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Estimation of the whole-plant CO_2 compensation point of tobacco (*Nicotiana tabacum* L.)

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Abstract

The whole-plant CO_2 compensation point (Γ_{plant}) is the minimum atmospheric CO_2 level required for sustained growth. The minimum CO_2 requirement for growth is critical to understanding biosphere feedbacks on the carbon cycle during low CO_2 episodes; however, actual values of Γ_{plant} remain difficult to calculate. Here, we have estimated Γ_{plant} in tobacco by measuring the relative leaf expansion rate at several low levels of atmospheric CO_2, and then extrapolating the leaf growth vs. CO_2 response to estimate CO_2 levels where no growth occurs. Plants were grown under three temperature treatments, 19/15, 25/20 and 30/25 °C day/night, and at CO_2 levels of 100, 150, 190 and 270 μmol CO_2 mol^{-1} air. Biomass declined with growth CO_2 such that Γ_{plant} was estimated to be approximately 65 μmol mol^{-1} for plants grown at 19/15 and 30/25 °C. In the first 19 days after germination, plants grown at 100 μmol mol^{-1} had low growth rates, such that most remained as tiny seedlings (canopy size <1 cm^2). Most seedlings grown at 150 μmol mol^{-1} and 30/25 °C also failed to grow beyond the small seedling size by day 19. Plants in all other treatments grew beyond the small seedling size within 3 weeks of planting. Given sufficient time (16 weeks after planting) plants at 100 μmol mol^{-1} eventually reached a robust size and produced an abundance of viable seed. Photosynthetic acclimation did not increase Rubisco content at low CO_2. Instead, Rubisco levels were unchanged except at the 100 and 150 μmol mol^{-1} where they declined. Chlorophyll content and leaf weight per area declined in the same proportion as Rubisco, indicating that leaves became less expensive to produce. From these results, we conclude that the effects of very low CO_2 are most severe during seedling establishment, in large part because CO_2 deficiency slows the emergence and expansion of new leaves. Once sufficient leaf area is produced, plants enter the exponential growth phase and acquire sufficient carbon to complete their life cycle, even under warm conditions (30/25 °C) and CO_2 levels as low as 100 μmol mol^{-1}.

Keywords: CO_2 compensation point, low CO_2, *Nicotiana tabacum*, photosynthesis, temperature, tobacco

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Introduction

Currently, atmospheric CO_2 is 375 μmol CO_2 mol^{-1} air and is expected to rise above 600 μmol mol^{-1} later this century (IPCC, 2001). These levels of CO_2 are well above the norm for recent geological time. During the preindustrial Holocene epoch (100 years ago to 10 thousand years ago, ka), atmospheric CO_2 was 260–280 μmol mol^{-1}, while during the latter part of the Pleistocene epoch (12–500 ka), CO_2 levels ranged between 180 and 300 μmol mol^{-1}, with the majority of time (67%) spent below 240 μmol mol^{-1} (Petit *et al.*, 1999). This predominance of low CO_2 in recent geological time has important implications for the evolution of photosynthetic physiology. Low CO_2 is thought to be a major factor affecting the evolution of C_4 photosynthesis (Ehleringer *et al.*, 1991; Sage, 2004), while in C_3 plants, patterns of carbohydrate use may reflect adaptations for CO_2 levels below current values. Low CO_2 adaptations may constrain plant responses to rising CO_2, for example by imposing ceilings

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on sink strength and growth potential (Sage & Coleman, 2001). By affecting primary productivity, low CO_2 levels may have affected community interactions and biome structure in ways that are fundamentally different than today. Ecosystems were proposed to be more open during low CO_2 periods, with grasslands expanding at the expense of woodland during the lowest CO_2 episodes – low CO_2 was a factor in this, in addition to a drier climate (Prentice & Jolly, 2000; Sage, 2001). Seed yields were almost certainly affected during CO_2 deficiency, which would have caused a cascade affecting the feeding patterns of numerous animal species, including our own. For example, the origin of agriculture may have been delayed by the low CO_2 of the late Pleistocene (Sage, 1995).

Importantly, the strength of the carbon cycle would have been affected by CO_2 fluctuations, and it may have experienced strong biospheric feedbacks during episodes of CO_2 depletion. Lovelock & Whitfield (1982) for example, suggest that below about 150 $\mu mol\,mol^{-1}$, net productivity of C_3 photosynthesis should be minimal, such that the carbon flux into C_3 dominated ecosystems would have been negligible.

Most of the studies examining C_3 plant performance at low CO_2 were performed on plants grown at levels no lower than 150 $\mu mol\,mol^{-1}$ (Allen *et al*., 1991; Polley *et al*., 1992; Polley *et al*., 1993; Dippery *et al*., 1995; Gesch *et al*., 2000; Maherali *et al*., 2002). At some CO_2 level below this, which is not currently well defined, photosynthesis in C_3 plants is inhibited to such a degree that respiratory and photorespiratory processes consume all of the assimilated carbon and thus plant growth is reduced to zero. This point of zero growth is the whole-plant CO_2 compensation point (Γ_{plant}). Photorespiration increases at low atmospheric CO_2 (Farquhar & von Caemmerer, 1982; Sage & Reid, 1992) and there is some evidence that dark respiration also increases (Baker *et al*., 1992), meaning that Γ_{plant} cannot be extrapolated from photosynthetic measurements and construction costs alone. The whole-plant CO_2 compensation point is difficult to measure because it requires growing multiple plants at a range of low CO_2 concentrations for several days, and then extrapolating the observed patterns to zero growth. Limited facilities, a requirement for large sample size, and the need to precisely control CO_2, light and temperature are major obstacles to estimating Γ_{plant}. By contrast, the instantaneous CO_2 compensation point of photosynthesis (Γ) is easily determined with a gas exchange system. The instantaneous CO_2 compensation points of C_3 plants average approximately 50 $\mu mol\,mol^{-1}$ at 25 °C (Krenzer *et al*., 1975), and are widely used as the reference for minimum CO_2 requirements.

