

Developmental change in CO₂ compensation concentrations in *Spartina alterniflora* results from sigmoidal photosynthetic CO₂ responses

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Abstract

We investigated seasonal patterns of photosynthetic responses to CO₂ concentrations in *Spartina alterniflora* Loisel, an aerenchymous halophyte grass, from a salt marsh of the Bay of Fundy (NB, Canada), and from plants grown from rhizome in controlled-environment chambers. From late May to August, CO₂ compensation concentrations (Γ) of field-grown leaves varied between 2.5–10.7 cm³(CO₂) m⁻³, with a mean of 5.4 cm³(CO₂) m⁻³. From September onwards field leaves showed CO₂ compensation concentrations from 6.6–21.1 cm³(CO₂) m⁻³, with a mean of 13.1 cm³ m⁻³ well into the C₃-C₄ intermediate range. The seasonal variability in Γ did not result from changing respiration, but rather from a sigmoidal response of net photosynthetic rate (P_N) to applied CO₂ concentration, found in all tested leaves but which became more pronounced late in the season. One explanation for the sigmoidal response of P_N to external CO₂ concentration could be internal delivery of CO₂ from roots and rhizomes to bundle sheath cells *via* the aerenchyma, but the sigmoidal responses in *S. alterniflora* persisted out to the tips of leaves, while the aerenchyma extend only to mid-leaf. The sigmoidicity persisted when CO₂ response curves were measured from low to high CO₂, or from high to low CO₂, and even when prolonged acclimation times were used at each CO₂ concentration.

Additional key words: aerenchyma; bundle sheath; *Carex*; leaf age; leaf anatomy; net photosynthetic rate; seasonal changes; species differences; *Zea*.

Introduction

Salt marshes cover over 600 000 ha of North America's Atlantic coast (Mann 1982) and are among the most productive ecosystems in the world. *Spartina alterniflora* Loisel (Gramineae) is a halophyte grass that is often the dominant primary producer in salt marshes in eastern North America. *S. alterniflora* has both short and tall ecotypes (Gallagher *et al.* 1988). Their substrate is hypoxic, and the plants contain extensive aerenchyma, large air channels in the leaves that allow exchange of gases between the roots and the atmosphere. The aerenchyma extend from the leaf base as far as 50 % along the length of the leaf, and can be found immediately adjacent to some bundle sheath cells. We therefore questioned the ability of *S. alterniflora* to act as an efficient C₄ photosynthesizer, as C₄ plants must create a steep CO₂:O₂ ratio in these bundle sheath cells to effectively minimize the

oxygenase activity of the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (RuBPCO) (*e.g.* Dai *et al.* 1995).

There are many diagnostic tests for C₄ photosynthesis, including anatomical, $\delta^{13}\text{C}$ (*e.g.* Bender 1971), and biochemical studies. Measuring CO₂ compensation concentration (Γ), the CO₂ concentration at which photosynthesis equals respiration and therefore net CO₂ uptake equals zero, is a convenient way to determine if a plant employs C₃ or C₄ photosynthesis. C₃ plants typically have a Γ near 50 cm³ m⁻³ CO₂, while C₄ plants have Γ of less than 5 cm³ m⁻³ (Canvin and Salon 1997). Plants with Γ of 7–30 cm³ m⁻³ (*e.g.* Hattersley *et al.* 1986, Moore *et al.* 1987, Wessinger *et al.* 1989), are termed as C₃-C₄ intermediates. According to Krenzer and Moss (1969) *Spartina* species have low Γ of less than 10 cm³ m⁻³.

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Abbreviations: P_N , net photosynthesis measured as CO₂ uptake; PEP, phosphoenol pyruvate; PPFD, photosynthetic photon flux density; RuBPCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; Γ , CO₂ compensation concentration.

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Giurgevich and Dunn (1979), on the other hand, found the tall ecotype of *S. alterniflora* had a Γ within the typical C_4 range, while Γ of the short ecotype was closer to the C_3 range. Bender (1971) found in the related *S. pectinata* and *S. cynosuroides* $\delta^{13}\text{C}$ values typical of C_4 plant values. In geothermal carbon dioxide spring area, Γ values of the C_4 plant *Echinochloa crus-galli* differed with plant height (Vodnik *et al.* 2002).

