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Differences in drought sensitivities and photosynthetic limitations between co-occurring C_3 and C_4 (NADP-ME) Panicoid grasses

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- *Background and Aims* The success of C_4 plants lies in their ability to attain greater efficiencies of light, water and nitrogen use under high temperature, providing an advantage in arid, hot environments. However, C_4 grasses are not necessarily less sensitive to drought than C_3 grasses and are proposed to respond with greater metabolic limitations, while the C_3 response is predominantly stomatal. The aims of this study were to compare the drought and recovery responses of co-occurring C_3 and C_4 NADP-ME grasses from the subfamily Panicoideae and to determine stomatal and metabolic contributions to the observed response.
- *Methods* Six species of locally co-occurring grasses, C_3 species *Alloteropsis semialata* subsp. *eckloniana*, *Panicum aequinerve* and *Panicum ecklonii*, and C_4 (NADP-ME) species *Heteropogon contortus*, *Themeda triandra* and *Tristachya leucothrix*, were established in pots then subjected to a controlled drought followed by re-watering. Water potentials, leaf gas exchange and the response of photosynthetic rate to internal CO_2 concentrations were determined on selected occasions during the drought and re-watering treatments and compared between species and photosynthetic types.
- *Key Results* Leaves of C_4 species of grasses maintained their photosynthetic advantage until water deficits became severe, but lost their water-use advantage even under conditions of mild drought. Declining C_4 photosynthesis with water deficit was mainly a consequence of metabolic limitations to CO_2 assimilation, whereas, in the C_3 species, stomatal limitations had a prevailing role in the drought-induced decrease in photosynthesis. The drought-sensitive metabolism of the C_4 plants could explain the observed slower recovery of photosynthesis on re-watering, in comparison with C_3 plants which recovered a greater proportion of photosynthesis through increased stomatal conductance.
- *Conclusions* Within the Panicoid grasses, C_4 (NADP-ME) species are metabolically more sensitive to drought than C_3 species and recover more slowly from drought.

Key words: C_3 and C_4 Panicoid grasses, NADP-ME subtype, drought response, stomatal and metabolic limitations, drought recovery.

INTRODUCTION

C_4 photosynthesis is the term used to describe the many combinations of anatomical, biochemical and physiological modifications that concentrate CO_2 in the bundle sheath, effectively saturating Rubisco at ambient CO_2 concentrations. This almost eliminates photorespiration and enables C_4 plants to reduce stomatal aperture while fixing CO_2 at rates equal to or greater than C_3 plants (Pearcy and Ehleringer, 1984). These C_4 characteristics and the resultant increased water-use efficiency led to the general view that C_4 photosynthesis was insensitive to drought and was advantageous in arid environments (Barbour *et al.*, 1987; Taiz and Zeiger, 1991; Haxeltine and Prentice, 1996).

Support for this tolerance comes both from the correlation between the increasing number of C_4 species with decreasing annual rainfall (e.g. Ellis *et al.*, 1980; Hattersley, 1992; Taub, 2000; Cabido *et al.*, 2008) and from the competitive success of C_4 species during periods of natural drought (Tilman and Downing, 1994). However, a general case for C_4 drought tolerance is questionable because of several lines of evidence.

First, the combined C_4 species relationship with rainfall masks the response of individual photosynthetic subtypes and unlike the overall C_4 correlation the numbers of NADP-ME species declines with aridity (Ellis *et al.*, 1980; Taub, 2000), suggesting that this subtype is drought-sensitive. This pattern is further complicated because the majority of NADP-ME species belong to the Panicoideae and hence drought sensitivity may be a characteristic of phylogenetic grouping rather than photosynthetic subtype (Taub, 2000; Osborne, 2008).

Secondly, physiological evidence indicates that C_4 photosynthesis is sensitive to drought. C_4 plants, like C_3 species, initially respond to drought by decreasing stomatal and mesophyll conductance to CO_2 (see reviews by Lawlor, 2002; Medrano *et al.*, 2002; Flexas *et al.*, 2004), although the quantification of the latter remains problematic (Lawlor and Tezara, 2009). A more severe water deficit further increases conductance limitations, but metabolic (biochemical) limitations become more important in decreasing photosynthetic potential (Ghannoum *et al.*, 2003; Marques da Silva and Arrabaça, 2004; Ripley *et al.*, 2007). The greater metabolic limitation in C_4 species is probably associated with drought effects on the CO_2-concentrating mechanism and may include impaired C_4 biochemistry and plasmodesmatal function. Additionally, drought is proposed to limit ATP synthesis, which would decrease the regeneration of substrates for both the C_3 and the C_4 cycle (Weiner *et al.*, 1988; Ghannoum, 2009; Lawlor and Tezara, 2009).

The severity of the water-stress influences the relative contributions of diffusional and metabolic limitations to photosynthesis and hence has important implications for the recovery of photosynthetic physiology (Ignace *et al.*, 2007). In C_3 species the initial limitations by stomatal and mesophyll conductance are rapidly and completely reversed by re-watering, but more severe metabolic limitations are slowly reversed and plants may therefore take more time to recover (Galle *et al.*, 2007). Hence, if C_4 species do suffer from increased metabolic limitation relative to C_3 species, this is likely to affect the rate at which they can recover from drought. This response would have important ecological implications influencing competitive interactions and plant distribution.

Data for direct comparisons of the drought responses of C_3 and C_4 species and of the differences between C_4 photosynthetic subtypes are limited, and experiments have largely not controlled for phylogenetic effects. This is despite the ecological relevance of such comparisons justified by the co-occurrence of C_3 and C_4 grasses (Gibbs Russell *et al.*, 1991) and because numbers of NADP-ME and/or Panicoid species decline with aridity. To begin to address this shortfall, the present study compares three C_4 (NADP-ME) species and three C_3 species, this comparison being limited to Panicoid grasses. All species grow in close proximity (in an area <10 m^2) in South Africa, and have similar perennial habits and growth phenologies characterized by spring and summer growth, and winter dormancy. Therefore, this experimental system represents a unique opportunity to remove the confounding effects that adaptations to different habitats may introduce and to control for phylogeny. The selected species were transferred to pots, subjected to a controlled drought and subsequently watered and used to investigate: (1) the relative contributions of stomatal and metabolic limitations to the decline of C_3 and C_4 (NADP-ME) photosynthesis under conditions of increasing drought; (2) the C_4 photosynthetic and water-use advantage under well-watered and drought conditions; and (3) the recovery rate of photosynthesis when plants were re-watered.

