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Altered night-time CO_2 concentration affects the growth, physiology and biochemistry of soybean

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ABSTRACT

Soybean plants (*Glycine max* (L.) Merr. c.v. Williams) were grown in CO_2 controlled, natural-light growth chambers under one of four atmospheric CO_2 concentrations ($[CO_2]$): (1) 250 μmol mol^{-1} 24 h d^{-1} [250/250]; (2) 1000 μmol mol^{-1} 24 h d^{-1} [1000/1000]; (3) 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours [250/1000] or (4) 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours [1000/250]. During the vegetative growth phase few physiological differences were observed between plants exposed to a constant 24 h $[CO_2]$ (250/250 and 1000/1000) and those that were switched to a higher or lower $[CO_2]$ at night (250/1000 and 1000/250), suggesting that the primary physiological responses of plants to growth in elevated $[CO_2]$ is apparently a response to daytime $[CO_2]$ only. However, by the end of the reproductive growth phase, major differences were observed. Plants grown in the 1000/250 regime, when compared with those in the 1000/1000 regime, had significantly more leaf area and leaf mass, 27% more total plant dry mass, but only 18% of the fruit mass. After 12 weeks of growth these plants also had 19% higher respiration rates and 32% lower photosynthetic rates than the 1000/1000 plants. As a result the ratio of carbon gain to carbon loss was reduced significantly in the plants exposed to the reduced night-time $[CO_2]$. Plants grown in the opposite switching environment, 250/1000 versus 250/250, showed no major differences in biomass accumulation or allocation with the exception of a significant increase in the amount of leaf mass per unit area. Physiologically, those plants exposed to elevated night-time $[CO_2]$ had 21% lower respiration rates, 14% lower photosynthetic rates and a significant increase in the ratio of carbon gain to carbon loss, again when compared with the 250/250 plants. Biochemical differences also were found. Ribulose-1,5-bisphosphate carboxylase/oxygenase concentrations decreased in the 250/1000 treatment compared with the 250/250 plants, and phosphoenolpyruvate carboxylase activity decreased in the 1000/250 compared with the 1000/1000 plants. Glucose, fructose and to a lesser extent sucrose concentrations also were reduced in the 1000/250 treatment compared with the 1000/1000 plants. These results indicate that experimental protocols that do not maintain elevated CO_2 levels 24 h d^{-1} can have significant effects on plant biomass, carbon allocation and physiology, at least for fast-growing annual crop plants. Furthermore, the results suggest some plant processes other than photosynthesis are sensitive to $[CO_2]$ and under ecologically relevant conditions, such as high night-time $[CO_2]$, whole plant carbon balance can be affected.

Key-words: *Glycine max*; carbohydrates; night-time CO_2; phosphoenolpyruvate carboxylase; photosynthesis; respiration; Ribulose-1,5-Bisphosphate carboxylase/oxygenase.

INTRODUCTION

Plant carbon fixation is a light-driven process, and thus it may seem logical to assume plant processes that respond to elevated CO_2 concentrations ($[CO_2]$) are sensitive only to the $[CO_2]$ around the leaves during daylight hours. However, short-term increases in ambient $[CO_2]$ are known to produce decreases in respiration (measured as CO_2 efflux in the dark, reviewed in Amthor 1997). This effect can result in a decrease of as much as 56% in respiration when the ambient $[CO_2]$ is doubled (Downton & Grant 1994; Begg & Jarvis 1968). Long-term effects of elevated $[CO_2]$ on apparent respiration have also been reported (Poorter *et al*. 1992; Thomas & Griffin 1994; Wullschleger, Ziska & Bunce 1994; Bunce 1995a; Amthor 1997). In a review of long-term $[CO_2]$ effects on leaf respiration published during 1992, Poorter *et al*. (1992) found apparent respiration rates increased in response to growth in elevated $[CO_2]$ by an average of 16% when expressed on a leaf area basis (μmol m^{-2} s^{-1}), but decreased by an average of 14% when expressed on a leaf mass basis (μmol g^{-1} s^{-1}). Any interactions between short- and long-term effects would complicate further the analysis of these results and our ability to predict whole plant responses to changes in atmospheric $[CO_2]$. Furthermore, the magnitude and even direction of these long-term responses can be influenced by leaf age (Thomas & Griffin 1994) and/or other environmental variables (Griffin, Ball & Strain 1996). Despite the fact that respiratory responses to elevated $[CO_2]$ are commonly reported, their overall impact

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on plant carbon balance and allocation has not been well quantified. Although the instantaneous rate of respiratory carbon loss is generally fairly small when compared with the instantaneous rate of photosynthetic carbon uptake, the net effect of even a small change in respiration when integrated over the course of a 24 h day and a full growing season could be quite substantial, as plant respiratory processes return roughly 50% of photosynthetically fixed carbon to the atmosphere annually (Amthor 1995).

