STOMATAL AND NONSTOMATAL ACCLIMATION TO A CO\textsubscript{2}-ENRICHED ATMOSPHERE

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STOMATAL AND NONSTOMATAL ACCLIMATION TO A CO₂-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 CO₂-enriched atmosphere. BIOTRONICS 23, 1-9, 1994. The relationship between stomatal conductance and leaf photosynthetic acclimation to long-term exposure to a CO₂-enriched atmosphere was examined in soybean cv. 'Jack'. For fully expanded leaves, net photosynthetic rates were lower in leaves grown at high CO₂ (about 800 µmol CO₂ mol⁻¹ air) than in leaves grown in ambient air (about 400 µmol CO₂ mol⁻¹), when measured in ambient air, indicating that leaf photosynthetic acclimation had occurred. The CO₂-enriched leaves frequently had lower stomatal conductances, when transferred to ambient CO₂ levels; similar Ci 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 2h after plants grown in enriched CO₂ were transferred to normal air, no differences were observed in stomatal patchiness between CO₂-enriched and ambient leaves. Stomatal closure in CO₂-enriched air was patchy and associated conductances were close to limiting CO₂ flux into the leaf in recent field tests.

Key words: CO₂ enrichment; photosynthetic acclimation; soybean; stomatal density; stomatal patchiness.

INTRODUCTION

A common phenomenon of leaf photosynthetic acclimation to long-term exposure to a CO₂-enriched atmosphere is the decline in net photosynthetic rate

Abbreviations: Ca, ambient CO₂ concentration, µmol CO₂ mol⁻¹; Ci, intercellular space CO₂ concentration, µmol CO₂ mol⁻¹; E, transpiration rate, mol H₂O m⁻²s⁻¹; Gs, stomatal conductance, mol H₂O m⁻²s⁻¹; Pn, net photosynthetic rate, µmol CO₂ m⁻²s⁻¹; PFD, photon flux density, µmol m⁻²s⁻¹; RuBPCase, ribulose-1,5-bisphosphate carboxylase; Ta, air temperature; Tl, leaf temperature.
per unit leaf area \((P_n)\), compared to that of plants grown in ambient air but measured at the same \(\text{CO}_2\) concentration \((19)\). In plants acclimated to high \(\text{CO}_2\), 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 \(\text{CO}_2\)-enriched plants, the decline in stomatal conductance could not by itself explain the reduced photosynthetic rate, because at any given external \(\text{CO}_2\) level the internal \(\text{CO}_2\) concentrations \((C_i)\) of the ambient and the \(\text{CO}_2\)-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 occurs the calculated \(C_i\) is correct only for those areas where stomata are open \((7,15)\), whereas \(C_i\) is, in fact, near the \(\text{CO}_2\) 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 \(\text{CO}_2\)-enriched atmosphere. For this purpose, it was attempted to determine whether: (1) stomatal density decreased for leaves grown in a \(\text{CO}_2\)-enriched atmosphere; (2) stomatal acclimation to long-term exposure to elevated \(\text{CO}_2\) occurred; (3) non-uniform stomatal closure occurred in a \(\text{CO}_2\)-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 \(\text{CO}_2\)-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°C by heaters in one mixing tank (diameter 95 cm, length 110 cm) supplying air to both chambers. During the daytime, \(\text{CO}_2\) was added to one growth chamber to give plants a \(\text{CO}_2\)-enriched atmosphere of about 800 \(\mu\text{mol}^\text{L}^{-1}\) 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
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separate room with restricted access; otherwise we were not able to maintain outside CO₂ concentrations in the ambient treatment. The light period was 12h with a photon flux density (PFD) of about 1,000 μmol photons m⁻²s⁻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) °C. The plants were watered daily. CO₂ concentrations were monitored by an infrared gas analyzer and were regulated daily by a flow meter in the CO₂ 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⁻²s⁻¹ and ambient CO₂ concentration, at least 2h after turning off supplemental CO₂ flow. Ci was obtained automatically from the LICOR system, based on equations shown in the LICOR manual.

Stomatal density measurement

Leaf segments (about 1×0.4 cm²) 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 standardized 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 5×10⁴ Pa was created in a desiccator using a hand-held vacuum pump in order to remove air inside the leaf through open stomata. Water subsequently infiltrated into the leaf through open stomata when

¹Mention 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.

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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 were 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 CO₂ did not cause significant changes in stomatal number and epidermal cell number per unit leaf area, or in their ratio, compared to

|                          | Grown at Ambient
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400 (400) µmol CO₂ mol⁻¹ Air</td>
</tr>
<tr>
<td></td>
<td>CO₂-enriched 800</td>
</tr>
<tr>
<td>Stomata (per square mm.)</td>
<td>390 (93)</td>
</tr>
<tr>
<td></td>
<td>375 (57)</td>
</tr>
<tr>
<td>Epidermal cells (per square mm)</td>
<td>1563 (356)</td>
</tr>
<tr>
<td></td>
<td>1444 (170)</td>
</tr>
<tr>
<td>Stomata/Epidermal cells</td>
<td>0.26 (0.07)</td>
</tr>
<tr>
<td></td>
<td>0.26 (0.04)</td>
</tr>
<tr>
<td>Leaf area (square cm per leaf)</td>
<td>36.47 (4.70)</td>
</tr>
<tr>
<td></td>
<td>48.08* (7.99)</td>
</tr>
</tbody>
</table>

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 CO₂. A representative sample of Gs values during these studies was 1.268 and 0.581 (n=10) for plants growing in the 400 and 800 treatments, respectively.

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Table 2. Effect of CO₂ enrichment on homogeneity in stomatal opening measured by the method of water-infiltration.