Several factors are known to affect the instantaneous, leaf-level CO_2 compensation point of C_3 plants. Elevated temperature increases both photorespiration (Leegood *et al*., 1995) and dark respiration (Amthor, 1984) and thus, in C_3 plants, the CO_2 compensation point increases with increasing temperature (Weber *et al*., 1985; Sage *et al*., 1990). At increasing scales of complexity, and over longer time intervals, the CO_2 compensation point must increase, because an increasing fraction of heterotrophic tissue will consume the daily income of photosynthate. Thus, as the scale of the CO_2 compensation point increases to encompass daily photosynthesis of a single leaf, the amount of carbon lost during the dark period must be accounted for. Daily carbon loss by both autotrophic and heterotrophic tissues at the whole plant level is estimated to expend 30–60% of carbon assimilated by healthy leaves (Amthor, 1984), indicating CO_2 requirements for whole plant performance are substantially greater than single leaf photosynthesis. Over the growing season, construction costs have to be integrated into the equation to estimate the CO_2 compensation point of whole plant growth. Presumably, growth CO_2 compensation points are substantially greater than single leaf compensation points, and even greater when the need for seed production is taken into account. How much greater remains uncertain, despite Lovelock's (1988) well publicized speculation that 180 $\mu mol\,mol^{-1}$ is near the lower limit tolerable for C_3 vegetation.

Identifying the whole plant CO_2 compensation point is therefore important for understanding low CO_2 thresholds for C_3 plant performance. Should the whole plant CO_2 compensation point be high, then the possibility exists that CO_2 levels in the atmosphere during the late Pleistocene may have approached values where C_3 plant performance was greatly impaired. This could have major significance, as it means that the terrestrial CO_2 sink weakened during low CO_2 episodes. A high CO_2 requirement for positive C_3 productivity would also indicate a greater potential for the emptying of niche space during low CO_2 episodes, thereby creating opportunity for the selection for carbon conservation mechanisms, such as CAM and C_4 photosynthesis.

In this study, we examine the ability of the C_3 annual tobacco to grow and photosynthesize under a range of subambient CO_2 conditions at three different temperatures. Growth potential was indexed as leaf area expansion using a digital camera that allowed for sequential, nondestructive measurements to be made over the course of 6 days. Tobacco seedlings were selected because they grow quickly, allowing several studies to be performed in rapid succession. Tobacco develops from a small seed so that the contribution of stored carbon to seedling growth is rapidly exhausted. In

addition, the initial leaf display of small seedlings is well suited to photographic analysis of leaf area. We limited our initial series of measurements to small seedling growth, in order to maximize sample size and avoid leaf overlap. A subset of seedlings at the lower CO_2 treatments were allowed to grow to fruiting in order to assess reproductive potential. We also examined photosynthetic performance and the content of Rubisco and chlorophyll in leaves to assess whether there may have been acclimation effects which may have compensated for limitations associated with CO_2 depletion. Optimal acclimation to low CO_2 is theoretically predicted to increase allocation to leaf area and Rubisco capacity (Sage & Coleman, 2001).

Materials and methods

Plant material and treatments

Seeds of *Nicotiana tabacum* L. (var. Havana) were sown into 500 mL pots of soil (Sunshine mix #3, Sun Gro Horticulture Inc., Bellevue, WA, USA). Because experiments examined seedling performance at subambient CO_2 levels for a relatively short duration (<5 weeks after planting), the relatively small pot size did not become a limitation. Seedlings were watered daily and fertilized every 4 days with a 0.5 strength Hoagland's solution modified to contain 9 mM nitrate-nitrogen. Seedlings were initially grown at 25/20 °C (day/night), 16 h photoperiod and 550 μmol photons $m^{-2} s^{-1}$ in controlled environment chambers (Bigfoot Model # GC-20, Enconair Ecological Chambers Inc., Winnipeg, MB, Canada), at atmospheric [CO_2], until 7 days after planting (DAP). At seven DAP, emerged seedlings were thinned to three plants per pot and the pots were transferred to experimental treatments in the same type of growth chambers. The total canopy area (i.e. the area covered by all of the leaves of each plant) of each seedling was typically about 1–3 mm^2 at the time of transfer, and the plants selected were ones that did not emerge until the day of transfer (i.e. the emergent canopies spent less than 24 h in ambient CO_2, much of this time during the night before transfer). Treatment CO_2 levels were 100, 150, 190 and 270 ± 10 μmol mol^{-1} CO_2; some experiments included 220 μmol mol^{-1}. Treatment temperatures were 19/15, 25/20 and 30/25 °C day/night. Light was maintained at 550 ± 50 μmol photons $m^{-2} s^{-1}$ at the upper leaf surface. The chamber CO_2 was controlled using a CO_2-controlling gas analyser (Model WMA-2, 3 or 4; PP Systems, Haverhill, MA, USA) that regulated the circulation of chamber air through a soda-lime scrubber located within the growth chamber (Cowling & Sage, 1998). The possibility that soil respiration may have elevated the [CO_2] near the soil could not be eliminated. However, as soil respiration should be present in the field under low [CO_2] conditions, it was felt that this small potential additional source of CO_2 was acceptable. This additional CO_2 might affect the initial growth measurements, but gas exchange and Rubisco measurements were carried out on older plants, and on leaves that had developed away from the soil surface, in the air column which was thoroughly mixed with the rest of the chamber air.

To assess the ability of plants to complete their lifecycle under severe carbon depletion conditions, 12 plants in separate pots in the 100 and 150 μmol mol^{-1} CO_2 treatments at 30/25 °C were transplanted to 8 L pots and maintained until 15 weeks after planting. The resulting plants were harvested and dried for growth and reproduction analysis. Germination rates were determined for sets of 100 seeds from each plant in each of the two CO_2 treatments; this trial was performed twice and the results pooled, since there was no significant difference between the two.

Determination of leaf area expansion rate

Beginning 14 DAP, plants were photographed with a Nikon Coolpix 5000 digital camera (Nikon, Mississauga, ON, Canada). A millimetre scale was placed on the soil of each pot prior to photographing so that the image could be calibrated. Images were filtered through a program designed by John Campbell (St Paul, MN, USA) to enhance the contrast between the plant and the soil behind it, which allowed for the quantification of canopy area using software by Image Pro Plus (Media Cybernetics, Carlsbad, CA, USA). Photos were taken every morning before the photoperiod began to minimize exposure of photosynthesising plants to elevated CO_2. Directly after photography on 19 DAP, one plant was harvested per pot and dried for assessment of leaf mass per area. Leaf overlap was not great during this measurement period, and what little overlap did occur was estimated by extrapolating the leaf edge of the covered leaf to provide a complete outline. Relative leaf expansion rate (RLER) was calculated as the slope of the relationship between the natural logarithm of the leaf area vs. the time in days. Areas from days 14 to 19 were used, as the RLER was constant over this period in all of the treatments.

