Functional Ecology

CHANDRA BELLASIO (Orcid ID : 0000-0002-3865-7521)

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C$_4$ savanna grasses fail to maintain assimilation in drying soil under low CO$_2$ compared with C$_3$ trees despite lower leaf water demand

Joe Quirk[1]*, Chandra Bellasio[1-4]*, David A. Johnson[1], Colin P. Osborne[1] and David J. Beerling[1]

[1]*Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, UK*
[2]*Research School of Biology, Australian National University, Acton, ACT, 2601 Australia;*
[3]*University of the Balearic Islands 07122 Palma, Illes Balears, Spain;*
[4]*Trees and Timber institute, National Research Council of Italy, 50019 Sesto Fiorentino (Florence).*
Correspondence: chandra.bellasio@anu.edu.au; *authors contributed equally

Summary

1) C$_4$ photosynthesis evolved when grasses migrated out of contracting forests under a declining atmospheric CO$_2$ concentration ([CO$_2$]$_a$) and drying climate around 30 million years ago. C$_4$ grasses are hypothesised to benefit from improved plant–water relations in open habitats like savannas, giving advantages over C$_3$ plants under low [CO$_2$]$_a$. But experimental evidence in a low CO$_2$ environment is limited and comparisons with C$_3$ trees are needed to understand savanna vegetation patterns.

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2) To test whether stomatal conductance (g_S) and CO_2 assimilation (A) are maintained in drier soil for C_4 grasses than C_3 trees, particularly under low $[CO_2]_a$, we investigated photosynthesis and plant–water relations of three C_3 tree and three C_4 grass species grown at 800, 400 or 200 ppm $[CO_2]_a$ over moderate wetting–drying cycles.

3) C_4 grasses had a lower soil–to–leaf water potential gradient than C_3 trees, especially at 200 ppm $[CO_2]_a$, indicating reduced leaf water demand relative to supply. Yet the dependence of g_S and A on predawn leaf water potential (a measure of soil water availability) was greater for the C_4 grasses than trees, particularly under low $[CO_2]_a$.

4) Our findings establish that g_S and A are not maintained in drier soil for C_4 grasses compared with C_3 trees, suggesting that this mechanism was not prevailing in the expansion of C_4–dominated grasslands under low $[CO_2]_a$. This inherent susceptibility to sudden decreases in soil water availability justifies why C_4 grasses have not evolved a resistant xylem allowing operation under drought, but instead shut down below a water potential threshold and rapidly recover. We point to this capacity to respond to transient water availability as a key overlooked driver of C_4 grass success under low $[CO_2]_a$.

Introduction

The C_4 photosynthetic pathway concentrates CO_2 around the ancestral C_3 photosynthetic machinery, allowing high CO_2 assimilation (A) for relatively low stomatal conductance (g_S) and canopy evapotranspiration (E), which leads to higher water–use efficiency (WUE=A/E) and potential soil water savings (Ward *et al.* 1999; Anderson *et al.* 2001; von Caemmerer & Furbank 2003; Taylor *et al.* 2011). The C_4 pathway evolved independently around 30 million years ago in multiple angiosperm lineages, after a sharp reduction (~75%) in atmospheric CO_2 concentration ($[CO_2]_a$) towards 180 ppm (Sage 2004; Edwards *et al.* 2010; Christin & Osborne 2014). Under declining $[CO_2]_a$, g_S increases to allow greater CO_2 diffusion into the leaf, causing higher E, a direct reduction of WUE and higher risk of reduced leaf water status (Temme *et al.* 2013; Pinto *et al.* 2014). Compared with the C_3 type, C_4 photosynthesis is advantageous when $[CO_2]_a$ is low, and temperature and irradiance are high, leading to the view that falling $[CO_2]_a$ selected for C_4 photosynthesis (Ehleringer & Björkman 1977; Ehleringer *et al.* 1991; Ehleringer, Cerling & Helliker 1997). This view is supported by constraints on the timing of C_4 evolution (Christin *et al.* 2008; Edwards *et al.* 2010) and

multi–proxy evidence for relatively low $[CO_2]_a$ over the same period (Beerling & Royer 2011). It is now thought that the evolution of C_4 photosynthesis and the subsequent diversification of C_4 lineages was also driven, in part, by the benefits of reduced water use in open, seasonally dry environments characterised by high evaporative demand and risk of hydraulic failure, such as grasslands and savannas (Osborne & Freckleton 2009; Osborne & Sack 2012; Griffiths *et al.* 2013; Spriggs, Christin & Edwards 2014).

