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H. Maherali
Duke University

C. D. Reid
Duke University

H. W. Polley
USDA-ARS, wayne.polley@ars.usda.gov

H. B. Johnson
USDA-ARS

R. B. Jackson
Duke University, jackson@duke.edu

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Stomatal acclimation over a subambient to elevated CO₂ gradient in a C₃/C₄ grassland

H. MAHERALI,¹ C. D. REID,¹ H. W. POLLEY,² H. B. JOHNSON² & R. B. JACKSON^{1,3}

¹Department of Biology, Duke University, Durham, NC 27708-0340, USA, ²USDA-ARS, Grassland, Soil and Water Research Laboratory, Temple, TX 76502, USA and ³Nicholas School of the Environment and Earth Sciences, Duke University, Durham, NC 27708, USA

ABSTRACT

An investigation to determine whether stomatal acclimation to [CO₂] occurred in C₃/C₄ grassland plants grown across a range of [CO₂] (200–550 μmol mol⁻¹) in the field was carried out. Acclimation was assessed by measuring the response of stomatal conductance (g_s) to a range of intercellular CO₂ (a g_s-C_i curve) at each growth [CO₂] in the third and fourth growing seasons of the treatment. The g_s-C_i response curves for *Solanum dimidiatum* (C₃ perennial forb) differed significantly across [CO₂] treatments, suggesting that stomatal acclimation had occurred. Evidence of non-linear stomatal acclimation to [CO₂] in this species was also found as maximum g_s (g_{smax}; g_s measured at the lowest C_i) increased with decreasing growth [CO₂] only below 400 μmol mol⁻¹. The substantial increase in g_s at subambient [CO₂] for *S. dimidiatum* was weakly correlated with the maximum velocity of carboxylation (V_{max}; r² = 0.27) and was not associated with CO₂ saturated photosynthesis (A_{max}). The response of g_s to C_i did not vary with growth [CO₂] in *Bromus japonicus* (C₃ annual grass) or *Bothriochloa ischaemum* (C₄ perennial grass), suggesting that stomatal acclimation had not occurred in these species. Stomatal density, which increased with rising [CO₂] in both C₃ species, was not correlated with g_s. Larger stomatal size at subambient [CO₂], however, may be associated with stomatal acclimation in *S. dimidiatum*. Incorporating stomatal acclimation into modelling studies could improve the ability to predict changes in ecosystem water fluxes and water availability with rising CO₂ and to understand their magnitudes relative to the past.

Key-words: grassland ecosystems; stomata; stomatal limitation of photosynthesis; subambient CO₂; water use efficiency.

INTRODUCTION

Stomatal conductance (g_s) is generally expected to decline in herbaceous plants with an increase in atmospheric CO₂ above the current concentration (Field, Jackson & Mooney

1995; Knapp *et al.* 1996; Drake, Gonzalez-Meler & Long 1997; Wand *et al.* 1999). There are many important physiological and ecological implications of such a decline. For example, lower g_s may alter ecosystem hydrology by reducing transpiration and could increase the surface temperature through reduced evaporative cooling (Sellers *et al.* 1996; Jackson *et al.* 1998; Bounoua *et al.* 1999). Reduced g_s, coupled with increased photosynthesis may also improve the water use efficiency (WUE) of many plants, which is of particular importance for productivity in arid and semi-arid regions (Polley, Johnson & Mayeux 1992; Polley *et al.* 1993; Sage 1995; Field *et al.* 1995; Owensby *et al.* 1999; Smith *et al.* 2000).

A major factor that could alter stomatal responses to CO₂ is the degree to which stomata acclimate to growth CO₂ concentration. Stomatal acclimation to CO₂ would be ecologically important if it either tempered or enhanced the reduction in g_s with rising CO₂ (Šantrůček & Sage 1996; Morison 1998). Acclimation is defined here as a change in stomatal function that occurs when plants are grown in contrasting CO₂ concentrations (Šantrůček & Sage 1996; Morison 1998). For example, physiological acclimation would be demonstrated if the stomatal behaviour of plants grown at contrasting CO₂ concentrations differed when measured at the same CO₂ concentration. Stomatal acclimation to CO₂ may occur in several ways, including changes in maximum g_s and stomatal sensitivity to CO₂. Other responses to CO₂, such as changes in stomatal morphology (density and size) may also influence g_s independently of physiological acclimation. Despite its potential importance for regulating plant water loss at high CO₂, stomatal acclimation has only been examined in a few species (Drake *et al.* 1997; Morison 1998).

The often dramatic response of terrestrial vegetation to elevated CO₂ has led to greater interest in plant responses to past CO₂ increases (Sage & Coleman 2001). Atmospheric CO₂ was as low as 180 μmol mol⁻¹ during the last glacial maximum and has risen by 37% since the 1700s (Barnola *et al.* 1987; Jouzel *et al.* 1993). Past CO₂ increases have been implicated in shifting global distributions of C₃ and C₄ plants and increases in ecosystem productivity (Polley *et al.* 1993; Ehleringer, Cerling & Helliker 1997). Although studies suggest that carbon assimilation may be affected by subambient CO₂ (Sage & Reid 1992; Tissue *et al.* 1995; Anderson *et al.* 2001; Sage & Coleman 2001), com-

Correspondence: H. Maherali. Fax: +1 919 660 7425; e-mail: maherali@duke.edu

paratively little is known about stomatal responses to past atmospheric CO₂ concentrations and their underlying mechanisms.

Recent empirical and modelling studies suggest that plant and ecosystem responses to increasing CO₂ may be non-linear (Ackerly & Bazzaz 1995; Luo, Sims & Griffin 1998; Luo & Reynolds 1999; Anderson *et al.* 2001). However, a majority of studies have examined plant responses to step increases in atmospheric CO₂, generally comparing ambient to twice ambient manipulations (Drake *et al.* 1997; Wand *et al.* 1999). Because atmospheric CO₂ is increasing gradually, results from step change experiments cannot be easily interpolated to intermediate CO₂ concentrations, and threshold or non-linear responses to CO₂ may go undetected (Luo & Reynolds 1999). Observations of plants grown across a range of CO₂ (e.g. Polley *et al.* 1993) may therefore help refine predictions of ecosystem responses to future CO₂ increases (Ackerly & Bazzaz 1995) and determine the magnitude of changes that have occurred since the start of the Industrial Revolution.

