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Elevated CO_2 concentration has independent effects on expansion rates and thickness of soybean leaves across light and nitrogen gradients

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Abstract

The rate and extent of leaf thickness and area development are important determinants of whole plant photosynthetic capacity. The interactive effects of photon flux density (*PFD*), nitrogen supply and CO_2 concentration on leaf expansion rate were measured as well as final leaf size and thickness of soybean. Leaf thickness and final area were not correlated with leaf relative expansion rate (*RER*) suggesting that these parameters are controlled by different mechanisms and that final leaf dimensions are determined by the duration rather than the rate of leaf expansion. Carbohydrate supply did not explain the variation in leaf *RER* since *RER* increased with increasing CO_2 concentration, but decreased with increasing *PFD*. Leaf thickness and final area were related to resource supply but not in a simple fashion. Both positive and negative correlations between leaf thickness and carbohydrate and nitrogen concentrations were obtained depending on the environmental variable responsible for the variation. In contrast, there was a simple proportional relationship between whole plant relative growth rate and a correlate of leaf thickness (leaf water content per unit area), suggesting that leaf thickness responds to the balanced supply of all resources, in the same fashion as *RGR*, rather than to any individual resource.

Key words: Anatomy, carbohydrates, *Glycine max*, microscopy, water content.

Introduction

Leaves which develop at elevated CO_2 concentrations are often thicker than those on ambient CO_2 plants. In a study of four *Populus* clones, Radoglou and Jarvis (1990) found increases in leaf thickness of 8% to 16% in response to elevated CO_2. Similar results were reported by Leadley *et al.* (1987) for soybeans where they found a 10% increase in leaf thickness for elevated CO_2-grown plants. Growth at high photon flux density (*PFD*) also increases leaf thickness (Boardman, 1977; Björkman, 1981). The increase in leaf thickness with high *PFD* is often closely correlated with increases in photosynthetic capacity per unit leaf area, suggesting that the high photosynthetic capacities of sun leaves result from increased volume of mesophyll tissue, and thus increased quantities of photosynthetic enzymes, without a change in photosynthetic capacity per unit volume of tissue (Louwerse and Zweerde, 1977; Patterson *et al.*, 1977, 1978; Sims and Pearcy, 1992).

Whereas *PFD* acclimation generally has little effect on photosynthetic capacity per unit leaf thickness, elevated CO_2 appears to decrease photosynthetic capacity per unit thickness since thickness increases while photosynthetic capacity per unit area generally decreases (Gunderson and Wullschleger, 1994). However, elevated CO_2 does not always decrease photosynthetic capacity per unit area and in some cases even increases it, at least in soybean (Campbell *et al.*, 1988). This could result from the combination of increased leaf thickness and decreased photosynthesis per unit volume if the increase in thickness

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was greater than the decrease in photosynthesis per unit volume. Luo *et al.* (1994) developed a Photosynthetic Acclimation to CO_2 (PAC) model that made use of the opposing changes in leaf thickness and photosynthetic capacity per unit thickness to explain the wide variation in acclimation responses to CO_2.

Considerable effort has been expended to understand the mechanisms controlling the biochemically-based changes in photosynthetic capacity per unit volume of tissue in response to CO_2 concentration (Stitt, 1991). In contrast, there have been few studies of the factors controlling changes in leaf thickness. The response of leaf thickness to elevated CO_2 suggests that carbohydrate supply is involved in the regulation mechanism. However, this does not preclude the involvement of other factors such as nitrogen supply and direct effects of *PFD*. Here the effects of *PFD*, nitrogen supply and CO_2 concentration on leaf thickness are compared in order to explore the mechanisms controlling leaf thickness.

The effect of light, nitrogen and CO_2 on the rate of leaf expansion was also measured. Acceleration of leaf development by elevated CO_2 may contribute to the variability in photosynthetic acclimation responses to CO_2. Besford *et al.* (1990) found that photosynthesis of tomato leaves exposed to ambient and elevated CO_2 reached the same maximum value during leaf development, but that elevated CO_2 leaves developed more rapidly and reached the maximum sooner. Consequently, elevated CO_2 appeared first to increase and then decrease photosynthetic capacity depending on the stage of leaf development. Increased rates of leaf expansion are often observed for elevated CO_2 plants (Cure *et al.*, 1989; Leadley and Reynolds, 1989; Ferris and Taylor, 1994; Gay and Hauck, 1994; Taylor *et al.*, 1994; Gardner *et al.*, 1995). Leaf expansion rates and final leaf areas and thicknesses were measured to determine whether CO_2 effects on these parameters are related and thus might be controlled by the same mechanisms.

Materials and methods

Plant material and growth conditions

Seeds of a non-nodulating variety of soybean, *Glycine max* (L.) Merr. (line D68–0099, Group VI maturity in a 'Lee' background, Soybean Production Research, Stoneville, MS, USA, Hartwig, 1994), were planted in March in 8 l pots in a 50/50 (v/v) mixture of fine sand and sandy loam topsoil. Four seeds per pot were planted initially and then thinned to one per pot after emergence. Sixteen to 23 pots were placed in each of four naturally lit growth chambers inside a greenhouse at the Desert Research Institute in Reno, NV, USA. The chambers had glass walls and tops (1×1 m base and 1.75 m in height). The greenhouse walls and roof were constructed from rigid, double-walled acrylic sheets ('Exolite', Cryo Industries, Orange, CT, USA). Temperatures were controlled to 28 ± 2 °C in the daytime and 20 ± 1 °C at night. Relative humidity at midday was 66 ± 7%.