Human activities are resulting in a rapid increase in the global atmospheric [CO_2] (Keeling *et al*. 1995), making an understanding of the effects of [CO_2] on respiration essential. Additionally, there are a number of situations in which [CO_2] can vary greatly during the course of a single day. For example, in agricultural fields night-time [CO_2] can be extremely variable and as high as 800 μmol mol^{-1} (Allen 1971; Bunce 1995a). Night-time [CO_2] can be even higher in the understory of forest canopies or in orchards (Fuller 1948; Sparling & Alt 1965; Garrett, Cox & Roberts 1978; Bazzaz & Williams 1991) where the CO_2 from soil and plant respiration can accumulate in still air masses. Finally the high CO_2 cost associated with Free Air CO_2 Enrichment (FACE) technology has caused investigators to consider whether they can discontinue CO_2 fumigation at night. Here we present the results of a growth chamber study that examined the sensitivity of plant growth, biomass allocation, physiology and biochemistry to different night-time [CO_2].

MATERIALS AND METHODS

Individual soybean plants (*Glycine max* (L.) Merr. c.v. Williams) were grown from seed in 15 L pots filled with a decomposed granite and topsoil mix (1 : 1 by volume). The seeds were sown, germinated and grown continuously in naturally lit, temperature- and CO_2-controlled growth chambers located in the Great Basin Environmental Research Laboratory of the Desert Research Institute in Reno, Nevada. Environmental conditions within the chambers were 28 °C day-time and 17 °C night-time temperatures, approximately 60% relative humidity, a natural photoperiod averaging 13·5 h. of daylight and one of four atmospheric [CO_2] treatments: (1) 250 μmol mol^{-1} 24 h d^{-1}; (2) 1000 μmol mol^{-1} 24 h d^{-1}; (3) 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours; or (4) 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours. These treatments will be abbreviated as follows: 250/250, 1000/1000, 250/1000, 1000/250, representing the day/night [CO_2] treatment. The elevated [CO_2] treatments were created by adding pure CO_2 to a mixing fan within the chambers. The low [CO_2] treatments were created by placing two scrubbers within each chamber and cycling a portion of the air contained within the chamber through the scrubber. Each scrubber consisted of a 4-inch-diameter PVC pipe filled with 4–6 in of indicating soda lime and capped with a fine mesh on the bottom and a fan on the top. The [CO_2] within the chambers was controlled by cycling the fan on and off as needed. The day/night switching of the [CO_2] within the chambers was controlled by the detection of 'sun-up' or 'sun-down' by an unobstructed gallium arsenide photodiode placed on the roof of the Great Basin Environmental Research Laboratory and monitored with a data logger (CR10X; Campbell Scientific, Logan, UT, USA) that also provided monitoring and control of the growth chambers. The atmospheric [CO_2] in the chambers were monitored with an infrared gas analyzer (Li-6262; Li-Cor Inc., Lincoln, NE, USA).

The seeds were planted in early March 1996 and were grown for approximately 80 d. All plants were well watered each day with deionized water, and each pot contained 17 g of Osmocote slow-release fertilizer (8·2% ammoniacal nitrogen, 5·8% nitrate nitrogen, 14% P_2O_5, 14% K_2O, Scotts-Sierra Horticultural Products Company, Marysville, OH, USA). One month after emergence a liquid fertilizer containing the major micronutrients was applied. On 5 June 1996 all plants were rapidly senescing and were harvested. All leaves were removed and the leaf area was measured (Li-3000; Li-Cor Inc.). The aboveground portion of the plant was separated into leaves, stems and fruits and dried to a constant mass in a 65 °C oven and weighed. The roots were recovered by washing away the sand/soil mix in a wooden frame with a fine nylon mesh lining and similarly dried and weighed.

On 8 May 1996, 8 weeks after planting, gas-exchange measurements were made with a portable open flow gas exchange system with a CO_2 control system (Li-6400; Li-Cor Inc.). The centre leaflet of the youngest fully expanded trifoliate of the main stem was placed in the cuvette. Environmental conditions within the cuvette mimicked the growth conditions. Photosynthetic rates were measured between 0900 h and noon on five replicate plants from each chamber. The net photosynthetic rate, stomatal conductance, and intercellular [CO_2] were recorded for ambient [CO_2] of both 250 and 1000 μmol mol^{-1} for each leaf. The respiration measurements were made during the evening following the photosynthetic measurements on the same trifoliate. These measurements were initiated no sooner than 2 h after dusk (and therefore the CO_2 switch). The following morning the leaf area of this trifoliate was measured and sampled by removing leaf punches of a known area and quickly plunging them into liquid nitrogen. Similarly on 6 June 1996, 12 weeks after planting, these measurements were repeated. All leaf samples were stored in a –80 °C ultra-low freezer until the analysis of the enzymes could take place.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and content were measured as described in Evans & Seemann (1984). Leaf material was homogenized and extracted in a buffer containing 100 mM Bicine [pH 7·8], 5 mM $MgCl_2$, 1 mM Na_2EDTA, 5 mM DTT (dithiothreitol) and 10% w/w PVPP (polyvinyl polypyrroliodone). The extracted material was centrifuged at 2500 *g* for 5 min, and the resulting supernatant was assayed in triplicate. The assay medium contained 100 mM Tricine-KOH (pH 8·1), 20 mM $MgCl_2$ and 20 mM $NaH^{14}CO_2$. (All chemicals from Sigma Chemical Co., St. Louis, MO, USA). Each assay was started by adding 1 mM RuBP (ribulose-1,5-bisphosphate) and ran

for 60 s at 25 °C. The reaction was terminated with 2N HCl, the reaction medium was evaporated and the activity was determined in a scintillation counter. The total number of Rubisco active sites was determined using a [^{14}C]CABP (carboxyarabinatol bisphosphate) assay by adding crude leaf extract to a buffer of 100 mM Bicine [pH 8·0], 20 mM $MgCl_2$, 4 nmol [2–^{14}C]CABP and 100 μL of antisera to RuBP carboxylase from chicken eggs. The protein was precipitated, collected on filters and washed using 0·85% NaCl and 10 mM $MgCl_2$. The bound [^{14}C] was determined with a scintillation counter and was a direct measure of the molar concentration of the enzyme active sites.