In this study, the influence of atmospheric CO₂ on stomatal physiology was examined using experimental chambers that maintain a continuous gradient of CO₂ from 200 to 550 $\mu\text{mol mol}^{-1}$ (Johnson, Polley & Whitis 2000). A previous study in this C₃/C₄ grassland ecosystem reported 40–80% declines in g_s along the gradient for several species (Anderson *et al.* 2001). Stomatal acclimation was examined in three dominant species of this grassland. First, we tested whether stomatal acclimation to CO₂ occurred and examined whether there was a threshold or non-linear response to the CO₂ gradient. Second, the widespread occurrence of photosynthetic acclimation to rising CO₂ suggests that stomata may also acclimate to maintain the tight coupling between photosynthesis and conductance. We therefore determined whether stomatal acclimation was associated with changes in photosynthetic acclimation (e.g. Jarvis, Mansfield & Davies 1999). Finally, we examined the implications of variation in stomatal behaviour for carbon gain by assessing its effects on relative stomatal limitation of photosynthesis (Jones 1985) and intrinsic WUE.

METHODS

Study site and experimental system

The measurements were conducted in a grassland area near the USDA-ARS Grassland, Soil and Water Research Laboratory in Temple, TX (31°05' N, 97°20' W). The site has been managed as grassland for the last 50 years and was last grazed by cattle in 1992. Soils are in the Austin black soil series, classified as a fine-silty, carbonatic, thermic Udorthentic Haplustoll with 35–55% clay in the top 40 cm (Johnson *et al.* 2000). To determine the potential for interspecific variation in responses to CO₂, the study focused on three abundant species with different growth forms and photosynthetic pathways: *Solanum dimidiatum* Raf., a C₃ perennial forb; *Bromus japonicus* L., a C₃ annual grass; and *Bothriochloa ischaemum* (L.) Keng, a C₄ perennial grass.

Other dominant species at the site include *Solidago canadensis* L. and *Ratibida columnaris* (Sims) D. Don. Mean annual precipitation (1913–99) is 877 mm and the mean minimum and maximum annual temperatures are 13.2 and 25.9 °C, respectively. C₃ species are mostly active early in the growing season, and C₄ species dominate by mid-summer.

The experimental system that was used consisted of two elongated chambers over parallel and adjacent plots of grassland, each 60 m in length, 1 m wide, and 1 m tall. Air was introduced into one end of each chamber and was progressively depleted of CO₂ by photosynthesis as it was moved down the chamber by a blower. The desired CO₂ concentrations were maintained by automatically varying the rate of air flow. During daytime, the CO₂ concentration gradients in the subambient and superambient chambers ranged from 360 to 200 $\mu\text{mol mol}^{-1}$ and from 550 to 360 $\mu\text{mol mol}^{-1}$, respectively. At night, CO₂ gradients were maintained at 150 $\mu\text{mol mol}^{-1}$ above daytime levels by reversing air flow and using respiratory CO₂ releases to create the gradient. Treatments began in May 1997 and operated each growing season (mid-February to mid-November) up to and including 2000.

The chambers were divided into 10 sections, each 5 m in length, with chilled-water cooling coils between sections to control temperature and humidity. Each section was enclosed in polyethylene film (which transmitted 85–95% of incident PPFD), and a rubber-coated barrier extended 1 m deep into the soil along the sides of each chamber. To maintain environmental conditions at ambient levels in chambers, air temperature and humidity were controlled by cooling and dehumidifying air before it entered each 5 m section of chamber. Irrigation was applied equally to each 5 m section to match ambient rainfall through July 1999. Thereafter, water was applied such that soil water content in sections matched that of adjacent grassland exposed to ambient CO₂ as measured by neutron attenuation. During the drought years of 1999 and 2000, the total water applied to the chambers was 349 and 381 mm, respectively. There were no consistent effects of CO₂ treatment on soil water content during these years (Polley *et al.* unpublished results). Therefore, stomatal responses to CO₂ were not confounded by CO₂-induced variation in soil water availability along the gradient. However, it should be noted that interspecific differences might have been affected by water availability because each species was measured at a different time of the year. Additional details of design, construction and operation of these chambers can be found in Johnson *et al.* (2000).

Steady-state gas exchange and stomatal acclimation

Steady-state leaf gas-exchange was measured at saturating irradiance with an open gas-exchange system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) between 0900 and 1500 h central standard time. Incident irradiance during all measurements was provided by red-blue light-emitting diodes.

Measurements were taken when each species was at its peak abundance, which was June 2000 for *S. dimidiatum*, April 2000 for *Br. japonicus*, and August 1999 for *Bo. ischaemum*. The youngest fully expanded leaf was sampled on three to four plants per section at a minimum of six treatment CO₂ concentrations (six chamber sections). Stomatal conductance (g_s) was measured at saturating light levels of 1200, 1600, and 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for *S. dimidiatum*, *Br. japonicus* and *Bo. ischaemum*, respectively. A Peltier cooling module maintained leaf temperatures at 20–23, 27–30 and 29–32 °C for *Br. japonicus*, *S. dimidiatum* and *Bo. ischaemum*, respectively, matching the approximate ambient conditions at the time of the measurement. Leaf-to-air vapour pressure deficits (LAVPD) were maintained at levels that permitted the measurement of maximum g_s , which were 0.9–1.1 kPa for *S. dimidiatum* and *Br. japonicus* and 1.4–1.6 kPa for *Bo. ischaemum*. To calculate g_s and intercellular CO₂ concentration (C_i) a boundary layer conductance of 4.86 $\text{mol m}^{-2} \text{s}^{-1}$ was used for the grasses and 1.42 $\text{mol m}^{-2} \text{s}^{-1}$ was used for *S. dimidiatum*. Boundary layer conductance was calculated on the basis of leaf area and fan speed using the energy balance algorithms of the LI-6400. Leaf area was measured with a portable leaf area meter (LI-3100; Li-Cor Inc.) or from leaf dimensions, depending on sample morphology. To examine long-term consequences of CO₂ for stomatal function, measurements were made during the third and fourth growing season of the experiment.

