Annals of Botany **74**: 595–599, 1994

Effect of Elevated CO_2 on Stomatal Density and Distribution in a C_4 Grass and a C_3 Forb under Field Conditions

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Received: 18 April 1994 Accepted: 23 June 1994

Two common tallgrass prairie species, *Andropogon gerardii*, the dominant C_4 grass in this North American grassland, and *Salvia pitcheri*, a C_3 forb, were exposed to ambient and elevated (twice ambient) CO_2 within open-top chambers throughout the 1993 growing season. After full canopy development, stomatal density on abaxial and adaxial surfaces, guard cell length and specific leaf mass (SLM; mg cm^{-2}) were determined for plants in the chambers as well as in adjacent unchambered plots. Record high rainfall amounts during the 1993 growing season minimized water stress in these plants (leaf xylem pressure potential was usually $> -1{\cdot}5$ MPa in *A. gerardii*) and also minimized differences in water status among treatments. In *A. gerardii*, stomatal density was significantly higher (190 ± 7 mm^{-2}; mean ± s.e.) in plants grown outside of the chambers compared to plants that developed inside the ambient CO_2 chambers (161 ± 5 mm^{-2}). Thus, there was a significant 'chamber effect' on stomatal density. At elevated levels of CO_2, stomatal density was even lower ($P < 0{\cdot}05$; 121 ± 5 mm^{-2}). Most stomata were on abaxial leaf surfaces in this grass, but the ratio of adaxial to abaxial stomatal density was greater at elevated levels of CO_2. In *S. pitcheri*, stomatal density was also significantly lower when plants were grown in the open-top chambers (235 ± 10 mm^{-2} outside *vs.* 140 ± 6 mm^{-2} in the ambient CO_2 chamber). However, stomatal density was greater at elevated CO_2 (218 ± 12 mm^{-2}) compared to plants from the ambient CO_2 chamber. The ratio of stomata on adaxial *vs.* abaxial surfaces did not vary significantly in this herb. Guard cell lengths were not significantly affected by growth in the chambers or by elevated CO_2 for either species. Growth within the chambers resulted in lower SLM in *S. pitcheri*, but CO_2 concentration had no effect. In *A. gerardii*, SLM was lower at elevated CO_2. These results indicate that stomatal and leaf responses to elevated CO_2 are species specific, and reinforce the need to assess chamber effects along with treatment effects (CO_2) when using open-top chambers.

Key words: *Andropogon gerardii*, elevated CO_2, *Salvia pitcheri*, stomatal density, tallgrass prairie.

INTRODUCTION

Many studies have documented responses of stomata to elevated levels of CO_2. In general, stomatal conductance to water vapour diffusion declines as CO_2 concentration increases (Eamus and Jarvis, 1989; Tyree and Alexander, 1993) and some evidence indicates that density of stomata on leaf surfaces also declines (Woodward, 1987; Woodward and Bazzaz, 1988; Paoletti and Gellini, 1993). However, there are conflicting studies that show no consistent effect of CO_2 on stomatal density (Thomas and Harvey, 1983; Korner, 1988; Ryle and Stanley, 1992; Malone *et al.*, 1993; Ferris and Taylor, 1994) and some that indicate that stomatal density increases as CO_2 increases (Apel, 1989). As a result of the variability in responses, Apel (1989) stressed the need for evaluations of stomatal responses to CO_2 in individual species, and even for varieties of agricultural plants, rather than relying on generalizations. Methodologies used to assess responses in stomatal density to increased CO_2 also vary significantly, and some of the diversity in responses can be attributed to the different methods used (Eamus and Jarvis, 1989). To minimize such problems, it is preferable to expose plants to a given CO_2 level throughout the development of leaves (Eamus and Jarvis, 1989), to do so under field conditions since light levels in growth chambers typically are much lower, and spectrally distinct, compared to the field (Tibbits and Langhans, 1993), and to control as many other factors as possible to isolate the direct response of stomatal density to CO_2 from indirect effects. Plant water status is an example of a factor that almost always is affected by elevated CO_2 (Dahlman, 1993; Kimball *et al.*, 1993; Morison, 1993) and that can also affect stomatal densities (Thakur, 1990). Indeed, leaf water potentials are typically higher in plants grown at elevated *vs.* ambient CO_2 (Frederick *et al.*, 1990; Nie *et al.*, 1992; Knapp, Hamerlynck and Owensby, 1993*b*; Tschaplinski, Norby and Wullschleger, 1993) and greater epidermal cell expansion at higher turgor levels may account for decreases in stomatal density (although not stomatal indices) independent of direct effects of CO_2.