MATERIALS AND METHODS

Plant collection and growth conditions

Six grass species (Poaceae) were selected as C_3 and C_4 (NADP-ME) representatives within the subfamily Panicoideae. C_3 species were *Alloteropsis semialata* (R. Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell, *Panicum aequinerve* Nees and *Panicum ecklonii* Nees. C_4 (NADP-ME) species were *Heteropogon contortus* (L.) Roem. & Schult., *Themeda triandra* Forssk. and *Tristachya leucothrix* Nees. Gibbs Russell *et al.* (1991) and Clayton *et al.* (2006) provide full descriptions of these species. They represent the dominant Panicoid grasses that co-occur naturally in grasslands around Grahamstown, South Africa, and can be collected from within a small area (10 m^2). All species share a common summer growing season and winter dormancy period. Fourteen plants of each species were carefully excavated at Faraway Farm, 8 km from Grahamstown (33°19′S, 26°28′E) on 25 June, 2006, thinned to five tillers, and planted into 10-L pots with 6·7 kg of topsoil similar to that of the field site. The potted plants were maintained in a naturally lit, clear polyethylene tunnel where average daily temperatures ranged between approx. 16 and 30 °C, with an average of 25 °C. The plants were kept well-watered for the month leading up to the experiment and once a week were watered with 0·1 % (v/v) hydroponic fertilizer (Chemicult Products, Cape Town, South Africa). Seven plants of each species were maintained well-watered for the duration of the experiment and seven were subjected to drought.

Soil water content and controlled pot drought

To subject plants to drought conditions representative of those encountered in the field where these species co-occur, the soil water content (SWC) at Faraway Farm was monitored over a 6-month period from August, 2006 to January, 2007. A soil moisture probe (ECH20, Decagon Devices Inc., Pullman, WA, USA) automatically recorded hourly SWC over a 20-cm soil profile and on nine occasions during the period ten randomly positioned SWC measurements were made at a depth of 6 cm with a dielectric probe (ThetaProbe, ML2X, Delta-T Devices, Cambridge, UK). These data were correlated to the gravimetric SWC of samples collected at depths >6 cm and this relationship was used to calculated the actual gravimetric SWC.

Low rainfall from 16 December to 24 January decreased SWC at Faraway farm from approx. 25 to 5 % and the magnitude and duration of this drought was replicated in the pot experiment as follows. To prevent evaporation from the soil surface, a 4-cm-deep layer of pre-weighed fine stone (0·5–1 cm in diameter) was spread across the soil surface. ThetaProbe measurements of SWC, in conjunction with pot weighing, were used as a guide either to maintain pots at constant SWC or to allow them to dehydrate in a controlled fashion. Well-watered pots were maintained at 15–20 % gravimetric SWC for the duration of the experiment, while drought-treated pots were initially maintained at this level for 4 weeks, and then allowed to dry by approx. 0·4 % d^{-1} for a period of 48 d. At the end of the 48-d drought plants were re-watered and SWC was maintained above 15 % for a further 26 d. To attain the standard rate of dehydration, SWC of pots was measured every second day and water was added as required. Pot weights were recorded each time the probe readings were taken and were used calculate the actual gravimetric SWC. This calculation was only possible after the final dry soil masses were measured at the end of the experiment and the pots were oven dried at 60 °C. This approach allowed all plants, regardless of total leaf area, to dry down at similar rates and to have SWCs that were not different between species.

Midday leaf water potentials

Prior to and after 36 and 48 d of drought, the leaf previously used for gas-exchange measurements was immediately excised and the leaf water potential (Ψ_{leaf}) was determined using a Scholander pressure chamber. This procedure was followed during the drought but not re-watering phase of the experiment. To assess the relevance of the pot-imposed drought, Ψ_{leaf} of ten randomly selected individuals of each species of grass was measured at Faraway Farm on 24 January when SWC was approx. 5 %.

Leaf gas exchange

Gas exchange measurements were made on the control and treatment plants prior to imposing the drought treatment on 10 April, 2007, and after 20, 36 and 48 d of drought. Plants were then re-watered and recovery was measured after 3, 4, 20 and 24 d. Gas exchange was measured between 1100 and 1500 h on fully expanded leaves using a Li-6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Cuvette conditions were: photosynthetic photon flux density (PPFD) of 2000 μmol m^{-2} s^{-1}, air temperature of 25 °C and vapour pressure deficit (VPD) of 1–2·5 kPa. On repeated occasions during the measurement period the empty Li-Cor leaf chamber was held open to prevailing ambient conditions to record VPD and air temperature. Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981) and instantaneous water-use efficiency was calculated as the ratio between net photosynthetic rate and stomatal conductance to water vapour (A/g_{ST}).

Photosynthetic recovery after re-watering

The photosynthetic rates (A) of individual control and drought-stressed plants were measured on five occasions during a 27-d recovery period after re-watering. The length of time required for drought-stressed plants to recover their pre-drought photosynthetic rates involved fitting the data from each replicate water-stressed plant with the following function: $A = a \times [1 - \exp(b - c \times \text{number of days of recovery})]$ and all fits had $r^2 > 0{\cdot}92$. Photosynthetic rates of control plants were combined for each species and fitted with a linear equation to produce a single average response over the five measurement occasions. The intercept of the individual plant drought recovery curve with the average linear control response was used to define the number of days required for each plant to recover. Some individuals of *Th. triandra* and *Tr. leucothrix* did not recover and were assigned a conservative recovery period of 27 d, the duration of the recovery experiment.

A/C_i *curves*

The response of photosynthesis (A) to intercellular concentrations of CO_2 (C_i) were measured using the Li-6400 photosynthesis system on well-watered plants with SWC of 15–20 % and after 36 d of drought when SWCs were decreased to 4 %. Plants were transferred to the laboratory and gas exchange measures were made on a fully expanded, first non-apical leaf after it had adjusted to the environment of the cuvette ($T_L = 25$ °C, PPFD = 2000 μmol m^{-2} s^{-1}, VPD = approx. 1·3 kPa). External concentrations of CO_2 (C_a) were supplied in the sequence 37, 25, 15, 10, 5, 3·5, 37, 50, 75, 100, 130 and 160 Pa and photosynthetic parameters were calculated. CO_2 response curves for the C_3 grasses were analysed using the models of von Caemmerer (2000) and temperature corrections were performed using the equations from Bernacchi *et al.* (2001, 2003). C_4 curves were modelled according to Collatz *et al.* (1992). Further measurements were made after 48 d of drought when SWC was reduced to 3 %, but rather than constructing full A/C_i curves, measures were made at ambient C_a and then C_a was increased so that C_i equalled 38 Pa; this allowed stomatal and metabolic limitations to be calculated.

Relative stomatal limitation (R_{SL}) and relative metabolic limitation (R_{ML}) were calculated to explain how drought reduced A relative to the average value for well-watered plants at an ambient CO_2 concentration of 38 Pa (X), according to:

$$\begin{aligned} \text{0 d of drought: } R_{SL} &= [(A - X)/X] \\ &\quad \times 100 \text{ and } R_{ML} \text{ cannot be calculated;} \\ \text{36 d of drought: } R_{SL} &= [(B - Y)/X] \times 100 \text{ and } R_{ML} \\ &= [(X - B)/X] \times 100; \\ \text{48 d of drought: } R_{SL} &= [(C - Z)/X] \times 100 \text{ and } R_{ML} \\ &= [(X - C)/X] \times 100. \end{aligned}$$

A, B and C are photosynthetic rates at an atmospheric CO_2 concentration of 38 Pa, with no stomatal limitation, at 0, 36 and 48 d of drought, respectively. X, Y and Z are photosynthetic rates at an atmospheric CO_2 concentration of 38 Pa, with prevailing stomatal limitations, at 0, 36 and 48 d of drought, respectively (see Fig. 4).