Plants were grown in four growth chambers, two controlled to 350 ppm CO_2 and the other two at 700 ppm CO_2. Carbon dioxide concentration in each chamber was measured once every 6 min by an infrared gas analyser (model 6262, Li-Cor Inc, Lincoln, NE, USA). A datalogger (model CR10, Campbell Scientific, Logan, UT, USA) collected the data and controlled the duration of CO_2 injection into the chambers on a 30 s cycle to maintain the CO_2 setpoint. Because the ambient CO_2 concentration within the greenhouse was quite variable, CO_2 scrubbers were used in the 350 ppm chambers to maintain a constant CO_2 concentration. The scrubber boxes measured $14 \times 45 \times 56$ cm, and were constructed from Plexiglas with two fans in the top and an open grill covered by screening in the bottom. CO_2 in the air flowing through the boxes was absorbed by soda lime ('Sodasorb', WR Grace & Co. Atlanta, GA, USA). These boxes were placed inside the chambers and the fans were operated continuously. Control to the 350 ppm setpoint was achieved through additions of CO_2.

Within each pair of chambers four *PFD* environments were established. The highest and lowest *PFD* environments were in one chamber and the two intermediate *PFD* environments were in the other chamber. Each chamber was divided into high and low *PFD* halves. The front (south) half received full sun or slightly reduced *PFD* under neutral density black plastic shade cloth (40% grade). The back (north) half received lower *PFD*s under thicker shade cloth (60 and 90%). *PFD* was measured continuously throughout the experiments with one gallium arsenide photodiode (model G1118, Hamamatsu Corp., Bridgewater, NJ, USA) in each *PFD* treatment connected to a datalogger (model CR10, Campbell Scientific, Logan, UT, USA). These sensors were previously calibrated against a quantum sensor (model 190s, Li-Cor Inc., Lincoln, NE, USA). The mean daily *PFD* over the 4–6 week growing period of the plants was used in the data analysis.

Nutrient treatments were randomly assigned within the light and CO_2 treatments and were begun 3 d following seedling emergence. All plants were watered to excess daily with either a 1/2 strength Hoagland solution (7.5 mmol NO_3, 0.5 mmol PO_4, 3 mmol K, 2.5 mmol Ca, 1 mmol Mg, 1 mmol SO_4, 0.067 mmol Fe-EDTA, plus micronutrients) or a similar solution modified to reduce nitrate concentrations while keeping all other ions constant, except for SO_4 and Cl which were used in equal portions to maintain charge balance. Five nitrogen treatments (0, 0.9, 1.9, 3.8, and 7.5 mM nitrate) were applied to the plants in the highest *PFD* treatment whereas the other *PFD* treatments received only 0.9 or 7.5 mM nitrate treatments. Pots were flushed with deionized water once per week to avoid salt build-up.

There were four plants per treatment except for the five nitrogen levels in the highest *PFD* where there were only three plants.

Leaf expansion

After 4 weeks growth, an expanding trifoliate leaf with a central leaflet measuring between 0.5 and 3 cm in length was marked on each plant with a small piece of wire tied around the petiole. Marked leaves were mostly at trifoliate node 4 for the high light/high nutrient plants but ranged down to node 2 for the low light/low nutrient plants since the later plants were smaller. Some studies have reported increases in leaf mass per unit area and thickness with increasing nodal position in soybean (Lugg and Sinclair, 1979, 1980). In measurements of a similar set of plants (data not shown) increases in leaf dry mass per unit area were found with increasing leaf nodal position but significant changes in leaf thickness were not found. Consequently, sampling of leaves at different nodes for this experiment should

have had little effect on the conclusions regarding leaf thickness. The length of each of these leaflets was measured daily for 1 week and then every 2 d for another week until all the leaflets had completed expansion. Since preliminary measurements showed that the square of leaf length was directly proportional to leaflet area, this was used to estimate the rate of leaf area expansion.

Leaf characteristics

One to 2 weeks following the completion of the central leaflet expansion measurements, the same leaflets were harvested. The plants were now about 7 weeks old and were starting to fill pods. This work was done at least 1 h after dark to ensure full hydration of the leaves. After excision of the midrib and a small section from the middle of the blade to be used for leaf anatomy measurements, the remaining leaflet was quickly weighed to determine fresh mass, leaf area was measured with an area meter (model 3000, Li-Cor Inc., Lincoln, NE, USA) and the samples were dried for 48 h at 60 °C prior to determination of dry mass. Preliminary measurements showed that water loss from the leaves between the time of excision and fresh mass determination was less than 1% of total fresh mass.

Leaf cross-sections were prepared by hand with a razor blade from the small section cut from the middle of the leaf blade. The cross-sections were then photographed with a photomicroscope (model Nos BX60 and PM-30, Olympus America Inc, Lake Success, NY, USA) and the photographs used to determine total leaf thickness as well as the thickness of the epidermal, palisade and spongy cell layers.

