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STOMATAL AND NONSTOMATAL ACCLIMATION TO A CO_2–ENRICHED ATMOSPHERE

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XU D. –Q, TERASHIMA K., CRANG R. F. E., CHEN X. –M. and HESKETH J.D. *Stomatal and nonstomatal acclimation to a CO_2–enriched atmosphere.* BIOTRONICS **23**, 1–9, 1994. The relationship between stomatal conductance and leaf photosynthetic acclimation to long–term exposure to a CO_2–enriched atmosphere was examined in soybean *cv.* 'Jack'. For fully expanded leaves, net photosynthetic rates were lower in leaves grown at high CO_2 (about 800 μmol CO_2 mol^{-1} air) than in leaves grown in ambient air (about 400 μmol CO_2 mol^{-1}), when measured in ambient air, indicating that leaf photosynthetic acclimation had occurred. The CO_2–enriched leaves frequently had lower stomatal conductances, when transferred to ambient CO_2 levels; similar *C*i values and plots of net photosynthesis *vs.* stomatal conductance indicated that this reduction in conductance was not a major cause for the reduction in photosynthesis in our experiments. More than 2 h after plants grown in enriched CO_2 were transferred to normal air, no differences were observed in stomatal patchiness between CO_2–enriched and ambient leaves. Stomatal closure in CO_2–enriched air was patchy and associated conductances were close to limiting CO_2 flux into the leaf in recent field tests.

Key words: CO_2 enrichment; photosynthetic acclimation; soybean; stomatal density; stomatal patchiness.

INTRODUCTION

A common phenomenon of leaf photosynthetic acclimation to long–term exposure to a CO_2–enriched atmosphere is the decline in net photosynthetic rate

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Abbreviations: *C*a, ambient CO_2 concentration, μmol CO_2 mol^{-1}; *C*i, intercellular space CO_2 concentration, μmol CO_2 mol^{-1}; *E*, transpiration rate, mol H_2O $m^{-2}s^{-1}$; *G*s, stomatal conductance, mol H_2O $m^{-2}s^{-1}$; *P*n, net photosynthetic rate, μmol CO_2 $m^{-2}s^{-1}$; *PFD*, photon flux density, μmol $m^{-2}s^{-1}$; RuBPCase, ribulose–1,5–bisphosphate carboxylase; *T*a, air temperature; *T*l, leaf temperature.

per unit leaf area (*P*n), compared to that of plants grown in ambient air but measured at the same CO_2 concentration (*19*). In plants acclimated to high CO_2, the decrease in net photosynthetic rate is often accompanied by a decline in stomatal conductance (*G*s). Therefore, the decline in stomatal conductance has been considered to be the main cause of acclimation (*11*). However, for CO_2-enriched plants, the decline in stomatal conductance could not by itself explain the reduced photosynthetic rate, because at any given external CO_2 level the internal CO_2 concentrations (*C*i) of the ambient and the CO_2-enriched plants were similar (*19*). The similar *C*i may have been an artifact due to non-uniform stomatal closure (patchiness) over a leaf surface. When patchiness cccurs the calculated *C*i is correct only for those areas where stomata are open (*7,15*), whereas *C*i is, in fact, near the CO_2 compensation point for areas of the leaf where stomata are closed. The calculated value for *C*i, therefore, is higher than the mean for both areas where stomata are open and closed. An overestimated *C*i leads to an apparent but false assumption of a non-stomatal limitation to leaf photosynthesis.

Although there have been many studies on leaf photosynthetic acclimation, the relationship between stomatal conductance and leaf photosynthetic acclimation has not been clear. The main object of this study was to understand the role of stomata in leaf photosynthetic acclimation to long-term exposure to a CO_2-enriched atmosphere. For this purpose, it was attempted to determine whether: (1) stomatal density decreased for leaves grown in a CO_2-enriched atmosphere; (2) stomatal acclimation to long-term exposure to elevated CO_2 occurred; (3) non-uniform stomatal closure occurred in a CO_2-enriched atmosphere; and (4) a decline in stomatal conductance was a part of the cause for leaf photosynthetic acclimation to long-term exposure in a CO_2-enriched atmosphere.