Savannas are meta–stable, disturbance–driven ecosystems and can transition rapidly between open, C_4–dominated grassland with scattered C_3 trees, and the alternative state of closed broad–leaf forest. The dominant disturbance in most contemporary savannas is fire – the frequency and intensity of which depends on the amount and seasonality of rainfall – but disturbance from large–bodied mammalian herbivores was also important before Quaternary megafaunal extinctions (Edwards *et al.* 2010; Edwards & Smith 2010; Hirota *et al.* 2011; Staver, Archibald & Levin 2011; Charles-Dominique *et al.* 2016). Under low Neogene $[CO_2]_a$, productive C_4 grasses supported large–scale grazing and fuelled fire activity that are thought to have suppressed woody plant recruitment, promoting open habitats (Beerling & Osborne 2006; Bond 2008; Bond & Midgley 2012; Hoetzel *et al.* 2013; Charles-Dominique *et al.* 2016). Subsequently, C_4–dominated grasslands expanded from mixed C_3 and C_4 grasslands *around* 8–10 million years ago, and in some places, C_4 grasses displaced C_3 trees (Osborne & Beerling 2006; Edwards *et al.* 2010; Strömberg 2011). Recent long–term field observations, however, indicate that effects of soil properties on plant water availability and use by trees and grasses are as important as fire disturbance for determining savanna vegetation patterns (Staver, Botha & Hedin 2017). Declining $[CO_2]_a$ modifies plant–water relations, but how $[CO_2]_a$ affects the stomatal and photosynthetic responses of savanna trees and C_4 grasses to drying soils is poorly resolved.

Osborne and Sack (2012) hypothesised that, alongside its benefits for carbon fixation, C_4 photosynthesis was selected for to conserve water and protect the vascular system by reducing *E*. Using comparative studies of C_3 and C_4 grasses to inform and parameterise simple models, Osborne and Sack (2012) reasoned that better water conservation during soil and atmospheric drying allows C_4 plants to maintain *A* under moderate water shortage. Further, they hypothesised that for C_4 grasses, maintenance of g_S (water demand) at lower Ψ_{soil} would be linked with higher *A* compared with C_3 grasses, especially under low $[CO_2]_a$, but only if soil–to–leaf water supply remained relatively high. However, their simulations suggested that, to benefit from this mechanism, C_4 grasses needed to maintain a similar or higher supply of water from soil and roots to that in C_3 grasses. The Osborne and Sack (2012) proposal helps explain competitive advantages of C_4 grasses over C_3 grasses in

ecosystems subject to periodic soil drying under low $[CO_2]_a$. However, empirical comparisons between C_3 and C_4 plants under low $[CO_2]_a$ have largely focused on grasses and other herbaceous plants (Polley, Johnson & Mayeux 1992; Ward *et al.* 1999; Anderson *et al.* 2001; Ripley, Cunniff & Osborne 2013) and the comparisons between C_4 grasses and C_3 trees that are needed to understand savanna vegetation patterns have not been made.

We identified four hypotheses arising from Osborne and Sack (2012) to test whether improved plant–water relations for C_4 grasses translate into physiological advantages over trees under low $[CO_2]_a$ and variable soil water availability. Firstly, 1) water demand, relative to supply, will be lower for C_4 grasses compared with C_3 trees. Osborne and Sack (2012) characterised leaf water demand to supply as the ratio of g_S to plant conductance (K_{plant}). As leaf E is the product of g_S and the leaf–to–boundary layer water mole fraction gradient (D_S), and because K_{plant} is E divided by the soil–to–leaf gradient in water potential ($\Delta\Psi$), by definition $g_S/K_{plant} \equiv \Delta\Psi/D_S$ – a convenient proxy for the ratio of water demand to supply. Secondly, 2) the dependence of g_S and A on predawn leaf water potential, Ψ_{PD} (a proxy for soil water potential) will be lower for C_4 grasses than trees, such that 3) g_S and A will be maintained in drier soil for the C_4 grasses compared with trees. Finally, 4) the combined effects of Hypotheses 2 and 3 will favour C_4 grasses relative to trees under low $[CO_2]_a$.