To examine stomatal acclimation to growth CO₂ concentrations, we measured the response of g_s and the C_i/C_a ratio to a manipulation of C_i (by changing the external CO₂ concentration, C_a) within the leaf cuvette. Stomatal conductance versus C_i (g_s-C_i) curves were measured on each species during the same period as steady-state gas exchange. Once clamped in the cuvette, leaves were exposed to the light, LAVPD, and temperature levels described above and the respective growth CO₂. After steady-state conditions were achieved, the first measurement was taken, and C_i was then reduced to the CO₂ compensation point and raised in steps (Šantrůček & Sage 1996). Stomatal conductance was recorded after steady-state conditions were re-established at each CO₂ level. Steady state was determined when the coefficient of variation of change in water vapour was < 0.05% (generally after 30–50 min).

To measure stomatal density (the number of stomates per mm²) and the size of stomata, casts were made of leaves sampled in the field during April 2000. Stomatal size was defined as the length in micrometres between junctions of the guard cells at each end of the stomate (Malone *et al.* 1993), and was therefore related to the maximum potential opening of the stomatal pore, and not the amount of opening that occurs at a given C_i . Measurements of stomatal density and stomatal size were made on leaf casts (e.g. Williams & Green 1988) for at least six individuals per species at each of six CO₂ concentrations (a total of at least 36 plants per species). For each impression, a leaf section located 2–3 cm from the petiole was pressed onto a micro-

scope slide covered with polyvinylsiloxane dental impression material ('Extrude' Medium; Kerr Manufacturing Co., Orange, CA, USA). After the polymer hardened (approximately 5 min), the leaf was removed and the resulting leaf mould was later used as a cast for clear nail polish. Each impression was analysed at 400 \times (for *S. dimidiatum* and *Bo. ischaemum*) or 100 \times (for *Br. japonicus*) using a light microscope interfaced with a solid-state TV camera (Model CCD-72-SX; DAGE-MTI Inc., Michigan City, IN, USA) using NIH Image 1.58 (U.S. National Institutes of Health; <http://rsb.info.nih.gov/nih-image/>). The stomatal density and stomatal size were sampled on three to six fields-of-view per slide, depending on the variation in the counts, and averaged for each slide.

The relative stomatal limitation of photosynthesis (I_g) was calculated, based on the response of net photosynthesis (A) to variation in C_i ($A-C_i$ curves). These curves were recorded concurrently with g_s-C_i curves on sampled plants and fitted with a non-linear regression model describing an exponential rise to a maximum:

$$A = a[1 - \exp(-bC_i)] + c \quad (1)$$

where c is the y intercept, $1/b$ is the rate constant, and $a + c$ is CO₂ saturated A (A_{max}). This model provided a good fit to our data ($r^2 > 0.97$) and has been used previously for herbaceous plants (Jacob, Greitner & Drake 1995; Reid & Fiscus 1998). The relative stomatal limitation (I_g) was calculated using the differential method of Jones (1985):

$$I_g = r_g / (r_g + r^*) \quad (2)$$

where r_g is the gas-phase resistance to CO₂ uptake (the supply function) and r^* is the slope of the $A-C_i$ curve (demand function). We calculated r^* as the first derivative of Eqn 1 at the operating C_i and calculated r_g as $(C_a - C_i)/A$ at the operating C_i (Jones 1985). This model was also used to calculate I_g for the C₄ species because A was not saturated at high CO₂ (550 $\mu\text{mol mol}^{-1}$; Anderson *et al.* 2001), permitting the calculation of a positive slope for the $A-C_i$ curve at the operating C_i .

To examine whether variation in stomatal conductance was associated with the maximum velocity of carboxylation (V_{cmax}) and photosynthetic capacity (Jarvis *et al.* 1999), we compared maximum g_s (g_{smax}), measured at the lowest CO₂ concentration to V_{cmax} and A_{max} (Eqn 1). V_{cmax} was estimated from the biochemical model of von Caemmerer & Farquhar (1981):

$$V_{\text{cmax}} = CE\{I^* + K_C[1 + [O_2]/K_O]\} \quad (3)$$

where the constants were $K_C = 43.5$ Pa, $K_O = 23.3$ kPa, and I^* (CO₂ compensation point in the absence of mitochondrial respiration in the light) = 4.44 Pa at a temperature of 26 °C (Harley, Webber & Gates 1985; Reid & Fiscus 1998) and an [O₂] of 21 kPa. Carboxylation efficiency (CE), which is the first derivative of Eqn 1 at the CO₂ compensation point, was calculated as

$$CE = ab \times \exp(-bC_i) \quad (4)$$

where $C_i = (1/b) \ln[a/(a - A + c)]$ at $A = 0$ (Reid & Fiscus 1998).

We note that because the $A-C_i$ curves were measured during the construction of the g_s-C_i curves, our calculations of V_{cmax} may be influenced by changes in the activation state of Rubisco over the course of the measurement. However, our observations of V_{cmax} using the 'slow' approach were similar to values derived from standard $A-C_i$ curves measured on these species previously (Anderson *et al.* 2001).

Statistical analyses

To examine whether CO_2 treatments influenced g_s-C_i and $(C_i/C_a)-C_i$ curves, we used the analysis of repeated measures (ANOVAR) for physiological response curves (e.g. Potvin, Lechowicz & Tardif 1990) in SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). C_i was the within subjects factor whereas growth CO_2 was the between subjects factor. The statistical significance of relationships between measured variables and growth CO_2 was determined using linear, hyperbolic and power functions in SPSS 10.0. As multiple measurements (three to five) were taken in each treatment section, there was more than one Y for each X (where $X =$ growth CO_2). Although means (± 1 SE) are presented in figures, all analyses were carried out using individual plants.

RESULTS

Steady-state g_s declined strongly with increasing growth CO_2 for all species (Fig. 1). The decline was non-linear for *S. dimidiatum* [$y = a + (b/x^2)$ where $a = 191.99$ and $b = 9.27 \times 10^6$; $r^2 = 0.94$; $P < 0.001$], decreasing by 81% from 200 to $550 \mu\text{mol mol}^{-1} CO_2$. Three-quarters of this decline occurred from subambient to ambient growth CO_2 . The decline in g_s with growth CO_2 was linear for *Br. japonicus* ($r^2 = 0.74$; $P < 0.0001$); a 49% decline from 215 to $550 \mu\text{mol mol}^{-1}$ and weakly non-linear for *Bo. ischaemum* ($y = ax^b$, where $a = -0.64$ and $b = 8.30 \times 10^3$; $r^2 = 0.76$; $P < 0.001$); a 46% decline from 215 to $540 \mu\text{mol mol}^{-1}$. *Solanum dimidiatum* had the highest overall g_s among the three species, followed by *Br. japonicus* and *Bo. ischaemum* (Fig. 1).