The purpose of this study was to compare responses in stomatal density, distribution (adaxial *vs.* abaxial), guard cell length and specific leaf mass at ambient and elevated (twice ambient) CO_2 concentrations in two common, but distinctly different, tallgrass prairie species. Studies were conducted under field conditions in open-top chambers

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0305-7364/94/120595+05 $08.00/0

during a year when record high levels of precipitation fell in this system. This minimized treatment-induced differences in plant water status during leaf development that could alter stomatal density and distribution independent of the direct effect of CO_2.

MATERIALS AND METHODS

Study site

Research was conducted in native tallgrass prairie within the Flint Hills of north-eastern Kansas (USA) near Manhattan. This grassland type is dominated by C_4 grasses such as *Andropogon gerardii* Vitman and *Sorghastrum nutans* L., but numerous broad-leaf C_3 forbs (perennial herbs) are also common (Freeman and Hulbert, 1985). The two species selected for study were the dominant grass, *A. gerardii* (Poaceae), and the common forb, *Salvia pitcheri* Torr. (Lamiaceae). The response of tallgrass prairie communities, and *A. gerardii* in particular, to elevated CO_2 have been well-documented (Nie *et al.*, 1992; Owensby *et al.*, 1993; Knapp *et al.*, 1993*b*; Knapp, Fahnestock and Owensby, 1994), but less is known about responses in forbs.

Undisturbed portions of the tallgrass prairie community were exposed to elevated (double ambient levels) or ambient CO_2 concentrations continuously throughout the 1993 growing season (approx. mid-April until mid-October) in large open-top chambers. Precipitation in 1993 was 61·5 cm above the 30-year mean of 82·1 cm. Chambers were 4·5 m in diameter and 4·0 m in height with a cone-top baffle that reduced the top opening to 1·5 m. UV resistant polyethylene film (6 mm) was used to cover the aluminium structural frame of the chambers. Precipitation intercepted by the baffle was collected in gutters at the top of the chambers and pumped through a centrally located sprinkler within each chamber. Plants selected for measurements came from two chambers each with ambient or elevated CO_2 concentrations and from two adjacent field plots without chambers. Additional details on the experimental design and microclimatic characteristics of the chambers can be found in Owensby *et al.* (1993).

Plant water relations

Midday (approx. 1300 h CDT) leaf xylem pressure potential (ψ) in *A. gerardii* was measured at about 10-d intervals throughout the 1993 growing season to document potential differences in water status due to CO_2 treatment. Mature, upper canopy leaves ($n = 5$–7) of *A. gerardii* were collected from the four chambers and the two field plots, immediately placed in plastic bags with wet filter paper and quickly transported to a Scholander-type pressure chamber (model 1000, Plant Moisture Stress, Corvallis, Oregon, USA) for ψ determinations. Because of the limited number of *S. pitcheri* plants in each chamber, ψ in this species could not be measured.

Stomatal measurements

During a period from mid-July (day of year 197) through mid-August (day of year 232), a total of 15 *A. gerardii* and 10 *S. pitcheri* leaves were collected in the morning from near the top of the grass canopy in each chamber. Leaves were stored in humidified plastic bags and returned to the laboratory where the tissue was hydrated in distilled water. Central portions of these leaves (approx. 1 cm^2) were selected for estimates of stomatal density on abaxial and adaxial surfaces and measurements of guard cell length. Cellulose acetate impressions of leaf surfaces were made (Payne, 1970) and scanned at $\times 100$ and $\times 450$ magnification with a light microscope equipped with a calibrated ocular micrometer. Densities were estimated in two randomly selected regions per leaf by counting all stomata within a $0{\cdot}5 \times 0{\cdot}5$ mm area ($\times 100$ magnification) for both abaxial and adaxial surfaces. After densities were estimated, five measurements of guard cell length were made at $\times 450$ magnification for each leaf. In *S. pitcheri*, stomata were numerous on abaxial and adaxial surfaces, but in *A. gerardii*, the number of estimates of guard cell length on adaxial surfaces was limited by low stomatal densities on this leaf surface. Finally, the areas of ten, 1-to-2-cm^2 portions of leaves from each treatment were determined with a leaf area meter (model CI-202, CID, Inc., Moscow, Idaho, USA) and then the leaf segments were oven-dried to a constant weight to determine specific leaf mass (mg cm^{-2}).