Materials and methods

Plants: Blocks of mud substrate (*ca.* 25×25×25 cm) containing *S. alterniflora* Loisel plants were collected from Peck's Cove, New Brunswick (45°50'N, 64°23'W), a salt marsh located within the Cumberland Basin of the Bay of Fundy, brought back to the lab in buckets and watered with sea water. Measurements referred to as field data were recorded within 48 h of collection. Some of these buckets were also transferred to controlled-environment growth chambers (model CMP 3244, Conviron, Winnipeg, MB, Canada) equipped with Philips F72T12/CW/VHO fluorescent and Royale-Country 40-W incandescent lamps, and periodically watered with seawater. Measurements referred to as growth chamber data were recorded from mature leaves that had broken the surface of the sediment since the time they were placed in the growth chambers. Plants in the growth chamber were subjected to a simulated day/night cycle (PPFD = 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 22:30 until 06:00; PPFD = 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 08:00 until 20:30; ramping between these times). Temperature within the chambers varied between 16 °C (dark) and 20 °C (maximum PPFD), and humidity was set at 50 %. Field leaves referred to as senescing were leaves collected and measured late in the growing season (*i.e.* early October–early November) which were yellowing; growth chamber leaves referred to as senescing were also yellowing.

Blocks of mud containing the comparison species *S. patens*, *S. pectinata*, or *Carex paleacea* were collected from the estuary of a tidal river *ca.* 10 km from Peck's Cove, brought back to the lab, and watered with salt water. Measurements were recorded within 24 h of collection. Maize, barley, wheat, and pea plants were grown from seed outdoors, and watered with half-strength Hoagland nutrient solution (1.5 mM $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$, 1 mM $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 1 mM KNO_3 , 1 mM KH_2PO_4 , 9 μM $\text{Fe}(\text{NH}_4)\text{SO}_4 \times 6 \text{H}_2\text{O}$, 20 μM H_3BO_4 , 3.8 μM $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$, 0.4 μM ZnCl_2 , 0.3 nM $(\text{NH}_4)_6\text{Mo}_4\text{O}_7 \times 4 \text{H}_2\text{O}$; Hoagland and Arnon 1950). All field plants were collected in 2000 or 2001.

CO₂ response curves: An open gas exchange system incorporating infra-red CO₂ and water vapor gas analyzers (CIRAS 1, PP Systems, Hitchin, Herts., UK) was connected to a PC and controlled remotely using the supplied program (CIRAS.exe) was used to subject leaves to various concentrations of CO₂ while recording net CO₂

The intent of this study was thus to examine Γ of *S. alterniflora* in a northern saltmarsh. We found this population of *S. alterniflora* has variable Γ , with an increase in Γ well into the C_3/C_4 intermediate range late in the season. This variation resulted from a pattern of sigmoidal response of CO₂ uptake to atmospheric CO₂ concentrations in *S. alterniflora*. In contrast, typical CO₂ response curves for C_3 and C_4 plants exhibit a hyperbolic shape.

uptake. CO₂ was varied between *ca.* 650 and 0 $\text{cm}^3 \text{m}^{-3}$. In most cases, leaves were first subjected to the highest CO₂ concentration and allowed to equilibrate to reach a stable CO₂ uptake rate. The CO₂ was then decreased in a stepwise fashion and the leaves allowed to equilibrate at each concentration prior to measurement. The equilibration time depended on the species and plant age: *Spartina* species often took 30 min or longer. In some runs, leaves were first subjected to the lowest CO₂ concentrations, allowed to equilibrate, and then subjected to increasing concentrations to test for hysteresis effects in the CO₂ response. The flow rate and humidity were 2.5 $\text{cm}^3 \text{s}^{-1}$ and 95 % of ambient, respectively. Measurement temperature was 23 ± 1 °C.

At all CO₂ concentrations the leaves were subjected to PPFD of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which was sufficient to drive P_N but low enough to avoid photoinhibition during the runs, which in some cases extended over several h to allow equilibration at each CO₂ concentration. CO₂ response curve measurements on 44 field samples of *S. alterniflora* leaf were made in 2000 or 2001. The CO₂ responses and seasonal patterns were similar in both years and the values from the two years were therefore pooled. Γ values of these field samples were extrapolated from the CO₂ response curves. The CO₂ response curves for *Spartina* plants from growth chambers and from the comparison species were performed at various times over 2000 and 2001, using the same experimental system and techniques as for *S. alterniflora*. Net respiration rate was measured as rate of CO₂ release at 0 $\text{cm}^3 \text{m}^{-3}$ applied CO₂ under 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, taken from the CO₂ response curve measurements.