Total non-structural carbohydrate (TNC) contents of mature central leaflets collected from the same plants during the period of expansion of the leaves used for the anatomy measurements were measured by an enzyme-coupled colorimetric technique (Hendrix, 1993). These leaves were collected in late afternoon, when TNC contents should be maximal. Half of each leaf was immediately frozen in liquid nitrogen, and stored at −80 °C until TNC analysis. The other half was dried for 48 h at 60 °C prior to measurement of total nitrogen content (model 2400 CHN analyser, Perkin Elmer, Norwalk, CT, USA).

Whole plant relative growth rate

Plants were grown under conditions similar to those described above except that there were only two levels of each environmental factor (*PFD* of 25 or 3.7 mol m^{-2} d^{-1}, nitrogen supply of 7.5 or 0.75 mM nitrate and CO_2 concentration of 350 or 700 ppm). The initial harvest was made after 4 weeks growth in the treatments. The final harvest was made 1 week later for the high *PFD* plants and 2 weeks later for the low *PFD* plants. Roots were washed free of soil and the plants were divided into root, stem and individual leaf fractions. Leaf area (model 3000, Li-Cor Inc., Lincoln, NE, USA) and fresh mass were determined separately for each leaf. Dry mass (following drying for 48 h at 60 °C) was determined for each leaf and for the root and stem fractions. For the comparison of *RGR* with leaf water content per unit area, values for the first trifoliate leaves, which were all fully expanded, were used.

Statistical analysis

The results were analysed by ANOVA (SAS, Cary, NC, USA). Since limited growth chamber space made it impossible to replicate each of the light and CO_2 treatments some caution is warrented in interpreting the interaction terms. In particular, effects observed only for the two middle *PFD*s but not at the highest and lowest *PFD*s (which were in a different chamber) could be artefactual. However, since the conclusions are based on the main effects of light and CO_2 rather than the interaction terms this was not felt to be a serious concern.

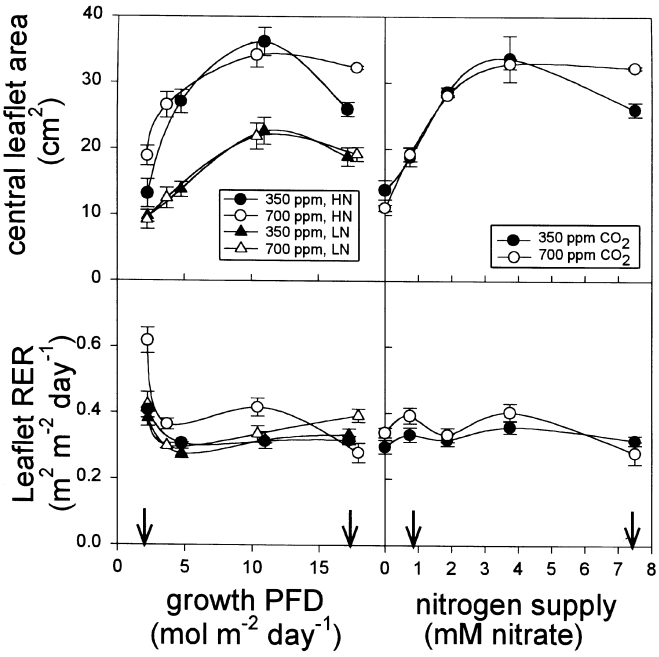

Fig. 1. Trifoliate central leaflet area and leaflet relative expansion rate (*RER*) for soybean plants growing in a range of photon flux densities (*PFD*), nitrogen supplies and CO_2 concentrations. Symbols: 700 ppm CO_2 (open symbols), 350 ppm CO_2 (filled symbols), 7.5 mM nitrate (circles) and 0.9 mM nitrate nitrogen (triangles). Arrows indicate resource levels used in the other gradient.

Results

Increasing *PFD* and nitrogen supply increased fully expanded leaflet area, but had surprisingly little effect on relative expansion rate (*RER*, calculated for the point at which the leaflet was 30% of final area) of the leaflets (Fig. 1). *RER* was actually greatest at the lowest *PFD* where final leaf areas were the smallest. Elevated CO_2 had no effect on final leaflet area in most treatments, except for the highest and lowest *PFD*s at high nitrogen supply where an increase in leaf area for elevated CO_2 plants was observed. Elevated CO_2 increased *RER* in most of the treatments but there did not appear to be any relationship between changes in *RER* and final leaf area.

Total leaf thickness increased strongly with increasing *PFD* whereas increasing nitrogen supply resulted in smaller but also significant increases in leaf thickness (Figs 2, 3). Increased leaf thickness resulted primarily from increases in the mesophyll cell layers with much smaller changes in total epidermal thickness (Figs 4, 5). All leaves appeared to have two palisade cell layers, but the size of the cells in the second layer were greatly reduced in the low *PFD* plants (Fig. 2). There was a