Gas exchange measurements

At 28 DAP for the plants of the 30/25 °C treatment and 33 DAP for the 19/15 °C treatment, photosynthetic rates were measured using a Li-Cor 6400 photosynthesis system (Li-Cor Inc., Lincoln, NB, USA), with the red-

blue light source (6400-02B) chamber. This allowed enough time for the plants in most treatments to reach a size suitable for gas exchange measurements to be taken. Photosynthesis was measured at both chamber light levels (550 μmol photons $m^{-2} s^{-1}$) and at saturating light (1750 μmol photons $m^{-2} s^{-1}$) for all treatments except for the 100 μmol mol^{-1}-grown plants at 19/15 °C and the 100 and 150 μmol mol^{-1}-grown plants at 30/25 °C, which were too small to be measured at this time. Initial slopes were calculated by regression using the values obtained at air CO_2 levels (C_a) from 50 to 270 μmol mol^{-1} CO_2; adjusted R^2 values varied from 0.989 to 0.999. After gas exchange had been performed, leaf punches were taken from all plants, frozen in liquid nitrogen, and stored in a −80 °C freezer for chlorophyll and Rubisco content determination.

Extraction and Rubisco and chlorophyll determination

Rubisco content was assayed by determining the amount of ^{14}C-labeled 2-carboxyarabinitol-1,5-bisphosphate ([^{14}C]-CABP) that was bound to Rubisco extracted from frozen leaf discs (harvested immediately after gas exchange measurements were performed) and assuming 6.6 mol of CABP were bound per mol Rubisco (Seemann & Sharkey, 1986; Collatz *et al*., 1990; Zhu & Jensen, 1990). Combined chlorophyll *a* and *b* concentration was determined on samples from the same extraction, using methanol extraction (Porra *et al*., 1989).

Experimental design and statistical analysis

Because only four growth chambers were available, trials had to be staggered to give the desired treatments and replication. In total, there were 11 treatment combinations (four CO_2 × three temperatures, −100 μmol mol^{-1} at 25/20 °C). For the seedling growth trials, each treatment was replicated twice except for the 100 μmol mol^{-1} treatment at 30/25 °C. The 100 μmol mol^{-1} treatments were added late in the study when initial results showed insufficient growth reductions at 150 μmol mol^{-1} to allow for a reasonable estimation of Γ_{plant}. Trials had either 15 or 22 pots, with three seedlings per pot. Statistical tests were performed on averages of plant parameters pooled for all plants growing in individual pots within a given chamber.

Gas exchange measurements were determined on data from nine plants per treatment within a single treatment replication. For the assessment of reproductive output at the lowest CO_2 conditions, the final replication of the 30/25 °C treatments at 100 and 150 μmol mol^{-1} were maintained for 12 weeks beyond the 4-week period. This experiment could not be replicated because of the length of the growth period and the demand for chamber space.

Statistical analysis was carried out using Sigma Stat Version 2.01 (SPSS Inc., Chicago, IL, USA) or SPSS Version 11.0.1 (SPSS Inc.) with $P = 0.05$ as the critical level of significance. Where appropriate, data were evaluated using a two-way ANOVA, with means differentiated using Tukey's Honestly Significant Difference which allows multiple comparisons of means while minimizing the probability of type I errors. For data that were not normally distributed, the Kruskal–Wallis one-way ANOVA based upon ranks was used, with Dunn's pair-wise comparison for the differentiation of means of individual treatments. The RLER data were analysed using the General Linear Model, which is appropriate with unbalanced data sets. The General Linear Model was also used for the data in Table 1, which were also unbalanced. The germination rates were analysed using a χ^2 test. Sigma Stat automatically tests for normality and homogeneity of variance with each statistical test.

Results

Growth of immature plants (<3 weeks of age)

Declining CO_2 significantly reduced the RLER in both the 19/15 and 30/25 °C day/night temperature treatments (Figs 1 and 2a). The reduction in RLER from 270 to 100 μmol mol^{-1} was approximately 60–75%. Growth temperature, however, had no significant effect on RLER. We used regression analysis to extrapolate the RLER to the *x*-axis, in order to estimate the minimum CO_2 level at which leaf growth could occur (and hence estimate Γ_{plant}, assuming leaf growth stops when total plant growth stops). Estimated values of Γ_{plant} were 63–66 μmol mol^{-1} at 19/15 and 30/25 °C. The lack of a data point at 100 μmol mol^{-1} for the 25/20 °C treatment precludes estimating Γ_{plant} at this growth temperature with any certainty.

As a result of differences in leaf growth rate, total canopy area remained very low in the plants in the 100 μmol mol^{-1} treatments at both 19/15 and 30/25 °C, and in those in the 150 μmol mol^{-1} treatment at 30/25 °C (Fig. 1). Canopy size (as measured by the total projected leaf area per plant) in most plants in these three treatments failed to exceed 1 cm^2 by 19 DAP (Table 1). For example, at 100 μmol mol^{-1} CO_2 and both 19/15 and 30/25 °C, approximately 85% of the seedlings remained tiny with less than 1 cm^2 of leaf canopy. With the high mortality rates at 30 °C, this means that almost no plants had a leaf area larger than 1 cm^2 in this treatment. At 150 μmol mol^{-1}, 59% of seedlings grown at 30/25 °C remained tiny, and the average canopy size