MATERIALS AND METHODS

Plant materials and growing conditions

Soybean [*Glycine max* (Merr.) L.] *cv.* 'Jack' was planted in plastic pots (diameter 20 cm, height 28 cm) containing a mixture of soil, peat, and perlite (v/v/v/=1:1:1). The pots were positioned at the bottoms of two open-top growth chambers (each 125 cm long, 75 cm wide, and 90 cm deep) surrounded by walls of clear plastic film. There were 10 pots, with one plant per pot, in each chamber. Six successive sets of plants were grown to pod set between September 1992 and March 1993, with seedlings established in a nearby greenhouse. Air from outside the building was pumped continuously into the two growth chambers, after warming to about 20℃ by heaters in one mixing tank (diameter 95 cm, length 110 cm) supplying air to both chambers. During the daytime, CO_2 was added to one growth chamber to give plants a CO_2-enriched atmosphere of about 800 μmol^{-1} air, or about twice that of ambient air in the other growth chamber during the winter months. The open top chambers were close together but the chamber flushed with ambient air was placed in a

separate room with restricted access; otherwise we were not able to maintain outside CO_2 concentrations in the ambient treatment. The light period was 12h with a photon flux density (*PFD*) of about 1,000 μmol photons $m^{-2}s^{-1}$ at the fourth trifoliolate level from two 1,000 W multi-vapor lamps. Frequently, the humidity of the two growth chambers was increased by placing an ultrasonic humidifier in each chamber and by wetting the chamber floor. Variable levels of atmospheric humidity permitted the measurement of leaf photosynthetic acclimation at different stomatal conductances, with the latter depending upon humidity. Enhanced relative humidities were 40–50% depending upon outside conditions; air temperatures of the two growth chambers were about 26 (day)/18 (night) ℃. The plants were watered daily. CO_2 concentrations were monitored by an infrared gas analyzer and were regulated daily by a flow meter in the CO_2 stream. Fertilizer (20:20:20) was added (2.27 g per pot) when the third trifoliolate leaves appeared.

Gas exchange measurement

A portable photosynthetic measurement system LI-6200 (Li-Cor Ltd., Lincoln, NE)[1] was used for leaf net photosynthetic measurements. Its chamber was clamped onto attached, fully-expanded, outer-canopy, unshaded leaves. Measurements of leaf photosynthetic acclimation were made at 1,000 μmol photons $m^{-2}s^{-1}$ and ambient CO_2 concentration, at least 2h after turning off supplemental CO_2 flow. *Ci* was obtained automatically from the LICOR system, based on equations shown in the LICOR manual.

Stomatal density measurement

Leaf segments (about $1\times0.4\ cm^2$) were observed with scanning electron microscopy (SEM) following fixation in 2.5% buffered glutaraldehyde overnight, dehydration in a graded ethanol series, critical point drying using carbon dioxide as a transition fluid, and sputter coating with gold/palladium. From SEM micrographs stomatal numbers per unit leaf area were counted for different samples at standarized angles of tilt and magnification. Values reported represent means and standard deviations from measurements on five leaves.