Statistical analysis

Nested General Linear Models (GLM) were used to detect the effects of drought, date, photosynthetic type, species and their interactions on SWC, Ψ_{leaf}, A, g_{ST} and A/g_{ST}. Species were treated as nested within photosynthetic type and separate analyses were conducted for the drought (days 0–48) and re-watering (days 48–75) phases of the experiment. R_{SL} and R_{ML} were compared between drought-treated plants at 0, 36 and 48 d, photosynthetic type, species and their interactions. A/C_i parameters were similarly compared, but were analysed separately for C_3 and C_4 photosynthetic types, and the number of days required for the recovery of photosynthetic rates were compared between species and photosynthetic type. Homogeneity of variance for all models was determined with Levene's test and data transformations were performed as needed. Statistical differences between means were determined by Fisher-LSD *post-hoc* tests (at $P < 0{\cdot}05$) if the GLM effect was significant.

A comparison of the initial linear relationship of A to g_{ST} measured on different days of drought was made using the standardized major axis (SMA) technique and tested for heterogeneity from a common slope. Where a common slope was found across all days, axis scores were used to determine shifts along this common axis due to drought using the WALD test (Warton *et al.*, 2006). Common slope or individual slopes were fitted with linear regressions.

RESULTS

Field soil water content and pot drought experiment

Field SWC declined from October to February with episodes of rain temporarily increasing values. During the period 16 December to 22 January the field site SWC showed an average decline of 0·4 % d^{-1} (Fig. 1A); these drought conditions were approximately replicated in the pot experiment by decreasing SWC by a similar rate and extent (Fig. 1B). The pot drought decreased SWC from 20 to 3 % over the 48-d period and then subsequent re-watering rapidly returned SWC to above 15 %, the level that was maintained for the rest of the experiment.

Comparisons of control and drought-treated plants

On day 0, control and drought-treated plants had similar values of Ψ_{leaf}, A, g_{ST} and A/g_{ST}, but values for control plants showed fluctuations over the subsequent 75-d period (Fig. 2 and Supplementary Data, available online). Hence, all further comparisons of these parameters were made between control and drought-treated plants at each measurement occasion. Similar analysis was not possible for all parameters as A/C_i curves, R_{SL} and R_{ML} were measured only on drought-treated plants at 0, 36 and 48 d. Hence, the response of these parameters includes the effects of drought and to a lesser extent those related to time.

Midday leaf water potential and gas exchange response to drought

Drought reduced SWC relative to controls leading to values of midday Ψ_{leaf} that were significantly different between treatments (Fig. 2A–D, Table 1). The rate of decrease was initially different between photosynthetic types and was less in the C_4 than C_3 species (Fig. 2A, B). On days 0 and 36, the C_4 species had less negative Ψ_{leaf} than C_3 species, with the exception of *P. aequinerve* (Fig. 2C, D), but by day 48 values were similar for all species.

At high SWC, before the drought treatment, the average stomatal conductance (g_{ST}) of C_3 and C_4 plants was not different (Fig. 2E, F), although this was largely due to the low value for *A. semialata* that reduced the average for the C_3 species (Fig. 2G). g_{ST} declined with drought more in C_3 than C_4 species and at the advanced stages of drought (days 36–48), the C_4 species maintained higher g_{ST} than the C_3 species (Fig. 2E, F, Table 1). The g_{ST} of individual species within a photosynthetic type did not respond uniformly to drought, with certain species responding earlier than others, as is evident from the day on which species became different to well-watered controls (Fig. 2G, H).

Photosynthetic rates of the C_4 species were significantly higher than those of the C_3 species and this difference was maintained on all but the severest day of drought (Fig. 2I, J, Table 1). Photosynthesis declined at a similar rate in both photosynthetic types until day 36; thereafter, A declined more abruptly in the C_4 than C_3 species (Fig. 2I, J). All species within a photosynthetic type responded uniformly to drought, although *P. ecklonii* had photosynthetic rates that were higher than those of the other two C_3 species.

Among the well-watered plants, the average A/g_{ST} values for C_4 species were higher than those of the C_3 species (Fig. 2M, N). Initially drought increased A/g_{ST} in both photosynthetic types and this was sustained until day 36 in the C_3 species, and only decreased on the most severe day of drought to values comparable with those of the well-watered controls (Fig. 2M). In contrast, the C_4 plants showed a decrease in A/g_{ST} after day 20, and by day 48 these values were lower than those of the controls (Fig. 2N). Not all the species within each photosynthetic type responded uniformly to drought, with *Tr. leucothrix* and *P. ecklonii* responding more slowly than the other species within their type (Fig. 2O, P).

Photosynthetic recovery

After re-watering, the g_{ST} of drought-treated C_3 species increased by more than that of C_4 species (Fig. 2E–H), but individual species took different numbers of days to recover

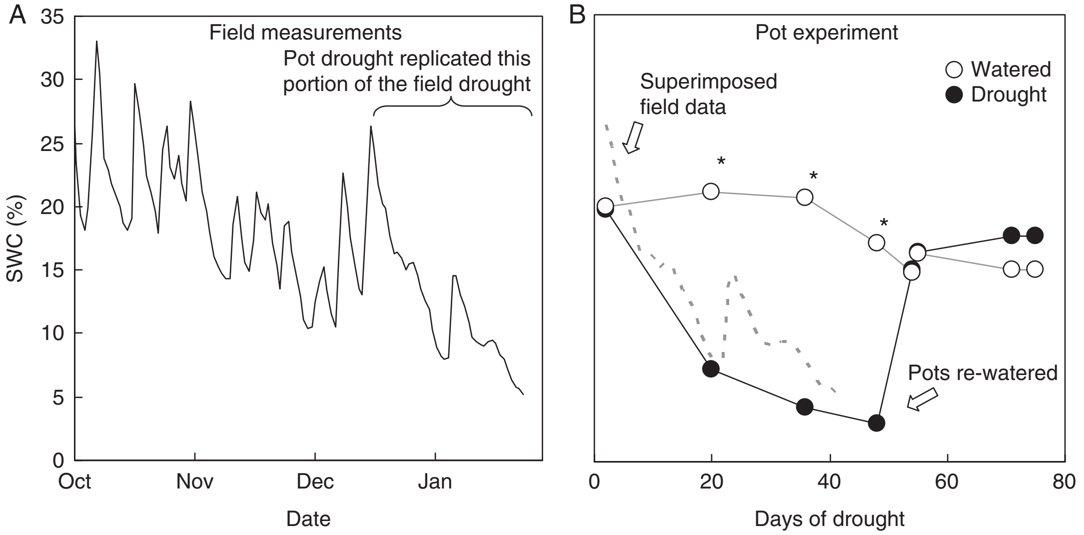

FIG. 1. (A) Soil water contents (SWC) at the field site (solid line). (B) SWC of pot-cultivated plants that were watered or subjected to drought, as indicated. For the pot experiment the data presented are for measurements made prior to the adjustment of pot water content (see Methods for details). The pot dehydration lasted 48 d and SWC was decreased at a similar rate to that observed for field measures over the period 16 December to 24 January. This period of field data (—) has been superimposed on the pot SWC response to demonstrate this similarity. The pots were re-watered on the 28 May and an asterisk indicates significant differences in SWC between well-watered and drought-treated pots at $P < 0{\cdot}05$ (Fisher LSD test).

