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Drought constraints on C₄ photosynthesis: stomatal and metabolic limitations in C₃ and C₄ subspecies of *Alloteropsis semialata*

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Abstract

The C₄ photosynthetic pathway uses water more efficiently than the C₃ type, yet biogeographical analyses show a decline in C₄ species relative to C₃ species with decreasing rainfall. To investigate this paradox, the hypothesis that the C₄ advantage over C₃ photosynthesis is diminished by drought was tested, and the underlying stomatal and metabolic mechanisms of this response determined. The effects of drought and high evaporative demand on leaf gas exchange and photosynthetic electron sinks in C₃ and C₄ subspecies of the grass *Alloteropsis semialata* were examined. Plant responses to climatic variation and soil drought were investigated using a common garden experiment with well-watered and natural rainfall treatments, and underlying mechanisms analysed using controlled drying pot experiments. Photosynthetic rates were significantly higher in the C₄ than the C₃ subspecies in the garden experiment under well-watered conditions, but this advantage was completely lost during a rainless period when unwatered plants experienced severe drought. Controlled drying experiments showed that this loss was caused by a greater increase in metabolic, rather than stomatal, limitations in C₄ than in the C₃ leaves. Decreases in CO₂ assimilation resulted in lower electron transport rates and decreased photochemical efficiency under drought conditions, rather than increased electron transport to alternative sinks. These findings suggest that the high metabolic sensitivity of photosynthesis to severe drought seen previously in several C₄ grass species may be an inherent characteristic of the C₄ pathway. The mechanism may explain the paradox of why C₄ species decline in arid environments despite high water-use efficiency.

Key words: *Alloteropsis semialata*, C₄ photosynthesis, C₄ plant biogeography, electron sinks, evaporative demand, leaf photosynthesis, non-stomatal limitations, photoinhibition, soil water.

Introduction

C₄ photosynthesis typically increases the water-use efficiency (WUE) of leaves compared with the C₃ type (Downes, 1969) because its carbon concentrating mechanism (CCM) counteracts the limitation of photosynthesis by CO₂ diffusion. This allows maximal rates of net leaf photosynthesis (A) at a lower stomatal conductance (g_{ST}) than in C₃ species, lowering transpiration and conserving water, especially in hot conditions when evaporative demand is high (Pearcy and Ehleringer, 1984). The mechanism should therefore confer significant selective and competitive benefits for C₄ plants over C₃ species in arid climates; however, at the regional scale, the relative abundance of grasses utilizing the C₄ pathway declines with decreasing annual rainfall (Paruelo and Lauenroth, 1996). This paradox results from climatic correlations for the individual biochemical and anatomical subtypes of C₄ photosynthesis, first recognized more than 25 years ago (Ellis *et al.*, 1980).

Regional relationships with rainfall are positive for the percentage of C₄ species with the NADP-malic enzyme (NADP-ME) subtype, but negative for the NAD-malic enzyme subtype, and may be positive, negative, or absent for the PEP-carboxykinase enzyme (PCK) subtype (Ellis

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et al., 1980; Hattersley, 1992; Taub, 2000). Interpretation of these relationships is complicated by the strong association of photosynthetic subtypes with subfamilies of grasses, with partial regression analysis showing that relationships are positive for the Panicoideae and negative for the Chloridoideae independently of C_4 subtype (Taub, 2000). Together, these observations raise important questions about the costs and advantages of C_4 photosynthesis in drought conditions, and the interacting role of the phylogenetic background. This paper addresses the question of whether C_4 photosynthesis is inherently more limited by drought conditions than the C_3 type, and investigates the mechanisms responsible.

Decreases in stomatal conductance (g_{ST}) control the initial responses of C_3 and C_4 photosynthesis to drought by limiting the diffusion of CO_2 from the atmosphere to mesophyll cells (Cornic, 1994). Stomatal limitation could potentially decrease C_4 photosynthesis to a greater degree than the C_3 type, because A typically operates at (or close to) the inflexion point of the photosynthetic CO_2 response (Ghannoum *et al.*, 2000; Wand *et al.*, 2001), and the initial slope of the CO_2 response is steeper in C_4 than C_3 leaves (Björkman, 1971). Thus, in the absence of metabolic limitations, large decreases in g_{ST} under drought conditions will elicit proportionally greater decreases in photosynthesis than the same change in a C_3 species.

However, during prolonged or extreme drought, metabolic limitations to photosynthesis become more important than reduced g_{ST} (Lawlor, 2002; Ghannoum *et al.*, 2003; Flexas *et al.*, 2006). Decreased photosynthetic electron consumption is one important direct consequence of the drought-induced reduction in CO_2 supply, causing a down-regulation of photosynthetic efficiency and increased requirement for additional electron sinks such as photorespiration and the Mehler reaction (Valentini *et al.*, 1995; Cornic and Fresneau, 2002). Since the C_4 pathway minimizes photorespiration (Osmond and Harris, 1971), drought is expected to reduce photochemical efficiency or increase alternative electron sinks to a greater extent than in C_3 leaves.

The South African grass *Alloteropsis semialata* was used as a model species for investigating the direct consequences of C_4 pathway evolution on photosynthetic drought responses. *A. semialata* is unique in having C_3 and C_4 subspecies (Gibbs-Russell, 1983) and, used as a model species, thus avoids the confounding effects of phylogenetic distance usually encountered in C_3/C_4 comparisons. Preliminary analysis of the chloroplast DNA sequence *ndhF* suggests that the species is monophyletic, with a recent divergence between subspecies (DG Ibrahim, CP Osborne, TA Burke, unpublished data).

Two factors lead to the *a priori* expectation that C_4 photosynthesis in *A. semialata* gives its greatest advantage in high rainfall climates: (i) *A. semialata* is a member of the subfamily Panicoideae, in which C_4 species abundance shows a strong positive correlation with rainfall (Taub, 2000); and (ii) South African populations of the C_4 subspecies express high levels of both NADP-ME and PCK enzymes (Frean *et al.*, 1983; Ueno and Sentuko, 2006). Grass subtypes characterized by these enzymes show positive correlations with rainfall in southern Africa and, where PCK species do occur in arid regions, they are confined to moist habitats among boulder outcrops and drainage lines (Ellis *et al.*, 1980).

The experiments carried out tested the hypothesis that, although photosynthesis in warm, well-watered conditions is higher in the C_4 than the C_3 subspecies of *A. semialata*, this advantage is diminished during severe drought episodes. Photosynthetic responses to drought were first investigated by applying soil water manipulations in a common garden experiment. The underlying stomatal and metabolic limitations were subsequently analysed by using potted plants subjected to controlled episodes of soil drying or high atmospheric vapour pressure deficit (VPD).

Materials and methods

Plant materials

Established plants of the C_4 subspecies *A. semialata* (R.Br.) Hitchc. subsp. *semialata* and C_3 subspecies *A. semialata* (R.Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell were collected from grasslands in February 2004, in the surrounds of Middelburg (near Pretoria, Mpumalanga, South Africa) and Grahamstown (near Port Elizabeth, Eastern Cape, South Africa), respectively. These were used to establish a common garden experiment and a collection of potted plants at the Botany Department, Rhodes University, Grahamstown (33° S 27° E).

The common garden experiment applied watering and control treatments to plots in 10 replicate blocks. Each plot was separated from its neighbours by 2 m, and consisted of a 1×1 m area of undisturbed soil. Plots were planted with three evenly spaced clumps of each subspecies, each consisting of 15 tillers, and were kept free of other plants by weekly weeding. To avoid edge effects, all experimental measurements were made on the two central clumps. The control plots received only natural rainfall, which follows a bimodal pattern in the Grahamstown area, with maxima in the spring and autumn and minima in summer and winter (annual mean 681 mm; Stone *et al.*, 1998). The watering treatment was initiated after a 4 month establishment period, and applied at a rate of $\approx 20 \, l \, m^{-2}$ (≈ 20 mm) when soil water content (SWC) fell below 20% at 0.2 m depth. Excavated plants showed that there was a high density of roots at this depth, consistent with literature reports suggesting that >57% of roots in savanna grasslands are located in the surface 0.3 m of soil (Jackson *et al.*, 1996). The 20% SWC threshold was based on preliminary measurements of natural drying in the control plots, and represented an approximate 60% depletion of the available water (field capacity–wilting point). SWC was measured using a dielectric technique (PR1 Profile Probe; Delta-T Devices, Cambridge, UK) via tubes sunk permanently into the centre of each plot. Surface atmospheric conditions (radiation, temperature, humidity, precipitation) were monitored at 3.5 m using a weather station (Vantage Pro Weather Station; Davis Instruments, Hayward, CA, USA).