Data analysis

Stomatal densities were expressed as number mm^{-2} abaxial, adaxial, total and adaxial/abaxial ratios. ANOVA was used to assess the statistical significance ($P < 0{\cdot}05$) of treatments on ψ, stomatal density, guard cell length and specific leaf mass. Non-parametric tests (Kruskal–Wallis) were used to compare stomatal ratios (Zar, 1974).

RESULTS

The experimental design of this study allowed for two types of comparisons. First, by comparing data from the ambient chamber to data from outside the chambers, the 'chamber effect' on leaf characteristics could be quantified. Second, by comparing data from within the chambers, the effect of CO_2 concentration could be assessed. In *A. gerardii*, ψ was maintained at relatively high levels ($> -1{\cdot}5$ MPa) for most of the 1993 growing season and throughout most of the period that leaf samples were collected (Fig. 1). In years with more typical rainfall patterns, ψ is often reduced to $< -2{\cdot}5$ MPa (Knapp *et al.*, 1993*a*) and extremes in ψ to $< -6{\cdot}0$ MPa have been recorded in this grassland (Knapp, 1984). Despite the lack of apparent water stress in 1993, ψ in *A. gerardii* in elevated CO_2 chambers was always slightly higher (seasonal mean $\pm$ s.e. $= -0{\cdot}99 \pm 0{\cdot}11$ MPa) than in the ambient chambers ($-1{\cdot}13 \pm 0{\cdot}12$), with the lowest ψ usually measured in leaves from the unchambered control plots ($-1{\cdot}22 \pm 0{\cdot}17$; Fig. 1). At individual dates, there were significant differences ($P < 0{\cdot}05$) in ψ two times during the season; at day 183 prior to measurements of stomatal density and at day 234 after sampling was completed. However, over the entire growing season there were no significant chamber and CO_2 effects on ψ. Significant differences in water status due to the open-top chamber and CO_2 concentration have been documented previously and were more pronounced in years with more typical (lower)

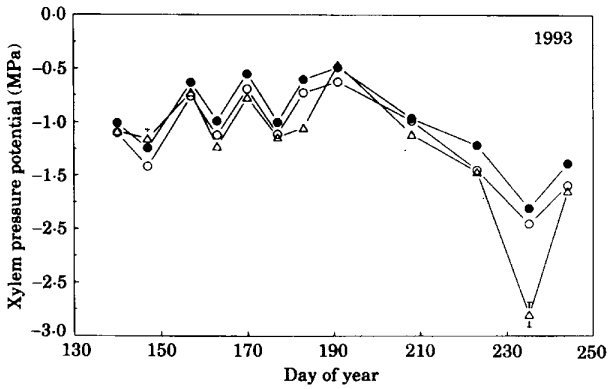

FIG. 1. Seasonal course of midday (approx. 1300 h CDT) leaf xylem pressure potential in *Andropogon gerardii* grown in undisturbed tallgrass prairie (control, △) and in open-top chambers with ambient (○) and twice ambient (●) (elevated) CO_2 concentrations. Vertical bars indicate ±1 s.e. of the mean (most error bars are smaller than the symbols). Rainfall in 1993 during the months of this study exceeded the 30-year average by 63·9 cm.