Phosphoenolpyruvate carboxylase (PEPc) activity was assayed as described in Lane, Maruyama & Easterday (1969). The leaf material was homogenized and extracted as described above. The extracted material was centrifuged at 2500 *g* for 5 min and resulting supernatant was assayed in triplicate. The assay medium contained 100 mM Bicine (pH 7·8), 5 mM $MgCl_2$, 1 mM EDTA, 550 μM NADH, 15 units MDH, 2 mM PEP and 20 mM $H^{14}CO_3$. Each assay ran for 5 min at 25 °C at which time it was terminated with 2N HCl. The reaction medium was evaporated, the residue was dissolved in Universol (ICN Pharmaceuticals Inc., Costa Mesa, MO, USA), and the activity was counted in a liquid scintillation counter.

Chlorophyll was extracted in ethanol and determined spectrophotometrically following the protocol of Wintermans & De Mots (1965). Nonstructural carbohydrates (glucose, fructose, sucrose and starch) were determined using the methods of Hendrix (1983) as follows: leaf tissue was extracted in 80% ethanol at 80 °C and portions of the extract were placed in microtitre plate wells. The plates were dried at 60 °C, re-suspended in 20 μL water and 100 μL of glucose analysis solution (glucose kit 115 A; Sigma Chemical Co.) and the absorbance at 490 nm was read on a plate reader. Fructose and sucrose were measured as the increase in absorbance following respective additions of phosphoglucose isomerase, 1 EU mL^{-1} of 0·2 M HEPES buffer, and invertase, 4260 EU mL^{-1} of 0·1 M citrate buffer, 20 μL to each well. The starch in the extracted tissue was converted to glucose and measured as above. For starch digestion, the tissue was boiled in 0·1 M KOH for 1 h, the pH of the solution was lowered to 7 with acetic acid, and heated to 85 °C for 30 min in the presence of (alpha) amylase. The pH was then lowered to 4·5 with additional acetic acid and heated at 50 °C for 1 h with amyloglucosidase.

The effects of growth-CO_2 concentration and leaf age on all measured parameters and calculated sensitivities were tested by ANOVA (Data Desk 5·0 statistical software, Data Description Inc., Ithaca, NY, USA). Means separation, based on planned comparisons, were accomplished with a protected LSD test. Treatment effects and means separation were considered significant only when $P \leq 0{\cdot}05$. Statistical outliers, calculated as points whose value is either > upper quartile + 1·5 x interquartile distance or < lower quartile – 1·5 x interquartile distance, are shown and included in the descriptive statistics, although excluded from the analysis of variance and means separation (gas-exchange characteristics only).

RESULTS

The total biomass accumulation and partitioning was responsive to daytime [CO_2]. When averaged across the switching treatments, the plants grown in 1000/1000 and 1000/250 were 2·4 times larger than the 250/250 and 250/1000 plants. The plants grown in high daytime CO_2 had more leaf mass, stem mass and root mass, higher root-to-shoot ratio, more than twice the leaf area, and a significant increase in leaf area per unit leaf mass (SLM). Of greater interest here was the comparison between the plants grown in constant 24 h CO_2 and their night-switching counterparts (250/250 compared with 250/1000 and 1000/1000 compared with 1000/250). For example the 250/1000 versus the 250/250 plants had 18% higher SLM, but 21% lower leaf area per unit total plant mass (LAR), with the balance of the plant mass resulting in a nonstatistically significant 9% increase in root mass (Table 1).

Table 1. Biomass accumulation and allocation in soybean plants grown from seed to senescence in one of four [CO_2] treatments: 250 μmol mol^{-1} 24 h d^{-1}, 1000 μmol mol^{-1} 24 h d^{-1}, 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours or 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours

	[CO_2] treatment – day-time: night-time (μmol mol^{-1})			
	250/250	250/1000	1000/1000	1000/250
Leaf mass (g)	2·21 ± 0·39a	2·08 ± 0·08a	5·59 ± 0·93b	8·05 ± 0·91c
Stem mass (g)	1·08 ± 0·20a	0·90 ± 0·04a	3·07 ± 0·62b	4·26 ± 0·67b*
Fruit mass (g)	2·75 ± 0·19c	3·08 ± 0·13c	1·02 ± 0·25b	0·18 ± 0·09a
Shoot mass (g)	6·04 ± 0·66a	6·05 ± 0·22a	9·68 ± 1·45b	12·49 ± 1·55b*
Root mass (g)	2·87 ± 0·74a	3·12 ± 0·69a	9·70 ± 2·03b	12·19 ± 1·06b
Total mass (g)	8·91 ± 1·39a	9·18 ± 0·57a	19·38 ± 3·16b	24·69 ± 2·38b*
R : S (g : g)	0·45 ± 0·06a	0·53 ± 0·13a	1·01 ± 0·14b	1·01 ± 0·10b
LAR (cm^2 g)	47·25 ± 3·92b	37·28 ± 2·98a	43·35 ± 2·23ab	47·82 ± 2·48b
Leaf area (cm^2)	432·17 ± 87·8a	337·69 ± 11·6a	831·96 ± 128·5b	1175·85 ± 116·6b
SLM, (cm^2 g^{-1})	52·42 ± 2·27a	61·74 ± 2·37b	66·69 ± 1·16bc	68·13 ± 1·24c