There was strong evidence for stomatal acclimation in *S. dimidiatum*. The g_s-C_i response curves for this species differed significantly across CO_2 treatments. Maximum g_s (g_{smax} ; g_s measured at the lowest C_i), increased and the g_s-C_i curves were progressively steeper for growth CO_2 concentrations below ambient levels ($P < 0.05$, ANOVAR, Fig. 2A). We also found evidence of a non-linear response of stomatal acclimation to CO_2 as the g_s-C_i curves did not differ among plants exposed to growth $CO_2 > 400 \mu\text{mol mol}^{-1}$. These differences in g_s-C_i curves also indicated that stomatal sensitivity to C_i was greater in plants grown at less than $400 \mu\text{mol mol}^{-1} CO_2$. Although the $(C_i/C_a)-C_i$ curves did not differ significantly ($P = 0.10$) among CO_2 treatments (Fig. 2B), average C_i/C_a was generally higher in subambient CO_2 -grown plants measured at $C_i < 400 \mu\text{mol mol}^{-1}$. This

trend is consistent with stomatal acclimation observed in Fig. 2A. Modification of stomatal behaviour in *S. dimidiatum* was not strongly associated with the maximum velocity of carboxylation (V_{cmax}). The g_{smax} was weakly, although significantly, correlated with V_{cmax} ($r^2 = 0.27$; $P = 0.05$, Fig. 3A). Variation in CO_2 saturated photosynthesis (A_{max}), however, was not correlated with g_{smax} ($r^2 = 0.05$; $P = 0.32$; Fig. 3B). In contrast to *S. dimidiatum*, there was no evidence of stomatal acclimation in *Br. japonicus* or *Bo. ischaemum*, as the g_s-C_i curves (Figs 4A & B) and $(C_i/C_a)-C_i$ curves (data not shown) were statistically indistinguishable across growth CO_2 concentrations.

Interestingly, the stomatal density (Fig. 5A) increased linearly with growth CO_2 in *S. dimidiatum* ($r^2 = 0.30$; $P < 0.001$) and *Br. japonicus* ($r^2 = 0.41$; $P < 0.0001$), but decreased linearly with growth CO_2 in *Bo. ischaemum* ($r^2 = 0.15$; $P < 0.05$). Stomatal size (Fig. 5B), measured as the length between junctions of the guard cells at each end of the stomate, decreased linearly with growth CO_2 in *S. dimidiatum* ($r^2 = 0.49$; $P < 0.0001$). In contrast, stomatal size increased weakly with growth CO_2 in *Br. japonicus* ($r^2 = 0.14$; $P < 0.05$) and was not associated with growth CO_2 in *Bo. ischaemum* ($r^2 = 0.06$; $P = 0.13$).

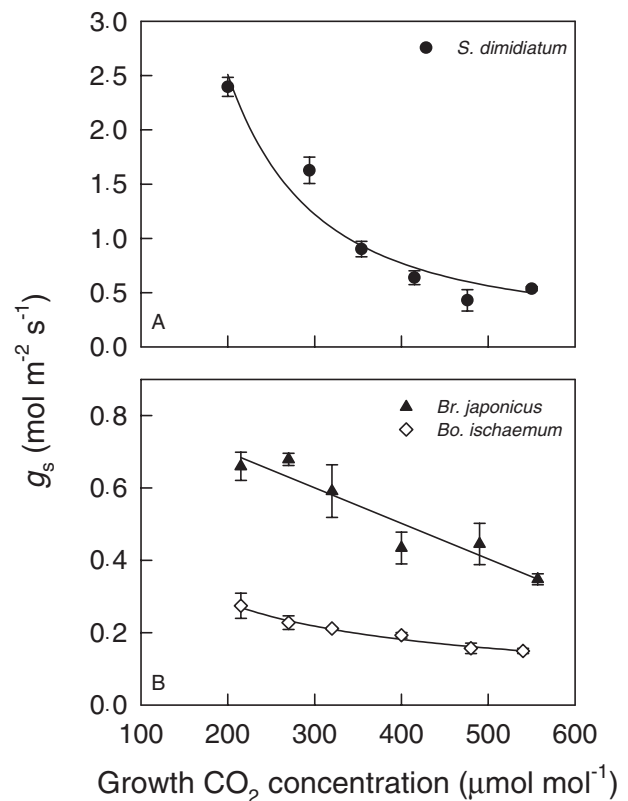


Figure 1. Mean (± 1 SE) values of steady-state stomatal conductance (g_s) for *S. dimidiatum* (A), *Br. japonicus* and *Bo. ischaemum* (B) plants grown across a range of atmospheric CO_2 . Note the difference in scales on the Y-axis between (A) and (B).

Despite higher g_s , plants grown at lower CO₂ concentrations had greater relative stomatal limitation of photosynthesis (I_g) than plants grown at higher CO₂ concentrations (Fig. 6). The strongest effect was for *Br. japonicus*, in which the relationship was non-linear (second-order polynomial, $r^2 = 0.82$; $P < 0.0001$). Stomatal limitation of photosynthesis decreased linearly with increasing growth CO₂ for *S. dimidiatum* ($r^2 = 0.51$; $P < 0.001$) and *Bo. ischaemum* ($r^2 = 0.26$; $P = 0.03$). Although *S. dimidiatum* and *Br. japonicus* had similar I_g at elevated growth CO₂, they diverged at subambient CO₂. Among species, stomatal limitation of photosynthesis was inversely proportional to g_s , being greatest in *Bo. ischaemum*, followed by *Br. japonicus* and *S. dimidiatum*.