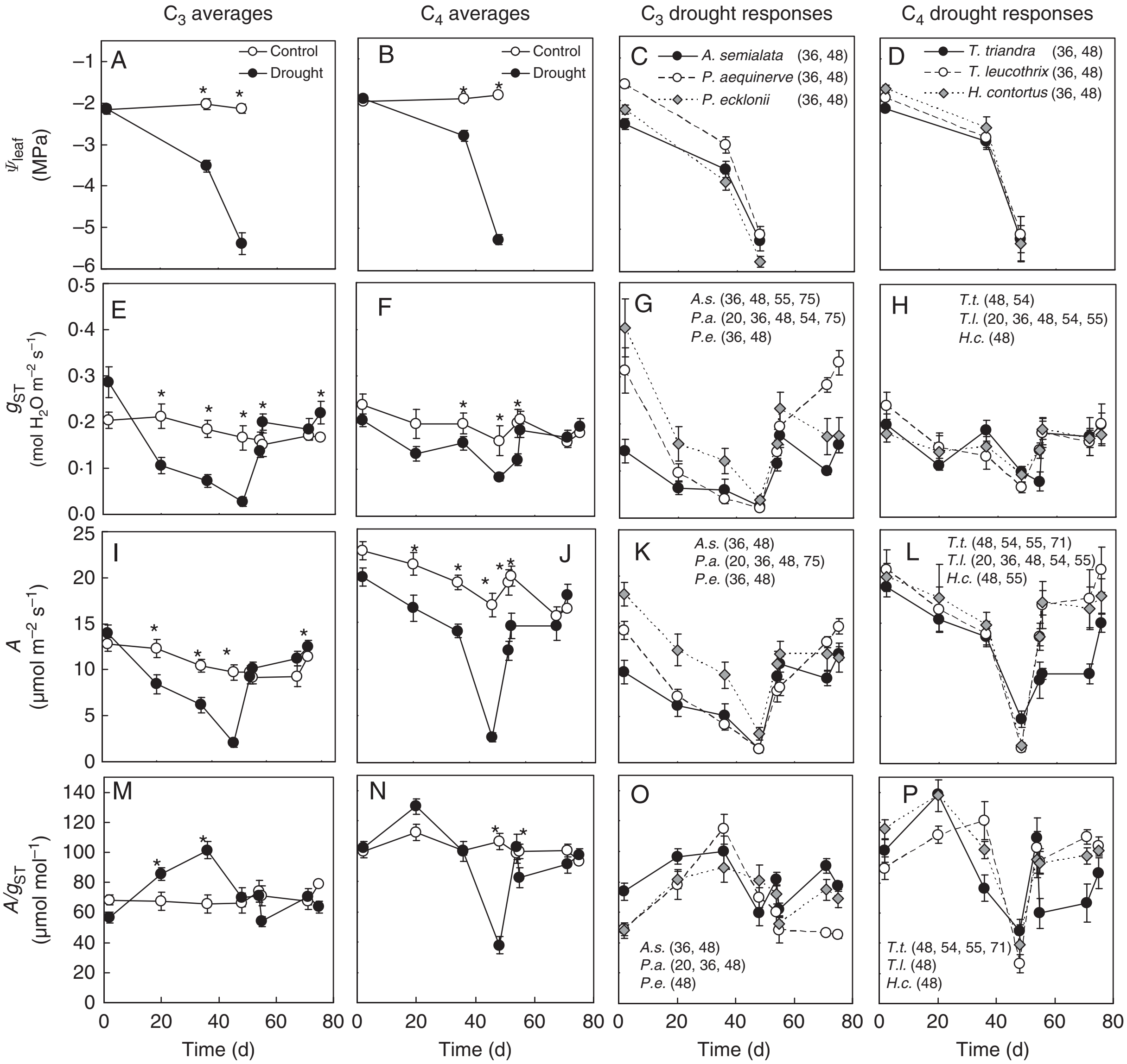

FIG. 2. (A–D) Midday leaf water potential (Ψ_{leaf}), (E–H) net leaf photosynthesis (A), (I–L) stomatal conductance (g_{ST}) and (M–P) instantaneous water-use efficiency (A/g_{ST}) of C_3 and C_4 Panicoid grasses subjected to controlled drought. Data are presented for individual C_3 and C_4 species or averaged by photosynthetic type and include the average photosynthetic type responses of the well-watered controls. The individual species control responses are available as Supplementary Data, available online. Plants were subjected to a 48-d period of drought after which pots were re-watered and maintained at an SWC above 16 %. Values are means ± s.e. and $n > 5$ for each species. For the average C_3 and C_4 responses, an asterisk indicates significant differences between means for control and drought-stressed plants. Likewise, in the individual species responses these differences are indicated by the numbers included after the species legends indicating for which days these differences were significant. Differences are considered significant at $P < 0{\cdot}05$ (Fisher LSD test).

irrespective of photosynthetic type. Two of the C_3 species, *A. semialata* and *P. aequinerve*, recovered such that g_{ST} values exceeded those of the controls by day 75 (Fig. 2K).

Irrespective of g_{ST} recovery, the photosynthetic rates of all the C_3 species recovered by day 54 (Fig. 2I, K), within 6 d of re-watering. In contrast, the average photosynthetic rate of the C_4 species had not recovered by day 55 (Fig. 2J) and *Th. triandra* did not recover by day 71 (Fig. 2L). The slow C_4 recovery, and particularly that of *Th. triandra*, meant that the average C_4 photosynthetic superiority was only attained by day 75 (Fig 2I, J). However, comparing averages in this way limits resolving recovery times because of the sampling intervals used, which can be avoided by comparing the intersection of curves fitted to control and recovery data (see Methods). This approach estimated that the C_4 species took on average 23 d to recover (Fig. 3A) and individual C_4 species recoveries ranged from 16 d for *H. contortus* to recoveries that were incomplete within the duration of the experiment (Fig. 3C). In contrast, the average C_3 species recovered within 9 d (Fig. 3A), while individual species recoveries ranged from 4 to 12 d (Fig. 3B).