Shoot mass = leaf mass + stem mass + fruit mass; R : S, root-to-shoot ratio; SLM, leaf area per unit leaf mass; LAR, leaf area ratio or leaf surface area per gram of total plant mass. All data are presented as means ± 1 SE, $n = 5$. Within each row, values followed by the same letter are not statistically different at the 0·05 level of significance. Marginally significant differences are denoted with a * ($P = 0{\cdot}08$).

There were several differences in biomass allocation in the 1000/250 plants compared with the plants grown in 1000/1000 (Table 1). When the elevated [CO_2] was not continued in the dark hours of the day, the average 1000/250 plant was 25% larger ($P = 0{\cdot}08$) and had significantly more leaf mass than the average plant grown in 1000 /1000. Nearly significant 30% increases in stem mass and total above-ground mass also were found in these plants. However, SLM, LAR and root-to-shoot ratio were unaffected by low [CO_2] at night (1000/250 versus 1000/1000). This specific treatment, 1000/250, also resulted in a highly significant decrease in fruit mass (Table 1). In fact, only one of five plants in this treatment had any significant accumulation of fruit mass (data not shown). All plants flowered at roughly the same time, yet there was little seed set.

Physiologically, it was difficult to distinguish the switched plants from the nonswitched plants after 8 weeks of growth (250/250 versus 250/1000 and 1000/1000, compared with the 1000/250, Fig. 1). The net photosynthetic rates at the day-time growth [CO_2] were approximately 5 μmol m^{-2} s^{-1} higher in the elevated [CO_2], 1000/1000 and 1000/250, than they were in the low [CO_2], 250/250 and 250/1000. Dark respiration was 35% lower in elevated [CO_2] compared with the low [CO_2] (1000/1000 versus 250/250), and increased when the [CO_2] was reduced at night (1000/250 as compared to the 1000/1000). The ratio between instantaneous leaf-level carbon gain and carbon loss averaged 10 : 1 and was not significantly affected by [CO_2]. Interestingly, the sensitivity of photosynthesis to a change of atmospheric [CO_2] from 250 to 1000 μmol mol^{-1} was correlated to the daytime [CO_2], while sensitivity of respiration to the same change correlated to the night-time [CO_2] (Table 2). Respiration decreased about 25% in response to a short-term change in ambient [CO_2] in the 250/250 and 1000/250 treatments. The 250/1000 and 1000/1000 plants showed no significant respiratory sensitivity to short-term changes in ambient [CO_2]. The photosynthetic rates of plants grown in high daytime [CO_2] were more sensitive to short-term changes in atmospheric [CO_2], increasing an average of 140% between 250 and 1000 μmol mol^{-1} compared with the plants grown in low daytime [CO_2]. The photosynthetic rates of the 250/250 and 250/1000 plants increased an average of 35% over the same range of ambient [CO_2]. Stomatal conductance was highest in the low [CO_2] day-time plants and the 250/1000 switched plants had a significantly lower conductance than the 250/250 plants. This reduction in stomatal conductance did not result in a significant difference in intercellular [CO_2] due to small differences in the rate of net carbon uptake. During the dark period the inverse was found; stomatal conductance was not sensitive to [CO_2] switching but intercellular [CO_2] was, in this case, as a result of changes in the rate of respiration.

After 12 weeks of growth, physiological differences between the constant 24 h treatments and their night-time switching counterparts were more apparent. Net photosynthetic rates had decreased significantly in the 4 week period since the first measurements were made, presumably due to the rapid onset of senescence, particularly in the high