<table>
<thead>
<tr>
<th>Grown at</th>
<th>Ambient (400)</th>
<th>CO₂-enriched (800)</th>
<th>Enriched/Ambient Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol CO₂ mol⁻¹ Air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured at their growth [CO₂]</td>
<td>0.9076 (0.0134)</td>
<td>0.4895* (0.0482)</td>
<td>0.5393</td>
</tr>
<tr>
<td>Measured at same ambient [CO₂]</td>
<td>0.8482 (0.0711)</td>
<td>0.8411 (0.0701)</td>
<td>0.9916</td>
</tr>
</tbody>
</table>

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 CO₂ enrichment during growth on net photosynthetic rate as well as related parameters measured at ambient CO₂.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>PFD</th>
<th>Ta</th>
<th>Tl</th>
<th>Ca</th>
<th>Pn</th>
<th>Gs</th>
<th>Ci</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>1031</td>
<td>28.22</td>
<td>27.09</td>
<td>399.7</td>
<td>18.04</td>
<td>0.7916</td>
<td>340.2</td>
<td>0.0122</td>
</tr>
<tr>
<td>SD</td>
<td>15</td>
<td>0.32</td>
<td>0.58</td>
<td>2.7</td>
<td>1.82</td>
<td>0.2119</td>
<td>10.0</td>
<td>0.0012</td>
</tr>
<tr>
<td>Enriched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>1037</td>
<td>28.05</td>
<td>27.99</td>
<td>403.2</td>
<td>12.09</td>
<td>0.4691</td>
<td>340.8</td>
<td>0.0098</td>
</tr>
<tr>
<td>SD</td>
<td>12</td>
<td>0.85</td>
<td>0.90</td>
<td>2.0</td>
<td>2.18</td>
<td>0.0394</td>
<td>8.9</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

The values are means from 8 leaves. Measurements were made at about 1000 μmol photons m⁻²s⁻¹.

leaves grown in ambient air (Table 1).

High CO₂ led to stomatal aperture patchiness for leaves grown and measured at high CO₂. However, no significant difference was observed in the extent of stomatal patchiness between leaves grown at ambient air conditions and leaves from a CO₂-enriched atmosphere exposed to ambient air conditions for several hours (Table 2).

Effects of CO₂ enrichment on leaf CO₂ flux in ambient air

Figs. 1 and 2 show the dynamics of Gs, Pn and Ci after changes in the atmospheric CO₂ level. In Fig. 1 both sets of plants had been transferred to ambient CO₂ levels for 15-h or more before exposure to 800 μmol CO₂ mol⁻¹ air at time zero. After this equilibration period, Gs in ambient-growth plants was more sensitive to an abrupt increase in CO₂ and did not recover within 1 h after plants were returned to ambient (initial) conditions; whereas Gs in enriched-CO₂ plants did recover. Fig. 2 shows how long it took for Gs (30 min), Pn (20 min) and Ci (10 min) to equilibrate after CO₂-enriched plants had been transferred to ambient conditions. These experiments were repeated three times.

Long-term exposure to high CO₂ resulted in a decline in Pn measured in
Fig. 1. Time course of stomatal response to change in CO₂ concentration for soybean leaves.

ambient air, as compared to Pn for leaves grown in ambient air, Table 3. The decreased Pn and Gs were accompanied by no differences in Ci. In another experiment (10 leaves), Gs was 0.58 (S.D.=0.11) mol H₂O m⁻²s⁻¹ in leaves grown in enriched CO₂ compared to 0.85 (0.18); in the same comparison Pn values were 16.3 (2.4) vs. 20.4 (16.3) umol m⁻²s⁻¹, with little difference again in Ci or E. Leaf transpiration E did not change because of the increased Tl of leaves grown in enriched CO₂. Fig. 3 shows the relationship between Pn and Gs for leaves grown at the two atmospheric CO₂ concentrations; low Gs 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 Gs was smaller than 0.3 mol H₂O m⁻²s⁻¹, Pn decreased linearly with a decline in Gs, indicating a stomatal limitation to photosynthesis, but Pn did not increase when Gs was higher than 1 mol H₂O m⁻²s⁻¹.

DISCUSSION

In our study, long-term CO₂ 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 CO₂ (Table 3 and Fig. 3). These data are consistent with earlier reports (e.g. 1, 9, 10, 11). Reduced stomatal conductance (II, 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.
We had to wait for leaves to equilibrate to ambient conditions before making comparisons between plants grown in enriched CO₂ 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 CO₂-enriched leaves with ambient leaves at about 400 μmol CO₂ 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 Ci to that of ambient-grown leaves, although they had a lower Gs value (0.47–0.58 vs. 0.79–0.85 in ambient-grown leaves); the lower Gs values were close to the threshold Gs value limiting net photosynthesis, as see from a Pn vs. Gs plot, Fig. 3, although this threshold value might change with growing conditions; (2) No difference in the extent of stomatal patchiness was observed in ambient air between CO₂-enriched and ambient air-grown leaves (Table 2), indicating that the calculated Ci values from gas exchange data were correct; (3) Pn was also lower in CO₂-enriched leaves than in ambient leaves at identical Gs values (Fig. 3).

The lack of complete recovery of Gs under ambient air for CO₂-enriched leaves can not be explained by decreased stomatal density because no significant difference in stomatal density was observed between CO₂-treatments (Table 1). Our results showing no effect of CO₂ 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 experimentally, before analyzing for stomatal and non-stomatal limitations to photosynthesis based on Ci calculations from leaf gas exchange data, otherwise overestimated Ci 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, disease 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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