaves in order to estimate whether photosynthesis of the growing leaf itself can affect phasing and shape of the diel growth cycle (Fig. 8). An unusually low growth rate in the middle of the day and a strong temporal fluctuation of growth intensity was observed in shaded leaves, but the overall diel growth pattern – a clear decline of RGR during the night and an increase in RGR during the day – was the same as in sunlit leaves. These data prompted investigations into photosynthesis and carbohydrate concentrations of growing leaves in 2003. Although fewer imaging measurements were performed in parallel with the investigations in 2003, these measurements confirmed the occurrence of an afternoon trough in leaf growth in the elevated CO_2 treatment towards the end of the season.

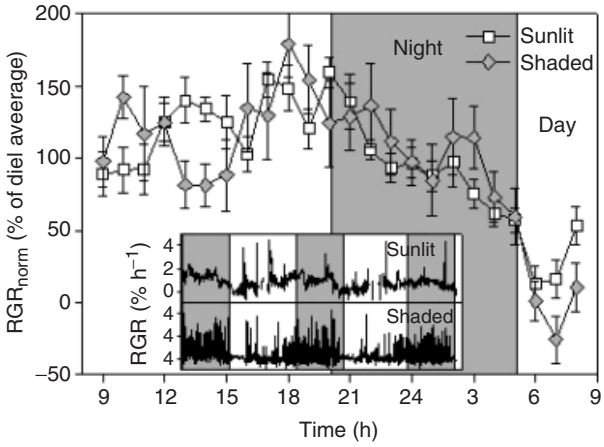

Fig. 8 Comparison of leaf growth in sunlit and shaded leaves of *Populus deltoides* in July 2002 at elevated CO_2 (inset: raw data; main panel: mean value and SE, $n = 7$).

The diel course of stomatal conductance and net CO_2 exchange at elevated CO_2 did not show any fluctuations that might correlate with the afternoon trough in leaf growth (Figs 9a, b). In growing leaves, stomatal conductance was very low but comparable between treatments, while net CO_2 exchange rates were higher in leaves at elevated CO_2 and were almost reaching

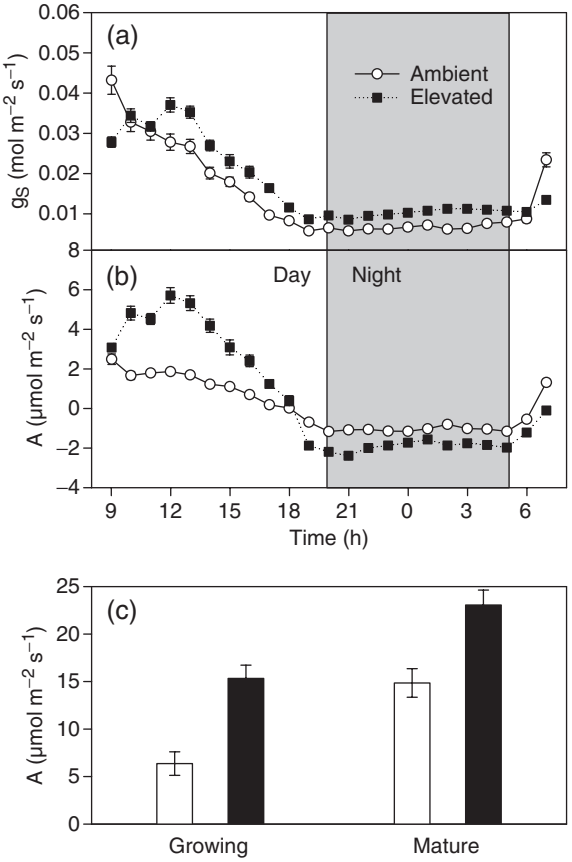

Fig. 9 Photosynthesis of *Populus deltoides*. (a) Diel course of the stomatal conductance (g_s) of growing leaves measured in July 2003 (mean value and SE, $n = 3$). (b) Diel course of the net CO_2 exchange rates (a) of growing leaves measured in July 2003 (mean value and SE, $n = 3$). (c) Daytime net CO_2 exchange rates (a) for growing and mature leaves in October 2003 (mean value and SE, $n = 3$).