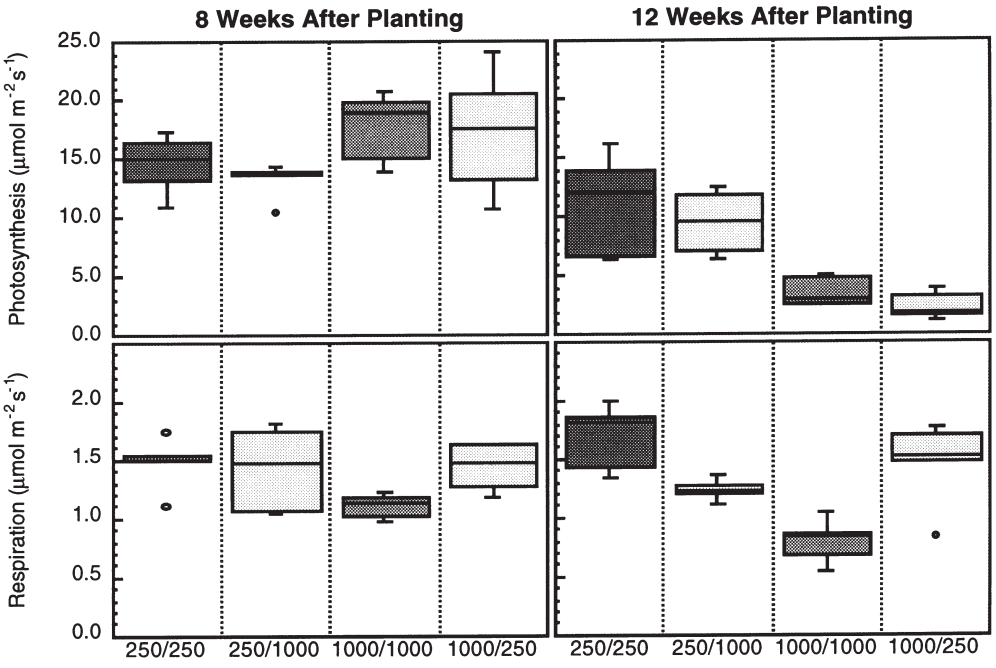

Figure 1. Net photosynthetic rates (CO_2 uptake in the light) and apparent respiration rates (CO_2 efflux in the dark) from fully expanded soybean leaves grown for either 8 or 12 weeks in one of four atmospheric [CO_2] treatments: 250 μmol mol^{-1} 24 h d^{-1}, 1000 μmol mol^{-1} 24 h d^{-1}, 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours or 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours. The median value for each treatment is the horizontal line within the box. The shaded box contains the upper and lower quartiles (UQ and LQ) and the whiskers contain the data range. Statistical outliers, calculated as points whose value is either > UQ + 1.5 * (UQ-LQ) or < LQ –1.5 * (UQ-LQ), are plotted as open circles. $n = 5$.

Table 2. Gas-exchange characteristics of soybean leaves grown in one of four [CO_2] treatments: 250 μmol mol^{-1} 24 h d^{-1}, 1000 μmol mol^{-1} 24 h d^{-1}, 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours or 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours

	[CO_2] treatment – day-time: night-time (μmol mol^{-1})			
	250/250	250/1000	1000/1000	1000/250
8 weeks				
Photosynthesis:respiration	9·81 ± 0·50a	9·89 ± 1·39a	12·96 ± 1·56a	11·77 ± 2·08a
Respiratory sensitivity (R_{1000}/R_{250})	0·74 ± 0·03a	1·00 ± 0·08b	0·99 ± 0·05b	0·73 ± 0·04a
Photosynthetic sensitivity (A_{1000}/A_{250})	1·22 ± 0·12a	1·52 ± 0·10a	2·36 ± 0·07b	2·42 ± 0·24b
Daytime stomatal conductance (mmol $^{-2}$ s^{-1})	1976 ± 345·8c	821 ± 89·6b	284 ± 12·5a	362 ± 48·6ab
Daytime intercellular [CO_2] (p.p.m.)	225·6 ± 1·8a	210·0 ± 6·6a	864·2 ± 12·1b	883·8 ± 23·1b
Night-time stomatal conductance (mmol m^{-2} s^{-1})	37·2 ± 3·2a	35·6 ± 7·3a	24·9 ± 2·4a	31·2 ± 4·5a
Night-time intercellular [CO_2] (p.p.m.)	307·4 ± 3·4a	1044·0 ± 8·7b	1076·0 ± 11·2c	328·8 ± 12·9b
12 weeks				
Photosynthesis:respiration	6·53 ± 1·04c	7·65 ± 0·88c	4·91 ± 1·17b	1·70 ± 0·29a
Respiratory sensitivity (R_{1000}/R_{250})	0·59 ± 0·10a	0·70 ± 0·04a	0·78 ± 0·09a	0·65 ± 0·06a
Photosynthetic sensitivity (A_{1000}/A_{250})	1·9 ± 0·12a	1·7 ± 0·16a	6·8 ± 1·85b	10·1 ± 3·10b
Daytime stomatal conductance (mmol $^{-2}$ s^{-1})	1108 ± 47·2b	940 ± 124b	42·4 ± 5·32a	31·2 ± 3·01a
Daytime intercellular [CO_2] (p.p.m.)	222 ± 3·04a	221 ± 3·37a	805 ± 16·41b	813 ± 20·02b
Night-time stomatal conductance (mmol m^{-2} s^{-1})	34·0 ± 6·7ab	46·7 ± 6·58b	31·26 ± 3·64a	40·12 ± 5·90ab
Night-time Intercellular [CO_2] (p.p.m.)	333 ± 12·9b	1048 ± 6·63c	1054 ± 10·3c	300 ± 8·8a

All data are presented as means ± 1 SE, n = 5. Within each row, values followed by the same letter are not statistically different at the P = 0·05 level of significance.