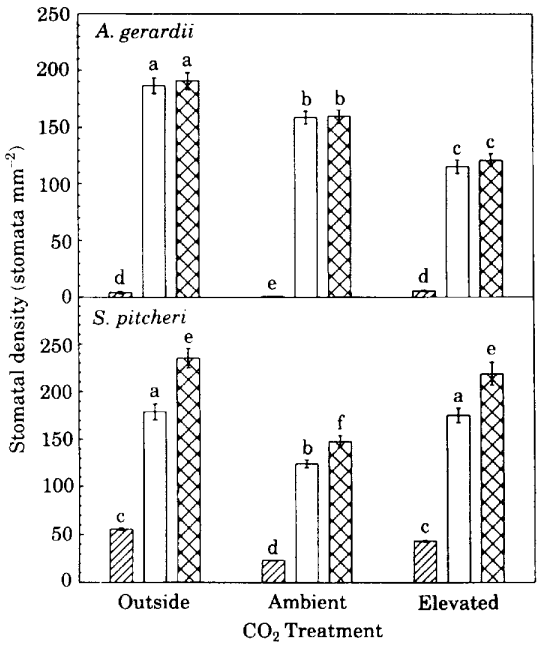

FIG. 2. Comparison of stomatal densities on adaxial (▨) and abaxial (□) leaf surfaces, and total density (⊠) (both surfaces combined) in *A. gerardii*, a C_4 grass and *Salvia pitcheri*, a C_3 forb. Treatments are 'outside' (plants grown in undisturbed tallgrass prairie), 'ambient' and 'elevated' [plants grown in open-top chambers with ambient and elevated (twice ambient) CO_2 concentrations]. Error bars indicate ±1 s.e. of the mean and means with the same letter are not significantly different at the $P = 0{\cdot}05$ level.

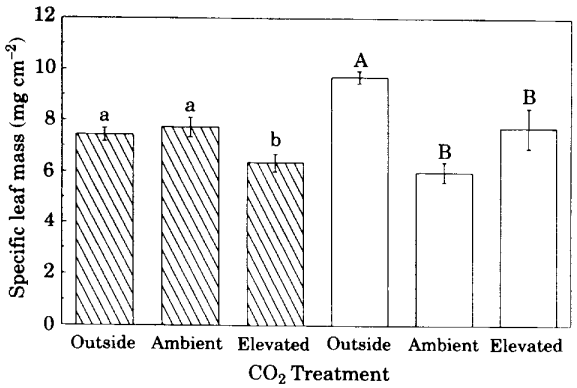

FIG. 3. Comparison of specific leaf mass in *A. gerardii* (▧) and *S. pitcheri* (□) growing in undisturbed tallgrass prairie (outside) or within open-top chambers at ambient or elevated (twice ambient) levels of CO_2. Error bars indicate ±1 s.e. of the mean and means with the same letter are not significantly different at the $P = 0{\cdot}05$ level.

rainfall amounts (Owensby *et al.*, 1993). Overall, the level of ψ in *A. gerardii* in this study likely resulted in little physiological stress prior to, or during the period of leaf sampling. For example, previous studies have shown that net photosynthetic rates in this grass are not negatively impacted until ψ declines to $< -1{\cdot}7$ MPa (Knapp *et al.* 1993*a*).

In *A. gerardii*, total stomatal density (adaxial+abaxial) was significantly lower in the open-top chamber compared to field plants. Moreover, stomatal density was even lower in plants grown at elevated levels of CO_2 (Fig. 2). Most of the stomata were located on abaxial surfaces and thus, abaxial stomatal density responded in an identical manner. In contrast, adaxial surfaces had fewer stomata, and although chamber growth conditions resulted in significantly lower total density, stomatal density on adaxial surfaces was slightly higher at elevated *vs.* ambient CO_2. As a result, when stomatal ratios (adaxial/abaxial) were compared within *A. gerardii*, this ratio was significantly higher (ratio = 0·058) at elevated CO_2 compared to plants grown at ambient CO_2 either inside or outside chambers (ratio = 0·015). Guard cell length in *A. gerardii* varied little when abaxial *vs.* adaxial surfaces were compared or due to treatment (data not shown). Overall, guard cell length varied between 36 and 42 μm, with adaxial guard cells usually slightly longer than those on abaxial surfaces.