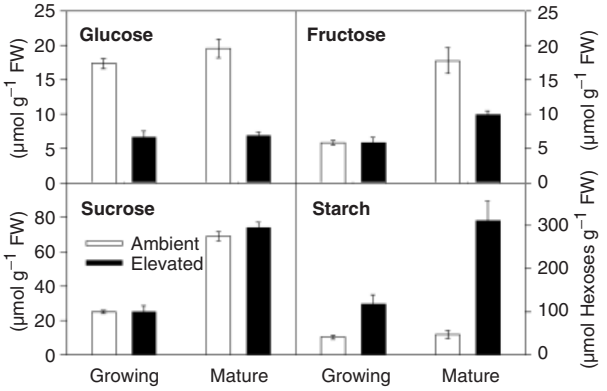

Fig. 10 Soluble sugar and starch concentration of growing and mature leaves of *Populus deltoides* at 16:00 hours, measured in July 2003 (mean value and SE, $n = 15$).

values of mature leaves (Fig. 9c). Daytime net CO_2 exchange rates were significantly increased ($P < 0.001$) by more than a factor of two at elevated CO_2 in growing leaves; in mature leaves the increase was less pronounced.

A possible hint for the observed growth anomaly at 16:00 hours came from carbohydrate analysis. Carbohydrate analysis showed that glucose, one of the most important substrates for growth, was significantly reduced in the afternoon in leaves at elevated CO_2 ($P < 0.001$, Fig. 10). Fructose and sucrose were comparable in growing leaves from both conditions ($P > 0.05$, respectively), while starch was significantly increased at elevated CO_2 ($P < 0.01$). Investigations of the diel variation of carbohydrate concentrations in an independent, second set of growing leaves confirmed the afternoon trough in glucose concentrations (Fig. 11). At elevated CO_2, a very low value of glucose concentration was found in the afternoon, which might also be interpreted as a transient minimum with two enclosing higher values. No such drastic, transient minimum was found for any of the other carbohydrate time series. Glucose concentration decreased during the afternoon at elevated CO_2 to comparable values reached during the phase of minimal growth at the end of the night, and was two times lower than that of leaves from ambient conditions. The time series of starch from leaves grown at elevated CO_2 reached its maximal value later than that of leaves from ambient conditions. The breakdown of this starch surplus during the second half of the night provided carbohydrates that might have been utilized for the stronger growth of leaves at elevated CO_2 during the night.

Discussion

The increase in final leaf area under elevated CO_2, found at the end of the vegetation period, confirms responses reported for other species of *Populus* (Ferris *et al*., 2001). Moreover our experiments also confirm that the increase in leaf area in elevated CO_2 was not because of increased leaf size at emergence, but because of an increase in growth during later phases of leaf development (Fig. 7), analogous to the enhancement of postemergent developmental stages of the leaf reported by Taylor *et al*. (2003). Our findings that this difference developed throughout the season (compare Figs 2 and 6), and was accompanied by the appearance of a trough in growth in the afternoon at elevated CO_2, and by an increased coefficient of growth variation (Fig. 5) are novel. The trough in leaf growth rate recorded by our relatively high-resolution imaging analysis correlates with a reduction in the glucose content of the growing leaf (Figs 10 and 11), leading us to speculate that