day-time [CO_2] plants (1000/1000 and 1000/250, Fig. 1). Carbon gain was unaffected by the switching treatments (250/250 compared with the 250/1000, and 1000/1000 compared with the 1000/250). However, respiration rates were affected significantly by the switching treatments. Plants grown with low night-time [CO_2] (250/250 and 1000/250) had higher leaf-level respiration rates than the corresponding plants grown in high night-time [CO_2] (250/1000 and 1000/1000, respectively). The combination of these differences in carbon exchange resulted in a significant change in the ratio of instantaneous carbon gain to carbon loss. Plants grown in 1000/250 had the lowest ratio of photosynthesis to respiration (1·7) and plants grown in a constant 1000 μmol mol^{-1} atmosphere had a ratio closer to 5. Interestingly, the respiratory sensitivity to a change in ambient [CO_2] was similar between all treatments after 12 weeks of growth. This was not the case after 8 weeks of growth, when the plants grown with elevated [CO_2] at night showed no sensitivity, suggesting that this response can be developmentally and/or environmentally controlled. Although the photosynthetic rates of the plants grown in high day-time [CO_2] were significantly more sensitive to ambient [CO_2] than those grown in low day-time [CO_2], this result is a bit misleading since the plants grown in elevated [CO_2] had low initial photosynthetic rates. Day-time stomatal conductance and intercellular [CO_2] were sensitive to day-time [CO_2] and were insensitive to night-time [CO_2], while night-time conductance and intercellular [CO_2] were related more closely to night-time [CO_2].

Table 3. Soluble sugars and starch in soybean leaves grown in one of four [CO_2] treatments: 250 μmol mol^{-1} 24 h d^{-1}, 1000 μmol mol^{-1} 24 h d^{-1}, 250 μmol mol^{-1} during daylight hours and 1000μmol mol^{-1} during night-time hours or 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours

	[CO_2] treatment – day-time: night-time (μmol mol^{-1})			
	250/250	250/1000	1000/1000	1000/250
Glucose (mg g^{-1} FW)	0·188 ± 0·015a	0·160 ± 0·009a	0·448 ± 0·170b	0·216 ± 0·011a
Fructose (mg g^{-1} FW)	0·108 ± 0·016a	0·098 ± 0·008a	0·401 ± 0·188b*	0·142 ± 0·018ab
Sucrose (mg g^{-1} FW)	2·478 ± 0·447a	2·085 ± 0·148a	2·80 ± 0·246a*	2·108 ± 0·112ab
Starch (mg g^{-1} FW)	40·5 ± 7·155a	56·4 ± 6·261a	138·3 ± 10·733b	140·8 ± 9·615b

*, P = 0·07; FW, fresh weight. All data are presented as means ± 1 standard error, n = 5. Within each row, values followed by the same letter are not statistically different at the 0·05 level of significance.

The soluble sugars, glucose, fructose and sucrose, were higher in plants grown in higher day-time [CO_2] (Table 3). When the elevated [CO_2] was limited to the daylight hours, the levels of all three sugars were lower (1000/250 compared with the 1000/1000). Starch concentrations were significantly higher in the elevated day-time treatments compared with the low day-time treatments, but not affected by switching between 1000 and 250, compared with the 1000/1000. Starch concentrations increased in the 250/1000 plants compared with those of the 250/250 plants.

Chlorophyll concentrations were related most closely to day-time [CO_2] (Table 4). The Rubisco content was three- to four-times higher in low [CO_2] day-time treatments than in the high day-time [CO_2]. Increasing the [CO_2] at night decreased Rubisco (250/1000 compared with the 250/250). When expressed per mg of chlorophyll, there were few significant differences in Rubisco content. The plants grown in the 250/250 treatment had nearly twice the activity of PEPc as plants grown in the 1000/1000 treatment, and there were strong interactions between day-time and night-time [CO_2] (Table 4). Reducing the [CO_2] at night cut the activity of PEPc in half (1000/250 compared with the 1000/1000) on both per unit fresh weight or on a chlorophyll-normalized basis.

DISCUSSION

After 12 weeks of growth in the switching [CO_2], we found dramatic differences in the biomass allocation and physiology of plants grown in high day-time CO_2/low night-time CO_2 (1000/250) conditions compared with plants grown in constant high [CO_2] (1000/1000). The most striking of these results was a nearly complete lack of reproductive material (fruits) in these plants. There also were large changes in leaf area and mass, as well as smaller changes in LAR. These changes in plant biomass were accompanied by physiological differences that occurred only late in the reproductive phase of growth. During this period, respiration rates increased and photosynthetic rates decreased and the associated ratio of leaf-level carbon gain to carbon loss therefore decreased in the 1000/250 plants, compared with the 1000/1000 plants. Similarly, physiological differences were found when comparing the 250/250 plants with the 250/1000 plants. In this case the respiration was reduced in the switching plants and photosynthesis was increased, resulting in an increased ratio of leaf-level carbon gain to carbon loss compared with the 250/250 plants. However, unlike the comparison between the plants grown in constant high [CO_2] and their high/low counterparts, we found no important differences in biomass or biomass allocation between the constant low and the low/high switching plants.