Stomatal patchiness observation

A slightly modified water infiltration method (*3*) to observe the degree of stomatal aperture patchiness was used, in which a leaf sample was put into water in a pyrex desiccator (diameter 16 cm) immediately after the leaf was excised from a plant. A vacuum of 5×10^4 Pa was created in a desiccator using a hand-held vacuum pump in order to remove air inside the leaf through open stomata. Water subseqently infiltrated into the leaf through open stomata when

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[1]Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the Shanghai Inst. Plant Physiol., the Univ. Illinois or the USDA and does not imply the approval of the named product to the exclusion of other products that may be suitable.

the vacuum was removed. From the abaxial leaf surface it could be clearly seen that some areas of the leaf where water infiltrated became dark green, while other areas where water did not infiltrate due to stomatal closure remained light green. Images of the abaxial leaf surface were immediately recorded onto video tape for subsequent image analysis. Each video-taped image was digitized (640 ×480 8-bit pixels) using an image-capture circuit-board and stored on a computer diskette. Thresholding software ("Image", Version 1.22, National Center for Supercomputer Applications, Champaign IL) was used to obtain the total area of water-infiltrated leaf tissue by summing individual pixels in the video image. A ratio consisting of total dark green to total leaf area was calculated for each leaf. The ratio approximated the percentage of open stomata for each leaf. However, it was always lower than 100% even if all stomata wre open over a leaf, due to error caused by leaf veins which were included in the total leaf area but not in the water-infiltrated area because of their light color. This error led to an underestimation of the ratio of at least 5%.

RESULTS

Comparisons of stomatal density and patchiness

Growth at high CO_2 did not cause significant changes in stomatal number and epidermal cell number per unit leaf area, or in their ratio, compared to

Table 1. Effects of CO_2 enrichment on stomatal density in soybean leaf bottom surfaces.

	Grown at		
	Ambient (400)	CO_2-enriched (800)	Enriched/ambient Ratio
	μmol CO_2 mol^{-1} Air		
Stomata (per square mm.)	390 (93)	375 (57)	0.9615
Epidermal cells (per square mm)	1563 (356)	1444 (170)	0.9239
Stomata/Epidermal cells	0.26 (0.07)	0.26 (0.04)	1.0000
Leaf area (square cm per leaf)	36.47 (4.70)	48.08* (7.99)	1.3183

The middle leaflets of fourth trifoliolate from base of plants were used in the experiment. The leaf area values represent the means of 5 leaves with standard deviation in parentheses. The stomata and epidermal cell values represent the means of 14 observations from 5 leaves with standard deviation in parentheses. $*p<0.05$, ambient vs. high CO_2. A representative sample of *G*s values during these studies was 1.268 and 0.581 (n=10) for plants growing in the 400 and 800 treatments, respectively.

Table 2. Effect of CO_2 enrichment on homogeneity in stomatal opening measured by the method of water-infiltration.

	Grown at Ambient (400) μmol CO_2 mol^{-1} Air	Grown at CO_2-enriched (800) μmol CO_2 mol^{-1} Air	Enriched/Ambient Ratio
Measured at their growth [CO_2]	0.9076 (0.0134)	0.4895*** (0.0482)	0.5393
Measured at same ambient [CO_2]	0.8482 (0.0711)	0.8411 (0.0701)	0.9916

The values are ratios of water-infiltrated area over total leaf area, and represent the means with standard deviations in parentheses from 3 to 6 leaves. ***$p < 0.001$

Table 3. Effect of CO_2 enrichment during growth on net photosynthetic rate as well as related parameters measured at ambient CO_2.

Growth conditions	*PFD*	*T*a	*T*l	*C*a	*P*n	*G*s	*C*i	*E*
Ambient								
mean	1031	28.22	27.09	399.7	18.04	0.7916	340.2	0.0122
SD	15	0.32	0.58	2.7	1.82	0.2119	10.0	0.0012
Enriched								
mean	1037	28.05	27.99	403.2	12.09	0.4691	340.8	0.0098
SD	12	0.85	0.90	2.0	2.18	0.0394	8.9	0.0007

The values are means from 8 leaves. Measurements were made at about 1000 μmol photons $m^{-2}s^{-1}$.

leaves grown in ambient air (Table 1).

High CO_2 led to stomatal aperture patchiness for leaves grown and measured at high CO_2. However, no significant difference was observed in the extent of stomatal patchiness between leaves grown at ambient air conditions and leaves from a CO_2-enriched atmosphere exposed to ambient air conditions for several hours (Table 2).