We tested these hypotheses by comparing leaf gas exchange and plant–water relations of three tree species, spanning broad–leaf tropical forest and open savanna habitats, and three C_4 savanna grass species, grown in replicated controlled environments at 800, 400 or 200 ppm $[CO_2]_a$. We isolated stomatal and photosynthetic responses to soil wetting–drying cycles, as opposed to evaporative demand, by maintaining constant atmospheric vapour pressure deficit (VPD) within the growth chambers and focussed our analyses at leaf–level to avoid confounding influences of canopy size and growth rates of the different plant functional types.

Materials and Methods

Plants and growth conditions

Seeds of *Vachellia karroo* (Hayne) were obtained from the Desert Legume Program, (Tucson, AZ, US), and both *Combretum apiculatum* (Sond.) and *Celtis africana* (N.L.Burm.) from Silverhill Seeds (Cape Town, ZA). Seeds of the C_4 grasses *Eragrostis curvula* ([Schrad.] Nees) (accession number PI–155434), *Heteropogon contortus* ([L.] P.Beauv. ex Roem. & Schult.) (PI–228888) and *Themeda triandra* (Forssk.) (PI–208024) were obtained from the Germplasm Resources Information Network (Agricultural Research Service, USDA,

Washington DC, US). *V. karroo* is a fire–tolerant nitrogen–fixing leguminous tree typical of dry savannas; *Combretum* spp. are common in miombo closed savanna woodland; and *C. africana* is a representative forest tree. The C_4 grasses span a range of adaptations to fire and drought and are broadly representative of open African savannas (O'Connor 1994; Fynn & Naiken 2009; Kgope, Bond & Midgley 2010).

The trees and grasses were grown in 3 dm^3 pots (one plant per pot $n = 4$–10) filled with three–parts loam–free top soil (Boughton Ltd. Kettering, GB) plus one part John Innes No.3 compost (John Innes Manufacturers Association, Reading, GB). This substrate mixture was chosen because, of a range of soil, sand and compost mixtures tested, it had the steadiest decline in soil matric potential from field capacity within a plant free pot under regular cabinet conditions (SI Figure S1). Tree seedlings were randomly distributed between six controlled environment growth chambers (Conviron BDR16, Conviron, Manitoba, CA) and grown for 18 months prior to measurements. Meanwhile a plant from each grass species was randomly selected, split into individuals at the rhizome, distributed between the six growth chambers and grown for six months prior to measurements. From the outset, plants were watered to gravimetrically determined 80% of pot capacity three times per week after 24–32 photoperiod hours since last watering and all pots were provided with 150 ml of 3:1:2 N:P:K soluble nutrient mix (Miracle–Gro® All Purpose Plant Feed, Scotts Miracle–Gro, Marysville, OH, US) diluted to 5g nutrient mix L^{-1} water every two or three weeks as part of the watering volume.

Growth chambers were maintained at 200, 400, or 800 ppm $[CO_2]_a$ (two chambers per $[CO_2]_a$ treatment) and constant conditions of 26 : 17 °C and 70 : 50 % relative humidity (day : night). A 12–hr photoperiod with a midday maximum photosynthetic photon flux density (PPFD) of 800 µmol m^{-2} s^{-1} was imposed at canopy level provided from 48 fluorescent tubes in a 3:1 mix of 39–W white (Master TL5, Philips, Eindhoven, NL) and 39–W red–blue (Grolux T5, Havells–Sylvania, Newhaven, GB) tubes, augmented with six 105–W halogen bulbs (GLS, Havells–Sylvania). This combination provided suitable common growing conditions for both trees and C_4 grasses in dedicated pilot trials. Low $[CO_2]_a$ was achieved by routing chamber air supply through a scrubbing unit of sodalime (Sofnolime, 1–2 mm, Molecular Products, Essex, GB) and $[CO_2]_a$ was monitored using a CO_2 sensor (Carbocap GMP242, Vaisala, FI) linked to a feedback system regulating air inlet between the scrubbing unit and ambient air. The 400 ppm $[CO_2]_a$ treatment was supplied with ambient air and 800 ppm $[CO_2]_a$ was achieved with automated injections of pure CO_2. Chamber $[CO_2]_a$ was checked with an infrared gas analyser (IRGA, LI6400XT, Li–Cor Biosciences, Lincoln,