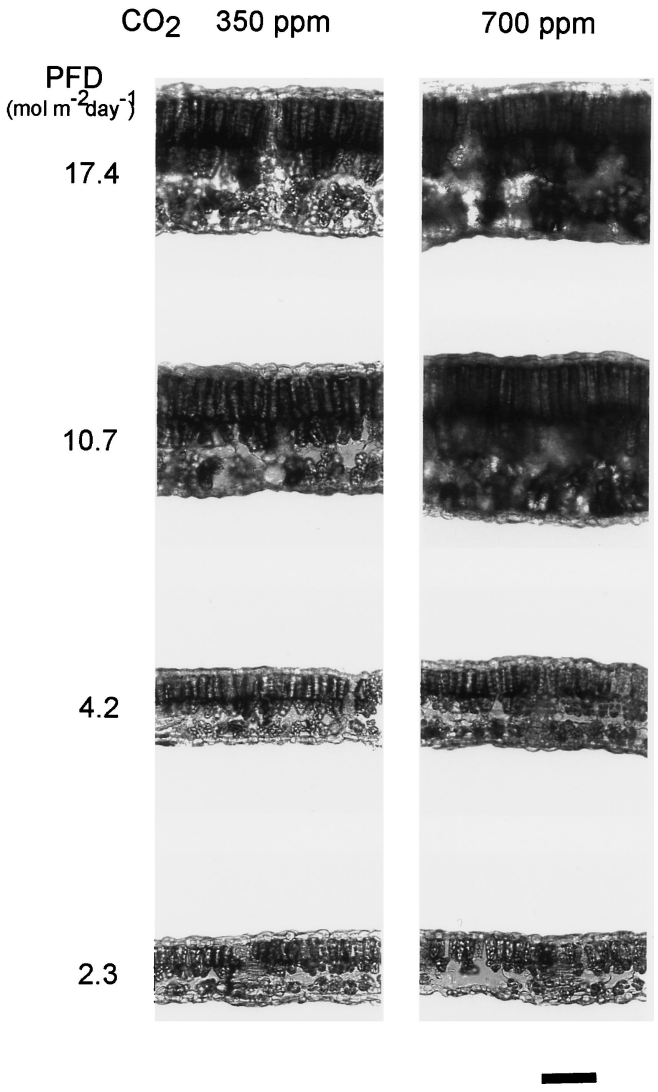

Fig. 2. Representative leaf cross-sections from plants grown at high nitrogen (7.5 mM nitrate), a range of photon flux densities (*PFD*) and two CO_2 concentrations. Black bar at bottom represents 0.2 mm.

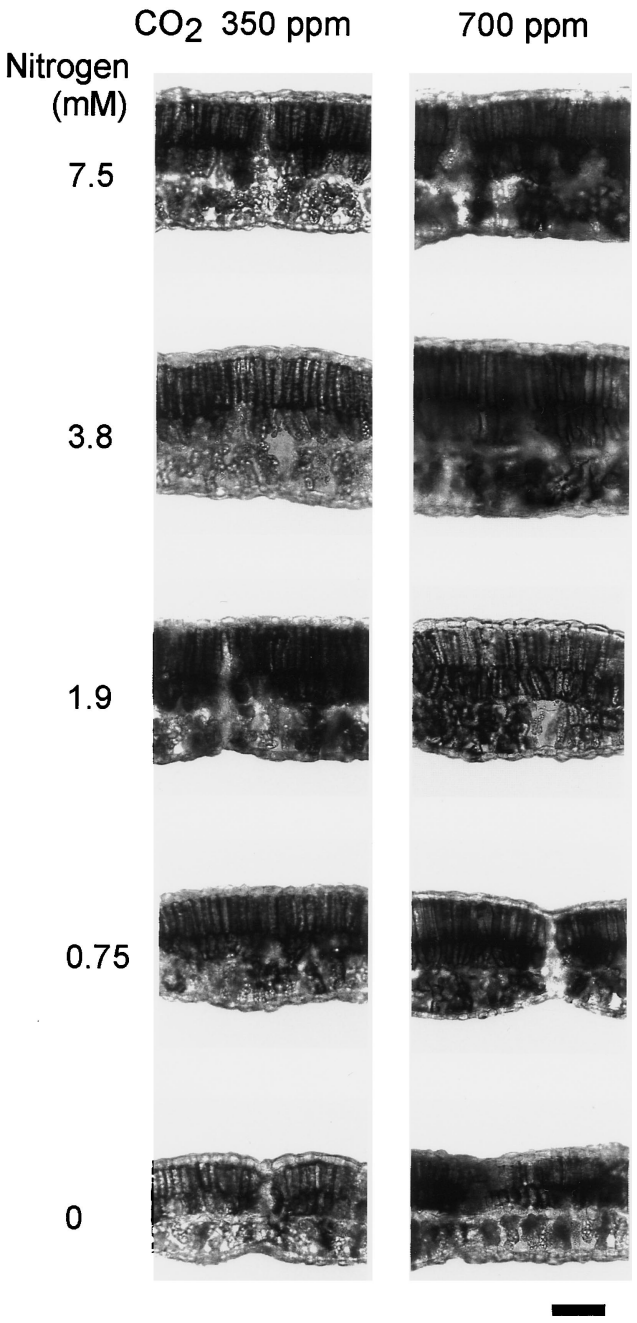

Fig. 3. Representative leaf cross-sections from plants grown at high photon flux density (17.4 mol m^{-2} d^{-1}), a range of nitrogen supplies and two CO_2 concentrations. Black bar at bottom represents 0.2 mm.

significant interaction between nitrogen supply and CO_2 concentration such that elevated CO_2 increased total leaf thickness in the high nitrogen treatments, but had no significant effect on thickness at low nitrogen (Fig. 4; Table 1). Whereas *PFD* effects on thickness were similar for the palisade and spongy mesophyll layers (Fig. 4), CO_2 had a significant effect only on spongy mesophyll thickness (Table 1). CO_2 did not have any significant effect on total epidermal thickness.