Effects of CO_2 enrichment on leaf CO_2 flux in ambient air

Figs. 1 and 2 show the dynamics of *G*s, *P*n and *C*i after changes in the atmospheric CO_2 level. In Fig. 1 both sets of plants had been transferred to ambient CO_2 levels for 15-h or more before exposure to 800 μmol CO_2 mol^{-1} air at time zero. After this equilibration period, *G*s in ambient-growth plants was more sensitive to an abrupt increase in CO_2 and did not recover within 1 h after plants were returned to ambient (initial) conditions; whereas *G*s in enriched-CO_2 plants did recover. Fig. 2 shows how long it took for *G*s (30 min), *P*n (20 min) and *C*i (10 min) to equilibrate after CO_2-enriched plants had been transferred to ambient conditions. These experiments were repeated three times.

Long-term exposure to high CO_2 resulted in a decline in *P*n measured in

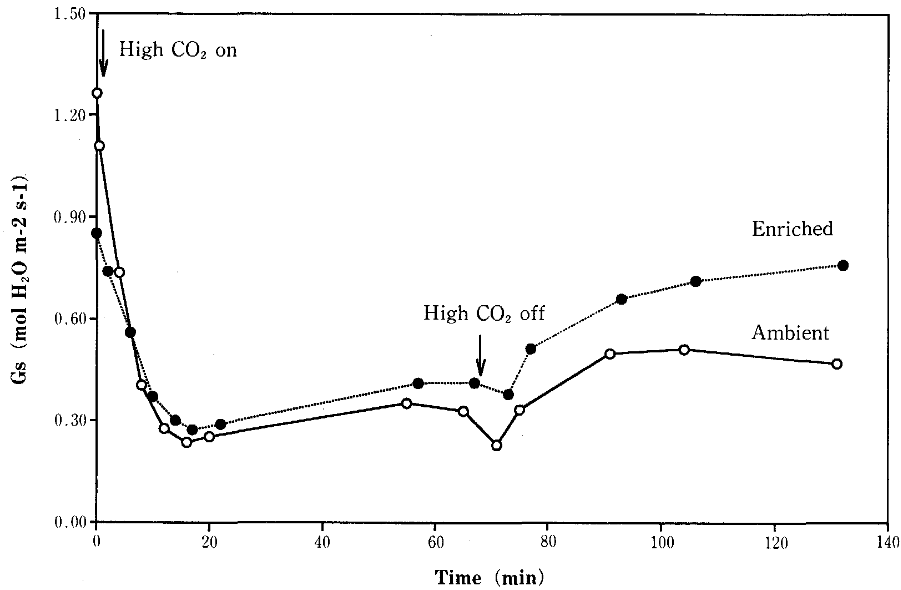

Fig. 1. Time course of stomatal response to change in CO_2 concentration for soybean leaves.

ambient air, as compared to *P*n for leaves grown in ambient air, Table 3. The decreased *P*n and *G*s were accompanied by no differences in *C*i. In another experiment (10 leaves), *G*s was 0.58 (S.D.=0.11) mol H_2O $m^{-2}s^{-1}$ in leaves grown in enriched CO_2 compared to 0.85 (0.18); in the same comparision *P*n values were 16.3 (2.4) *vs.* 20.4 (16.3) umol $m^{-2}s^{-1}$, with little difference again in *C*i or *E*. Leaf transpiration *E* did not change because of the increased *T*l of leaves grown in enriched CO_2. Fig. 3 shows the relationship between *P*n and *G*s for leaves grown at the two atmospheric CO_2 concentrations; low *G*s values were induced by exposing leaves to low atmospheric humidities, which were available when the air outside was very cold. Each value shown represented a mean for 8 to 10 leaves; comparisons were made over a 14 day period. When *G*s was smaller than 0.3 mol H_2O $m^{-2}s^{-1}$, *P*n decreased linearly with a decline in *G*s, indicating a stomatal limitation to photosynthesis, but *P*n did not increase when *G*s was higher than 1 mol H_2O $m^{-2}s^{-1}$.