Statistical analyses: A hyperbolic curve (1-site binding hyperbola) and two sigmoidal curves (logistic and Boltzmann) were fit to each CO₂ response curve (Motulsky 1999). Fitting of data to these equations was done with non-linear regression, an iterative process. An F-test was used to test which of the three equations gave the best fit. This tested whether the decrease in the sum of squares due to choosing a more complex model was worth the cost of additional parameters which results in a loss of degrees of freedom (Bärlocher 1999).

Microscopy: Leaf slices were fixed in 4 % paraformaldehyde in phosphate buffered saline, pH 7, overnight on

ice, then dehydrated in a graded ethanol series (45 min in 30 %, then 90 min in each of 50, 70, 85, and 95 % ethanol, followed by three 60 min treatments in 100 % ethanol). The tissue was transferred to 50 % *Histoclear* (National Diagnostics, Atlanta) for 60 min, then given three changes in 100 % *Histoclear* (60 min each). Tissues

Results

CO₂ compensation concentrations: Γ of field-grown *S. alterniflora* leaves were variable. From mid May to August (day 135–238) field leaves showed Γ from 1.5–10.7 cm³ m⁻³ CO₂, with a mean of 5.4 cm³ m⁻³ ($n = 29$) just above the 5 cm³ m⁻³ definition of Γ for C₄ plants (Canvin and Salon 1997). Over this period only a minority of measured leaves (12) achieved Γ below 5 cm³ m⁻³ thereby showing full C₄ performance. From September (day 255 onwards) field leaves showed Γ of 6.6–25.5 cm³ m⁻³ CO₂, with a mean of 13.1 cm³ m⁻³ CO₂ ($n = 15$), well in the C₃–C₄ intermediate range (Fig. 1A). A two-tailed *t*-test with Welch's correction for unequal variance and a Mann-Whitney non-parametric test (*Prism 3.0*, *Graph-Pad Software*) both showed that the late season rise in Γ (day 255 onwards) was statistically significant ($p < 0.0001$).

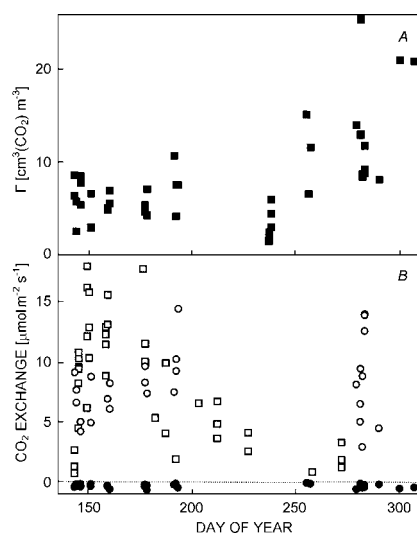


Fig. 1. Seasonal changes in CO₂ compensation concentration (Γ) and rates of respiration and photosynthesis for field-grown *S. alterniflora* from the 2000 and 2001 seasons. (A) Γ extrapolated from CO₂ response curves under 250 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ (closed squares). This irradiance was high enough to drive near-maximal P_N but low enough to avoid photoinhibition during the long measurement periods required to reach steady state gas exchange. (B) Respiration rate at 0 cm³ m⁻³ applied CO₂ (closed circles, values from the same CO₂ response curves used to estimate Γ) and net photosynthetic rate, P_N at applied saturating CO₂ under 250 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ (open circles, values from the same CO₂ response curves used to estimate Γ ; open squares, values from separate measurements of irradiance response curves under saturating CO₂).

were then infiltrated with 50 : 50 *Histoclear* : *Paraplast* (Sigma Chemical, St. Louis, USA) overnight, followed by infiltration with 100 % *Paraplast* changed each day for 6 d. Embedded tissue was sectioned on a rotary microtome at 7 μm , and stained with Toluidine Blue O for 10 s.