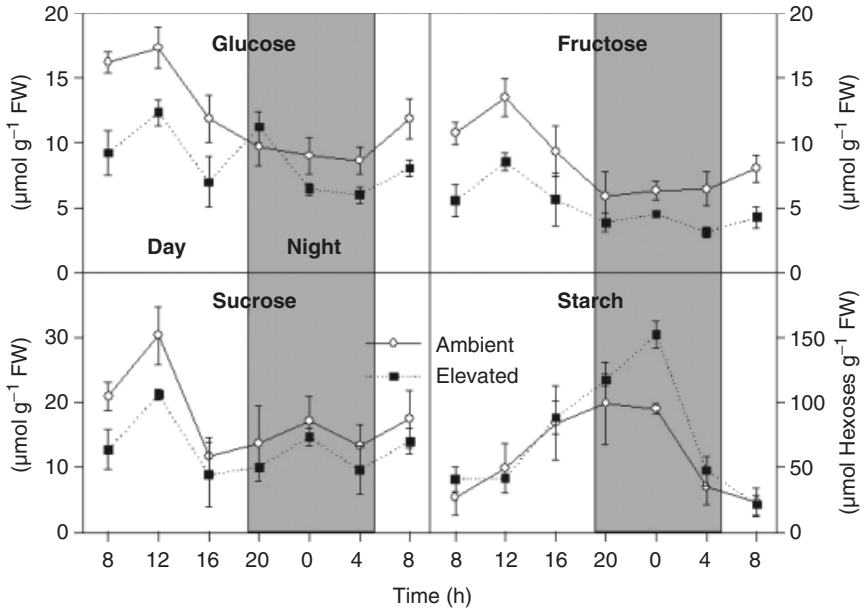

Fig. 11 Diel cycle of soluble sugar and starch concentrations in growing leaves of *Populus deltoides* measured in July 2003 (mean value and SE, $n = 3$; data from a different set of leaves as shown in Fig. 10).

glucose availability might be an important regulatory factor that can dampen growth response under elevated CO_2. Yet, it has to be noted that systematical temperature differences between treatments observed in the afternoon might affect the results as discussed later on. In the second half of the night, glucose availability, but not concentration, might be increased in growing leaves under elevated CO_2, when starch concentration decreases rapidly (Fig. 11). At night, the increased growth rate compensated for more than the afternoon growth trough, leading to an overall higher final leaf area in elevated CO_2. As other investigations show, aboveground tree biomass, as well as system respiration, were stimulated in the *P. deltoides* plantations in elevated CO_2 (Murthy *et al*., 2003, 2005; Barron-Gafford *et al*., 2005). The increase in leaf dry weight per tree and of leaf area in the stand in response to elevated CO_2 produced increases in total plant dry weight well within the limits reported in literature (Poorter & Navas, 2003), even though the 'elevated' CO_2 treatment used in this study exceeded that used in most other studies. As reported in Barron-Gafford *et al*. (2005), the 800 ppm growth treatment of *P. deltoides* produced almost the same stimulation of above- and belowground biomass as the 1200 ppm treatment. It is conceivable that effects of elevated CO_2 on leaf longevity (Craine & Reich, 2001; Herrick & Thomas, 2003) and on specific leaf weight (Wait *et al*., 1999; Harmens *et al*., 2000), although sometimes contradictory in the literature, also contributed to the simple interpretation given here.

As glucose is the central substrate for respiration, starch and cellulose synthesis, a decrease in glucose concentration below a certain threshold could have dramatic effects on cell and leaf expansion. The finding that starch concentration increased at elevated CO_2 at the same time when glucose concentration decreased (Fig. 10) indicates that CO_2 affects the balance of carbohydrate metabolism and pathways of starch synthesis. Carbohydrates diverted to starch in the light are evidently not rapidly remobilized, leading to a reduction in afternoon growth rate. Indeed, starch concentration continues to increase during the first hours of night, after assimilation has ceased. In all other respects, such as increased starch at elevated CO_2 (Lindroth *et al*., 1993; Will & Ceulemans, 1997; Loewe *et al*., 2000) and increased assimilation rate in growing leaves (Wait *et al*., 1999), our results of *P. deltoides* fit well with other reports. Several other photosynthetic parameters, such as stomatal conductance, can respond strongly to elevated CO_2 (e.g. Pearson & Brooks, 1995; Miller *et al*., 1997; Wait *et al*., 1999), but seem unlikely to contribute to the afternoon trough in leaf growth reported here. We found no indication of an afternoon depression of assimilation that might cause the decline in glucose concentration. In contrast to several other reports (Ludewig *et al*., 1998; Grimmer *et al*., 1999), sucrose concentrations were higher in mature, than in growing leaves, but did not respond to CO_2 treatments. This may reflect the long acclimation of plants in our study to elevated CO_2, compared with plants in other investigations.