Further plants were established in topsoil in large pots (6.0l), and maintained in a clear polythene tunnel. During the experimental

period average day/night tunnel temperatures were 28/21.5 °C, and humidity ranged between 32% and 48%. Pots were well watered until the beginning of the experiment, when water was withheld from half of them. SWC was measured daily using a dielectric probe (ThetaProbe, type ML2x; Delta-T Devices), to maintain the well-watered pots close to field capacity and to select water-limited plants of similar SWC for experiments. Watering treatments resulted in well-watered plants with SWC=20.6±5.3% and drought plants with SWC=7.8±2.1%.

Photosynthesis measurements

Leaf CO_2 and H_2O exchange were measured for the youngest mature leaf on north-facing (sunlit) tillers, using a portable photosynthesis system with a blue–red LED light source (LI-6400; Li-Cor, Inc., Lincoln, NE, USA). Measurements were made on plants in the common garden experiment at 12.20–14.50 h on cloudless days, with conditions in the leaf cuvette set to match the ambient atmospheric conditions in full sunlight. The measurements were repeated on five occasions over a 43 d rainless period (October to November 2004), when the soil dried from field capacity (*c.* 33%) to 12% in the natural rainfall plots. Following the first set of photosynthesis measurements, leaves from the watered treatment were oven-dried to constant mass at 70 °C, and ground to a homogeneous powder using a ball mill (800M mixer/mill; Glen Creston, Middlesex, UK). The stable carbon isotope ratio ($\delta^{13}C$) was determined in dried leaf samples using a mass spectrometer (PDZ Europa 20-20; Cheshire, UK).

Light and CO_2 responses of photosynthesis were constructed using simultaneous gas exchange and PAM chlorophyll *a* fluorescence measurements with the LI-6400 photosynthesis system. Pot plants were transferred each morning to a controlled environment cabinet (Conviron CMP3244; Controlled Environments Ltd, Winnipeg, Canada) and maintained at an air temperature of 25 °C, VPD of 0.9 kPa, and photosynthetic photon flux density (PPFD) of 400 µmol m^{-2} s^{-1}. This treatment was necessary as the VPD experienced by the whole plant strongly influenced the stomatal response of the leaf being measured; i.e. stomata responded to leaf–leaf signals. Measurements were made on single leaves at an average leaf temperature of 26 °C and cuvette VPD <1.6 kPa. Light-response curves were constructed at 370 µmol mol^{-1} CO_2, with either 1% or 21% O_2, and PPFD was sequentially decreased from a value of 2000 µmol m^{-2} s^{-1}. Responses of *A* to intercellular CO_2 concentration (C_i) were constructed according to Long and Bernacchi (2003) with 21% O_2 and a PPFD of 2000 µmol m^{-2} s^{-1}. Following the completion of photosynthesis measurements, dark respiration rates (R_D) were recorded after 1 h of adaption to the dark.

Simultaneous gas exchange and chlorophyll fluorescence measurements were made to ascertain the effects of dry soil and high VPD on photosynthetic CO_2 assimilation, electron production, and electron sinks. Each morning, well-watered or dry-soil plants were moved to a warm (>25 °C), humid (VPD <1.0 kPa) laboratory and placed under high intensity sodium lamps at a PPFD of 1400 µmol m^{-2} s^{-1}. Measurements were made on fully expanded leaves at a PPFD of 2000 µmol m^{-2} s^{-1}, with chamber conditions as above, and either 21% or 1% O_2 with 370 µmol mol^{-1} CO_2. Data were recorded when g_{ST} and fluorescence output reached steady-state values. Dark respiration rates were recorded as above. The measurements were repeated on well-watered plants after exposure for several hours of whole plants to a high VPD >2 kPa. High VPD-treated plants displayed reduced photosynthetic rates, despite SWC that remained above 21%.

For both plot and pot experiments, leaf water potentials (Ψ_{leaf}) of the experimental leaves were measured subsequent to photosynthetic measurements using a pressure chamber.

Photosynthesis calculations

Photosynthetic parameters were calculated according to von Caemmerer and Farquhar (1981), and stomatal limitation of photosynthesis (L_S) following Farquhar and Sharkey (1982). Relative stomatal limitation (RL_S) and relative metabolic limitation (RL_M) were also calculated to explain how drought reduced *A* relative to the well-watered controls at an ambient CO_2 concentration of 380 µmol mol^{-1} (equations 3 and 4, Table 1).

The quantum yield of CO_2 assimilation (ΦCO_2) was calculated as the ratio of gross CO_2 fixation (A_G) to absorbed PPFD (Genty *et al.*, 1989; equation 1, Table 1), where leaf PPFD absorption was 0.85 for both subspecies, measured using a Taylor integrating sphere. The quantum yield of PSII ($\Phi PSII$) and the electron transport rate (J_F) were calculated according to Genty *et al.* (1989) (equations 2 and 13, Table 1), and the relative optical cross-section of PSII chlorophyll (a_{II}) following Laisk and Edwards (1998). Briefly, ΦCO_2 was plotted against $\Phi PSII$ for data from light responses constructed at 1% O_2 on well-watered plants, and a_{II} calculated as four times the linear slope of this relationship. As a minimum of four electrons are required per molecule of CO_2 fixed under non-photorespiratory conditions, $J_F/4$ was assumed equal to A_G (Valentini *et al.*, 1995).

Table 1. *Definitions of chlorophyll fluorescence and gas-exchange parameters*

Equation 1 has units of mol CO_2 mol^{-1} absorbed photons, equations 2–5 are dimensionless, equations 6–16 have units of CO_2 flux (µmol CO_2 m^{-2} s^{-1}), equation 17 defines the empirical curve fit to photosynthetic CO_2 response data, equation 18 calculates chloroplast CO_2 concentration of C3 leaves in µmol mol^{-1}.

Parameter	Definition	Equation
ΦCO_2	$=A_G/Q$	1
$\Phi PSII$	$=(F'_m - F_s)/F'_m$	2
R_{SL}	$(Y-Z)/X$	3
R_{ML}	$(X-Z)/X - R_{SL}$	4
L_S	$(W-X)/W$ (well-watered) or $(Y-Z)/Y$ (drought)	5
W	*A* at C_i=380 µmol mol^{-1} on well-watered plants	6
X	*A* at C_a=380 µmol mol^{-1} on well-watered plants	7
Y	*A* at C_i=380 µmol mol^{-1} on drought plants	8
Z	*A* at C_a=380 µmol mol^{-1} on drought plants	9
A	=Measured net CO_2 fixation	10
A_G (21%)	$=A(21\%)+R_D$	11
A_G (1%)	$=A(1\%)+R_D$	12
$J_F/4$	$=(\Phi PSII \times a_{II} \times Q \times 0.85)/4$	13
V_O	$=2/3[A_G(1\%) - A_G(21\%)]$	14
V_C	$=A_G(21\%)+1/2(V_O)$	15
$J_{ALT}/4$	$=J_F/4(21\%) - (V_C + V_O)$	16
A	$=a \times [1 - \exp(b - c \times C_i)]$	17
C_C	$=\Gamma_* [J_F + 8 \times A_G(21\%)]/[4 \times A_G(21\%)]$	18

Abbreviations: ΦCO_2, quantum yield of CO_2 assimilation; A_G, gross photosynthetic CO_2 fixation; Q, absorbed photosynthetic photon flux density; $\Phi PSII$, PSII quantum yield; F'_m, maximum fluorescence yield during illumination; F_s, steady-state fluorescence during illumination; R_{SL}, relative stomatal limitation; R_{ML}, relative metabolic limitation; L_S, stomatal limitation; *A*, net photosynthetic CO_2 fixation; R_D, dark respiration; $J_F/4$, linear photosynthetic electron transport rate; a_{II}, the relative optical cross-section of PSII chlorophyll; V_O, photorespiration; V_C, photosynthetic carbon reduction; $J_{ALT}/4$, alternative electron sinks; C_i, intercellular CO_2 concentration; C_c, chloroplast CO_2 concentration; Γ_*, photocompensation point in the absence of non-photorespiratory CO_2 evolution. The values of *a*, *b*, and *c* are determined by curve fitting. 21% and 1% indicate that gas exchange and fluorescence measures were made in an atmosphere of 21% or 1% O_2, respectively, and 370 µmol mol^{-1} CO_2.