NE, US). Plants were randomly distributed between cabinets with a maximum of ten plants per cabinet (over multiple growth cycles) to allow complete separation between plants. Plants were rotated weekly within, and monthly between, cabinets along with environmental settings to minimise block effects. We measured the biomass of selected species (*V. karroo*, *C. africana* and *E. curvula*) for which biomass was available at the end of the experiment

Operational leaf gas exchange

Instantaneous leaf gas exchange was measured in the afternoon on young, fully expanded leaves three times over six weeks under operational environmental conditions after a night and ~12 photoperiod hours since watering. The IRGA was fitted with a 6 cm^2 leaf chamber and a red–blue LED light source (6400–02B, Li–Cor Biosciences). Three to four grass leaves were aligned side by side, avoiding gaps and overlaps, and clamped between the gaskets to fill the entire leaf chamber. For tree leaves that did not fill the leaf chamber, leaf area was measured using scaled, digital images processed in ImageJ (NIH, Bethesda, MA, US). Flow rate was 235 µmol s^{-1}, chosen to increase signal / noise ratio following the recommendations of Bellasio, Beerling and Griffiths (2016), reference [CO_2] (supplied from cartridges, Liss, Répcelak, HU) was 200, 400 or 800 µmol mol^{-1}, block temperature was 26 °C, PPFD was 500 µmol m^{-2} s^{-1} and VPD was between 1.2 and 1.6 kPa, to match mid–afternoon growth conditions. After readings stabilised, a 10 s average was recorded as a single point measurement. To minimise environmental perturbations and error associated with CO_2 leakage from the IRGA, the leaf chamber was positioned inside, and supplied with air from within, the growth chambers.F

Plant water relations

Operational assimilation (A_{op}), E, g_S and the leaf–to–boundary layer water mole fraction gradient (D_S, mmol H_2O mol^{-1} air = 10×VPD) were obtained from instantaneous gas exchange measurements, and operational WUE was calculated as $WUE = A_{op}/E$. Leaf water potential at midday (Ψ_{MD}) and pre–dawn (Ψ_{PD}, a measure of soil water potential) were measured on leaves cut the day and night following instantaneous gas exchange, using a Scholander pressure chamber (PMS Instrument Company, Model 1000, Albany, OR, US) and the soil–to–leaf gradient in Ψ was calculated as $\Delta\Psi = \Psi_{PD} - \Psi_{MD}$.

Statistical analysis

A_{op}, g_{Sop}, Ψ_{PD}, Ψ_{MD}, $\Delta\Psi$, $\Delta\Psi/D_S$, WUE and aboveground and root biomass were subject to two–way ANOVAs using a general linear model (GLM) framework testing for effects of species (nested within photosynthetic type, PT), $[CO_2]_a$, and their interaction. Specific differences between means were tested with *post–hoc* Tukey pairwise comparisons. To satisfy assumptions of normality of variance within the GLMs we took the square root of A_{op} [$\sqrt{(A_{op})}$] and the natural log. (ln) of g_S, Ψ_{PD}, Ψ_{MD}, $\Delta\Psi$ and WUE. Separate three–way analyses testing the dependence of $\sqrt{(A_{op})}$ on either $\ln(g_S)$ or $\ln(\Delta\Psi)$, alongside $[CO_2]_a$ and PT were performed; and further regressions testing the dependence of $\sqrt{(A_{op})}$ and $\ln(g_S)$ separately on $\ln(\Psi_{PD})$ were conducted at the level of PT × $[CO_2]_a$. The level of biological replication was *n* = 4–10 (in which biological replicates are the mean of triplicate technical replicates) unless indicated otherwise. All GLMs and regressions were fitted and analysed in Minitab v.17 (Minitab Inc., State College, PA, US) with a significance threshold of 95%.