The diel growth cycle in *P. deltoides*, characterized by highest growth rates usually found in the afternoon, is markedly different from that of other dicot shrubs and herbs that have been investigated. In *Nicotiana tabacum* or *Ricinus communis*, maximal leaf growth activity is found at dawn (Schmundt *et al*., 1998; Walter & Schurr, 2005), and is presumably driven by remobilization of glucose from starch at night. The diel pattern in *P. deltoides* clearly places leaf growth in competition with other demands for photosynthates. Leaf growth is regulated by a network of factors (Dale, 1988; Cosgrove, 1999), including diel patterns of enzyme regulation. Unfortunately, most of the investigations of elevated CO_2 effects on enzyme activities have been conducted with tobacco (Geiger *et al*., 1998; Matt *et al*., 2001), a species that shows low growth rates in the afternoon (Walter & Schurr, 2005). Although it is known that the diel performance of several key enzymes can be altered by elevated CO_2, further studies of the diel regulation of carbohydrate metabolism are needed in *P. deltoides*. For example, it has also been shown that larger leaf and cell sizes in other poplar species in elevated CO_2 are because of increased cell wall extensibility (Ferris *et al*., 2001). This is controlled by enzymes such as xyloglucan-endotransglycosylase and expansins (Cosgrove, 1999) that act on the flexibility of the cell wall and require a controlled input of carbohydrates as substrates for leaf growth.

We hypothesize that the observed afternoon trough in leaf growth at elevated CO_2 is caused by a transient depletion of glucose and that this transient depletion is caused by altered enzyme activities in response to elevated CO_2. As leaf growth dynamics are controlled by a complex network of factors, several other explanations are conceivable as well, one of the most likely being a transient respiratory depletion of glucose in leaves of the elevated CO_2 treatment caused by higher afternoon temperatures that were present in the west bay in which the elevated CO_2 treatment was performed (Fig. 1; 1 °C on average at 16:00 hours). Higher temperature leads to higher respiration rate and hence to decreased carbohydrate content, but assuming a Q_{10} of a factor of two, we think it is unlikely that the observed small temperature difference has led to a depletion of glucose by a factor of two. Moreover, it is unlikely that a respiratory depletion would solely affect glucose and not the other carbohydrates that were investigated here. A thorough investigation of putative effects of temperature and CO_2 interactions based on existing models of photosynthesis and carbohydrate metabolism would certainly be beneficial to further elucidate the causal chain leading to the observed afternoon growth trough observed at relatively high afternoon temperatures, but such an analysis exceeds the scope of this study. Changes in leaf turgor can be ruled out as an explanation for the afternoon growth trough since diel time series for stomatal conductance were comparable (Fig. 9) and as leaf water potential and atmospheric vpd were comparable between treatments (Murthy *et al*., 2005). The observed growth patchiness might lead to transient growth peaks or troughs in the diel time series of an individual leaf, but the high number of replicate measurements in both treatments makes it very unlikely that statistical aberrations underlie the observed growth trough in data averaged throughout the entire season.

Support for a connection between the afternoon growth trough and reduced glucose availability comes from the observation that shaded leaves also showed a distinct trough in growth activity in the middle of the day compared with sunlit leaves (Fig. 8). However, shading the leaves also blocked wavelengths in the red and blue spectral range that are crucial for growth regulation via phytochrome and cryptochrome receptors (Smith, 1995). These wavelengths increase cell expansion rates by acting on apoplastic acidification via specific pathways (Van Volkenburgh, 1999). In future studies, it will be important to separate spectral effects on diel leaf growth cycle from those of overall irradiation intensity. These factors seem to be of second order when compared with the distinctive, afternoon maximum, diel growth pattern in leaves of *P. deltoides*. In addition to carbohydrate availability from contemporary photosynthesis, this pattern brings with it many potentially interesting implications for effects of leaf water relations on growth. A network of mechanisms is likely to be found in control of leaf growth on a temporal scale, making it necessary to combine results from growth analyses with investigations of enzyme activities and gene expression that often show strong diel fluctuations, and to test the hypothesis of a glucose-depletion-mediated growth response using transgenic plants.

Acknowledgements

Achim Walter was supported by a Feodor Lynen grant from the Alexander von Humboldt Stiftung, and Uwe Rascher was supported by a postdoctoral fellowship from Columbia University. Maja M. Christ was supported by a grant of the German Science Foundation (DFG Schu 946 2–2) and acknowledges support for her PhD thesis at the Heinrich-Heine University Düsseldorf. Other support for this project came from program enhancement grants through the office of the Executive Vice Provost, Columbia University (Dr Michael Crow), and through the generosity of Mr Edward P. Bass. We are grateful to Biosphere 2 Laboratory technical support and operations staff for maintaining the environmental regimes of the facility. We would like to thank Lisa Ainsworth and Barry Osmond for

helpful comments on the manuscript and Terrence Paige for assistance in the lab.

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