The apportioning of electron transport to A_G, photosynthetic carbon fixation (V_C), photorespiration (V_O), and alternative electron sinks ($J_{ALT}/4$) such as the Mehler reaction were calculated using CO_2 exchange and chlorophyll fluorescence data (Table 1, illustrated in Fig. 1), and all expressed as CO_2 equivalents (=4 electrons). V_O and V_C were calculated according to von Caemmerer (2000) and based on the assumption that under non-photorespiratory conditions (1% O_2) $J_F/4 \approx A_G$. Thus calculated, $J_{ALT}/4$ accounts for light intensity effects but cannot be attributed solely to Mehler reaction activity (Siebke *et al.*, 2003). Mesophyll CO_2 concentration (C_C) was calculated for C_3 plants according to Evans and von Caemmerer (1996) (equation 18, Table 1), with values of CO_2 compensation point in the absence of non-photorespiratory CO_2 evolution (Γ_*) from von Caemmerer (2000).

Statistics

Gas exchange and fluorescence response curves, and measurements on watered (control), dry-soil, and high-VPD plants were each replicated three to five times. Individual A–C_i curves were each fitted with a monomolecular equation (Table 1) and these were used to interpolate data to a common series of C_i values for the calculation of means and standard errors, and to calculate the CO_2 compensation point (Γ) and carboxylation efficiency (CE). L_S was calculated from individually fitted A–C_i replicates. RL_S and RL_M were derived by comparing each A–C_i replicate for the drought-treated plants with the average curve for controls.

In the common garden and pot experiments, A, g_{ST}, C_i, Ψ_{leaf}, Γ, CE, $\delta^{13}C$ isotope ratio, g_{ST}, C_i, L_S, $J_F/4$, V_C, V_O, $J_{ALT}/4$, and ΦPSII were compared between subspecies and treatments using factorial ANOVA. Values of RL_S and RL_M were compared by one-way ANOVA. Where main effects were significant at the 95% confidence level, Tukey *post hoc* comparisons were made.
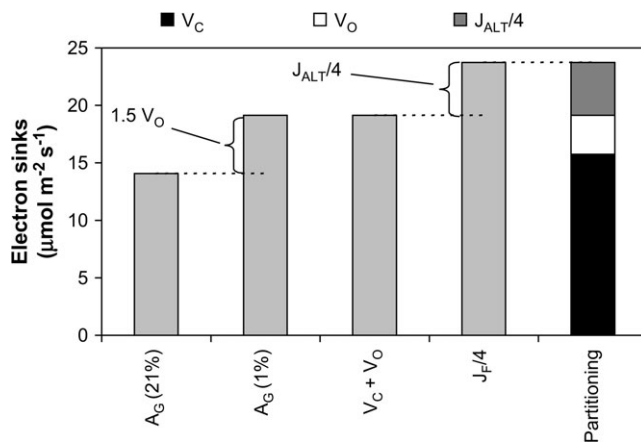

Fig. 1. Illustration of electron sink calculations for the C_3 *Alloteropsis* subspecies under well-watered conditions. Electron sinks were each quantified as CO_2 equivalents (μmol m^{-2} s^{-1}). Photosynthesis was first measured in air with 21% or 1% O_2 and used to estimate A_G (21%) and A_G (1%) according to equations 11 and 12, respectively (Table 1). Electron consumption by photosynthetic carbon reduction (V_C) was calculated with equation 15 (Table 1), and photosynthetic carbon oxidation (V_O) with equation 14 (Table 1). Total electron transport through PSII ($J_F/4$) was then estimated with equation 13 (Table 1), using measurements of Φ_{PSII} in air containing 21% O_2. Finally, the strength of alternative sinks for electron transport ($J_{ALT}/4$) was calculated as the difference between $J_F/4$ and electron consumption by carbon metabolism (equation 16, Table 1).

Results

Characterization of plants

The CE, Γ, and $\delta^{13}C$ isotopic ratio were all statistically different between the *A. semialata* subspecies, confirming a difference in photosynthetic pathways (Table 2). Moreover, they were within the range of values found in C_3 and C_4 species.

Drought constraints in the common garden experiment

SWC in natural rainfall and watered plots did not differ over the first 15 d of the experiment (Fig. 2A). The photosynthetic and leaf water potential data collected during this period were therefore averaged, and are presented as predrought measurements. In the natural rainfall plots, SWC began to differ from well-watered values on day 17, decreasing from 23% at the beginning of the experiment to 12% on day 43 (Fig. 2A). Leaf data for the last four dates are presented as average drought measurements.

Well-watered plots in the common garden experiment had an SWC that averaged above 17.5% (Fig. 2A). Values of A in this treatment were, on average, always higher in the C_4 than the C_3 plants (Fig. 2B). By contrast, g_{ST} for well-watered plants did not differ significantly between C_3 and C_4 subspecies before or during the drought period (Fig. 2C). This similarity implies that the higher Ψ_{leaf} noted for the C_4 subspecies (Fig. 2E) results from differences in whole-plant hydraulic conductance between subspecies. C_i values were lower in C_4 than C_3 plants (Fig. 2D).

Values of g_{ST} for both C_3 and C_4 subspecies were significantly lower during the drought than pre-drought period (Fig. 2C). The values of A also declined significantly during the soil-drying episode, and the C_4 subspecies completely lost its photosynthetic advantage over the C_3 subspecies as drought progressed, so that there was no significant difference between C_3 and C_4 leaves in the natural-rainfall plots during the drought period (Fig. 2B).

In the common garden experiment, the decrease in g_{ST} between watered and natural-rainfall plots was similar in both subtypes, but the decline in A was almost three times greater in C_4 than C_3 plants (Fig. 2B, C). In response to decreased g_{ST} and A, C_i in the C_3 plants remained unaffected by drought but significantly increased in the C_4 plants (Fig. 2D). Drought decreased Ψ_{leaf} and the effect was greater in the C_4 than the C_3 plants (Fig. 2E).

Mechanisms of drought limitation in pot plants

Values of A in the pot plants exposed to the dry-soil and high-VPD drought treatments were reduced by 40–85% compared with controls, replicating the drought-induced 35–85% reduction in A observed in the common garden experiment. Values of A (21%) for well-watered C_4 pot

Table 2. *Photosynthetic characterization of* Alloteropsis semialata *subspecies*

Mean ±SE ($n \geqslant 5$) are shown for the leaf CO_2 compensation point (Γ), carboxylation efficiency (CE), and the leaf $\delta^{13}C$ isotopic ratio of well-watered C_3 and C_4 subspecies from the common garden experiment. Within each column, values assigned the same letter (Tukey *post hoc*) represent homogeneous groups at the 95% confidence level. Italicized ranges indicate values typical for C_3 and C_4 species (Γ: C_3, Jordan and Ogren, 1984; Bernacchi *et al.*, 2001; C_4: e.g. Ghannoum *et al.*, 1997; Pittermann and Sage, 2000; Colom and Vazzana, 2003; CE: C_3 Wullschleger, 1993; C_4, Wand *et al.*, 2001; $\delta^{13}C$: Cerling, 1999).