A/g_{ST} values for the C_3 species during the recovery were not different from control values and this was consistent across all three species (Fig. 2M, O). This pattern contrasted with the response for C_4 species, where values were greatly reduced at the severest drought, essentially as a result of a faster

TABLE 1. *General Linear Model (GLM) results of a comparison of midday leaf water potential (Ψ_{leaf}), stomatal conductance (g_{ST}), photosynthetic rate (A) and water-use efficiency (A/g_{ST}) between C_3 and C_4 Panicoid species (represented as species nested in photosynthetic type) in response to conditions of adequate water supply, decreasing SWC and after re-watering*

	Treatment	Treatment × date	Treatment × date × type	Treatment × date × species
Plants subject to drought (Drought phase, days 0–48)				
$\Psi_{\rm leaf}$	$F_{1,239} = 505\cdot0$***	$F_{4,239} = 207\cdot9$***	$F_{6,239} = 5\cdot4$***	$F_{24,239} = 6\cdot8$***
$g_{\rm ST}$	$F_{1,254} = 115\cdot8$***	$F_{6,254} = 46\cdot0$***	$F_{8,254} = 9\cdot5$***	$F_{32,254} = 5\cdot5$***
A	$F_{1,254} = 235\cdot7$***	$F_{6,254} = 108\cdot6$***	$F_{8,254} = 40\cdot4$***	$F_{32,254} = 3\cdot9$***
$A/g_{\rm ST}$	$F_{1,254} = 1\cdot0$ n.s.	$F_{6,254} = 34\cdot6$***	$F_{8,254} = 34\cdot2$***	$F_{32,254} = 5\cdot0$***
Plants re-watered subsequent to drought (Re-watering phase, days 48–75)				
$g_{\rm ST}$	$F_{1,265} = 21\cdot6$***	$F_{8,265} = 34\cdot9$***	$F_{10,265} = 4\cdot5$***	$F_{40,265} = 5\cdot2$***
A	$F_{1,265} = 98\cdot4$***	$F_{8,265} = 84\cdot1$***	$F_{10,265} = 26\cdot0$***	$F_{40,265} = 4\cdot2$***
$A/g_{\rm ST}$	$F_{1,265} = 20\cdot7$***	$F_{8,265} = 11\cdot1$***	$F_{10,265} = 19\cdot7$***	$F_{40,265} = 4\cdot6$***

All parameters were compared between well-watered controls and drought-treated plants and the analyses tested for the interacting effects of treatment, date, photosynthetic type and species. Separate analyses were conducted for the drought and re-watering phases of the experiment and levels of significance are indicated as: n.s. (not significant) $P > 0\cdot05$; *$P < 0\cdot05$; **$P < 0\cdot01$; ***$P < 0\cdot001$.

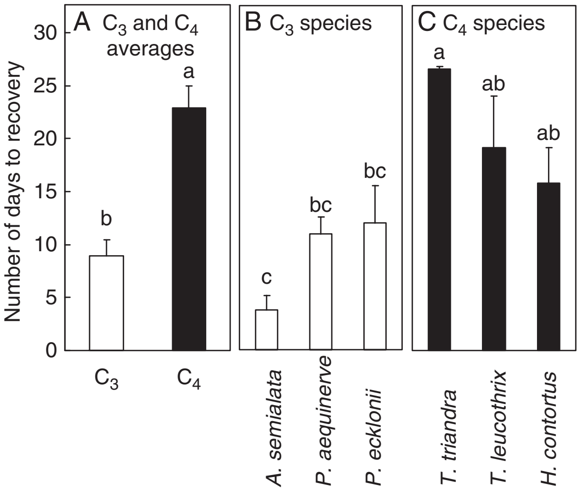

FIG. 3. The number of days required for re-watered C_3 or C_4 Panicoid grasses to recover photosynthetic rates. Data are averaged by photosynthetic type (A) or are presented for individual species (B, C). Values are means ± s.e. and $n \geq 5$ for each species. Different lower-case letters indicate significant differences between means for individual species or differences between averages for photosynthetic types at $P < 0\cdot05$ (Fisher LSD test).

decline in A than in $g_{\rm ST}$, but rapidly recovered on re-watering (Fig. 2N, P). Like photosynthetic rates, $A/g_{\rm ST}$ values for C_4 species only became superior to those for C_3 species by the final day of the experiment.

Responses of photosynthesis to stomatal conductance

A single straight-line relationship described the initial response of C_3 A to $g_{\rm ST}$ for all species, across all levels of drought, at $g_{\rm ST}$ values below $0\cdot15$ mol H_2O m^{-2} s^{-1} (Fig. 4A). As $g_{\rm ST}$ was decreased by drought, A was shifted significantly along this relationship (WALD test, $P < 0\cdot001$). In contrast, the C_4 species relationship of A to $g_{\rm ST}$ at values lower than $0\cdot25$ mol H_2O m^{-2} s^{-1} could not be described by an identical relationship across all treatments. The relationship of A to $g_{\rm ST}$ was similar for plants measured under well-watered conditions and after 20 d of drought, but the slope was successively decreased after 36 and 48 d of drought (Fig. 4B).

CO_2 response curves

CO_2 response curves of well-watered pot plants demonstrated typical C_3 and C_4 variation. The C_3 species had consistently higher CO_2 compensation points and lower carboxylation efficiencies than the C_4 species. C_3 photosynthesis saturated at a $C_i > 100$ Pa and at ambient conditions had an operating C_i of $22\cdot6$–$31\cdot4$ Pa (Fig. 5A–C). In contrast, the C_4 species were saturated at a C_i of approx. 40 Pa and operated with a C_i of $20\cdot5$–$21\cdot5$ Pa (Fig. 5D–F).

In all cases, drought significantly decreased both the estimated carboxylation efficiencies (k and $V_{\rm cmax}$) and CO_2-saturated photosynthetic rates ($V_{\rm max}$ and $J_{\rm max}$; Table 2). After 36 d of drought, k and $V_{\rm cmax}$ decreased by on average 64 and 54 %, respectively, while $V_{\rm max}$ and $J_{\rm max}$ decreased by 55 and 46 %, respectively. The magnitude of these reductions was not always consistent across species for each photosynthetic type, resulting in a significant species-by-drought interaction (Table 2). These parameters could not be calculated for the plants subjected to 48 d of drought as complete CO_2 response curves were not constructed for these plants. However, the selected points that were measured indicate further reductions in both carboxylation efficiencies and CO_2-saturated photosynthetic rates (Fig. 5A–F).

Thirty-six days of drought reduced average C_4 photosynthetic rate by 54 %, of which 6 % could be attributed to stomatal limitations ($R_{\rm SL}$) and 48 % to metabolic limitations ($R_{\rm ML}$; Fig. 6A). At the same stage of drought the C_3 average photosynthetic rates were similarly decreased by 50 %, but 24 % was due $R_{\rm SL}$ and 26 % due to $R_{\rm ML}$ (Fig. 6A).