Because plants were measured at the same LAVPD across treatments, the intrinsic water use efficiency (A/g_s) is representative of the water cost of photosynthesis in contrasting growth CO₂ environments. To illustrate the influence of stomatal acclimation to CO₂ on stomatal optimization of water loss relative to carbon gain, we plotted

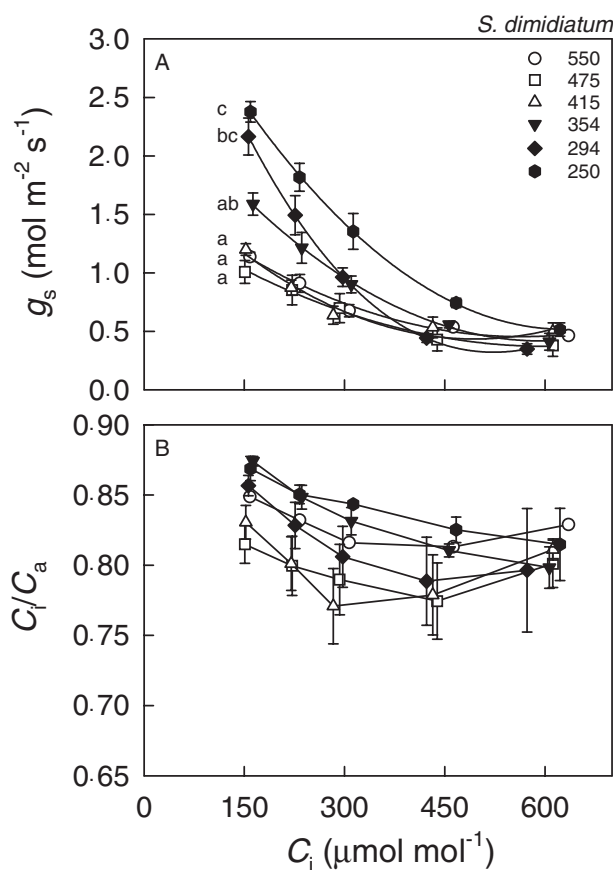


Figure 2. The response of mean (± 1 SE) g_s to C_i (g_s - C_i response curve; (A) and C_i/C_a to C_i [(C_i/C_a) - C_i response curve; (B)] for *S. dimidiatum* plants grown across a range of atmospheric CO₂. For g_s responses, a different letter next to each curve indicates that it is significantly different ($P < 0.05$, ANOVA) from the other curves. Symbols correspond to different growth CO₂ concentrations (μmol mol⁻¹).

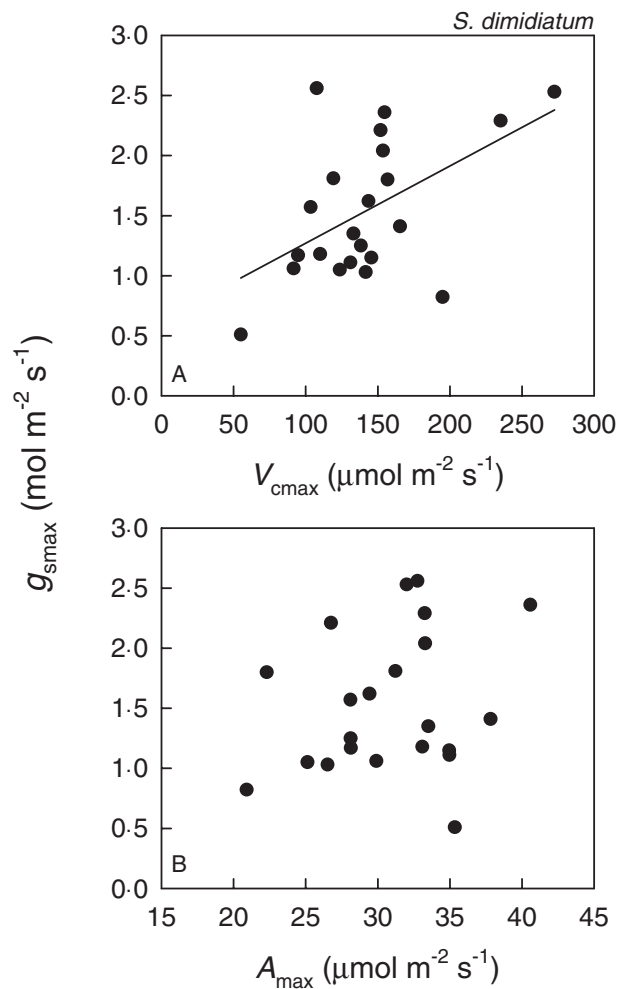


Figure 3. The relationship between maximum stomatal conductance (g_{smax} , measured at the lowest C_i) and V_{cmax} (A) and A_{max} (B) as determined from g_s - C_i response curves. The g_{smax} was significantly associated with V_{cmax} ($r^2 = 0.27$; $P = 0.05$), but was not correlated with A_{max} ($P > 0.05$).

A/g_s of all plants measured at a C_a of 200 μmol mol⁻¹ (where the strongest evidence for acclimation was observed, Fig. 2A) as a function of their growth CO₂ concentration (Fig. 7A-C). In *S. dimidiatum*, A/g_s increased significantly with growth CO₂ ($r^2 = 0.59$; $P < 0.0001$). Thus, when measured at 200 μmol mol⁻¹ CO₂, A/g_s for plants grown at 550 μmol mol⁻¹ was twice that of plants grown at 250 μmol mol⁻¹ CO₂. In contrast, growth CO₂ concentration had no effect on A/g_s measured at a common C_a for either grass species (Figs 7B & C).

DISCUSSION

We found clear evidence for stomatal acclimation to growth CO₂ in *Solanum dimidiatum*, a C₃ perennial forb, based on comparisons of the g_s - C_i curves among plants grown across a range of CO₂ concentrations. The strongest

response of g_s to C_i occurred in plants grown at 250 and 294 $\mu\text{mol mol}^{-1}$ CO_2 , followed by plants grown at 354 $\mu\text{mol mol}^{-1}$ CO_2 (Fig. 2A). In contrast, g_s - C_i curves did not differ for plants grown at greater than 400 $\mu\text{mol mol}^{-1}$ CO_2 . Although other studies have observed stomatal acclimation from ambient to elevated CO_2 (Morgan *et al.* 1994; Tubo, Szente & Koch 1994; Chen, Begonia & Hesketh 1995; Šantrůček & Sage 1996; Bunce 2001; Lodge *et al.* 2001), no evidence of such a pattern was found for any species in the present study (Figs 2A & 4). The results indicate that in *S. dimidiatum*, stomatal acclimation to growth CO_2 occurred non-linearly, below an apparent threshold of 350–400 $\mu\text{mol mol}^{-1}$ CO_2 . To our knowledge, this is the first study to document stomatal acclimation to subambient CO_2 in the field.