Subspecies	Γ (μmol mol^{-1})	CE (mmol m^{-2} s^{-1})	$\delta^{13}C$ (‰)
C_4	12±7 a (*<10*)	294±9 c (*210–480*)	−11.6±0.5 e (*−10 to −15*)
C_3	45±2 b (*41–45*)	97±6 d (*55–130*)	−26.0±0.5 f (*−24 to −30*)

Abbreviations: Γ, CO_2 compensation point; CE, carboxylation efficiency; $\delta^{13}C$, $\delta^{13}C$ isotope ratio.
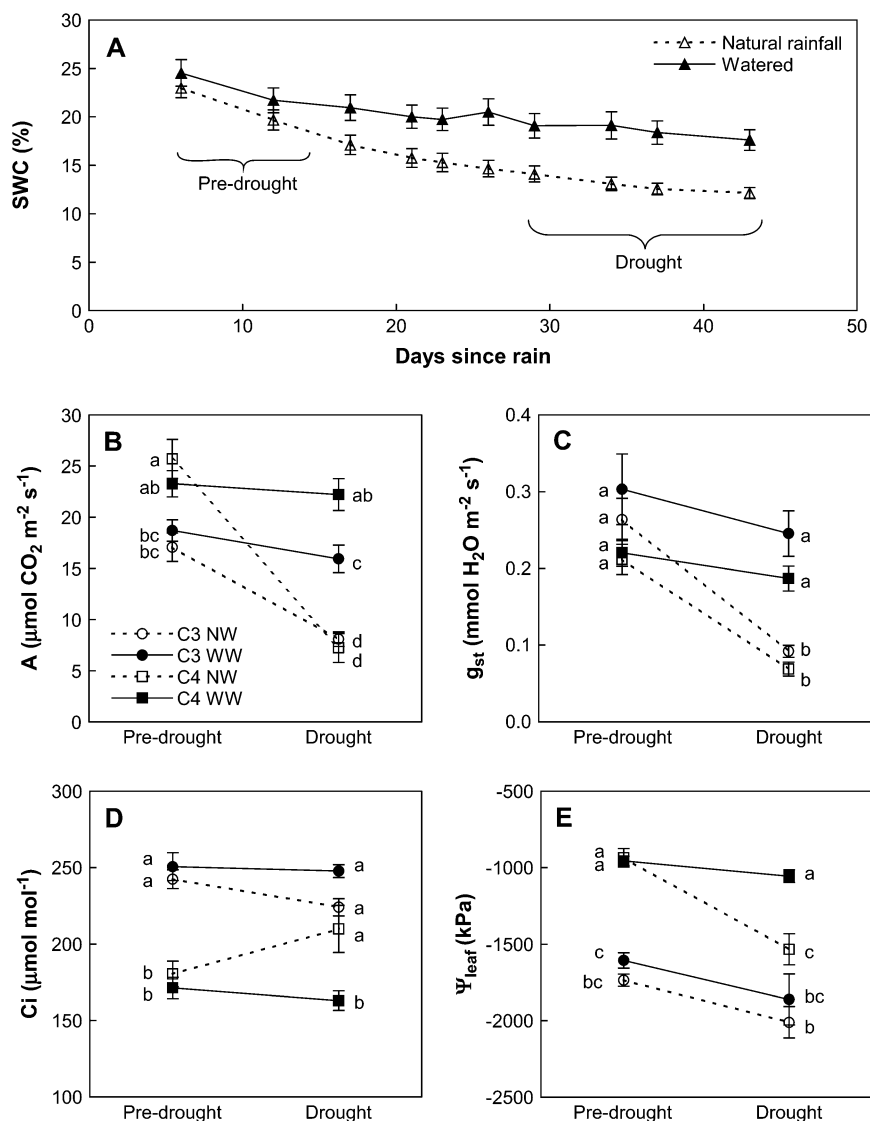

Fig. 2. (A) Soil water content (SWC) of watered and non-watered plots measured during a 43 d period of drought in October to November 2004. (B) Net photosynthetic CO_2 fixation (*A*), (C) stomatal conductance (g_{ST}), (D) intercellular CO_2 concentration (C_i), and (E) leaf water potential (Ψ_{leaf}) of leaves from well-watered control (WW) and non-watered (NW) C_3 and C_4 subspecies of *Alloteropsis semialata* grown in common garden plots. Values are means and vertical bars are standard errors ($n \geqslant 5$). Rates assigned the same letter represent homogeneous groups at the 95% confidence level (Tukey *post hoc*).

plants were significantly higher than those for C_3 plants, but this photosynthetic advantage was diminished under both dry-soil and high-VPD conditions (Fig. 3A). Al-though there was no significant difference between photosynthetic types in drought conditions, mean photo-synthetic rates were always higher for the C_4 subtype, and

a type-II error cannot be ruled out (Rohlf and Sokal, 1994). The loss of C_4 photosynthetic advantage was confirmed by measurements of A (21%) obtained from A–C_i responses in an independent set of plants under dry-soil conditions (Table 3). The photosynthetic advantage displayed by the C_4 pot plants under well-watered conditions was lost when measurements were made in an atmosphere of 1% O_2 (Fig. 3B), suggesting that it was due principally to the suppression of photorespiration.

The g_{ST} of well-watered pot plants was significantly lower in the C_4 than the C_3 subspecies, but drought conditions eliminated this difference (Fig. 4). Compared with well-watered conditions, g_{ST} was reduced by 68% and 76% under high VPD for the C_4 and C_3 subspecies, respectively (Fig. 4). Stomata were therefore significantly more sensitive to drought conditions in the C_3 subspecies, with the decrease of g_{ST} in dry-soil conditions of 81% for C_3 and 61% for the C_4 plants. The net effect of these contrasting drought responses was that the instantaneous leaf WUE (photosynthesis/transpiration) was significantly higher in the C_4 than C_3 subspecies, but showed no interaction with the drought treatments (data not shown).

The drought-induced reduction in g_{ST} significantly decreased C_i in the C_3, but not in the C_4 plants (Fig. 4B), and this reduction in C_i may explain the observed relative increase in photorespiration in the C_3 plants (Fig. 3). This contrasts with observations in the common garden drought experiment, where C_i was unchanged in the C_3 but increased in the C_4 plants (Fig. 2). It is not easy to explain this difference but it is suggested that the complex stomatal and photosynthetic response to drought may change the C_i response as drought progresses.

Ψ_{leaf} was the same for both subspecies under control conditions and significantly decreased by the dry-soil, and not the high-VPD treatment (Fig. 4C). Apparently, the observed stomatal response was not due solely to Ψ_{leaf} but was in direct response to VPD.

Table 3. *Photosynthetic rates, stomatal and metabolic limitations to photosynthesis for C_3 and C_4 subspecies of* Alloteropsis semialata *subjected to the treatments indicated*

Data were extracted from A–C_i curves and are means $\pm$SE ($n \geqslant 3$), and parameters listed in each column assigned the same letter (Tukey *post hoc*) represent homogeneous groups at the 95% confidence level.

Subtype and treatment	Photosynthetic rate at C_a=380 µmol m^{-2} s^{-1}	Stomatal limitation L_S (%)	Relative stomatal limitation RL_S (%)	Relative metabolic limitation RL_M (%)
C_4 control	20.5±1.0 a	11.4±2.1 c	–	–
C_3 control	13.1±0.7 b	21.8±0.8 bc	–	–
C_4 dry soil	8.8±1.1 bc	32.6±2.1 ab	20.4 b	36.5 a
C_3 dry soil	6.4±1.9 c	42.3±6.4 a	32.0 a	19.1 b

Abbreviation: C_a, ambient CO_2 concentration.

The stomatal and metabolic (non-stomatal) components of the photosynthetic limitation by drought were further analysed by constructing A–C_i curves for control and dry-soil pot plants (Fig. 5). A–C_i curves under well-watered conditions were typical of C_3 and C_4 plants (Table 2; Fig. 5). The C_4 curve saturated at C_i <400 µmol mol^{-1}, with an operating point at 200 µmol mol^{-1}, whilst the C_3 curve approached saturation at C_i=1000 µmol mol^{-1}, and had an operating point of 268 µmol mol^{-1} (Fig. 5). Drought had a much greater effect on the A–C_i response in C_4 than C_3 plants, via larger decreases in the CE, degree of curvature (c, equation 17), and CO_2-saturated assimilation rates. The largest effects of drought were decreases of 76% in CE and 70% in c for the C_4 plants, and 39% in CE and 30% in c for the C_3 plants.