DISCUSSION

In our study, long-term CO_2 enrichment resulted in a decline in net photosynthetic rates per unit leaf area, after plants were transferred to ambient conditions, or photosynthetic acclimation to atmospheres enriched in CO_2 (Table 3 and Fig. 3). These data are consistent with earlier reports (e.g. *1, 9, 10, 11*). Reduced stomatal conductance (*11, 19*), feedback inhibition of photosynthesis caused by excess accumulation of photosynthates (*5, 6, 8, 17*), and reduced RuBPCase activity (*4, 19, 20*) all have been hypothesized as mechanisms responsible for this kind of acclimation.

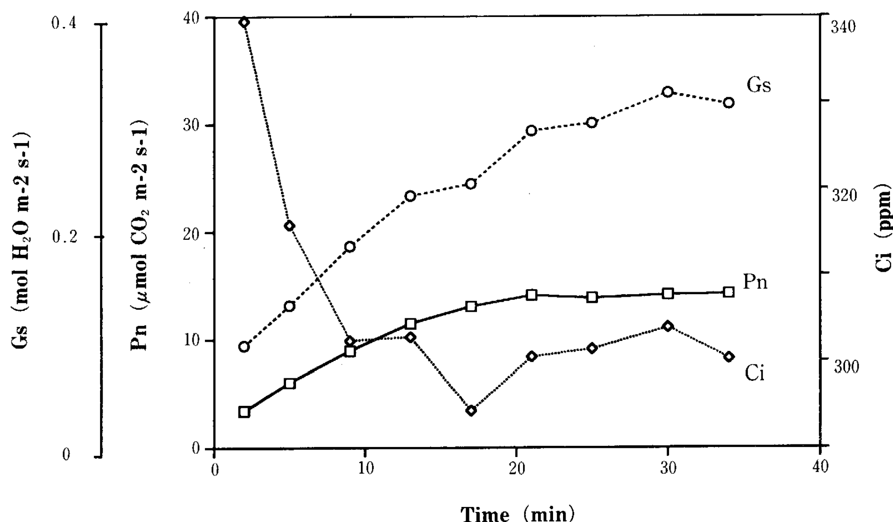

Fig. 2. Time course of changes in *P*n, *G*s, and *C*i after transferring a CO_2-enriched plant to ambient CO_2 conditions from high CO_2.

We had to wait for leaves to equilibrate to ambient conditions before making comparisons between plants grown in enriched CO_2 and those grown at ambient (Figs. 1 and 2). We also had to pay close attention to air humidity because of low humidities on cold days during the winter months; air humidity affected both the rate that leaves equilibrated to new conditions and the equilibrium stomatal conductance value.

Our results comparing CO_2-enriched leaves with ambient leaves at about 400 μmol CO_2 mol^{-1} air do not support the conclusion that decreased stomatal conductance is a major cause of leaf photosynthetic acclimation, for the following reasons: (1) Acclimated leaves always had a similar *C*i to that of ambient-grown leaves, although they had a lower *G*s value (0.47–0.58 vs. 0.79–0.85 in ambient-grown leaves); the lower *G*s values were close to the threshold *G*s value limiting net photosynthesis, as see from a *P*n vs. *G*s plot, Fig. 3, although this theshold value might change with growing conditions; (2) No difference in the extent of stomatal patchiness was observed in ambient air between CO_2-enriched and ambient air-grown leaves (Table 2), indicating that the calculated *C*i values from gas exchange data were correct; (3) *P*n was also lower in CO_2-enriched leaves than in ambient leaves at identical *G*s values (Fig. 3).