In summary, most field grown *S. alterniflora* leaves did not achieve a C₄ level Γ . To confirm the accuracy of our methods with comparison C₃ and C₄ grasses, using the same instrumentation and over the same period of time, we determined Γ of 1.4 cm³ m⁻³ (SD 0.4, $n = 5$) for mature *Zea mays* (maize, C₄); 3.5 cm³ m⁻³ (SD 0.4, $n = 4$) for mature *S. pectinata* (C₄); 44.1 cm³ m⁻³ (SD 3.4, $n = 10$) for mature *Hordeum vulgare* (barley, C₃); and 45 cm³ m⁻³ (SD 2.3, $n = 8$) for mature *Triticum aestivum* (wheat, C₃).

Respiration in *S. alterniflora* at 0 cm³ m⁻³ applied CO₂ was consistently small, as expected for a C₄ plant

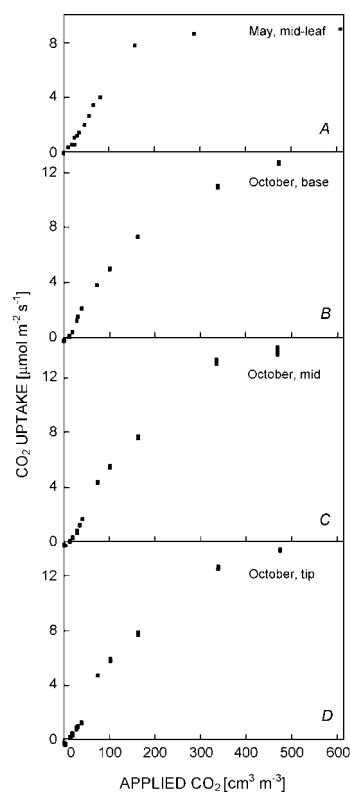


Fig. 2. Representative CO₂ response curves for field-grown *S. alterniflora*: (A) middle of leaf, 30 May 2001; (B) base, (C) middle, and (D) tip of two leaves, 9 October, 2000. A positive CO₂ uptake value indicates net photosynthesis; a negative value indicates net respiration. Γ is the value at which the curve crosses the x axis when respiration is equal to photosynthesis, and P_N equals 0. $n = 1$ –10 repeated measures of P_N at each applied CO₂ concentration on one (May) or two (Oct.) representative leaves, values of repeated measures are often within the symbols.

(Fig. 1B), nor did the variability and seasonal increase in Γ in *S. alterniflora* correlate with changes in P_N at saturating applied CO_2 . Instead, the variability in Γ in *S. alterniflora* resulted from leaves showing, to a variable extent, a sigmoidal pattern of response of CO_2 uptake to applied CO_2 .

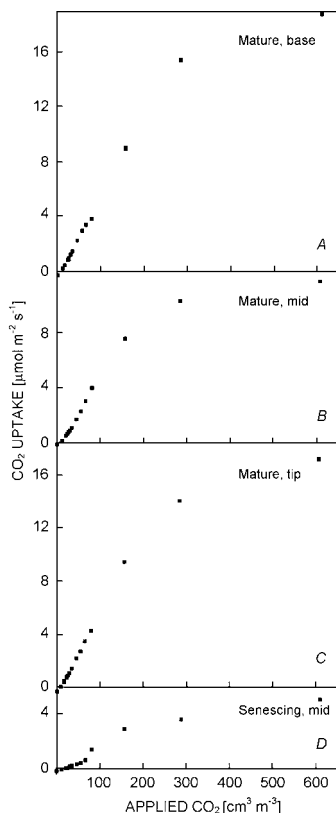


Fig. 3. Representative CO_2 response curves for *S. alterniflora* grown in a chamber: (A) base of mature leaf, (B) middle of mature leaf, (C) tip of mature leaf, (D) middle of senescing leaf. $n = 1-5$ repeated measures of P_N at each applied CO_2 concentration on a single representative leaf, values of repeated measures are generally within the symbols.

Patterns of CO_2 response curves: *S. alterniflora* leaves, mature or senescing, whether taken from the field immediately prior to measurement or grown in growth chambers, exhibited sigmoidal patterns of response of P_N to applied CO_2 concentration (examples in Figs. 2 and 3). The *S. alterniflora* P_N curves were fit by Boltzmann sigmoidal curves better than either logistic or hyperbolic curves (Table 1). This sigmoidicity was seen regardless of the position along the leaf where the readings were

Discussion

Seasonal variation in Γ : From May to August leaves from our northern population of *S. alterniflora* showed a range of Γ with an average near the $5 \text{ cm}^3 \text{ m}^{-3}$ borderline (Canvin and Salon 1997) separating C_4 and $\text{C}_3\text{-C}_4$ intermediate plants. From September onwards the Γ retained

taken, and became more prominent as the season progressed (Figs. 2 and 3), causing the observed pattern of higher Γ later in the season. We monitored exchange of water vapor between the leaf and the gas stream in the cuvette and found no apparent correlation between the shapes of the response curves of P_N at low ambient CO_2 levels and the water vapor exchange of the leaves (data not presented).