The rate of CO_2 supply permitted by g_{ST} and the 'no stomatal limitation' case were represented by 'supply functions' intersecting the A–C_i responses (Fig. 5), and used to calculate stomatal and metabolic limitations to A. Values of L_S were significantly greater in the C_3 than the C_4 subspecies in the well-watered plants, but not under drought conditions (Table 3; Fig. 5). This resulted mainly from the loss of the 'typical' well-watered A–C_i response
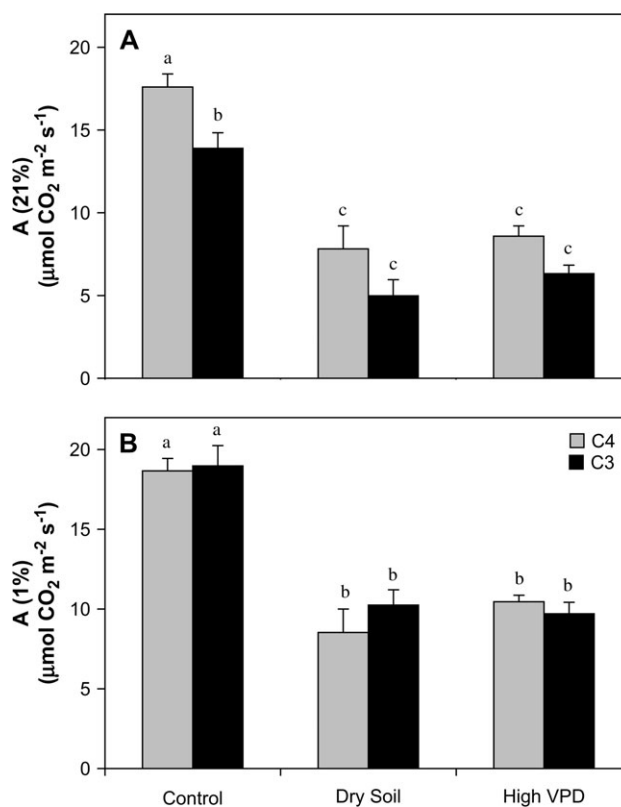

Fig. 3. Net photosynthetic CO_2 fixation (A) of pot-grown plants measured at (A) 21% or (B) 1% O_2 for C_3 and C_4 subspecies of *Alloteropsis semialata* subjected to well-watered control, dry-soil, and high-VPD treatments. Values are means and vertical bars standard errors ($n \geqslant 5$). Rates assigned the same letter represent homogeneous groups at the 95% confidence level (Tukey *post hoc*).
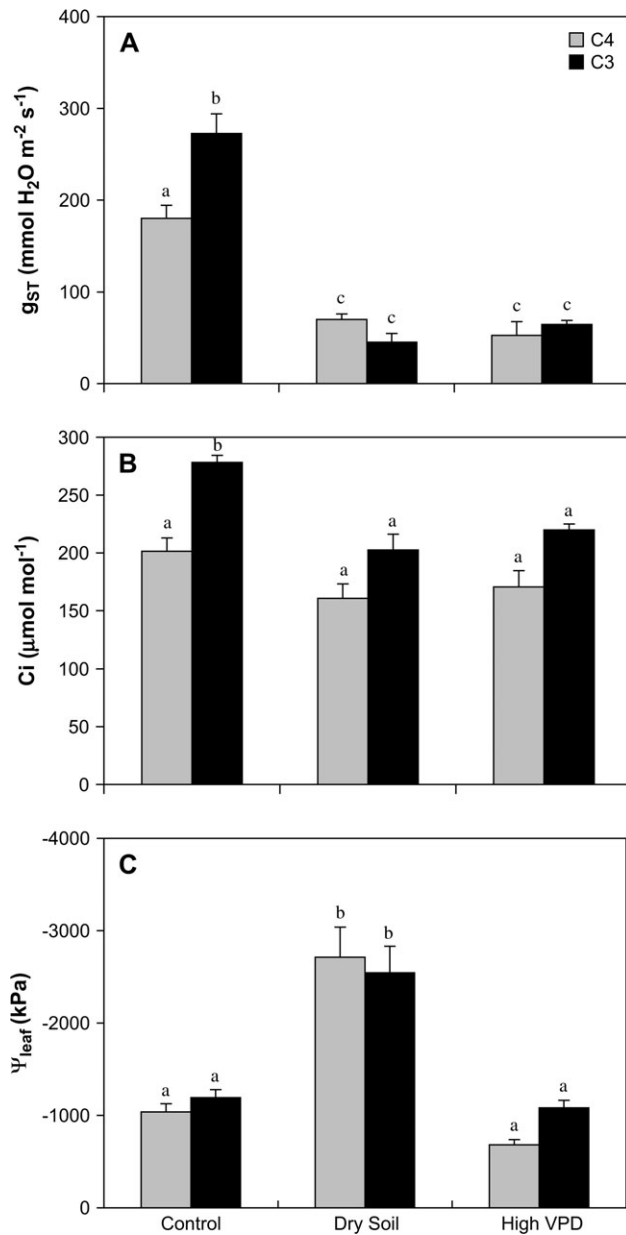
Fig. 4. (A) Stomatal conductance (g_{ST}), (B) intercellular CO$_2$ concentration (C_i), and (C) leaf water potential (Ψ_{leaf}) of pot-grown plants measured at 21% O$_2$ for C$_3$ and C$_4$ subspecies of *Alloteropsis semialata* subjected to well-watered, dry-soil, and high-VPD treatments. Values are means and vertical bars are standard errors ($n \geqslant 5$). Rates assigned the same letter represent homogeneous groups at the 95% confidence level (Tukey *post hoc*).

Fig. 5. Responses of net photosynthetic CO$_2$ fixation (*A*) to intercellular CO$_2$ concentration (C_i) for C$_3$ and C$_4$ subspecies of *Alloteropsis semialata*. Measurements were made on well-watered (closed squares) or dry soil-treated (closed diamonds) plants in an atmosphere of 21% O$_2$ and a PPFD of 2000 μmol m^{-2} s^{-1}, and curves fitted for replicate plants using equation 17 (Table 1), with $r^2 > 0.97$ for all fits. Also shown are the calculated photosynthetic rates at an atmospheric CO$_2$ concentration (C_a) of 370 μmol mol^{-1} with no stomatal limitation (W and Y). Dashed lines are supply functions representing the limitation on *A* imposed by CO$_2$ diffusion through the stomata for well-watered (X) or dry soil-treated (Z) plants. Each function has a slope set by the stomatal conductance and intercepts the C_i axis at C_a. Inset shows the initial response of *A* to chloroplast CO$_2$ concentration (C_C) for well-watered (open squares) and dry soil-treated (open diamonds) C$_3$ plants. For comparative purposes the initial *A*–C_i response of the dry-soil plants has been included (closed diamonds).