Neither grass species, *Br. japonicus* or *Bo. ischaemum*, showed evidence of stomatal acclimation to growth CO_2 ; there were no differences in the g_s - C_i response curves for either species across growth CO_2 treatments (Fig. 4A & B). This result was surprising, given systematic differences in absolute photosynthetic rates (Anderson *et al.* 2001) and g_s (Fig. 1) between the C_3 annual *Br. japonicus* and the C_4 perennial *Bo. ischaemum*. Our observations for *Br. japoni-*

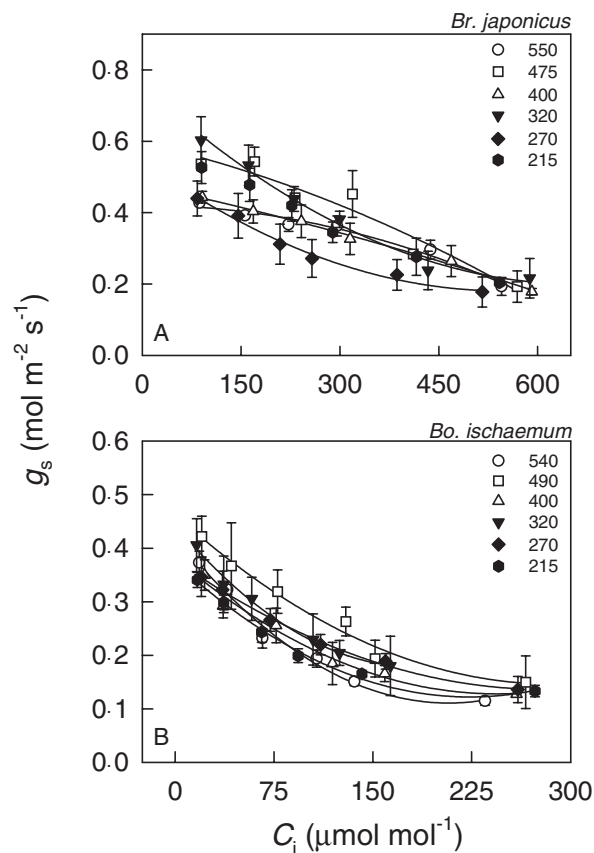


Figure 4. The response of mean (± 1 SE) g_s to manipulation of C_i (g_s - C_i response curve) for *Br. japonicus* (A) and *Bo. ischaemum* (B) plants grown across a range of atmospheric CO_2 . Symbols correspond to different growth CO_2 concentrations ($\mu\text{mol mol}^{-1}$).

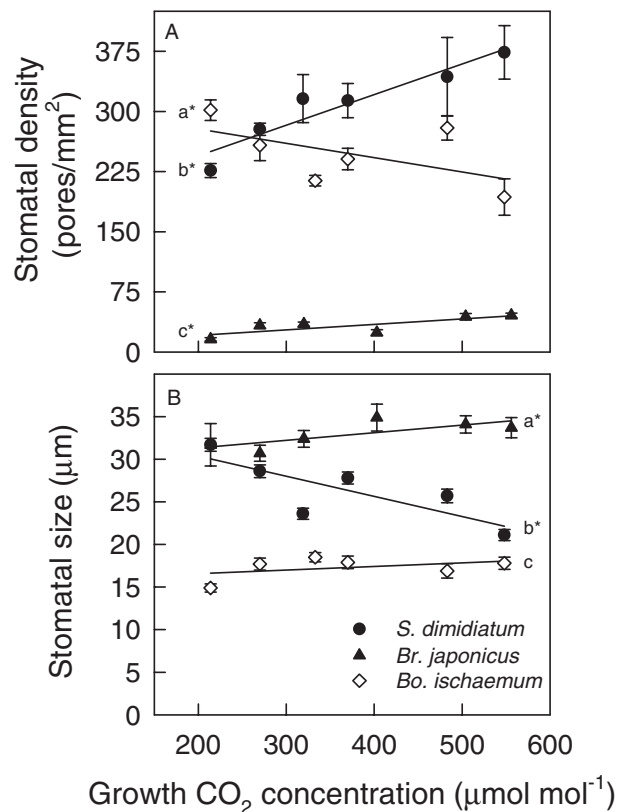


Figure 5. The response of mean (± 1 SE) stomatal density (A) and stomatal size (B) to a range of atmospheric CO_2 for the three study species. Statistically significant differences ($P < 0.05$) in the magnitude of each variable among species are indicated by different letters. A * next to each letter indicates that the slope of the relationship is significantly ($P < 0.05$) different from zero.

cus and *Bo. ischaemum* therefore suggest that stomatal responses to CO_2 in C_3 and C_4 grass species, like growth responses, may be quite similar (Wand *et al.* 1999). In contrast, stark differences in stomatal acclimation to CO_2 between *S. dimidiatum* and *Br. japonicus* indicate that species with similar photosynthetic pathways may have vastly different stomatal responses to CO_2 .

Previously, we observed significant up-regulation of the maximum velocity of carboxylation (V_{cmax}) at subambient growth CO_2 in *S. dimidiatum* (Anderson *et al.* 2001). The observation that g_s is often coupled to photosynthetic capacity (e.g. Wong, Cowan & Farquhar 1979) raises the possibility that stomatal acclimation in *S. dimidiatum* represents a response to photosynthetic acclimation rather than a direct response to growth CO_2 . A significant positive relationship was observed between g_{smax} and V_{cmax} (Fig. 3A), but variation in V_{cmax} could only account for 27% of the variation in g_{smax} . The CO_2 saturated photosynthetic rate (A_{max}), in contrast, was not related to g_{smax} (Fig. 3B). Although the mechanism for stomatal acclimation to atmospheric CO_2 concentration is not known, the present results suggest that stomatal acclimation to CO_2 may not be driven solely by adjustments in photosynthetic bio-

chemistry (e.g. Bunce 2001; Lodge *et al.* 2001; but see Jarvis *et al.* 1999).