Total leaflet thickness was not simply a function of increasing leaf size. Although there was a significant positive correlation between leaflet area and thickness for plants grown in the highest *PFD*, there was no correlation for the lower *PFD*s (Fig. 6). Leaves of the same total area in high and low *PFD* were about twice as thick in the high *PFD* treatment.

Since leaf thickness is difficult to measure, several easily measured parameters which might serve as a correlate of thickness were tested. Leaf dry mass per unit area showed responses to *PFD*, nitrogen and CO_2 which were very similar to those seen for leaf thickness (Fig. 7). However, it was not the best correlate for leaf thickness (Fig. 8). Leaf dry mass, fresh mass and water content (fresh mass minus dry mass) per unit area were all significantly correlated with leaf thickness, but the correlation was

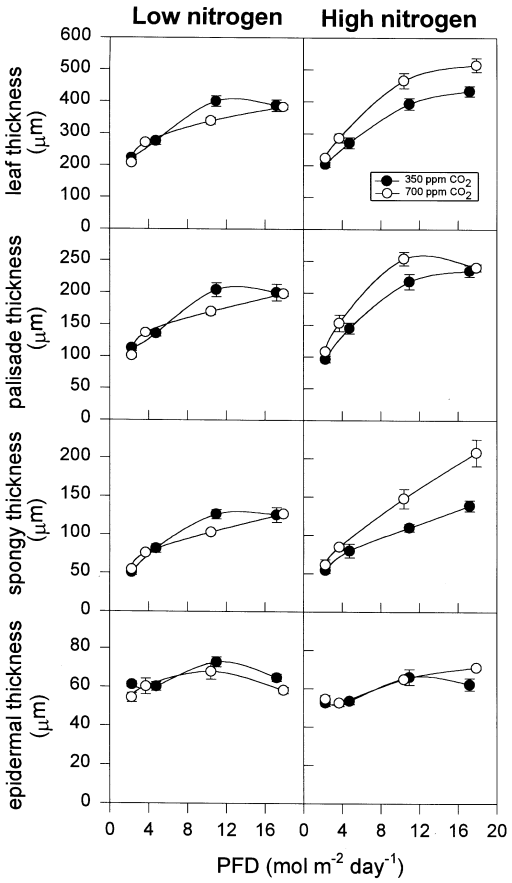

Fig. 4. Total leaf thickness and the thickness of the palisade, spongy and epidermal (upper and lower combined) cell layers for plants grown in a range of photon flux densities (*PFD*), two nitrogen supplies (7.5 or 0.9 mM nitrate) and two CO_2 concentrations (350 ppm (filled symbols) or 700 ppm (open symbols)).

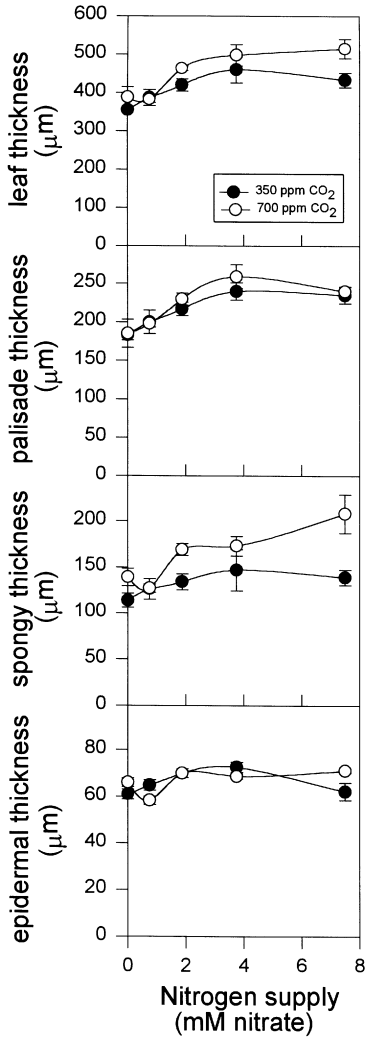

Fig. 5. Total leaf thickness and the thickness of the palisade, spongy and epidermal (upper and lower combined) cell layers for plants grown in a range of nitrogen supplies and two CO_2 concentrations (350 ppm (filled symbols) or 700 ppm (open symbols)).

best for leaf water content. Elevated CO_2 significantly reduced the ratio of leaf thickness to leaf dry mass per unit area (Table 1). This ratio was also significantly affected by *PFD* and nitrogen supply. The relationship between leaf thickness and fresh mass per unit area was better than that for dry mass, but *PFD* still had a significant effect. None of the treatments had a significant effect on the ratio of leaf thickness to water content, suggesting that leaf water content is the most robust predictor of leaf thickness.

Leaf thickness was not a simple function of either total non-structural carbohydrate content (TNC) or nitrogen content as percentage of dry mass (Fig. 9). TNC as a percentage of leaf dry mass in mature leaves was positively correlated with leaf thickness when variation was induced by variation in *PFD*, but was negatively correlated when the variation was induced by differences in nitrogen supply (Fig. 9). These relationships were exactly the opposite for nitrogen concentration. Variation due to variation in *PFD* resulted in a negative correlation whereas variation due to nitrogen supply resulted in a positive correlation (Fig. 9).