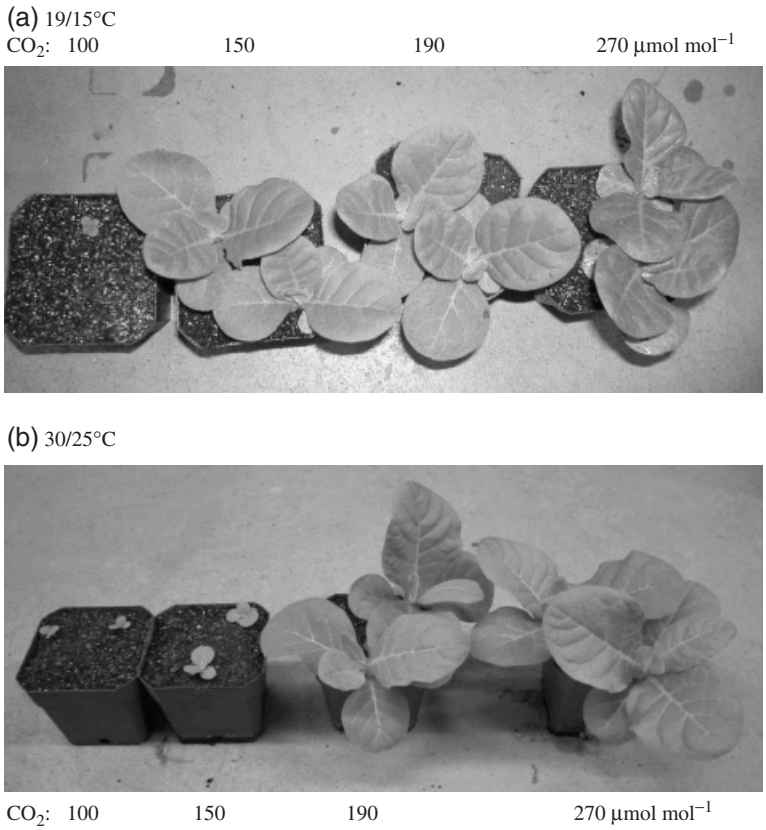

Fig. 1 *Nicotiana tabacum* var. Havana. (a) Plants grown at 19/15 °C at 28 days after planting (DAP). (b) Plants grown at 30/25 °C at 33 DAP. CO_2 levels, left to right, both photos: 100, 150, 190 and 270 μmol mol^{-1}. (Note: plants at this stage were not used in the growth assay because of leaf overlap).

of this treatment was less than 1 cm^2, which was about one-half and one-fifth of the canopy size of plants in the 150 μmol mol^{-1} treatments at 19/15 and 25/20 °C respectively (Table 1). These differences in canopy size were similarly reflected in total shoot biomass, which at 19/15 °C remained approximately 10 times lower in the plants grown at 100 μmol mol^{-1} CO_2 than both those at 150 and 190 μmol mol^{-1} and 15 times lower than those in the 270 μmol mol^{-1} treatment. At 30/25 °C, total shoot biomass in plants grown at both 100 and 150 μmol mol^{-1} CO_2 was about 10 times less than those grown at 190 μmol mol^{-1} and 20 times less than those in the 270 μmol mol^{-1} treatment (Table 1).

Leaf mass per area was unaffected by growth CO_2 except at 100 μmol mol^{-1} (19/15 and 30/25 °C) and 150 μmol mol^{-1} (30/25 °C only), where it was reduced by approximately 20% compared with values observed in the higher CO_2 treatments (Fig. 2b). Growth temperature had no effect on leaf mass per area except in plants grown at 150 μmol mol^{-1}, where leaf mass per area was depressed 20% in the warm compared with the cool-grown plants.

The number of plants that died between germination and harvest was counted at 19 DAP (Table 1). No plants died at 25/20 °C at any of the CO_2 levels measured. Mortality rates were low across all CO_2 treatments at 19/15 °C, on average less than 4%. The mortality rates were similar at 190 and 270 μmol mol^{-1} CO_2 for plants grown at 30/25 °C, but were significantly higher (>10%) at 100 and 150 μmol mol^{-1} (Table 1).

Leaf gas exchange and Rubisco and chlorophyll content

Photosynthetic rates measured under chamber growth conditions increased 30% from the 150 to the 190 μmol mol^{-1} conditions (Fig. 3a), and by about 15–30% from the 190 to the 270 μmol mol^{-1} treatment in both temperature treatments. Under light saturating conditions, the response of photosynthesis to intercellular CO_2 (Fig. 3b) showed no obvious effects of growth CO_2 between 150 and 270 μmol mol^{-1}; however, small plant size prevented us from measuring gas exchange in the low CO_2 treatments of 100 (19/15 and 30/25 °C) and 150 μmol mol^{-1} (30/25 °C). The initial slope of the

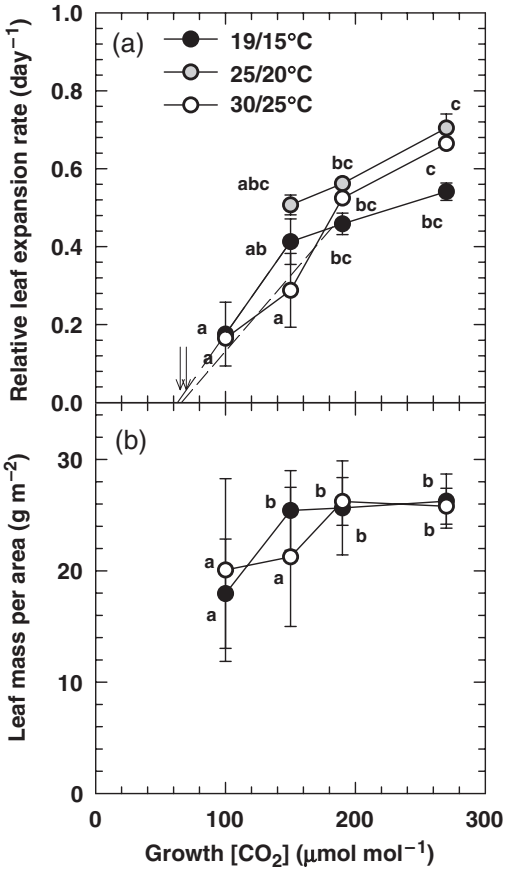

Fig. 2 Effects of growth CO_2 and temperature on (a) relative leaf expansion rate and (b) leaf dry mass per area at 20 days after planting (DAP). (a) Mean ± SE, $n = 2$ (25/20 and 30/25 °C) or 3 (19/15 °C) experiments. Regression lines added: arrows indicate projected Γ_{plant}. (b) Mean ± SD, $n = 22$ plants. Different letters indicate statistically significant differences at $P < 0.05$.

net CO_2 assimilation rate (A) vs. intercellular CO_2 (C_i), A/C_i, response (Fig. 4a), and the maximum CO_2 assimilation rate (Fig. 4b) were unaffected by growth CO_2 between 150 and 270 μmol mol^{-1}. In contrast to the effect on the initial slope and maximum rate of A, the CO_2 compensation point increased by 4 μmol mol^{-1} in plants grown at 270 relative to 150 μmol mol^{-1} at 19/15 °C (Fig. 4c). Increasing temperature increased the initial slope, the CO_2 compensation point, and the maximum rate of A by 15–25%.

Leaf Rubisco content per unit leaf area was similar in the leaves of all treatments except for those grown at the lowest CO_2 levels, where it declined by 40–50% relative to the values in plants grown at 270 μmol mol^{-1} (Fig. 5a). Growth temperature did not have a significant effect on Rubisco content, except at 150 μmol mol^{-1} CO_2, where the 30/25 °C-grown plants had about 25% less Rubisco than the 19/15 °C-grown plants. The chlorophyll content also declined by about a third in plants at the lowest CO_2 treatments (Fig. 5b). Like Rubisco content, growth temperature had a significant effect on chlorophyll content only at 150 μmol mol^{-1} CO_2, where 30/25 °C-grown plants had about 40% less chlorophyll than the 19/15 °C-grown plants (Fig. 5c). As a result of the reduction in both Rubisco content and chlorophyll, the Rubisco to chlorophyll ratios were little changed by the CO_2 or temperature treatments.