To validate the performance of our system we tested P_N response curves from a range of comparison species. The P_N curves for mature leaves of *Zea mays* (C_4 grass) *C. paleacea* (C_3 aerenchymous, halophytic sedge), and *S. patens* (C_4 non-aerenchymous relative of *S. alterniflora*) appeared hyperbolic (Fig. 4, Table 1), as did curves from *Hordeum vulgare* (C_3 grass) and from *Pisum sativum* (C_3 dicot) (values not shown). Even senescing leaves of *Z. mays* retained their characteristic hyperbolic shape (Table 1).

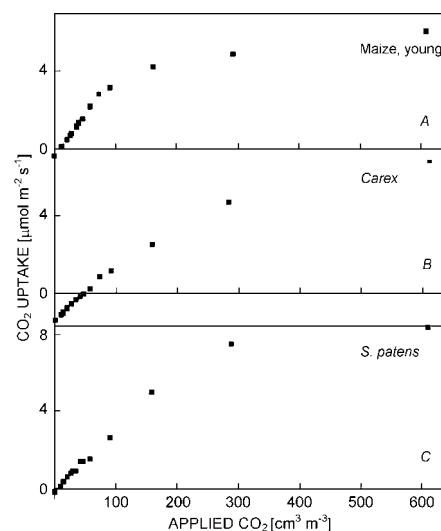


Fig. 4. Representative CO_2 response curves of leaves of: (A) mature *Zea mays* (C_4 non-aerenchymous grass), (B) *Carex paleacea* (C_3 aerenchymous, halophytic sedge), and (C) *S. patens* (C_4 non-aerenchymous, halophytic relative of *S. alterniflora*). $n = 1-5$ repeated measures of P_N at each applied CO_2 concentration on a single representative leaf, values of repeated measures are generally within the symbols.

Leaf cross sections: Aerenchyma were largest at the base of both *C. paleacea* (Fig. 5A) and *S. alterniflora* (Fig. 5D,G), declining with distance towards the tip. In general, aerenchyma became smaller with time at the middle positions of *S. alterniflora* leaves (Fig. 5E,H).

high inter-leaf variation but increased well into the $\text{C}_3\text{-C}_4$ intermediate range (Fig. 1). The inter-leaf and seasonal variation in Γ meant that some leaves in our population in mid-season achieved C_4 performance while most did not. This suggested that designation of *S. alterniflora* eco-

types as C₄ or C₃ plants (Giurgevich and Dunn 1979) should consider the developmental history of the plant material (Dai *et al.* 1995).

Respiration at 0 cm³ m⁻³ applied CO₂ remained low as expected for a C₄ plant, and did not show a seasonal pattern. CO₂ uptake capacity under saturating CO₂ was

highly variable among leaves but it did not show a seasonal pattern, nor did it correlate with the Γ . The inter-leaf and seasonal variation in Γ instead resulted largely from changes in the degree of sigmoidicity of the CO₂ response curves.

P_N and key enzymes involved in photosynthesis,

Table 1. Comparisons of curve fits to responses of CO₂ uptake to applied CO₂ for different classes of leaves. The three curve fit equations are ranked from 1 (best fit) to 3 (worst fit) for each class tested. Different ranks indicate $p \leq 0.01$ (pair-wise F-tests, adjusted with Bonferroni (Motulsky and Ransnas 1987). Representative examples of CO₂ responses of each leaf class are presented in Figs. 2 to 4. Replicates (n) for the curve fits are shown in parentheses.