In a separate experiment (unpublished data) using similar plants and treatments, leaf water content per unit area (shown here to be a good correlate of leaf thickness) was well correlated with whole plant relative growth rate (Fig. 10). This relationship was not significantly affected by any of the *PFD*, nitrogen or CO_2 treatments.

Discussion

The results suggest that elevated CO_2 has at least two independent effects on soybean leaf development. Growth of soybeans at elevated CO_2 concentrations accelerated leaf relative expansion rate (*RER*) in most of the *PFD* and nitrogen treatments. Elevated CO_2 also increased leaf thickness in the high *PFD* and high nitrogen treatments.

Table 1. *Significance levels for the effect of light, nitrogen and CO_2 treatments on leaf dry mass per unit area, leaf water content per unit area, total leaflet area, leaflet relative expansion rate (*RER*, calculated for the point when the leaf was 30% of final area), epidermal thickness (upper and lower epidermis combined), palisade mesophyll thickness, spongy mesophyll thickness, total leaf thickness, and the ratios of leaf thickness to leaf dry mass per unit area, fresh mass per unit area and water content per unit area; interaction terms for which there were no significant effects are not listed*

	Light (L)	Nitrogen (N)	CO_2 (C)	L × N	L × C	N × C
Leaf dry mass	***	***	***	***		
Leaf water	***	**		**		*
Leaf area	***	***				
RER	***	*	***	**		
Epidermis	***	**		*		
Palisade mesophyll	***	***		**		
Spongy mesophyll	***	*	**			
Total thickness	***	***		*		*
Per dry mass	***	*	***	*	*	
Per fresh mass	***					
Per water mass						

Significance levels; ***$P<0.001$, **$P<0.01$, *$P<0.05$.

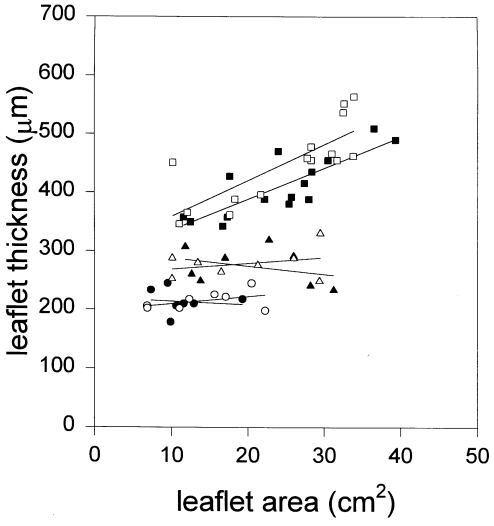

Fig. 6. Trifoliate central leaflet thickness as a function of total central leaflet area for soybeans grown at three photon flux densities (2.3 (circles), 4.2 (triangles) and 17.4 mol m^{-2} d^{-1} (squares), one intermediate *PFD* was left out for clarity) and two CO_2 concentrations (350 ppm (filled symbols) or 700 ppm (open symbols)).

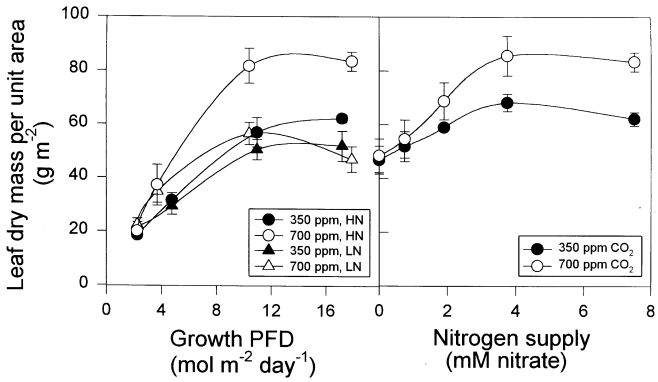

Fig. 7. Trifoliate central leaflet dry mass per unit area for soybean plants growing in a range of photon flux densities (*PFD*), nitrogen supplies and CO_2 concentrations. Symbols: 700 ppm CO_2 (open symbols), 350 ppm CO_2 (filled symbols), 7.5 mM nitrate (circles) and 0.9 mM nitrate nitrogen (triangles).

Increased leaf thickness (Thomas and Harvey, 1983; Leadley *et al.*, 1987; Vu *et al.*, 1989) and increased leaf expansion rates (Leadley and Reynolds, 1989; Cure *et al.*, 1989) have previously been reported for elevated CO_2 grown soybeans. However, we are not aware of any studies where the CO_2 effects on both these parameters were measured in the same experiment. It was found that the extent of the increase in leaf thickness was not correlated with the stimulation of leaf *RER*, suggesting that these CO_2 effects result from independent mechanisms.

Comparison of the light, nitrogen and CO_2 effects may provide some insight into these mechanisms. Both elevated CO_2 and high *PFD* increase photosynthesis and thus carbohydrate production. Comparison of the *PFD* and CO_2 responses suggest that leaflet *RER* is not a direct function of carbohydrate supply since elevated CO_2 stimulated *RER* but increasing *PFD* had a generally negative effect. Gay and Hauck (1994) reported a similar result for *Lolium temulentum* where leaf growth rate was greatest at low *PFD* and high CO_2. Whereas elevated CO_2 generally seems to stimulate leaf expansion (Cure *et al.*, 1989; Leadley and Reynolds, 1989; Ferris and Taylor, 1994; Gay and Hauck, 1994; Taylor *et al.*, 1994; Gardner *et al.*, 1995), the effect of *PFD* is less consistent. Taylor and Davies (1986) found no effect of *PFD* on early expansion rates of *Acer pseudoplatanis* and *Betula pendula* leaves, but increases in leaf expansion rates with increasing *PFD* have been reported in other studies (Taylor and Davies, 1988; Sims and Pearcy, 1992). It may be that this response varies between species, but it is also possible that differences in expansion rates resulted from variation in factors other than *PFD* and carbohydrate supply.