Prolonging the drought to 48 d resulted in a 92 % decline in C_4 photosynthetic rates with increases in $R_{\rm SL}$ to 17 % and $R_{\rm ML}$ to 75 %. Under the same conditions C_3 photosynthesis decreased by 88 %, of which 18 % was due to $R_{\rm SL}$ and 70 % to $R_{\rm ML}$ (Fig. 6A). These differences in the change in the magnitude of $R_{\rm SL}$ and $R_{\rm ML}$ with increasing drought resulted in significant type-by-date interactions, although the magnitude of these changes was not always consistent between species within a photosynthetic type (Table 3), the notable exception

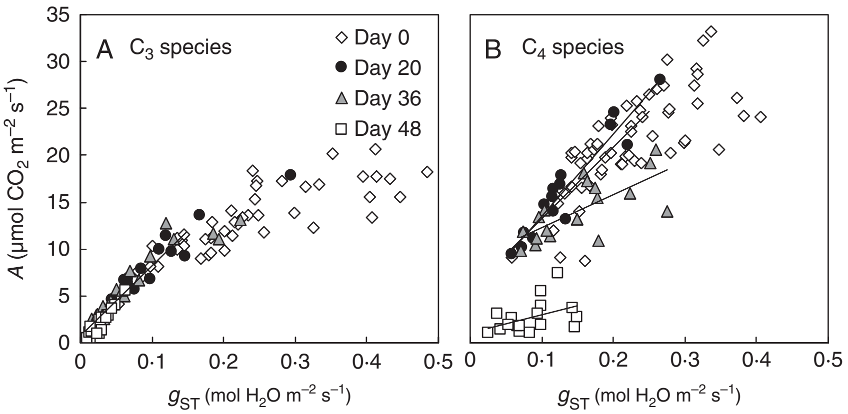

FIG. 4. Response of net leaf photosynthesis (*A*) to stomatal conductance (g_{ST}) of C_3 (A) and C_4 (B) plants subjected to controlled drought. Species data for each photosynthetic type were pooled and the different symbols indicate the number days for which water was withheld. For the C_4 species individual lines were fitted to the initial response of *A* to g_{ST} measured on each date because the SMA test (at $P < 0{\cdot}05$) indicated significant heterogeneity among slopes (Wald test, $P < 0{\cdot}01$). Individual linear regression statistics are as follows: day 0, $r^2 = 0{\cdot}55$, $F_{1,39} = 50$, $P < 0{\cdot}0001$; day 20, $r^2 = 0{\cdot}9$, $F_{1,13} = 133$, $P < 0{\cdot}0001$; day 36, $r^2 = 0{\cdot}5$, $F_{1,17} = 18$, $P < 0{\cdot}001$; day 48, $r^2 = 0{\cdot}2$, $F_{1,16} = 5$, $P = 0{\cdot}05$. A single slope described this response for the C_3 species (Wald test, $P > 0{\cdot}09$) and regression statistics are: $r^2 = 0{\cdot}86$, $F_{1,91} = 592$, $P < 0{\cdot}0001$.

being *P. aequinerve* which developed a large R_{SL} even at the severest level of drought (Fig. 6B, C).

DISCUSSION

Our results demonstrate that for co-occurring grasses within this single subfamily (Panicoideae), the sensitivity of photosynthesis to drought and the limitations responsible for this are different between C_3 and C_4 (NADP-ME) species. These differences between photosynthetic types are distinct despite interspecific variation and because our methods meant that slow-dehydrating species were subject to continuous drought, whereas others were occasionally given small volumes of water to ensure a uniform rate of drying. Under well-watered conditions, and consistent with an extensive literature (e.g. Long, 1999), the C_4 species had higher photosynthetic rates and A/g_{ST} than the C_3 species. This advantage was maintained during the initial period of the drought, but was lost as the drought became more severe, and took in excess of 20 d to be regained after re-watering. The loss of this advantage could be attributed to differences in the response of C_3 and C_4 stomatal conductance and photosynthetic potential to drought. The more sensitive and larger metabolic limitation in the C_4 plants was correlated with slower recovery from drought, while the predominance of stomatal limitation in the C_3 species allowed more rapid recovery. These findings provide comparative results between C_3 and C_4 grass species, data lacking in the current debate on the relative roles of these mechanisms in plant drought responses (Chaves *et al.*, 2009; Lawlor and Tezara, 2009). The results also suggest that the significance of the C_4 photosynthetic and water-use efficiency will depend both on the severity of the drought and frequency of rainfall events, determining both the degree of inhibition of gas exchange and its rate of recovery. This is important as C_4 water-use efficiency is considered a mechanism for maintaining soil water status and prolonging productivity after rainfall (Ehleringer and Monson, 1993; Kalapos *et al.*, 1996).

As has been characterized in many C_3 and C_4 species, drought decreased *A* through a combination of stomatal and metabolic limitations (Lawlor, 2002; Ghannoum *et al.*, 2003; Flexas *et al.*, 2006), but the magnitude of these responses differed in these C_3 and C_4 grasses, and changed as drought progressed. Under well-watered conditions, the C_3 species had R_{SL} values twice as high as those of the C_4 species and the importance of R_{ML} increased with drought. In the C_4 species, R_{ML} predominated under much less severe conditions, demonstrating the sensitivity of C_4 photosynthesis to drought. Similar differences were noted when comparing the drought response of C_3 and C_4 subspecies of *Alloteropsis semialata* (Ripley *et al.*, 2007) and, as with the present study, increased metabolic limitations were attributed to changes in the initial slope, curvature and CO_2-saturated values of the A/C_i curves. These changes indicate reductions in the rate of the C_3 and C_4 cycles, decreased bundle sheath or mesophyll conductance to CO_2, decreased Rubisco activity, and decreased rates of RuBP regeneration (Krieg and Hutmacher, 1986; von Caemmerer, 2000). The underlying mechanism responsible for these changes in C_3 species has been attributed to decreased ATP production (Lawlor and Tezara, 2009), although other mechanisms may be involved and have recently been reviewed by various authors (e.g. Flexas and Medrano, 2002; Lawlor, 2002; Flexas *et al.*, 2004; Lawlor and Tezara, 2009). Similar mechanisms have been proposed for C_4 species (Ghannoum, 2009; Lawlor and Tezara, 2009), and are probably complicated by the additional metabolism associated with the CO_2-concentrating mechanism, but as yet are unresolved. Furthermore, the interpretation of these A/C_i data are reliant on assumptions that do not necessarily hold under conditions of severe drought and may result in the overestimation of C_i (Terashima *et al.*, 1988). The interpretation is further complicated by the affects of drought on mesophyll conductance (g_M), the quantification of which remains controversial in C_3 species (Warren and Adams, 2006; Lawlor and Tezara, 2009) and is not yet possible for C_4 plants. However despite these concerns, A/C_i curves retain their value in