The lack of complete recovery of *G*s under ambient air for CO_2-enriched leaves can not be explained by decreased stomatal density because no significant difference in stomatal density was observed between CO_2-treatments (Table 1). Our results showing no effect of CO_2 on stomatal density during leaf growth are consistent with those recently reported in the literature (*13, 14, 16*). Therefore, we suggest that during the last several centuries decreases in stomatal density

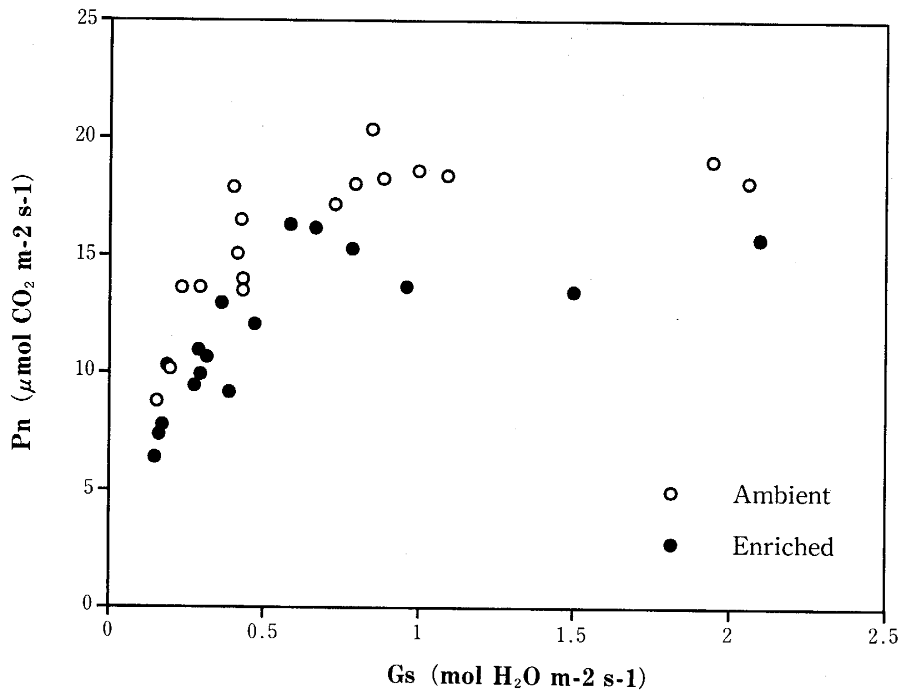

Fig. 3. Relationship between *G*s and *P*n for ambient and CO_2 enriched leaves. Measurements were made at ambient air during a period from October 1992 to February 1993. In this figure each point represents a mean from 8–10 leaves.

observed from leaf fossils of some plants (*12, 21*) are more likely due to other climatic factors (e.g., temperature) rather than an increase in atmospheric CO_2. Recently, Beerling and Chaloner (2) reported that leaves formed under warmer summer temperatures had reduced stomatal density, compared with their spring counterparts.

The discovery of non-uniform stomatal closure, or stomatal patchiness, represents important progress in the field of photosynthetic physiology. It has been widely recognized that the possibility of stomatal patchiness must be eliminated experimently, before analyzing for stomatal and non-stomatal limitations to photosynthesis based on *C*i calculations from leaf gas exchange data, otherwise overestimated *C*i values could lead to an apparent but false non-stomatal limitation to leaf photosynthesis. A variety of stresses due to water deficiency, low humidity, high light, desease infection (*18*), and ABA application (*7*) have been shown to lead to stomatal patchiness. However, there is no report of enriched CO_2 atmospheres resulting in stomatal patchiness, as we have shown here (Table 2). Of course as atmospheric CO_2 increases, stomatal conductances and associated characteristics become less and less limiting to leaf photosynthetic processes; however, recent studies at Urbana (X. -M. Chen, 1994, Ph. D. thesis) have suggested that *G*s values (0.44, n=10) of leaves growing at two times normal atmospheric CO_2 in open top chambers in the field were close to the threshold for becoming limiting to *P*n.

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