Temperature has a much stronger effect on leaf

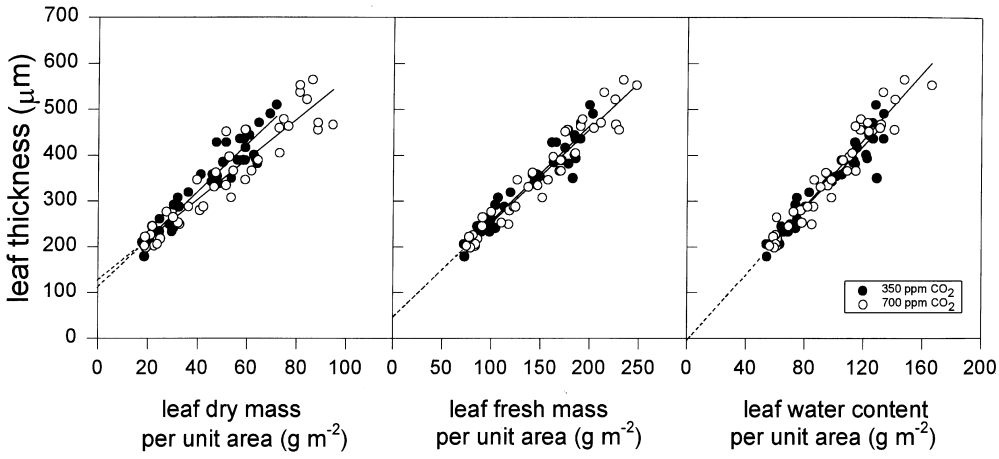

Fig. 8. Leaf thickness as a function of leaf dry mass, fresh mass and water content (fresh mass minus dry mass) per unit area for soybean plants grown at a range of photon flux densities, nitrogen supplies and two CO_2 concentrations (350 ppm (filled symbols) or 700 ppm (open symbols)).

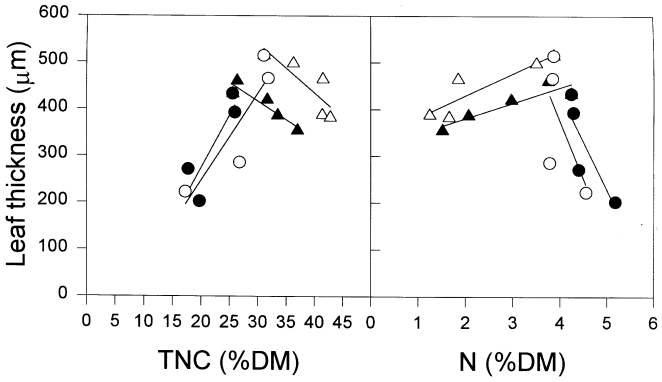

Fig. 9. Leaflet thickness as a function of total non-structural carbohydrates (TNC) and total leaf nitrogen per unit leaf dry mass for soybean plants grown at a range of photon flux densities (*PFD*, circles) and nitrogen supplies (triangles) and two CO_2 concentrations (350 ppm (filled symbols) or 700 ppm (open symbols)). The correlations were positive when the driving variable responsible for the variation was the same as the independant variable but were negative when the opposite resource provided the variation. For example, when *PFD*, which primarily affects carbohydrate uptake, varied there was a positive correlation between leaf thickness and TNC. However, when the variation resulted from changes in nitrogen supply at a constant *PFD* there was a negative correlation between leaf thickness and TNC.

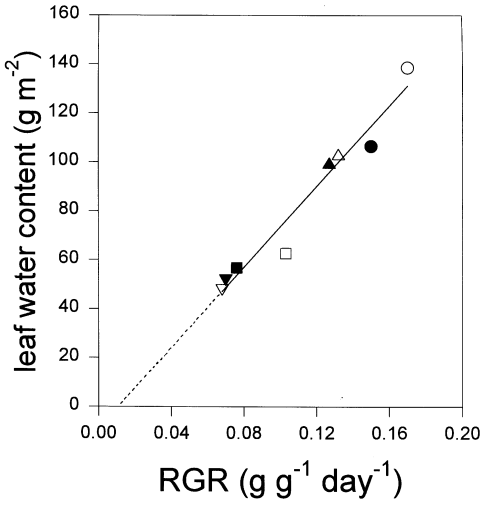

Fig. 10. Leaf water content as a function of whole plant relative growth rate (*RGR*) for soybeans grown at CO_2 concentrations of 350 ppm (filled symbols) or 700 ppm (open symbols), photon flux densities of 25 mol m^{-2} d^{-1} (circles and up triangles) or 4 mol m^{-2} d^{-1} (squares and down triangles) and nitrogen supplies of 7.5 mM nitrate (circles and squares) or 0.75 mM nitrate (up and down triangles).