As in previous studies in grasslands, increasing growth CO₂ reduced steady-state g_s in all species (Fig. 1; Johnson,

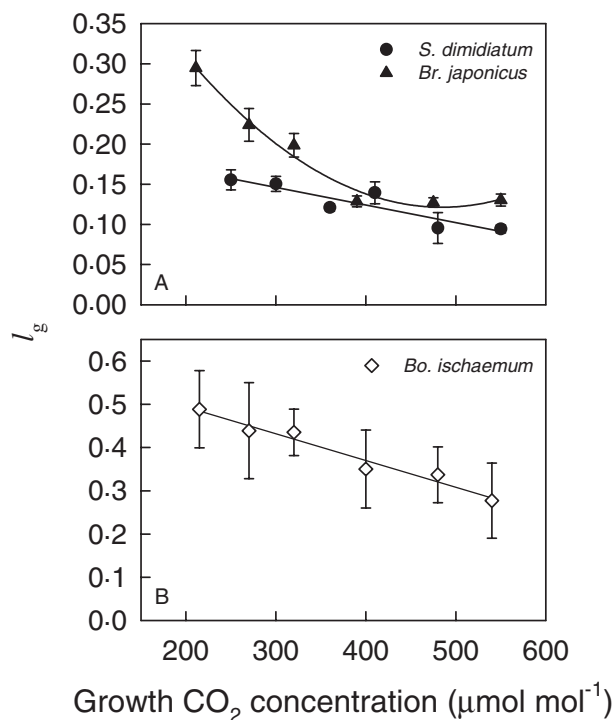


Figure 6. The response of mean (± 1 SE) relative stomatal limitation of photosynthesis (l_g) to increasing growth CO₂ for C₃ (A) and C₄ (B) species. Note the difference in scales on the Y-axis between (A) and (B).

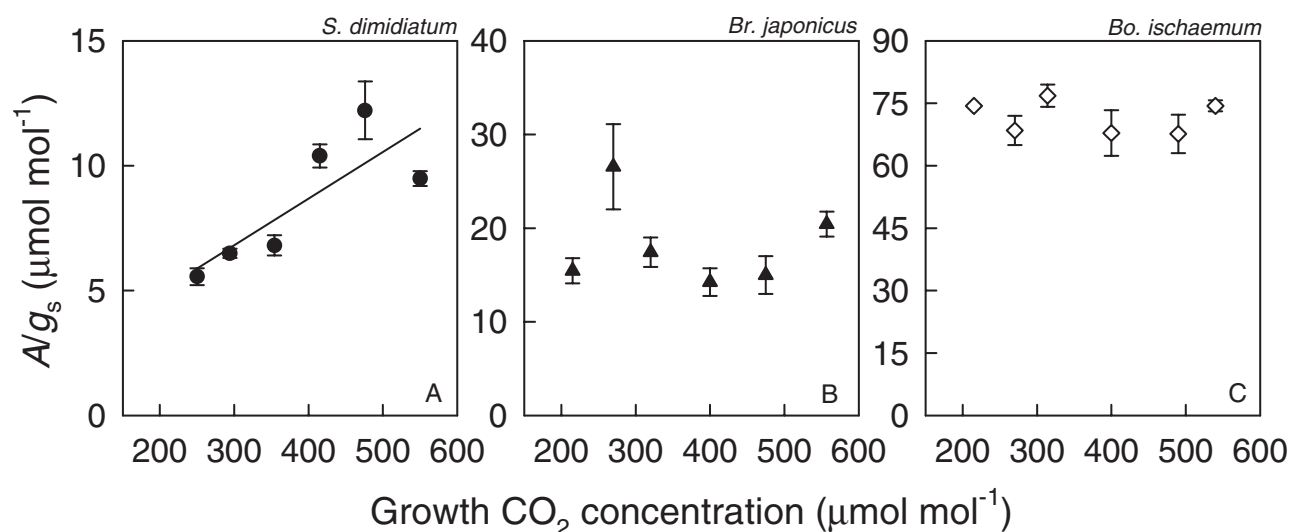


Figure 7. Mean (± 1 SE) intrinsic leaf water use efficiency (A/g_s) measured at 200 $\mu\text{mol mol}^{-1}$ C_a for each species in each growth CO₂ treatment. A/g_s increased significantly with growth CO₂ only in *S. dimidiatum*. Note the difference in scales on the Y-axis between (A), (B) and (C).

Polley & Mayeux 1993; Jackson *et al.* 1994; Knapp *et al.* 1996; Niklaus, Spinnler & Körner 1998; Lee *et al.* 2001). Our results suggest that stomatal acclimation to CO₂ in *S. dimidiatum* was responsible for driving both the large (81%) and non-linear decrease in steady-state g_s along the CO₂ gradient (Fig. 1A). For example, steady-state g_s at a CO₂ concentration of 200 $\mu\text{mol mol}^{-1}$ was 75% higher than it would have been if acclimation had not occurred (based on a comparison of the g_s - C_i curves of plants grown at 250 $\mu\text{mol mol}^{-1}$ CO₂ versus those grown at greater than 400 $\mu\text{mol mol}^{-1}$ CO₂; Fig. 2A). In contrast, g_s declined linearly and more modestly (46–49%) with CO₂ in the two grass species in which stomatal acclimation was not observed.

Some studies suggest that decreased stomatal density is a mechanism for reducing g_s in response to rising CO₂ (Woodward 1987; Beerling & Woodward 1993; Kurschner *et al.* 1997). Our results from a C₃/C₄ grassland provide little support for this pattern, as the species showing the strongest decline in g_s with rising CO₂ (*S. dimidiatum*, Fig. 1A), actually increased stomatal density with increasing growth CO₂ (Fig. 5A). Similarly, the relationship between stomatal density and growth CO₂ was very different between *Br. japonicus* and *Bo. ischaemum*, species that had nearly identical declines in g_s in response to increased CO₂ along the gradient. Of the three species measured only *Bo. ischaemum* decreased stomatal density in response to rising CO₂ (Fig. 5A), but the strength of this relationship was relatively weak ($r^2 = 0.15$). These results suggest that g_s responses to CO₂ need not correlate in magnitude or direction with stomatal density responses to CO₂. However, it should be noted that measurements of g_s and stomatal density were not made at the same time; therefore we cannot rule out the possibility that phenological variation in stomatal morphology also contributed to the lack of correlation between sto-

matal density and g_s . Nevertheless, our results suggest that the response of stomatal density to CO_2 is not necessarily generalizable across species or growth forms (e.g. Knapp *et al.* 1994).