Leaf tissue	source	age	Leaf position (n)	Sigmoidal: Boltzman	Sigmoidal: logistic	Hyperbola
<i>S. alterniflora</i>	field	mature	base (4)	1	2	3
			middle (4)	1	2	3
			tip (2)	1	2.5	2.5
		senescing	base (2)	1	2	3
			middle (6)	1	3	2
			tip (4)	1	2	3
<i>Carex paleacea</i>	chamber	mature	middle (3)	2	3	1
<i>Zea mays</i>	field	mature	middle (5)	2	3	1
		senescing	middle (3)	2	3	1
<i>S. patens</i>	field	mature	middle (2)	1.5	1.5	3

including RuBPCO, lose efficiency at lower temperatures (Wohlfahrt *et al.* 1998, Pittermann and Sage 2000). To test if changing field conditions of PPFD, humidity, or temperature caused the seasonal rise in Γ , we measured CO₂ response curves from *S. alterniflora* grown in the controlled-environment chambers (Fig. 3). The sigmoidicity and hence the Γ rose with plant age even under these constant growth conditions.

The observed variation in *S. alterniflora* Γ is not an indication of shifts towards C₃ photosynthesis in some leaves. The Γ values were taken from the x-intercept of the P_N curve with the applied CO₂ axis. This x-intercept varied without concomitant variation in the y-axis intercept of respiration at 0 cm³ m⁻³ applied CO₂, which remained near zero (Fig. 1B). This is seen in comparing *Carex paleacea* (Fig. 4B) with late season *S. alterniflora* (Fig. 2B,C,D). *Carex* showed a high Γ because of need for increased CO₂ to overcome significant net respiration observed at 0 cm³ m⁻³ CO₂, whereas for *S. alterniflora* little net respiration was observed even at 0 cm³ m⁻³ CO₂. Furthermore, unlike the comparison species, we observed significant heterogeneity in the Γ measured for *S. alterniflora* even among leaves sampled and measured on the same dates (Fig. 1), especially late in the season. This scatter resulted from inter-leaf variation in the degree of sigmoidicity of the curves. In some cases it was indeed difficult to evaluate the true Γ since the measured CO₂ uptake showed a plateau at 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ over a range of applied external CO₂ concentrations.

Sigmoidicity of *S. alterniflora* CO₂ response curves. The sigmoidal CO₂ response curves exhibited by *S.*

alterniflora leaves are unusual. CO₂ response curves in other species are generally hyperbolic, as we observed in comparison species measured using the same instrumentation and over the same period as our measurements of *S. alterniflora* leaves (Fig. 4, Table 1). We therefore tested to see if the sigmoidal response in *S. alterniflora* resulted from experimental artifacts. Our early runs began at the highest CO₂ concentration and decreased stepwise to the lowest CO₂ concentration. Comparison runs done from low to high CO₂ concentration showed the same response pattern: *S. alterniflora* leaves which showed a sigmoidal response from high to low CO₂ also showed a sigmoidal response from low to high CO₂. The comparison species that showed a hyperbolic response when measured from high CO₂ to low CO₂ continued to produce a hyperbolic response when measured from low CO₂ to high CO₂. *S. alterniflora* leaves exhibited a long equilibration time to reach stable P_N , as found previously for the related *S. anglica* (Steve Long, personal communication). We varied the length of equilibration times from 10 to 40 min in our experiments at each CO₂ concentration, with no effect on the curve shapes. For the majority of experiments the leaf was allowed to equilibrate at each concentration from high to low CO₂, often for longer than 30 min, before measurements of net CO₂ uptake were recorded (E. Ögren and S. Long, personal communication). Leaves of *S. alterniflora* have grooves along their adaxial surface. We tested if these grooves could have been responsible for the sigmoidicity *via* diffusion between the cuvette and the atmosphere by performing runs of maize leaves with induced leaks in the cuvette, using metal tubes with one end