expansion rate than either *PFD* (Milthorpe, 1959) or CO_2 (Ackerly *et al.*, 1992). Even when air temperatures are carefully controlled, leaf temperatures may vary substantially because of differences in radiant heat load and stomatal conductance, which affects transpirational cooling. Leaf temperatures of plants in high *PFD* can be either higher or lower than those of plants in low *PFD* depending on the balance between increased radiant load and the stomatal response. Elevated CO_2 generally increases leaf temperature (Campbell *et al.*, 1990), since it reduces stomatal conductance and thus transpirational cooling. Since photosynthesis of species with the C_4 photosynthetic pathway is unaffected by elevated CO_2, but stomatal conductance is reduced in the same fashion as C_3 species (Samarakoon and Gifford, 1996), comparison of the responses of C_3 and C_4 plant species can be used to test the importance of CO_2 effects on stomata as opposed to photosynthesis. Ackerly *et al.* (1992) found that elevated CO_2 stimulated leaf expansion rate in both *Abutilon theophrasti* (C_3) and *Amaranthus retroflexus* (C_4), suggesting that changes in expansion rate might have been related to stomatal effects.

Reduced stomatal conductance and thus transpiration rates in elevated CO_2 might also increase leaf water potential and turgor pressure which could stimulate leaf expansion (Morison, 1993; Tyree and Alexander, 1993). However, Gardner *et al.* (1995) found that leaf turgor pressure was actually reduced for several poplar clones

grown in elevated CO_2. They concluded that increased expansion rates of elevated CO_2 leaves resulted from increased cell wall extensibility rather than turgor pressure. Elevated CO_2 also increased the activity of xyloglucan endotransyglycosylase (Taylor *et al.*, 1994), an enzyme correlated with changes in growth rate and cell wall extension (Fry *et al.*, 1992; Heatherington and Fry, 1993). Changes in cell wall properties also appear to account for increased leaf expansion rate in other species in response to increased nitrogen (Taylor *et al.*, 1993) or *PFD* (Taylor and Davies, 1985, 1988), however, the signals controlling these changes in cell wall properties remain to be elucidated.

Cell divisions are largely complete prior to the rapid expansion phase of leaf development (Maksymowych, 1973), and there is some evidence that final cell size is also determined by the length of the cell cycle in meristems (Francis and Halford, 1995). Consequently, final leaf size and thickness may be determined quite early in leaf development by processes distinct from those controlling the rate of expansion during later stages of development. Two studies have recently demonstrated that elevated CO_2 has effects on development at very early stages. Kinsman *et al.* (1996) found that elevated CO_2 could increase the rates of cell division at the tip of the apical meristem, but this effect varied dramatically between the two genotypes of *Dactylis glomerata* that were measured. Robertson *et al.* (1995) found almost 3-fold increases in mitochondrial numbers in very young leaf cells of wheat.

In this study it was found that all the treatments had greater effects on mesophyll thickness than on epidermal thickness. In addition, the CO_2 treatments, but not *PFD* or nitrogen, had a greater effect on spongy mesophyll thickness than on palisade mesophyll thickness. A similar result for CO_2 treatment was reported for *Phaseolus vulgaris* by Radoglou and Jarvis (1992), but Thomas and Harvey (1983) found the opposite response, a greater increase in palisade than in spongy thickness with increasing CO_2, for soybean and sweet gum. All that can be concluded with certainty is that elevated CO_2 increases mesophyll thickness more than epidermal thickness. This suggests a well controlled response targeting specific cell types.

Whereas *RER* of leaves appeared to be largely independent of resource supply, final leaf size and thickness both increased with increasing *PFD* and nitrogen supply. However, the relationships between leaf thickness and carbohydrate and nitrogen concentrations in mature leaves were complex and dependent on whether the source of variation was *PFD* or nitrogen supply. It may be that resource concentrations in mature leaves do not accurately reflect the concentrations in young developing leaves. However, the strong correlation between leaf thickness and whole plant relative growth rate (*RGR*) suggests that leaf thickness responds to the balanced supply of all resources, in the same fashion as *RGR*, rather than to any individual resource. The relationship between *RGR* and leaf thickness did not simply result from the increased leaf size of more rapidly growing plants since only a weak relationship was found between leaf size and thickness. Regardless of whether leaf thickness is causally linked to *RGR* or whether they simply respond to the same signals, their close correlation suggests that the factors ultimately determining leaf thickness are complex. Given that leaf area development and leaf thickness are themselves important determinants of whole plant carbon gain and growth (Lambers and Poorter, 1992), it is clear that many feedback responses are possible which may result in quite complex responses at the whole plant level.

The capacity of plants to utilize increased carbohydrate supply under elevated CO_2 is frequently hypothesized to control plant responses. Decreased photosynthetic capacity and photosynthetic enzyme concentrations of leaves grown at elevated CO_2 are hypothesized to result from a feedback process when plants are unable to utilize the increased carbohydrate production (Stitt, 1991). These results suggest that the ability to utilize increased carbohydrate supply may also influence leaf thickness. Elevated CO_2 increased leaf thickness to the greatest extent in high nutrient conditions where there was also the greatest growth stimulation. This might increase photosynthetic capacity, because of increased photosynthetic tissue volume per unit area, under conditions where increased carbohydrate production could be used for increased growth. Further work should focus on the factors limiting growth and the signals linking growth potential to photosynthetic and leaf thickness responses.

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