Growth and Reproduction of Mature Plants (15 weeks old)

In the 30/25 °C trials at 100 and 150 μmol mol^{-1} CO_2, a subset of the plants were transplanted to 8 L pots rather than being harvested, and were subsequently grown for another 12 weeks to reproductive age. In both treatments, plants eventually put up a large leaf area and grew to a substantial height of 66 cm at 100 μmol mol^{-1} and 85 cm at 150 μmol mol^{-1} CO_2 (Table 2). Plants grown at 150 μmol mol^{-1} CO_2 produced approximately 50% more biomass and fruits than plants grown at 100 μmol mol^{-1} (Table 2). Seeds from the 150 μmol mol^{-1} treatment were about 15% heavier and had approximately 30% greater germination rate than seeds from the 100 μmol mol^{-1} treatment (Table 2).

Discussion

To overcome the limitations associated with growing many plants at a range of subambient CO_2 levels with precise environmental control, we developed a nondestructive technique that allowed the growth of the same individuals to be repeatedly measured over time. This allowed for a more precise estimation of RLER than possible with destructive harvests. In linearly extrapolating the response of tobacco RLER to decreasing levels of CO_2, we estimate Γ_{plant} for seedlings to be approximately 63–66 μmol mol^{-1} at the 19/15 and 30/25 °C day/night temperature regimes. This is about 10–20 μmol mol^{-1} CO_2 greater than the instantaneous leaf CO_2 compensation point (Γ), and is much lower than previous estimates (Lovelock, 1988). It is also unexpected that the two temperatures are predicted to have essentially the same Γ_{plant}. One possible explanation for this lack of temperature responsiveness is that the slowest growing plants in both treatments died, and as more plants died at higher temperatures, the growth curve would have been skewed to a higher growth rate than otherwise predicted. This does not change the final findings, as it is likely that some plants might still survive and grow, even at high temperature and very low CO_2, but the mortality rate may be assumed to be very high under these circumstances. That the Γ_{plant}

Table 1 Effect of growth CO_2 and temperature on percent mortality, average canopy area, percentage of plants with an area below 1 cm^2 and shoot dry weight at 19 DAP

		CO_2 ($\mu mol\,mol^{-1}$)			
	Temperature (°C)	100	150	190	270
Percent mortality at 19 DAP	19/15	3.0 ± 1.5^{a}	3.5 ± 3.5^{a}	2.7 ± 2.0^{a}	1.1 ± 1.1^{a}
	25/20	n.a.	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	30/25	14.5^{c}	11.6 ± 6.1^{bc}	4.4 ± 2.9^{ab}	2.0 ± 0.5^{a}
Average canopy area at 19 DAP (mm^2)	19/15	56 ± 30^{a}	210 ± 58^{ab}	213 ± 83^{ab}	344 ± 113^{b}
	25/20	n.a.	462 ± 334^{b}	490 ± 276^{b}	1095 ± 610^{c}
	30/25	39 ± 22^{a}	88 ± 85^{a}	370 ± 214^{b}	871 ± 416^{c}
Percent of plants below 1 cm^2 at 19 DAP	19/15	85.8 ± 15.8^{c}	8.8 ± 1.8^{a}	9.5 ± 2.2^{a}	0.8 ± 0.8^{a}
	25/20	n.a.	1.1 ± 1.1^{a}	1.1 ± 1.1^{a}	0 ± 0^{a}
	30/25	84.2^{c}	58.9 ± 29.3^{b}	4.4 ± 2.9^{a}	0.8 ± 0.8^{a}
Total shoot biomass at 19 DAP (mg)	19/15	0.63 ± 0.43^{a}	6.01 ± 2.38^{b}	5.58 ± 3.43^{b}	9.25 ± 4.00^{bc}
	25/20	n.a.	n.a.	n.a.	n.a.
	30/25	0.86 ± 0.72^{a}	0.97 ± 0.71^{a}	10.20 ± 5.70^{c}	19.80 ± 10.21^{d}

Mortality was calculated as the number of plants that had died by day 19 divided by the initial number of plants. The percent of plants with an area below 1 cm^2 was also calculated based on the initial number of plants. For percent mortality and percent below 1 cm^2, means are of one to three experiments ± standard error. Average canopy leaf area is from pooled data from all experiments, average ± standard deviation. Data for average dry weight were collected only from average ± standard deviation from plants within a single growth chamber trial. Different letters denote statistical differences at $P < 0.05$.
n.a., data not available; DAP, days after planting.

estimated here for both temperatures is so much lower than that previous estimates may be because these previous studies did not examine sufficiently low concentrations of CO_2, and thus did not take into account the potential of plants to acclimate to low CO_2. One of the mechanisms seen in this work is a change in leaf mass per area.

Leaf mass per area declined at low CO_2 in both the warm and cool grown plants, but this decline began at a higher growth CO_2 level in the 30/25 than 19/15 °C-grown plants. Relative to plants grown at 190 $\mu mol\,mol^{-1}$ and higher, leaf mass per area declined at 100 $\mu mol\,mol^{-1}$ in both the warm and cool-grown plants, and at 150 $\mu mol\,mol^{-1}$ in the 30/25 °C grown plants. As well, both Rubisco and chlorophyll content declined in the plants grown at 100 $\mu mol\,mol^{-1}$ CO_2, demonstrating a qualitative reduction of resource investment per leaf area. This, along with the reduction in leaf mass per area, indicates a shift to less expensive, albeit less photosynthetically active leaves per unit area. This shift allows the plants to maintain some leaf area production under severe carbon limitation, and thus reduce Γ_{plant}. An ability to maintain leaf growth is important in maintaining fitness, because eventually, enough leaf area and carbon was accumulated to allow the tobacco plants to gain sufficient carbon to enter the exponential growth phase. The plants then showed robust growth, even at 100 $\mu mol\,CO_2\,mol^{-1}$ air, and in time grew to a robust size and completed the lifecycle, producing an abundance of viable seed.