We know of few other studies that have examined the effects of altered day-time versus night-time [CO_2] on plant growth and physiology experimentally. Most of the existing studies examined only the effect of elevated [CO_2] at night (Reuveni & Gale 1985; Reuveni, Gale & Meyer 1993; Bunce 1995a; but see Reuveni, Gale & Zeroni 1997). Alfalfa, soybean, lemna and xanthium all had more biomass when plants were grown in elevated [CO_2] at night than when they were grown in 24 h ambient [CO_2] (approximately 350 μmol mol^{-1} CO_2). Bunce (1995a) found this increase to be the result of a higher leaf area ratio and suggested that decreased respiration was the physiological mechanism behind the increase in biomass since only small changes in leaf-level photosynthetic rates were found. In agreement with our conclusions, Bunce (1995a) suggested that plant developmental factors are important, and that some of the differences between plants grown in high 24 h [CO_2] and those grown in elevated [CO_2] only at night arise only after some extended period of time in the [CO_2] switching treatment. For example, Bunce (1995a) reported changes in leaf area growth did not become significant until 14 d after planting and the relative difference (comparison between 24 h 350 μmol mol^{-1} CO_2 plants and 350/700 μmol mol^{-1} CO_2 plants) in the net assimilation rate (g m^{-2} d^{-1}) decreased over the 20 d of the experiment.

We found that during the early vegetative phase of plant growth the day-time [CO_2] dominates the physiological

Table 4. Chlorophyll, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate carboxylase (PEPcase) activity in soybean leaves grown in one of four [CO_2] treatments: 250 μmol mol^{-1} 24 h d^{-1}, 1000 μmol mol^{-1} 24 h d^{-1}, 250 μmol mol^{-1} during daylight hours and 1000 μmol mol^{-1} during night-time hours or 1000 μmol mol^{-1} during daylight hours and 250 μmol mol^{-1} during night-time hours.

	[CO_2] treatment – day-time: nighttime (μmol mol^{-1})			
	250/250	250/1000	1000/1000	1000/250
Chlorophyll (mg g^{-1} FW)	1·80 ± 0·13c	1·40 ± 0·14b	0·44 ± 0·08a	0·39 ± 0·14a
Rubisco content (nmol sites g^{-1} FW)	67·8 ± 9·39c	47·5 ± 3·45b	17·9 ± 3·13a	15·5 ± 2·68a
Rubisco content (nmol sites mg^{-1} Chl)	37·20 ± 2·95 ab	34·34 ± 2·17a	41·25 ± 1·86b	39·75 ± 1·18ab
PEPcase activity (μmol g^{-1} FW h^{-1})	16·34 ± 7·42c	9·97 ± 1·25b	9·00 ± 1·87b	4·68 ± 2·04a
PEPcase activity (μmol mg^{-1} Chl h^{-1})	11·06 ± 3·92ab	9·31 ± 1·64a	30·28 ± 6·22c	14·76 ± 3·24b

FW, fresh weight; Chl, chlorophyll. All data are presented as means ± 1 SE, n = 5. Within each row, values followed by the same letter are not statistically different at the 0·05 level of significance.

responses, with plants grown in high day-time [CO_2] having higher photosynthetic rates than those grown in low day-time [CO_2]. The change in late reproductive phase leaf physiology demonstrates either substantial photosynthetic acclimation to day-time [CO_2] and/or an early onset of senescence relative to the plants grown in low day-time [CO_2]. The respiratory responses are consistent with reported changes in CO_2 efflux from leaves in response to immediate changes in ambient [CO_2] (Bunce 1990; Amthor, Koch & Bloom 1992; Thomas & Griffin 1994; Bunce 1995b; Griffin *et al*. 1996). When ambient [CO_2] increases, the leaf-level CO_2 efflux tends to decrease. Reconciling these changes in photosynthetic and respiratory rates with changes in plant growth and biomass is difficult. Both Bunce (1995a) and Reuveni *et al*. (1997) invoke respiratory mechanisms to explain changes in whole plant biomass but in some instances, with opposite conclusions. Bunce (1995a) suggests a negative correlation such that a decrease in respiration results in an increase in plant biomass for plants grown in high [CO_2] during the night-time versus those grown in 24 h low [CO_2]. Reuveni *et al*. (1997) on the other hand found in some cases, as respiration decreases, so does final biomass, but in other cases the inverse correlation of Bunce (1995a) holds. To explain this Reuveni *et al*. (1997) suggest there are two respiratory components, one that controls useful metabolism and another that is 'functionless' (not supporting growth or maintenance), and these two components can be affected individually by environmental conditions. Our results are most similar to those of Reuveni *et al*. (1997), showing a positive correlation between respiration and growth; in our case an increase in total biomass with an increase in leaf-level respiration, although this simple relationship did not hold for fruit mass, which is correlated inversely with leaf-level respiration.

Perhaps the most significant of our findings is the dramatic reduction in fruit mass in the 1000/250-grown plants. Many studies have shown that reproductive growth, seed mass, harvest index and yield are all sensitive to [CO_2] and can decrease at elevated [CO_2] (Madsen 1974, Wheeler *et al*. 1993; Grotenhuis & Bugbee 1996; Mackowiak & Wheeler 1996; Reuveni & Bugbee 1997). Many of these studies find a threshold near 1000 μmol mol^{-1}, above which fruit or seed mass starts to decrease. Wheeler *et al*. (1993) experimented with two varieties of soybean and found that cv. McCall had decreased seed yield when grown in ambient [CO_2] higher than 1000 μmol mol^{-1} but that seed yield of cv. Pixie was not affected by ambient [CO_2] over a range of 500–5000 μmol mol^{-1}. Our results indicate that cv. Williams has decreased fruit mass at 1000 μmol mol^{-1} and that lowering the [CO_2] level at night produced an even more dramatic effect. Obviously if this latter result is confirmed independently and found in other plant species it has important mechanistic implications, as well as dramatic implications for individual species responses and for population and community dynamics.