In the forb, *S. pitcheri*, total stomatal density as well as abaxial and adaxial density was lower in plants grown in the open-top chambers than in the field (Fig. 2). However, within chambers, elevated CO_2 resulted in significantly higher stomatal density. Stomatal ratios (0·27) were not

affected by the chamber or CO_2 concentration, and guard cell length in *S. pitcheri* varied little among treatments (range = 28 to 30 μm).

Specific leaf mass (SLM) was not affected by the open-top chamber in *A. gerardii*, but was significantly lower (38%) due to the chamber effect in *S. pitcheri* (Fig. 3). In contrast, elevated CO_2 resulted in significantly lower SLM (17%) in *A. gerardii*, but SLM was not affected by CO_2 in *S. pitcheri* (Fig. 3).

DISCUSSION

Both *A. gerardii* and *S. pitcheri* are long-lived perennials, as are most tallgrass prairie plants (Weaver, 1968). Thus, responses in leaf or stomatal characteristics to CO_2 in these species will be limited by the degree of phenotypic plasticity associated with these responses. Phenotypic plasticity in morphological or physiological characteristics is expected to be high in this grassland given the extreme interannual climatic variability characteristic of this system (Weaver, 1968; Knapp, 1984). Indeed, the two species studied did respond significantly to the treatments imposed. Overall, data from this study are consistent with previous studies of stomatal responses to CO_2 concentrations in that individual species responded differently (Apel, 1989; Eamus and Jarvis, 1989; Malone *et al.*, 1993). In studies of the effects of elevated CO_2 on the C_4 annual *Zea mays*, both increases and decreases in stomatal density have been reported (Thomas and Harvey, 1983; Apel, 1989); however, no consistent relationship between stomatal density and CO_2 concentration was found in the perennial C_4 grass *Schizachyrium scoparium* (Malone *et al.*, 1993). Stomatal conductance to water vapour diffusion is decreased by > 50% in *A. gerardii* at elevated CO_2 (Knapp *et al.*, 1994). Thus, the relatively small reductions in stomatal density (approx. 24%) at elevated CO_2 in this grass may play a lesser role in reducing water loss compared to reductions in stomatal pore aperture. No data are available on CO_2-induced responses in stomatal conductance in *S. pitcheri*, but measurements of transpiration in other tallgrass prairie forbs at elevated CO_2 have shown that marked reductions occur relative to plants grown at ambient CO_2 (Ham and Owensby, unpubl. res.).

Also noteworthy is the strong effect that open-top chamber growth conditions had on the measured leaf characteristics. Of the environmental alterations that plants are exposed to when grown in these chambers, reductions in wind speed and turbulence relative to outside (Owensby *et al.*, 1993) are probably the most important. Plant responses to reductions in wind can be dramatic, but are difficult to separate from correlated temperature and water relations responses (Grace, 1988). In general, responses in stomatal density and SLM in the tallgrass prairie dominant, *A. gerardii*, to growth in the open-top chambers were much less than in the forb (Figs 2 and 3), perhaps reflecting fundamental differences in responses in canopy-forming plants *vs.* isolated individuals. Previous studies in this tallgrass prairie system have detected only minor differences in SLM in this C_4 grass due to the chamber effect or CO_2 concentration (Knapp *et al.*, 1993*b*), however, most studies in other systems have shown that SLM is higher in plants grown at elevated CO_2 (Apel, 1989; Tyree and Alexander, 1993), particularly in C_3 species in which photosynthesis is enhanced by elevated CO_2. We conclude that although stomatal conductance is almost always reduced by elevated CO_2 (Dahlman, 1993), responses in stomatal density and distribution to CO_2 should be assessed on a species by species basis (Apel, 1989; Ferris and Taylor, 1994). Moreover, to insure that responses attributed to CO_2 are not due instead to alterations in growth conditions within chambers, the effects of chamber-induced environmental alterations should be assessed at the same time that CO_2 responses are evaluated.

ACKNOWLEDGEMENTS

Eric Kirchoffer is gratefully acknowledged for his assistance with measurements of plant water status. Research supported by the US Department of Energy Carbon Dioxide program, NSF grants DEB 90-11662 and 91-00164 (including an REU supplement that supported M. Cocke), and the Kansas Agricultural Experiment Station (95-193-J).

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