Earlier studies of the growth responses of C_3 plants to subambient CO_2 have also observed reductions in leaf mass per area. *Abutilon theophrasti* showed little leaf mass per area change between growth CO_2 levels of 350 and 270 $\mu mol\,mol^{-1}$ at 28/22 °C; however, at 150 $\mu mol\,mol^{-1}$, leaf mass per area declined about 30% relative to these higher CO_2 levels (Tissue *et al.*, 1995). In addition, Rubisco and chlorophyll levels declined in *A. theophrasti* grown at 150 $\mu mol\,mol^{-1}$ CO_2 when compared with plants grown at 270 and 250 $\mu mol\,mol^{-1}$. The leaf mass per area of soybean decreased approximately linearly with reduced growth CO_2 (Allen *et al.*, 1991). In tobacco selected for survival at the instantaneous Γ, the efficiency of carbon assimilation increased, partly as a result of reductions in leaf mass per area (Delgado *et al.*, 1992). Because this was a genotypic response to the selection pressure, reduced leaf mass per area may be an adaptive trait to low CO_2. Numerous other studies of plant responses to subambient CO_2 have not detected major shifts in leaf mass per area; however, these generally did not examine responses below 160 $\mu mol\,mol^{-1}$ (e.g. Rowland-Bamford *et al.*, 1991; Polley *et al.*, 1993). From these observations, we conclude that the production of thinner, less dense leaves is a common response to carbon deficiency, and one which likely enhances performance by lowering the cost of leaf area production. Production of thin, less expensive leaves is a common response to carbon deficiency brought about by low light availability (Lambers *et al.*, 1998). This could explain why predictions

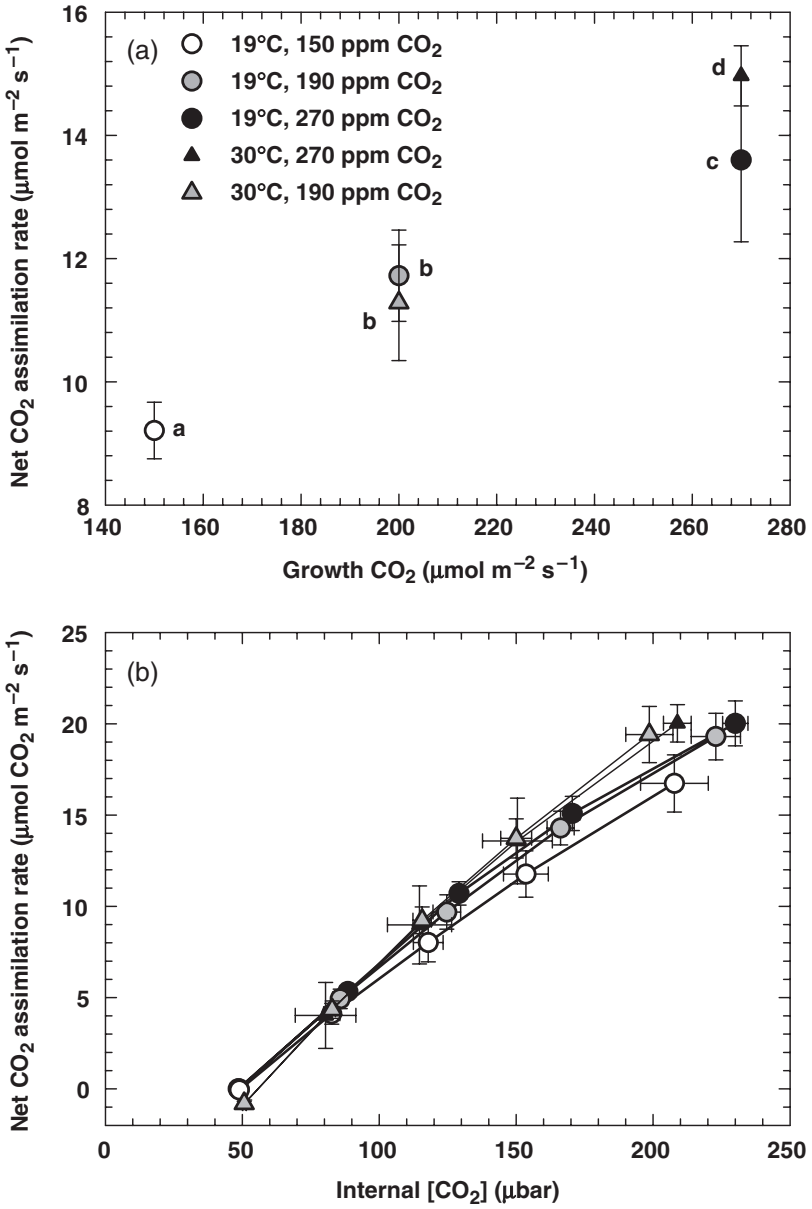

Fig. 3 Effects of growth CO_2 and temperature on (a) light saturated response of photosynthesis to intercellular CO_2 and (b) net CO_2 assimilation under growth light and a range of CO_2 levels. Light levels: (a) 1750 μmol photons $m^{-2} s^{-1}$ and (b) 550 μmol $m^{-2} s^{-1}$. Symbols: Temperature: circles, 19/15 °C; triangles, 30/25 °C. Growth CO_2: white, 150 μmol mol^{-1}; gray, 190 μmol mol^{-1}; black, 270 μmol mol^{-1}. Mean ± SD, $n = 9$ plants. Different letters indicate statistically different groups at $P < 0.05$.

based on higher concentrations of CO_2 fail to adequately predict Γ_{plant}, as this compensatory mechanism is not yet engaged at the CO_2 levels examined.

Effects of low CO_2 on photosynthesis

In plants grown under low CO_2, theoretical models predict an increase in allocation to processes controlling CO_2 fixation, mainly Rubisco, and a shift of resources away from nonlimiting process such as electron transport and sucrose export (Sage & Coleman, 2001). There was little evidence for this response in tobacco as judged by the response of the initial slope of photosynthesis to low CO_2, and the near constant Rubisco to chlorophyll ratio. However, at 19/15 °C, the instantaneous CO_2 compensation point declined by 4 μmol mol^{-1} CO_2 at 270 compared with 150 μmol mol^{-1}, indicating acclimation in a manner that enhances whole-plant carbon gain at low CO_2. This reduction in the instantaneous CO_2 compensation point under constant temperature and light levels reflects either a reduction in dark respiration, or an increase in carboxylation