in the C$_4$ plants during severe drought. Values of RL_S showed that 32% of *A* for the well-watered C$_3$ plants and 20% for the C$_4$ plants was lost as a consequence of reduced g_{ST} under drought conditions (Table 3). Thus, under drought, the limitation of *A* by CO$_2$ diffusion into the intercellular airspaces was significantly lower in the C$_4$ than C$_3$ subspecies, and cannot account for the greater drought-induced decrease in *A* for the C$_4$ leaves (Figs 3A, 5; Table 3).
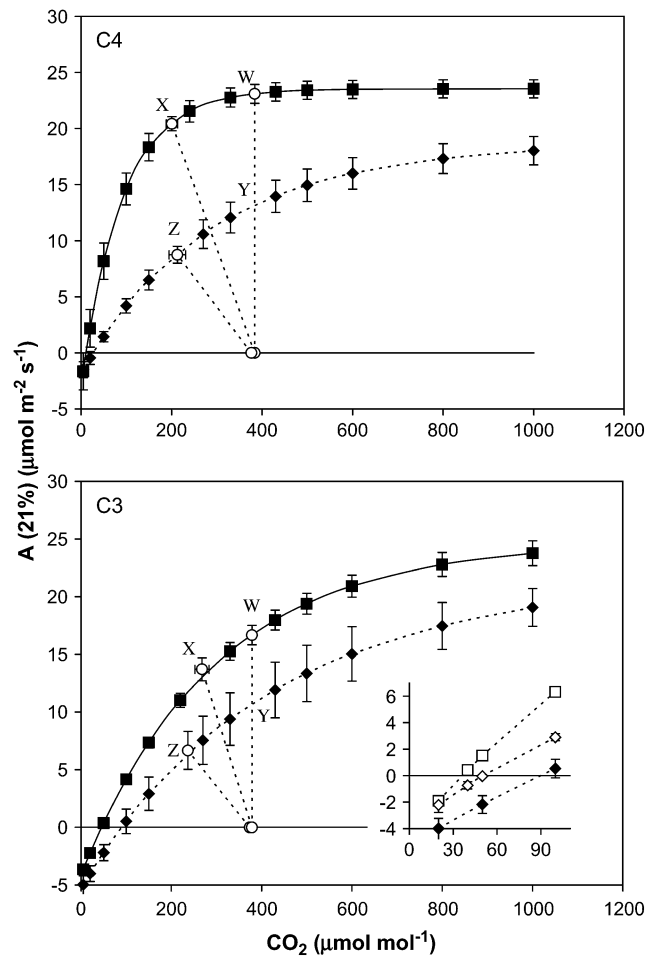
The difference can instead be attributed to RL_M, which was significantly higher in the C$_4$ than C$_3$ plants, accounting for 36%, as opposed to 19%, of the reduction in CO$_2$-assimilation rates during drought (Table 3). RL_M includes photoinhibition (photoprotection mechanisms and/or light-mediated damage to PSII; *sensu* Osmond, 1994), desiccation-linked changes in the biochemical capacity for leaf CO$_2$ fixation, and decreases in g_M. Expression of the photosynthetic CO$_2$ response on the basis of chloroplast CO$_2$ concentration (C_C), rather than C_i, allows the effects of g_M on CE to be quantified for C$_3$

species (von Caemmerer, 2000). When expressed on the basis of C_C, CE declined by 34% under drought compared with the well-watered treatment (Fig. 5, inset). This reduction is very similar to the value calculated using C_i, and suggests that decreases in g_M are not responsible for the drought limitation of A in the C_3 plants.

A–C_i curves were not constructed for the plants subjected to high-VPD treatments, since increasing CO_2 around their leaves caused dynamic responses in photosynthesis, precluding steady-state measurements, and suggesting a CO_2-mediated relaxation of photosynthetic limitations. Values of A in an ambient atmosphere of 21% O_2 and 380 μmol mol^{-1} CO_2 were the same in plants from the high-VPD and dry-soil treatments (data not shown). Together these observations show that, although atmospheric and soil drought cause similar decreases in photosynthesis, they are caused by different mechanisms of stomatal and metabolic limitation.

Drought constraints on electron sinks

The assumption that four electrons are required for each molecule of CO_2 fixed under non-photorespiratory conditions, and the subsequent estimation of a_{II} (Laisk and Edwards, 1998), results in a 1:1 relationship between $J_F/4$ and A_G (Fig. 6). The corresponding values for drought-treated and control plants did not deviate from this relationship, showing that treatments did not change PSII functioning and that $J_F/4 \approx A_G$ under non-photorespiratory drought conditions. This allows A_G to be used to estimate electron transport as CO_2 equivalents in the treated leaves (Fig. 6).

Across all of the treatments, a decrease in O_2 concentration from 21% to 1% caused C_i to increase from 178 to 197 μmol mol^{-1} in the C_4 subtype, and decrease from 234 to 205 μmol mol^{-1} in the C_3 subtype. Calculations based on the C_4 A–C_i curves (Fig. 5) show that increasing C_i could account for a large fraction of the 1.1, 0.7, and 1.9 μmol m^2 s^{-1} O_2 sensitivity of A measured in control, dry-soil, and high-VPD plants (Fig. 3). However, the corresponding changes in C_i for C_3 plants would decrease A by 1.1–1.4 μmol m^2 s^{-1}, and cannot account for the 5.0, 5.0, and 3.4 μmol m^2 s^{-1} O_2 sensitivity of control, dry-soil, and high-VPD plants (Fig. 3).

Under well-watered control conditions, $J_F/4$ and V_C did not differ between the subspecies (Fig. 7A, B). The major differences between subspecies were the 250% higher value of V_O and the 60% higher $J_{ALT}/4$ in the C_3 than the C_4 subspecies (Fig. 7C, D).

Dry soil and high VPD had similar effects to one another, both significantly reducing $J_F/4$ and V_C relative to well-watered controls. Drought (dry soil and high VPD) reduced $J_F/4$ on average by 33% for both subspecies and V_C by 48% and 53% in the C_4 and the C_3 subspecies, respectively. The larger decrease in V_C than in $J_F/4$ suggests electron flow to other sinks ($J_{ALT}/4$ and V_O). V_O
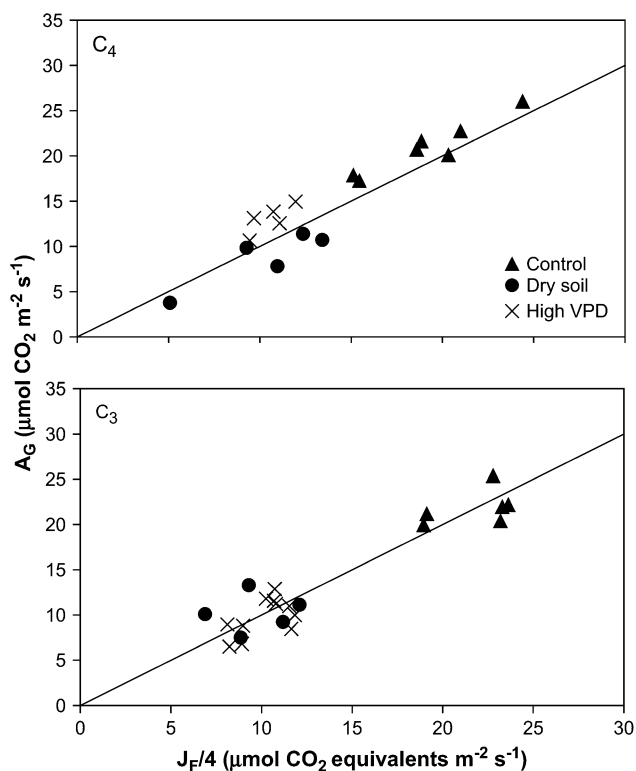

Fig. 6. Response of gross CO_2 fixation (A_G) to electron transport rate ($J_F/4$) for C_3 and C_4 subspecies of *Alloteropsis semialata* determined in an atmosphere of 1% O_2 and 370 μmol mol^{-1} CO_2 for plants from the control (closed triangles), dry-soil (closed circles), and high-VPD (crosses) treatments ($n \geqslant 5$). The continuous line shows $A_G = J_F/4$ which is assumed under non-photorespiratory control conditions.

showed no change between droughted and control plants, but $J_{ALT}/4$ tended to increase in the drought-treated plants relative to controls. However, this latter response was not statistically significant. $J_{ALT}/4$, particularly in the C_3, consumed a significant proportion of the electrons produced, and more than V_O, suggesting an important role for alternative sinks in the dissipation of excess electrons, particularly under the high light conditions used in these experiments.