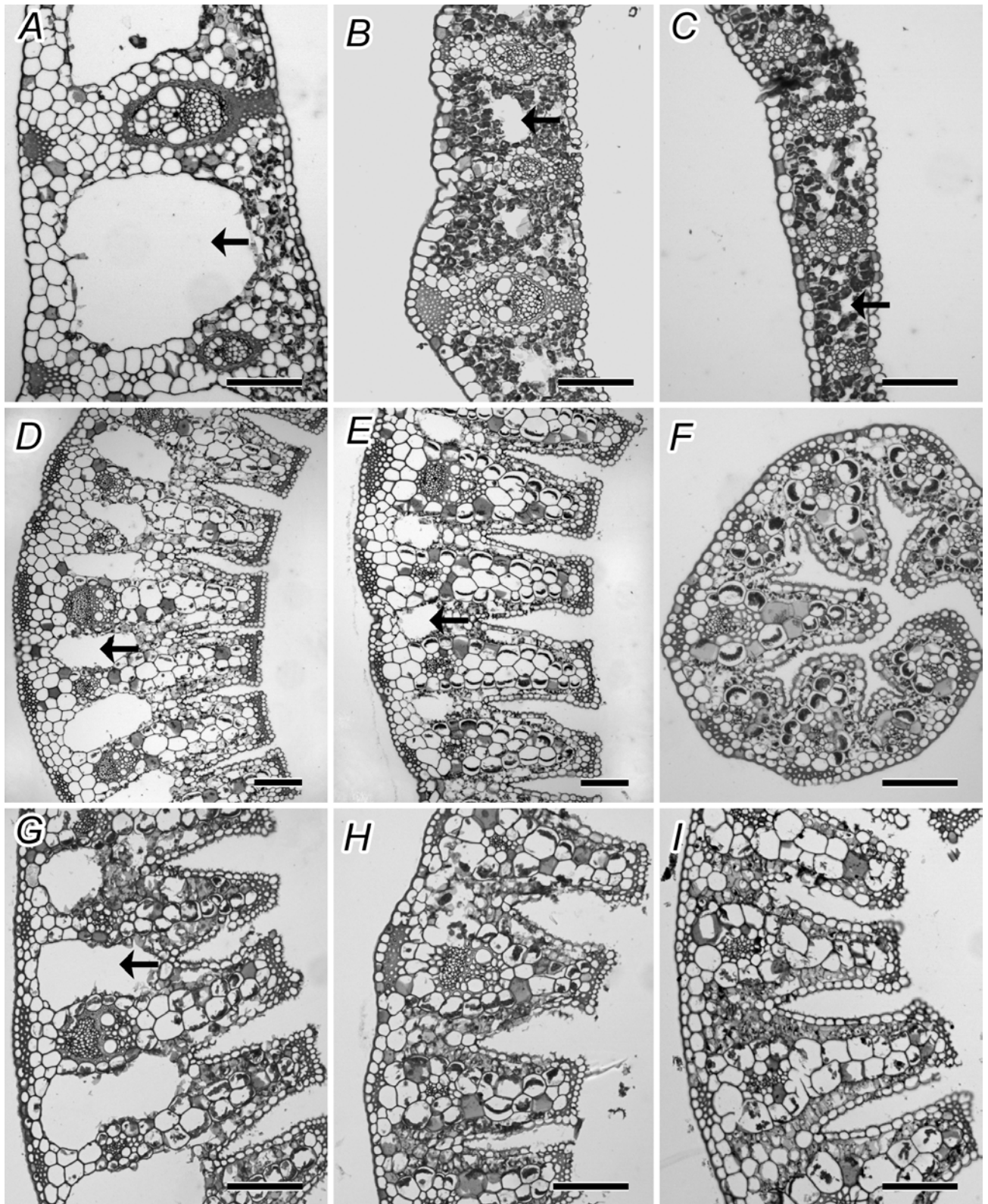


Fig. 5. Cross sections of: mature *C. paleacea* leaf at the base (A), middle (B), and tip (C) positions, young *S. alterniflora* leaf at the base (D), middle (E), and tip (F) positions, and senescing *S. alterniflora* leaf at the base (G), middle (H), and tip (I) positions. Tissues were stained with Toluidine Blue O. Arrows indicate positions of aerenchyma. Scale bars = 100 µm. Young leaf tip tissue (F) has not yet unfolded.

in the cuvette and the other exposed to ambient air. These maize curves measured with induced leaks did not become sigmoidal (values not shown). We also performed experiments where the grooves in the *S. alterniflora* leaves were filled in with high vacuum grease (Dow Corning) and again this had no effect on the shape of the response curve (values not shown).