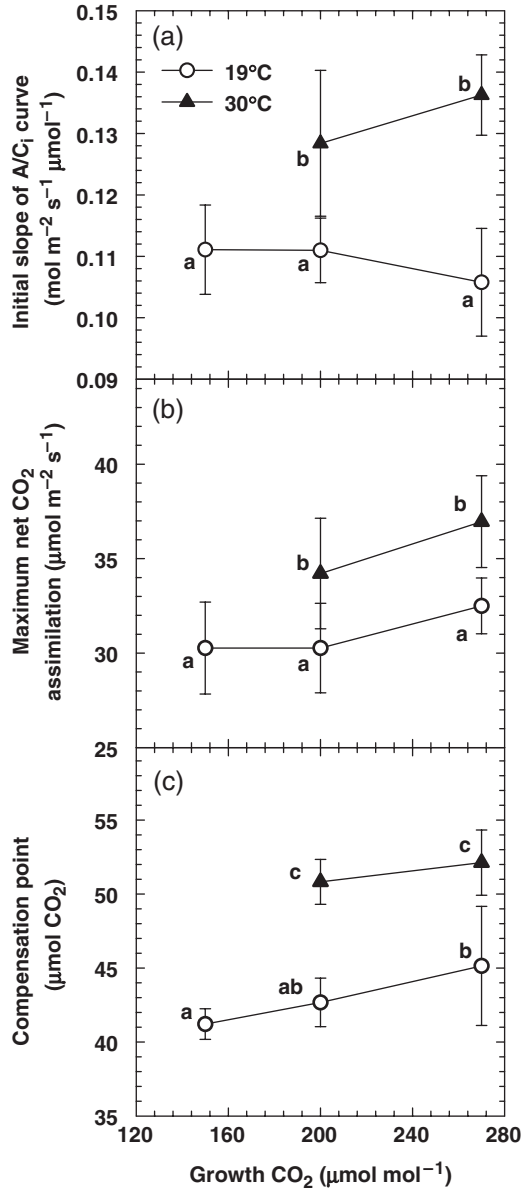

Fig. 4 Effect of growth CO_2 and temperature on (a) the initial slope of net CO_2 assimilation rate vs. intercellular CO_2, (b) the maximum net CO_2 assimilation rate and (c) the CO_2 compensation point of photosynthesis at saturating light. Light intensity = 1750 µmol photons m^{-2} s^{-1}. Mean ± SD, $n = 9$ plants. Different letters indicate statistically significant groups at $P < 0.05$.

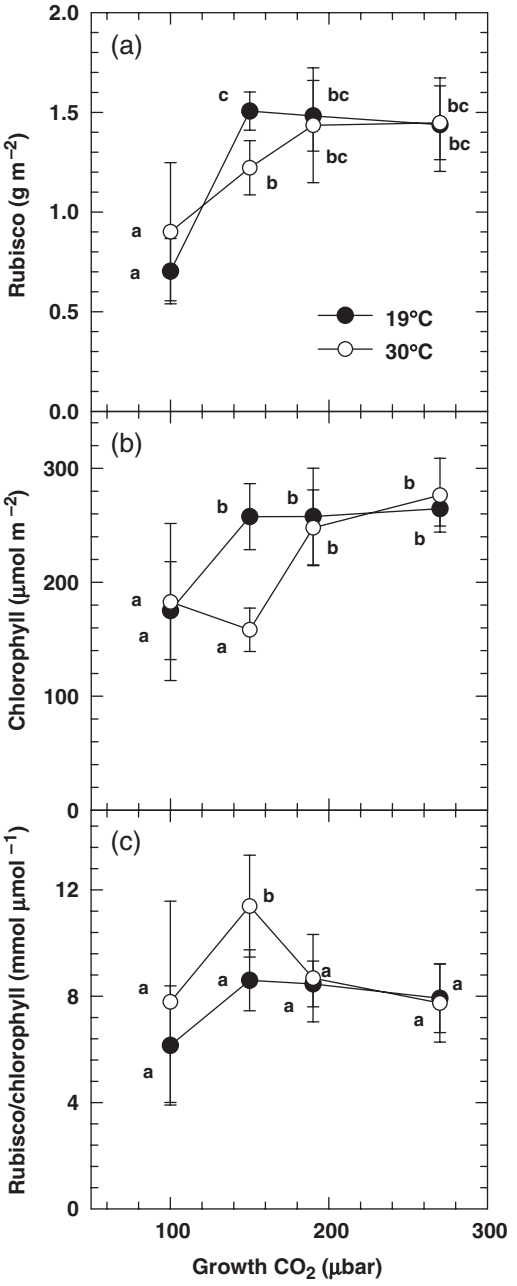

Fig. 5 Effects of growth CO_2 and temperature on (a) Rubisco content, (b) chlorophyll content and (c) the ratio of Rubisco to chlorophyll at 33 days after planting (DAP) at 19/15 °C and 28 DAP at 30/25 °C. Mean ± SD, $n = 9$ plants. Different letters indicate statistically significant differences at $P < 0.05$.

capacity (Smith *et al*., 1976; Farquhar & von Caemmerer, 1982; Sage *et al*., 1990). There was no evidence for increased carboxylation capacity, implicating respiration as the potential site of acclimation to low CO_2.

The lack of changes in Rubisco content at 180–270 µmol mol^{-1} are consistent with prior studies examining Rubisco responses to this range of CO_2 variation. For example, little photosynthetic acclimation was observed in rice, oats, wheat and beans grown between 350 and 160 µmol mol^{-1} CO_2 (Baker *et al*., 1990; Polley *et al*., 1992; Sage & Reid, 1992). Some species increase Rubisco levels when grown at low CO_2 compared with current levels, but these examples are much less common than the null response (Anderson *et al*., 2001; Sage & Coleman, 2001). By contrast to responses at low

Table 2 Growth parameters of tobacco plants at 15 weeks after planting

Growth CO_2	100 μmol mol^{-1} CO_2	150 μmol mol^{-1} CO_2
Shoot dry weight (g)	12 ± 2^a	18 ± 4^b
Height (cm)	66 ± 9^a	85 ± 13^b
Total fruit and flower number	10 ± 3^a	24 ± 10^b
Number of mature fruits	5.3 ± 3.3^a	8.5 ± 4.5^b
Seed weight (mg/100 seeds)	6.0 ± 0.4^a	7.0 ± 0.5^b
Germination rate of offspring (germinants/100 seeds)	59 ± 11^a	76 ± 18^b

Mean ± SD, $n = 12$ plants. Superscripted letters indicate statistically significant differences within a parameter at $P < 0.05$.

CO_2, at elevated CO_2, many species show reductions in Rubisco content, reflecting a feedback from high carbohydrate status in the plants (Sage & Coleman, 2001).