Growth responses to day–night switching also seem to be sensitive to other environmental stresses. For example a high night-time [CO_2] (900 or 1700 μmol mol^{-1} CO_2) had no effect on the growth of Xanthium plants unless they were exposed to a strong salinity stress (Reuveni *et al*. 1997). We suggest night-time [CO_2] responses may be controlled by the same carbohydrate signalling mechanism that has been suggested for the regulation of photosynthesis under elevated [CO_2] (Van Oosten & Besford 1995; Griffin & Seemann 1996; d Moore, Palmquist & Seemann 1997; Cheng, Moore & Seemann 1998). Accordingly gene expression, and therefore cellular metabolism, is responding to the level or rate of cytosolic sucrose cycling. Any environmental variable that influences the level of these sugars could have an effect on plant responses to elevated [CO_2], and since many plant stresses would result in changes in cellular sugar pools, the interaction between the salinity and night-time [CO_2] response reported by Reuveni *et al*. (1997) could be consistent with other findings (Reuveni & Gale 1985; Bunce 1995a) and with our results. It is logical to suspect the reported responses would therefore be interactive with other environmental variables such as light, temperature, nutrient availability and soil moisture conditions.

We observed significant effects of [CO_2] switching on the biochemistry of soybean leaves. The content of Rubisco, the main carbon fixing enzyme of C_3 plants, was significantly decreased by elevated day-time [CO_2] (250/250 and 250/1000 versus 1000/1000 and 1000/250), a result that is consistent with much of the elevated CO_2 literature (Sage, Sharkey & Seemann 1989; Besford, Ludwig & Withers 1990; Rowland-Bamford *et al*. 1991; Cheng *et al*. 1998). Increasing the [CO_2] at night (250/1000 versus 250/250) also decreased Rubisco. The reduced Rubisco protein content of the high day-time [CO_2] plants was not affected by night-time [CO_2] (1000/1000 and 1000/250), perhaps suggesting the concentration of Rubisco had reached a minimum threshold. Previous research has shown respiratory enzymes also can be sensitive to ambient [CO_2] (Azcón-Bieto *et al*. 1994; Gonzàles-Meler *et al*. 1996). Here we measured PEPc because of its potential role as a dark carbon fixing enzyme with subsequent effects on dark CO_2 exchange (Amthor 1997). We found PEPc activity was significantly increased (μmol mg^{-1} Chl h^{-1}) in the 1000/1000 and 1000/250 plants compared with the 250/250 and 250/1000 plants. The plants with the highest respiration rates had the lowest mass based PEPc activity (μmol g^{-1} FW h^{-1}), and the plants with lowest respiratory rates had the highest chlorophyll-based PEPc activity (μmol mg^{-1} Chl h^{-1}). Increased PEPc carbon fixation in the dark could help explain lower carbon efflux rates, but only if the products of the fixation (C_4 acids), or nondecarboxylated secondary products were accumulated; an interesting possibility that requires more attention. Similar to our results, Riviere-Rolland, Contard & Betsche (1996) found weight-based measures of PEPc activity decreased by 50% when pea plants were grown in 1000 μmol mol^{-1} CO_2, and suggest regulation of this

enzyme may be important in balancing carbon partitioning between amino acids and carbohydrates. Spencer & Bowes (1986) similarly found in water hyacinth that specific PEPc activity decreases when plants were grown in elevated $[CO_2]$, however, unlike our results, when they expressed the activity per unit chlorophyll, no significant differences between plants grown in 330 or 600 μmol mol^{-1} CO_2 were found. Anapleurotic carbon fixation is only one of several roles PEPc can play in C_3 plants (O'Leary 1982). The regulation of this enzyme has not been elucidated fully (Smith *et al*. 1996) and therefore its overall role in the carbon metabolism of C_3 plants grown in elevated $[CO_2]$ is still unknown.

Extrapolation of these observations to more general results should be done only with great care. While our experimental system was highly controlled and the level of detail known about the growth environment of the experimental plants is quite high, only one chamber per treatment was used. Obviously this work needs to be repeated and expanded upon, a task that is currently underway in our laboratory where we have found comparable results in a similar, but not identical preliminary experiment; after 45 d of growth in artificial light growth chambers, soybean plants grown under high day-time and low night-time $[CO_2]$ had no fruits while all other treatments did.

A small number of studies have now looked directly at the effect of day-time versus night-time $[CO_2]$ on plant growth and physiology. We have extended this work to include switching treatments (high day/low night and low day/high night) and found some striking results. Far too little research has been carried out on this subject to claim knowledge of the magnitude, variation or even the direction of the response, yet the implications of these responses are far-reaching ecologically, mechanistically and experimentally. All of the work done to date has been on herbaceous annuals, and with the exception of this study the published experiments stopped short of the reproductive stage of development. It is unknown if similar results would be found in perennial plants or any number of other combinations of plant species and environmental conditions. Furthermore we know almost nothing about the sensitivity of this response to diurnal changes in $[CO_2]$. In the current study we intentionally used a large and ecologically unrealistic range of $[CO_2]$ to try and identify potential mechanisms. Much more work is needed to verify these observations and to quantify the range of responses and experimental/environmental conditions under which they occur.

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