S. alterniflora leaves have another characteristic potentially relevant to their pattern of CO₂ response, in the presence of large air spaces (aerenchyma) along the leaf, which could potentially mediate internal delivery of CO₂ rich gas to leaf cells from the sub-surface tissues, resulting in the sigmoidal response of P_N to applied external CO₂. The air spaces are largest at the base of the leaf and decline in size until they end at or before the midway point of the leaf (Fig. 5G,H). To examine whether these aerenchymous spaces could be directly causing the sigmoidal CO₂ response, we carried out several experiments. First, we measured CO₂ response curves at the base, middle, and tip positions of young, mature, and senescing *S. alterniflora* leaves. If aerenchyma were directly responsible for the sigmoidal curve, then the tip of the leaf should exhibit a hyperbolic curve and the base a sigmoidal curve. This was not observed—leaf position in itself did not affect the shape of the curves (Figs. 2B,C,D and 3A,B,C). The second experiment involved testing *Carex paleacea*, a halophytic sedge with similar morphology and large aerenchyma (Fig. 5A,B,C). However, *C. paleacea* is a C₃ plant, as is evident from its high Γ (Fig. 4B). *C. paleacea* did not exhibit a sigmoidal CO₂ response (Fig. 4B), regardless of leaf position (data not shown for individual positions). CO₂ response curves for maize (Fig. 4A), a C₄ plant, were hyperbolic, as expected. CO₂ response curves were also created for *S. patens* (Fig. 4C), a relative of *S. alterniflora* which is also a C₄ halophyte but which has little or no aerenchyma. These curves appeared hyperbolic to the eye, although they were fit better statistically by a sigmoidal curve (Table 1). From these data, we concluded that the sigmoidal nature was not due simply to the presence or absence of aerenchyma, nor simply to the type of photosynthesis the leaf employs. Nevertheless, an interaction between aerenchyma and C₄ or C₃/C₄ intermediate metabolism might cause the sigmoidal response pattern, since *S. alterniflora* was the only one of our comparison plants to combine these characters.

One explanation for the sigmoidal CO₂ response curves could be stomata closure over a range of low CO₂ concentrations in spite of the 95 % humidity of the applied gas stream, which could flatten the CO₂ response curve by restricting gas exchange between the leaf and the gas stream in the cuvette. We did not, however, find

any correlation between the shapes of curves of CO₂ uptake over low applied CO₂ concentrations and the water vapor exchange characteristics of the *S. alterniflora* leaves (data not presented). Therefore although stomata effects on the CO₂ responses are a possibility our data do not support this interpretation. We did not attempt to estimate internal CO₂ concentrations in the leaves because the leaves contain aerenchyma and salt glands and can carry hygroscopic salt deposits on their surfaces that could interfere with the estimations.

The extent of the sigmoidicity of the CO₂ response curves of *S. alterniflora* leaves varied with age. CO₂ response curves of younger leaves (Figs. 2A and 3A,B,C) generally showed less sigmoidicity than older or senescing leaves (Figs. 2B,C,D and 3D). The shape of the CO₂ response curves for maize, on the other hand, did not vary with age (values not shown). Long *et al.* (1975) showed that Γ of *S. townsendii*, a related species, can vary slightly with measurement temperature. Our measurement temperatures were consistently 23±1 °C. In addition to our measures done on field grown *Spartina*, we did similar experiments with plants grown in controlled-environment chambers where the humidity, irradiance, and temperature were consistent throughout the leaf development. Nonetheless, a similar CO₂ response pattern was observed, with the CO₂ response of younger *S. alterniflora* leaves (Fig. 3A,B,C) being less sigmoidal than the CO₂ response of aging leaves grown in chambers (Fig. 3D).

Monocot leaves grow from the base so that the tip of the leaf is older than the base. Within a given leaf we found little difference between the CO₂ responses of the base and tip positions of *S. alterniflora* leaves (Figs. 2B,C,D and 3A,B,C), so tissue age *per se* is not directly related to sigmoidicity or Γ . Furthermore, the sigmoidal CO₂ response is not explained by reduced capacity for CO₂ uptake in aging tissue (Fig. 1B).

To seek correlations between morphology, the increasing Γ , and the increase in the sigmoidal character of the CO₂ response with age, we took cross-sections of young and senescing *S. alterniflora* leaves at various positions (Fig. 5). Fig. 5G,H,I shows *S. alterniflora* samples collected on September 19, with aerenchyma distribution similar to that observed in June (Fig. 5D,E,F). In general, however, by mid-season aerenchyma are absent or very small at the middle of the leaf as shown in Fig. 5H, further evidence that aerenchyma do not directly cause the increasing Γ and sigmoidal character of older tissue. We have begun an examination of seasonal changes in the subcellular localisation of enzymes of C₄ metabolism in *S. alterniflora* leaves, to see if they are associated with seasonal changes in Γ and the sigmoidal CO₂ response curves.

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