Given the relative lack of change at CO_2 levels between the current ambient and 190 μmol mol^{-1}, the reduction in Rubisco and chlorophyll content in tobacco at very low CO_2 is surprising. Reduced Rubisco content would reduce photosynthetic capacity at low CO_2, and thus, could reverse the reductions observed in the instantaneous CO_2 compensation point of photosynthesis (von Caemmerer & Farquhar, 1981). These possibilities could not be evaluated by gas exchange methods because the only plants to suffer reductions in Rubisco and chlorophyll content failed to accumulate sufficient leaf area through the course of the experiment. A fall off in Rubisco content at very low growth CO_2 may be a generalized response in C_3 plants. Majeau & Coleman (1996) found reduced Rubisco small subunit transcripts in pea grown at low (160 μmol mol^{-1}) CO_2, and Tissue *et al*. (1995) found reduced Rubisco content, as well as reduced chlorophyll and starch content, in *A. theorphrasti* grown at 150 μmol mol^{-1} CO_2, as compared with those grown at 350 μmol mol^{-1} CO_2. Dippery *et al*. (1995) suggest that this drop in leaf enzyme and chlorophyll content is because of reduced root growth under very low CO_2, which reduced nitrogen uptake and lowered nitrogen supply for photosynthesis. Alternatively, nitrogen metabolism is regulated by carbon supply, with the capacity for nitrogen reduction and assimilation being promoted by higher carbohydrate levels (Stitt & Krapp, 1999; Matt *et al*., 2001, 2002). At very low CO_2, carbon supplies may be insufficient to promote the same expression of nitrogen assimilation enzymes, leading to reduced leaf nitrogen and Rubisco levels. Unfortunately, this could not be directly assessed in these experiments.

Growth temperature increases CO_2 compensation points by increasing both photorespiration and dark respiration (Weber *et al*., 1985; Sage *et al*., 1990). Here, tobacco showed a greater CO_2 compensation point of instantaneous photosynthesis at 30/25 °C relative to 19/15 °C, but this was offset by a slightly higher initial slope of the A vs. C_i response such that the overall rate of carbon gain was similar at both temperatures above 150 μmol mol^{-1}. At 150 μmol mol^{-1} growth CO_2, tobacco plants at 30/25 °C had lower leaf mass per area and chlorophyll content relative to plants grown at 19/15 °C, indicating the warmer temperatures impaired growth and photosynthesis at low CO_2. Warm temperatures also increased seedling mortality, and, at 150 μmol mol^{-1} CO_2, reduced seedling growth such that seedlings remained restricted to a very small size (<1 cm^2) for the full 19 days of the trial. Given that temperatures above 30 °C would further promote photorespiration, we expect seedlings grown at temperatures above 30 °C to be restricted to the seedling stage for longer even under CO_2 conditions higher than 150 μmol mol^{-1}, including the 180 μmol mol^{-1} values of the late Pleistocene. Prior work with tobacco and *Phaseolus vulgaris* using established plants showed marked impairment of leaf expansion at 35/25 °C and 180–200 μmol mol^{-1} (Cowling & Sage, 1998; Sage & Cowling, 1999), indicating this temperature will cause serious problems for seedling establishment in atmospheric conditions of recent geological time. Earth's climate was colder during the Pleistocene; however, seedlings generally reside in the surface boundary layer where solar radiation can be trapped, thereby elevating the seedling temperatures to values where impaired growth would be expected. Thus, while temperature does not seem to affect Γ_{plant} directly, it does greatly affect the mortality rates of plants growing at low CO_2.

One of the popularized concepts associated with atmosphere–biosphere interactions is the Gaia hypothesis of Lovelock (1988). The Gaia hypothesis assumes the rate of CO_2 removal from the atmosphere by C_3 plants decreases at low CO_2, thereby acting as a self-regulatory check on atmospheric CO_2 depletion. A key assumption behind this aspect of the Gaia hypothesis is that the CO_2 compensation point of C_3 primary productivity is near 150 μmol mol^{-1} (Lovelock & Whitfield, 1982). Our results do not support this assumption, because we find the probable CO_2 compensation point is less than half this value in tobacco. As a result, the proponents of Gaia should re-evaluate the hypothesis in light of lower values of Γ_{plant} than previously assumed.

Implications for plant growth under low CO_2 atmospheres

Very low CO_2 severely retards growth, but does not stop it completely. There is some evidence, however,

that these slowly growing seedlings are more vulnerable to stochastic events. It was observed, in all experiments, that the plants grown at 30/25 °C were more likely to die over the course of the experiment. This may be because of increased vulnerability because of small root size, or just an increase in the length of the vulnerability window, as they were small for a longer period of time than the more rapidly growing plants in cooler treatments. Mortality rates are generally highest for smaller plants during seedling establishment, regardless of actual plant age, and seedling establishment generally has a higher mortality rate than other points in the plant life cycle (Grime & Hunt, 1975; Pino *et al*., 1997; Galen & Stanton, 1999; Hastwell & Facelli, 2003). One of the important mortality agents during seedling establishment is environmental stress, notably drought, disease and predation, and heat stress. By lengthening the vulnerability window, low CO_2 would greatly increase the time during which an episodic stress event could impact the seedling. When stress does occur, its severity should be greater, because low CO_2 is known to enhance intensity of both abiotic and biotic stress, while high CO_2 alleviates effects of many forms of stress (Cowling & Sage, 1998; Sage & Coleman, 2001). In low CO_2 conditions, the direct effects of low CO_2 on seedling establishment, combined with interactive effects between low CO_2 and environmental stress should have had profound effects on patterns of establishment in C_3 plant populations. This study also assessed the whole life cycle CO_2 compensation point in tobacco, which was found to occur between 65 and 100 $\mu mol\,mol^{-1}$ CO_2. The minimum limit here is defined by the plant-level CO_2 compensation point estimated here while the successful reproduction of the plants grown at 100 $\mu mol\,mol^{-1}$ CO_2 provides a maximum. This range, however, was determined under otherwise ideal conditions. During the Pleistocene, stressful conditions may have been enough to prevent successful reproduction of plants: herbivory, competition, nutrient and abiotic stresses may have exhausted the limited resource stores of plants growing at the time. In this experiment, the reduction in plant size and reproductive output of the plants grown at very low CO_2 suggest that fitness is reduced even at CO_2 concentrations above the life cycle CO_2 compensation point. The resulting increase in the mortality of C_3 seedlings could reflect intense selection pressure favoring adaptations that compensate for severe carbon deficiency during establishment. Among these are enhanced allocation to leaf area, increased seed size, and the development of carbon conservation mechanisms, including the scavenging of photorespiratory-derived CO_2 and eventually C_4 photosynthesis (Sage, 2004).

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