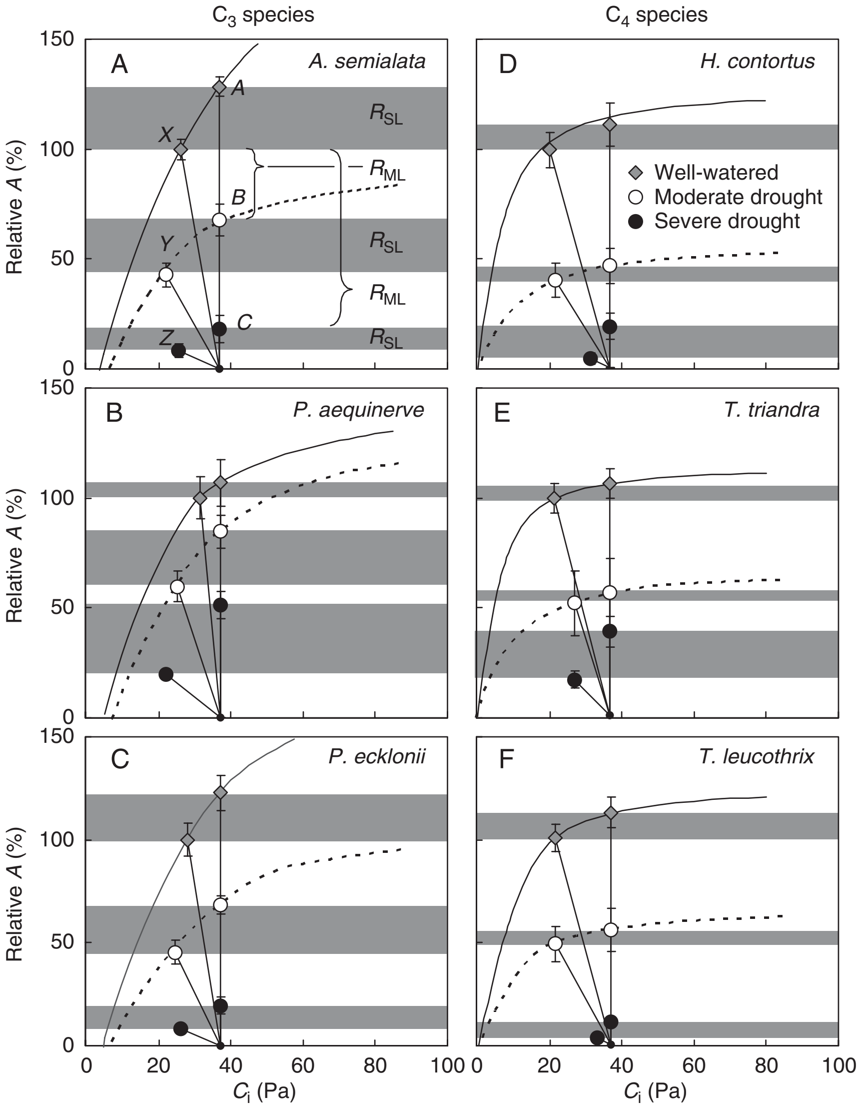

FIG. 5. A/C_i responses for C_3 (A–C) and C_4 (D–F) Panicoid grasses during three watering treatments: well-watered, moderate drought and severe drought, as indicated. Photosynthetic rates are expressed as a percentage of the rates measured at $C_a = 38$ Pa for well-watered plants (X). During the severe drought treatment, photosynthetic rates were only measured at a C_a of 38 Pa, or at a C_a corresponding to a C_i of 38 Pa. Also shown are the average photosynthetic rates at an atmospheric CO_2 concentration (C_a) of 38 Pa assuming no stomatal limitation (i.e. $C_a = C_i = 38$ Pa; A, B and C) and supply functions representing the limitation on A imposed by CO_2 diffusion through the stomata (X, Y and Z) for plants treated with three levels of drought. Each of these functions has a slope, which is set by the stomatal conductance and intercepts the C_i axis at C_a. $n \geq 4$ and vertical bars represent standard errors. Shading indicates relative stomatal limitations (R_{SL}) and brackets the relative metabolic limitations (R_{ML}).

demonstrating a drought-induced reduction in photosynthetic potential (Lawlor and Tezara, 2009).

Evidence for more complex mechanisms contributing to the metabolic limitations in C_4 plants comes from examination of the $A-g_{ST}$ responses of these drought-stressed grasses. In the C_3 species this response was described by a single relationship, demonstrating that irrespective of drought conditions, CO_2 supply and use are closely regulated, a phenomenon that is well described in the literature on C_3 plants (Schulze and Hall, 1982; Brodribb, 1996). In contrast, the C_4 grass species did not show this simple relationship and photosynthesis deviated from the initial $A-g_{ST}$ relationship as drought progressed, showing that this regulation was uncoupled under conditions of more severe drought.

The loss of superior C_4 photosynthetic rate only occurred under the most severe conditions of drought, while their A/g_{ST} advantage was lost under less severe conditions. The loss of the C_4 A/g_{ST} advantage could not be ascribed to differences in the contributions of R_{SL} and R_{ML} given that A declined by a similar amount in both C_3 and C_4 species over the initial 36 d. Instead the loss occurred because of differences in the stomatal responses and because C_4 species maintained higher g_{ST} than C_3 species. This might reflect differences in plant hydraulic architecture (Kocacinar and Sage, 2003) and strategies of water use (Long, 1999), and the higher g_{ST} in C_4 species combined with less negative values of Ψ_{leaf} implies a higher hydraulic conductance than was evident for the C_3 species. The initial response of A/g_{ST}

TABLE 2. *A/C_i parameters and GLM results of a comparison between C_3 and C_4 Panicoid species in response to conditions of adequate water supply and decreasing SWC measured on days 0 and 36*

Model parameter		Species			Species	Drought	Species × Drought
C_4 species		*H. contortus*	*Th. triandra*	*Tr. leucothrix*			
Control	k (mol m^{-2} s^{-1})	0·39 ± 0·10	0·28 ± 0·01	0·24 ± 0·03	$F_{2,30}$ = 0·86 n.s.	$F_{1,30}$ =21·6***	$F_{2,30}$ = 0·8 n.s.
Drought		0·10 ± 0·04	0·12 ± 0·07	0·10 ± 0·02			
Control	V_{max} (μmol m^{-2} s^{-1})	30·9 ± 3·7	22·4 ± 1·0	32·5 ± 2·6	$F_{2,30}$ = 4·1**	$F_{2,30}$ = 68·6***	$F_{1,30}$ = 0·9 n.s.
Drought		12·8 ± 1·6	11·0 ± 2·9	14·8 ± 2·5			
C_3 species		*A. semialata*	*P. aequinerve*	*P. ecklonii*			
Control	V_{cmax} (μmol m^{-2} s^{-1})	107·8 ± 7·3	57·2 ± 5·7	148·8 ± 10·2	$F_{2,30}$ = 3·0 n.s.	$F_{1,30}$ = 20·3***	$F_{2,30}$ = 0·9 n.s.
Drought		48 ± 6·5	36·9 ± 11·4	77·1 ± 10			
Control	J_{max} (μmol m^{-2} s^{-1})	44·9 ± 1·5	38·3 ± 15·4	64·0 ± 4·8	$F_{2,30}$ = 31·2***	$F_{1,30}$ = 56·0***	$F_{2,30}$ = 13·3***
Drought		23·9 ± 3·5	14·2 ± 5·3	31·5 ± 4·6			

For each photosynthetic type, these analyses tested for the interacting effects of species and drought. Abbreviations for A/C_i parameters are: initial slope of the C_4 photosynthetic response (k), C_4 maximum Rubisco capacity (V_{max}), C_3 maximum rate of Rubisco carboxylation (V_{cmax}) and the apparent C_3 maximum rate of photosynthetic electron transport (J_{max}). Levels of significance are indicated as: n.s. (not significant) $P > 0·05$;*$P < 0·05$;**$P < 0·01$;***$P < 0·001$.