The expected differences in photorespiration rates between C_3 and C_4 subspecies in the well-watered treatment were also evident when examining the response of ΦCO_2 to $\Phi PSII$, determined by varying PPFD (Fig. 8). In the C_3, but not the C_4 subspecies, the decrease in O_2 from 21% to 1% increased ΦCO_2 by a constant proportion across the full range of PPFD as a result of the inhibition of photorespiration. The slope of the relationship of ΦCO_2 to $\Phi PSII$ allows the photon requirement for CO_2 fixation to be calculated. The C_4 subspecies required, on average, 10.8 photons CO_2^{-1} under both photorespiratory and non-photorespiratory conditions, while the C_3 subspecies required 7.7 photons CO_2^{-1} under non-photorespiratory and 14.0 photons CO_2^{-1} under photorespiratory conditions (Fig. 8), values that are typical of

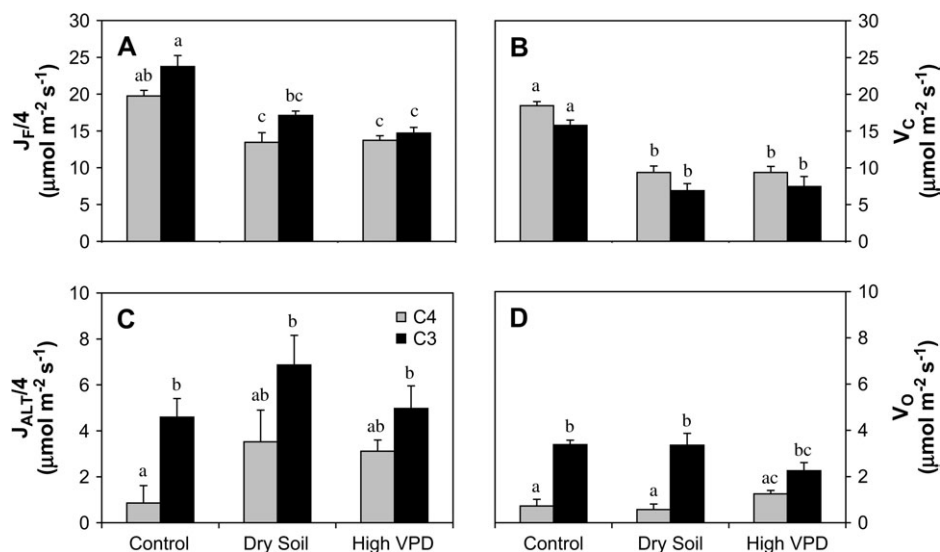*Drought constraints on C$_4$ photosynthesis* 1359

Fig. 7. Electron transport rate through PSII ($J_F/4$) and its calculated partitioning among photorespiration (V_O), photosynthetic carbon reduction (V_C), and alternative electron sinks ($J_{ALT}/4$) for C$_3$ and C$_4$ subspecies of *Alloteropsis semialata* subjected to the indicated treatments. All are mean values quantified as CO$_2$ equivalents (Table 1) with vertical bars representing standard errors ($n \geqslant 5$). Individual parameters assigned the same letter represent homogeneous groups at the 95% confidence level (Tukey *post hoc*).

C$_4$ and C$_3$ plants (Genty *et al.*, 1989; Valentini *et al.*, 1995; Laisk and Edwards, 1998; Cousins *et al.*, 2001).

Low values of ΦPSII indicate that only a small proportion of the photons absorbed were used in electron transport (Fig. 9), the majority being dissipated as heat. In both subspecies, drought significantly decreased ΦPSII (Fig. 9), indicating decreased electron transport and increased heat dissipation. Decreasing atmospheric O$_2$ to 1% exacerbated the decrease in ΦPSII in the C$_3$, but not in the C$_4$, subspecies (Fig. 9), demonstrating the role of photorespiration in dissipating energy.

Discussion

The results support the hypothesis that the C$_4$ photosynthetic advantage over the C$_3$ subspecies would be diminished during a natural drought of typical severity for South African grasslands. Controlled drying experiments showed that this response was due to a greater metabolic limitation of C$_4$ than C$_3$ photosynthesis. Previous studies have shown that such metabolic limitation is typical of the photosynthetic response to severe drought in C$_4$ (Ghannoum, 2002; da Silva and Arrabaça, 2004) and C$_3$ (Lawlor, 2002; Flexas *et al.*, 2006) species. The present findings advance this understanding in two important ways. (i) Unlike previous comparative studies (Kalapos *et al.*, 1996; Niu *et al.*, 2005), the choice of experimental species for the present study means that contrasting responses of C$_3$ and C$_4$ plants may be interpreted as the direct consequences of photosynthetic pathway evolution, modified only by the micro-evolutionary changes accompanying subspecies divergence. (ii) Since the species
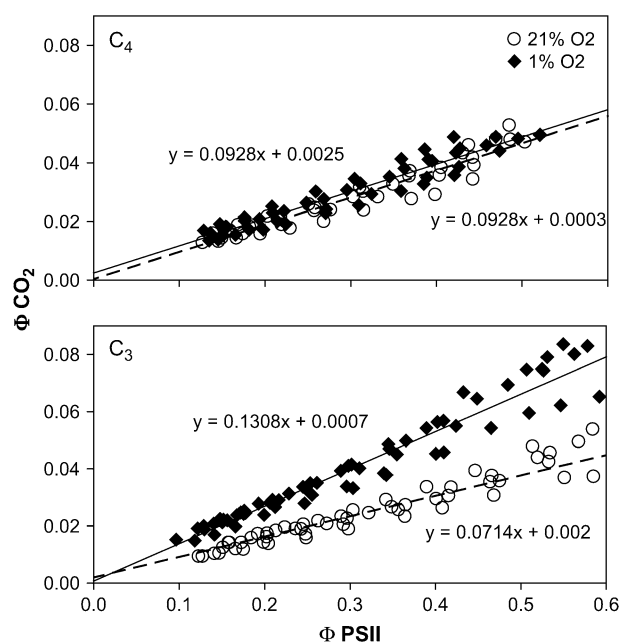

Fig. 8. Response of quantum yield of CO$_2$ assimilation (ΦCO$_2$) to PSII photochemical yield (ΦPSII) for C$_3$ and C$_4$ *Alloteropsis semialata* subspecies determined in an atmosphere of 21% (open circles) or 1% (closed diamonds) O$_2$ by varying PPFD ($n \geqslant 5$).

A. semialata is a member of a tropical grass subfamily (Panicoideae), the present experiments also avoid the confounding effects of 'climatic history' encountered when species from tropical (C$_4$) and temperate (C$_3$) phylogenetic groups are compared. The present data therefore indicate that C$_4$ photosynthesis may be inherently more susceptible than the C$_3$ pathway to metabolic limitations under severe drought conditions.
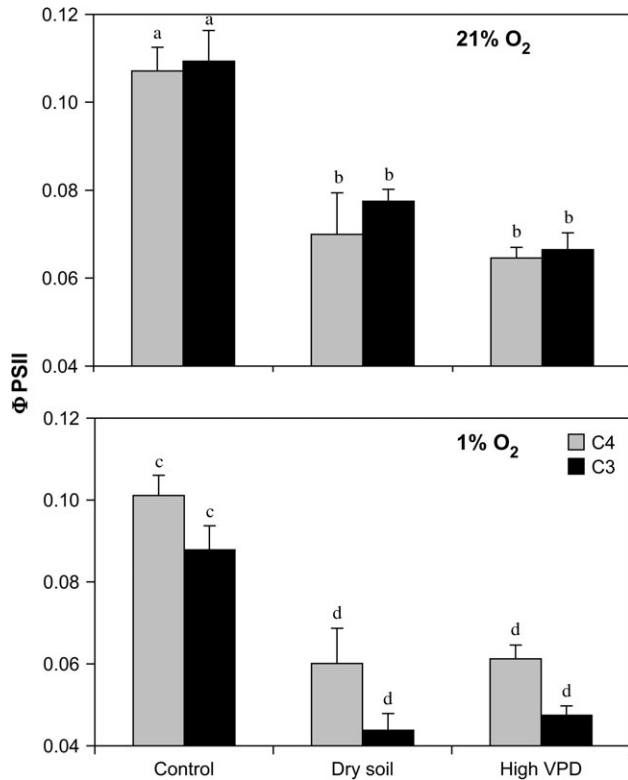
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Fig. 9. PSII photochemical yield (ΦPSII) for *Alloteropsis semialata* C_3 and C_4 photosynthetic subspecies subject to the treatments indicated. Measurements were made at 21% or 1% O_2 ($n \geqslant 5$), and vertical bars represent standard errors. ΦPSII, at each O_2 concentration assigned the same letter, represent homogeneous groups at the 95% confidence level (Tukey *post hoc*).