Stomates were significantly larger in *S. dimidiatum* plants grown at subambient CO_2 (Fig. 5B). As a potential developmental response to CO_2 starvation, the increased stomatal size facilitates CO_2 diffusion into the leaf (Parkhurst 1994) because conductance is proportional to the square of the effective radius of the stomatal pore (Nobel 1991). Therefore, larger stomatal size at subambient CO_2 may contribute to the substantial increases in g_s that were observed in *S. dimidiatum* (Fig. 1A). Similarly, small stomatal size at elevated CO_2 could constrain g_s upon exposure to subambient CO_2 , as was observed in our study (Fig. 2A). Large stomatal size in *S. dimidiatum*, however, does not appear to prevent guard cell control of water loss, as g_s measured at high C_i was the same for all plants regardless of growth CO_2 (Fig. 2A). A reduction in g_s for elevated CO_2 -grown *S. dimidiatum*, despite an increase in stomatal density, also suggests that increased stomatal size may be the primary morphological adjustment implicated in stomatal acclimation to CO_2 . The observation that stomatal size varied weakly with CO_2 treatment in *Br. japonicus* and *Bo. ischaemum* (e.g. Malone *et al.* 1993), where stomatal acclimation did not occur (Fig. 4), is consistent with this view.

To determine whether stomatal acclimation could influence CO_2 diffusion and carbon assimilation, relative stomatal limitation of photosynthesis (l_g ; Jones 1985) was calculated from $A-C_i$ curves measured concurrently with g_s-C_i curves (Fig. 6). In general, the magnitude of l_g was inversely proportional to g_s among species (*S. dimidiatum* < *Br. japonicus* < *Bo. ischaemum*). Stomatal closure at higher growth CO_2 , however, did not lead to an increase in l_g , suggesting that the stimulation of photosynthesis by CO_2 more than compensated for any diffusional limits imposed by stomata (Tissue *et al.* 1995; Drake *et al.* 1997). The value of l_g was approximately similar for both C_3 species at elevated CO_2 (approximately 10–15%) but l_g in *Br. japonicus* increased disproportionately relative to *S. dimidiatum* at subambient growth CO_2 concentrations. These results suggest that stomatal acclimation may have a significant role in reducing l_g in *S. dimidiatum* at subambient CO_2 when compared with the non-acclimating *Br. japonicus*.

Stomatal acclimation, by increasing water loss from the leaf surface, could alter intrinsic WUE (defined here as A/g_s). Although rising CO_2 increases WUE in this grassland (Anderson *et al.* 2001), stomatal acclimation appears to modify the trajectory of this response in *S. dimidiatum*. The influence of stomatal acclimation on WUE was apparent in the strong effect that growth CO_2 concentration had on A/g_s even when plants were measured at the same C_a . For example, A/g_s increased significantly with growth CO_2 in *S. dimidiatum* measured at $200 \mu\text{mol mol}^{-1}$ (Fig. 7A). In contrast, A/g_s measured at a common $200 \mu\text{mol mol}^{-1}$ C_a did not vary with growth CO_2 in either of the non-acclimating grass species (Fig. 7B & C).

The observation that A/g_s was lower in *S. dimidiatum* than it would have been without acclimation suggests that, in the past, this phenotype could have had a negative impact on plant productivity in water-limited grasslands (Polley *et al.* 1993; Sage 1995; Hsiao & Jackson 1999). Therefore, what is the ecological significance of stomatal acclimation to subambient CO_2 ? One possibility is that maximizing leaf A/g_s is not necessarily an adaptive response to variation in CO_2 . For example, maintaining a high A/g_s may be a disadvantage if conserved water that is stored in soil is lost to competitors or through soil evaporation (DeLucia & Schlesinger 1991; Jones 1993). It is also likely that the strength of stomatal regulation of water loss in a given species is dependent on other correlated physiological and morphological traits (Givnish 1986). For example, weak stomatal control of water loss may be associated with species that have access to stable sources of water via deep roots or that possess xylem that is resistant to drought-induced cavitation (Jones 1993; Jackson, Sperry & Dawson 2000). Given our limited understanding of the whole-plant context of stomatal responses to CO_2 and the mechanisms by which these responses occur, the adaptive significance of stomatal acclimation to subambient CO_2 remains uncertain.

It is not known to what degree comparisons of extant plants grown in subambient and elevated CO_2 concentrations are representative of actual changes in plant function from the past to the present and future. As we have no information on the genotypes of past populations, it is not known if our study species have evolved in response to rising CO_2 . We observed strong evidence of stomatal acclimation in *S. dimidiatum* at growth CO_2 concentrations as high $294 \mu\text{mol mol}^{-1}$, a level which occurred relatively recently (around 1900 AD). Although a step change in atmospheric CO_2 can act as a selective agent on plant populations (Ward *et al.* 2000), the relatively short time span involved suggests that populations of a perennial species such as *S. dimidiatum* may not have evolved substantially in response to the gradual increase in CO_2 . In consequence, our results suggest that the greatest stomatal response to CO_2 (approximately 76%) in *S. dimidiatum* has already taken place. Further reductions in g_s will likely occur as atmospheric CO_2 increases, but will be of much smaller magnitude. In contrast, the absence of stomatal acclimation in *Br. japonicus* and *Bo. ischaemum* suggests that future stomatal responses to rising CO_2 may mimic those of the past in these species.

Our results have implications for modelling efforts aimed at predicting how stomatal responses to CO_2 may feedback on ecosystem hydrology and climate (Henderson-Sellers, McGuffie & Gross 1995; Pollard & Thompson 1995; Sellers *et al.* 1996; Jackson *et al.* 1998). When stomatal acclimation is included in these models, it is usually assumed to occur linearly with CO_2 (e.g. Sellers *et al.* 1996). In contrast to these assumptions, we observed stomatal acclimation only at subambient CO_2 , a phenomenon that caused reductions in g_s from subambient to ambient CO_2 to be greater than those observed from ambient to elevated CO_2 . The

occurrence of stomatal acclimation and substantial reductions in g_s from pre-industrial to current ambient CO₂ concentration suggest that large changes in evapotranspiration and soil drainage in grassland ecosystems may have already occurred. Incorporating these physiological perspectives into modelling studies could improve our ability to predict changes in ecosystem water fluxes and water availability with rising CO₂ and to understand their magnitudes relative to the past.

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