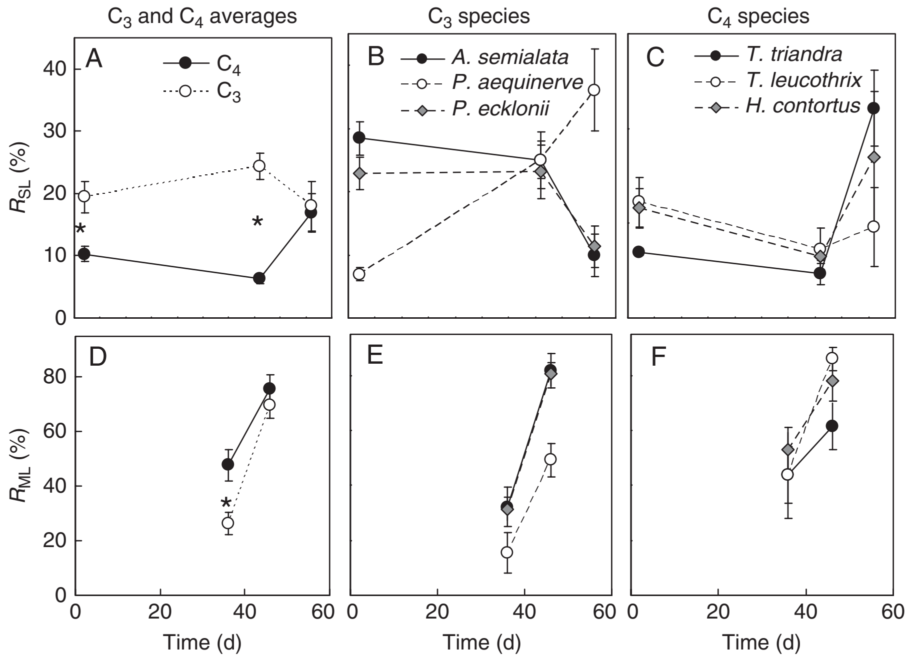

FIG. 6. Relative stomatal (R_{SL}) and metabolic (R_{ML}) limitations of photosynthesis for Panicoid grasses subjected to controlled drought. Data are averaged by photosynthetic type (A, D) or are presented for individual C_3 (B, E) or C_4 (C, F) species. Values are means ± s.e. and $n \geq 4$ for each species. *Significant differences between means on the different days at $P < 0·05$ (Fisher LSD test).

TABLE 3. *GLM results of a comparison of relative stomatal (R_{SL}) and metabolic (R_{ML}) limitations between C_3 and C_4 Panicoid species (represented as species nested in photosynthetic type) in response to conditions of adequate water supply and decreasing SWC measured on days 0, 36 and 48*

Parameter	Photosynthetic type	Species	Date	Type × date	Species × date
R_{SL}	$F_{1,66}$ = 6·7*	$F_{4,66}$ = 4·7**	$F_{2,66}$ = 53***	$F_{2,66}$ = 39***	$F_{8,66}$ = 1·6 n.s.
R_{ML}	$F_{1,37}$ = 43·7***	$F_{4,37}$ = 0·7 n.s.	$F_{1,196}$ = 1·3 n.s.	$F_{1,196}$ = 7·2**	$F_{4,37}$ = 10·0***

The analysis tested for the interacting effects of species, photosynthetic type and date. Levels of significance are indicated as: n.s. (not significant) $P > 0·05$;*$P < 0·05$;**$P < 0·01$;***$P < 0·001$.

contrasted with that noted under more severe conditions, when the reduction in A/g_{ST} was due to dramatically reduced C_4 photosynthetic rates combined with higher g_{ST}. Decreased instantaneous water-use efficiency in response to drought has been reported for various C_4 species (Marques da Silva and Arrabaça, 2004; Xu *et al.*, 2006), but data comparing closely related C_3 and C_4 species are limited to our work on *A. semialata*, where a similar loss of the A/g_{ST} advantage was demonstrated both in a controlled pot drought (Ripley *et al.*, 2007), and in a common garden experiment under natural rainfall conditions (Ibrahim *et al.*, 2008).

C_4 water-use efficiency contributes to a competitive advantage in certain environments (Ehleringer and Monson, 1993; Kalapos *et al.*, 1996) and its loss and the length of time required for its recovery would alter competition, and may constrain C_4 species distributions. The present study demonstrated the loss of this C_4 A/g_{ST} superiority, which took 20 d of well-watered conditions to be regained. The treatment applied was representative of field conditions and at day 36 produced a similar range of Ψ_{leaf} to those measured in the field ($-1{\cdot}8$ to -3 MPa), when SWC was approx. 5 %. Even the more severe conditions on day 48 are likely to occur in the field, and over the last 6 years the Faraway Farm site has experienced nine occasions when less than 10 mm month^{-1} of rain fell during the growing season (South Africa Weather Services). During the present study, when 17 mm of rain fell over the 29-d period (16 December to 24 January) SWC was reduced to 5 % (Fig. 1A), and hence these nine periods of more severe drought would probably have resulted in SWC of less than 5 %. Data on the recovery of other C_4 species are mainly available for maize (Lal and Edwards, 1996; Saccardy *et al.*, 1996; Foyer *et al.*, 1998) and sorghum (Loreto *et al.*, 1995), where the treatments imposed were mild and recovery was both rapid and complete. Hence, further research is needed on the responses of non-crop C_4 species to drought treatments of severities that are ecologically relevant.

Conclusions

The present results demonstrate that the loss of the photosynthetic advantage of C_4 NADP-ME Panicoid grasses relative to C_3 Panicoid grasses occurs only under conditions of severe drought, while the A/g_{ST} advantage was lost under less severe conditions. The underlying mechanisms for these responses were differences in the dominance of C_3 and C_4 stomatal and metabolic limitations and how these changed under conditions of drought. Differences in the $A-g_{ST}$ response to drought suggested the operation of alternative or additional mechanisms of photosynthetic inhibition in the C_4 species, which might explain why the recovery from drought differed between the C_3 and C_4 species.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and show midday leaf water potential (Ψ_{leaf}), net leaf photosynthesis (A), stomatal conductance (g_{ST}) and instantaneous water-use efficiency (A/g_{ST}) of well-watered C_3 and C_4 Panicoid grasses.

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