Metabolic limitations result from biophysical and biochemical processes that are to some degree resolved for C_3 plants (see reviews by Lawlor, 2002; Flexas *et al.*, 2006). They have been attributed to alterations in Rubisco content (Tezara *et al.*, 2002) and activity (Tenhunen, 1984), decreased ATP synthesis and RuBP regeneration (Flexas and Medrano, 2002), decreased chlorophyll content and lower photochemical efficiency (Ögren and Öquist, 1985; da Silva and Arrabaça, 2004). A role for decreased g_M (Chaves and Oliverira, 2004; Flexas *et al.*, 2006) and the possible involvement of aquaporin expression and regulation have also been recognized (Uehlein *et al.*, 2003; Hanba *et al.*, 2004). However, the present results showed that only part of the C_3 metabolic limitation under drought conditions could be attributed to this mechanism. Instead, decreases in the initial slope and saturated values of $A–C_i$ curves in droughted C_3 leaves implied reductions in Rubisco activity and the RuBP regeneration rate (von Caemmerer and Farquhar, 1981). This interpretation assumes uniform stomatal responses, which, if incorrect, would lead to significant overestimation of C_i and therefore photosynthetic limitations (Terashima *et al.*, 1988). Overestimation of C_i may also occur at very low values of g_{ST}, since cuticular conduc-

tance is significantly higher for CO_2 than H_2O vapour (Boyer *et al.*, 1997).

The reasons why drought-induced metabolic limitation was greater in the C_4 than C_3 leaves are unclear and require further investigation. Drought causes metabolic limitations to the C_4 cycle, previously attributed to reduced metabolite pool sizes and enzyme activity (Saccardy *et al.*, 1996), and osmotic effects on plasmodesmatal function (Weiner *et al.*, 1988). A decrease in the initial slope of the $A–C_i$ curves for the droughted C_4 plants in the present experiment implies reductions in the rate of the C_4 cycle (von Caemmerer, 2000). However, V_o did not increase in these plants (Fig. 7D), indicating that the CCM remained fully functional. Curvature and saturated values of the $A–C_i$ curves also decreased in the C_4 plants under drought, indicating increased bundle sheath conductance to CO_2, and decreased Rubisco activity, respectively (von Caemmerer, 2000). All of these interpretations are subject to the same caveats about C_i calculations as for the C_3 leaves, and assume minimal effects of drought on g_M. Furthermore, it was apparent from the differences in CO_2-mediated relaxation of photosynthetic limitations that dry soil and not high VPD was required to invoke the observed metabolic effects.

These findings suggest that a diminished C_4 photosynthetic advantage in drought conditions may be one reason why C_4 species are less abundant in arid than high-rainfall regions, and suggest an hypothesis to explain the WUE paradox. However, a number of important research questions remain to be addressed. Do C_4 grasses generally have greater metabolic limitations than co-occurring or closely related C_3 grasses under comparable drought conditions? Do photosynthetic sensitivity to drought and the contribution of metabolic limitations correlate with the differing climatic distributions of C_4 photosynthetic subtypes? What are the underlying mechanisms for the C_4 metabolic limitation? How do water acquisition and use, growth allocation patterns, and life-history vary amongst C_3 and C_4 grasses of different phylogentic clades and photosynthetic subtypes?

Electron sinks during drought

Despite differences in PCO and J_{ALT} between the subspecies, drought-induced limitations due to photoinhibition or changes in biochemical capacity resulted in a similar decrease in PSII photochemistry in both photosynthetic types. Reduced g_{ST} limits C_i, and hence V_C in drought conditions, increasing the potential for excess reductant production under high light conditions (Long *et al.*, 1994). Plants respond to this constraint in a number of ways: (i) by increasing V_O relative to V_C (Powles and Osmond, 1979; Valentini *et al.*, 1995; Noctor *et al.*, 2002); (ii) by increasing J_{ALT} via processes such as the Mehler reaction (Nakano and Edwards, 1987; Asada, 1999); (iii) by decreasing J_F via non-radiative energy

dissipation (Badger *et al.*, 2000); and (iv) by photodamage if the former mechanisms are inadequate in dissipating energy (Demmig *et al.*, 1988; Aro *et al.*, 1993). In subspecies of *A. semialata*, the decreased electron demand by V_C was not compensated for by increased J_{ALT} (Fig. 7), a response that has been observed in other species (Valentini *et al.*, 1995; Cornic and Fresneau, 2002). However, at the high light intensities used in this study, J_{ALT} dissipated 30% and 16% of $J_F/4$ in the C$_3$ and C$_4$ subspecies, respectively. This demonstrates the importance of alternative electron sinks as a mechanism of energy dissipation in the C$_3$ subspecies at ambient CO$_2$ levels, and agrees with the values calculated for drought-stressed wheat (Biehler and Fock, 1996). Absolute values of V_O did not increase to compensate for the decrease in V_C, and were not significantly affected by drought in either the C$_3$ or C$_4$ subspecies (Fig. 7). V_O in the C$_4$ subspecies accounted for only 6% of J_F, although the use of 1% O$_2$ during gas exchange measurements may have resulted in a slight underestimation of V_O. Maroco *et al.* (1997) have demonstrated that O$_2$ concentrations of 5–10% are optimal for C$_4$ photosynthesis, and concentrations below this may limit ATP supply to the C$_4$ cycle, although this limitation is not seen in all C$_4$ species (Ghannoum *et al.*, 1997). V_O in the C$_3$ subspecies of *A. semialata* consumed 17% of J_F and, if photorespiration was suppressed with 1% O$_2$, photochemical use of absorbed energy only decreased by 3% (Fig. 9). Hence photorespiration in the C$_3$ subspecies under high light conditions offered only limited protection against photoinhibition.

For both the C$_3$ and C$_4$ subspecies of *A. semialata* subjected to high light, Φ_{PSII} indicated that only 11% of absorbed light energy was used in photochemistry, the remainder being dissipated as non-radiative energy (Fig. 9). Φ_{PSII} was significantly decreased by drought; a response documented in other C$_3$ and C$_4$ species (Demmig *et al.*, 1988; Ghannoum *et al.*, 2003), apparently in response to decreases in V_C. Non-photochemical energy dissipation is considered the major process in reducing Φ_{PSII} to maintain a balance with the electron flow requirements of carbon metabolism (Krause and Weis, 1991). The major drought response in both the C$_3$ and C$_4$ subspecies was therefore a decrease in J_F, associated with decreased photochemical energy dissipation, rather than an increase in the sinks that consume electrons.

Photosynthetic characterization

Previous work with *A. semialata* suggested that populations of the South African C$_4$ subspecies may be 'C$_4$-like', due to the expression of the Rubisco large subunit in mesophyll cells, and the C$_3$ subspecies 'C$_3$-like' because it has anatomical and physiological characteristics partially resembling those of C$_3$–C$_4$ intermediates (Ueno and Sentoku, 2006).

Two complementary types of evidence suggest that the operation of subtle C$_3$–C$_4$ intermediate mechanisms was absent from the plant populations sampled in the present study. First, isotopic values for the C$_4$ subspecies show no evidence of the strong ^{13}C discrimination characterizing atmospheric CO$_2$ fixation by Rubisco, indicating that (even if it was present) the enzyme was not active in mesophyll cells (Table 2). Secondly, the calculated fraction of CO$_2$ lost to photorespiration in the well-watered C$_3$ plants was 22%, within the range of values typically reported for C$_3$ plants on empirical (Ehleringer and Björkman, 1977) and theoretical (Sage and Pearcy, 2000) grounds. This suggests that photorespiratory enzymes localized in the bundle sheath were not allowing significant re-fixation of photorespiratory CO$_2$, as previously suggested (Ueno and Sentoku, 2006). Other populations of *A. semialata* may contain C$_3$–C$_4$ intermediates (Ueno and Sentoku, 2006; PW Hattersley, personal communication), but the plants in the present study appear to be C$_3$ and C$_4$ types.

Conclusions

The present results show a loss of the C$_4$ photosynthetic advantage over the C$_3$ type during drought conditions caused by greater increases in metabolic limitation. It is suggested that this mechanism may partially explain the paradox of decreasing relative C$_4$ species abundance along regional gradients of declining rainfall, despite high WUE in C$_4$ leaves. Decreased electron flow to carbon reduction under drought conditions was not compensated for by increased photorespiration or alternative sinks in either the C$_3$ or C$_4$ leaves. Rather, photochemical efficiency was decreased in both subtypes.

Acknowledgements

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