Results

Stomatal effects on assimilation

Operational assimilation (A_{op}) was significantly lower for trees grown at 200 ppm compared with 400 or 800 ppm $[CO_2]_a$ (Figure 1; SI Figure S2, Tables S1 and S2). Responses of A_{op} to growth $[CO_2]_a$ for the grasses were species–specific: A_{op} for *E. curvula* was unaffected by $[CO_2]_a$, while both *T. triandra* and *H. contortus* operated higher A_{op} at 800 ppm than at lower $[CO_2]_a$. The slope of the relationship between $\ln(g_S)$ and $\sqrt{(A_{op})}$ was steeper for the grasses grown at 200 and 400 ppm $[CO_2]_a$ than for the trees (Figure 1), highlighting the higher sensitivity of the C$_4$ grasses to declines in g_S, in line with previous findings for C$_3$ and C$_4$ grasses (Ripley, Frole & Gilbert 2010; Taylor *et al.* 2010; Taylor *et al.* 2011). The steeper relationship between A and g_S for the C$_4$ grasses translated into 25% higher WUE on average across species at 200 ppm $[CO_2]_a$, and ~20% higher WUE on average for the grasses at 400 ppm and 800 ppm $[CO_2]_a$, compared with the trees (Table S1).

Regression analysis confirmed that the $\ln(g_S)$–$\sqrt{(A_{op})}$ slopes were steeper for trees grown at 400 ppm or 800 ppm than at 200 ppm $[CO_2]_a$, and that the opposite $[CO_2]_a$ trend was observed for the grasses (Figure 1; three–way interaction effect of $\ln(g_S)$ × $[CO_2]_a$ × PT on the response of $\sqrt{(A_{op})}$: $F_{2, 431} = 28.8$; $P < 0.0001$), indicating that the photosynthetic rates of the C$_4$ grasses growing under low $[CO_2]_a$ were most sensitive to stomatal responses. At 200 ppm $[CO_2]_a$, *E. curvula* attained at least double the A of the C$_3$ trees, but at 800 ppm $[CO_2]_a$,

A_{op} reached higher rates for C_3 trees than for C_4 grasses (Figure 1; SI Tables S1 and S2). For the C_4 grasses, the highest rates of A were only marginally higher between 200 ppm and higher growth $[CO_2]_a$, but increased by 40–60% for the trees (Figure 1; SI Tables S1 and S2).

Growth

The biomass of *V. karroo* and *C. africana* trees responded more than C_4 *E. curvula* to $[CO_2]_a$, consistent with previous comparative studies of C_3 and C_4 savanna trees and grasses (Ward *et al.* 1999; Kgope, Bond & Midgley 2010). At 200 ppm $[CO_2]_a$, shoot biomass for *V. karroo* and *C. africana* was 44% and 51% lower than at 400 ppm $[CO_2]_a$, compared with 12% lower for *E. curvula* (Table S3 and S4). At 800 ppm $[CO_2]_a$, shoot biomass for *V. karroo* and *C. africana* was 35% and 32% higher than at 400 ppm $[CO_2]_a$, compared with 5% higher for *E. curvula*. For roots, *V. karroo* and *C. africana* biomass was 10% and 56% lower at 200 ppm $[CO_2]_a$ than at 400 ppm $[CO_2]_a$, compared with 7% lower for *E. curvula*; and, at 800 ppm $[CO_2]_a$, root biomass for *V. karroo* and *C. africana* was 46% and 13% higher than at 400 ppm $[CO_2]_a$, compared with 1% higher for *E. curvula* (Table S3 and S4).

Water demand and supply

We focus on $\Delta\Psi$ as a convenient proxy measure of water demand–to–supply because D_S in the growth chambers was held constant at 10 mmol mol^{-1} (but see $\Delta\Psi/D_S$ in SI Figure S3). $\Delta\Psi$ was lower for the C_4 grasses than C_3 trees across $[CO_2]_a$: -19.3%, -16.0%, and -17.9% lower on average across species for grasses at 200 ppm, 400 ppm and 800 ppm $[CO_2]_a$, respectively (Figure 2; SI Tables S1 and S2). Although $\Delta\Psi$ varied among species, when averaged across trees and grasses, values were 18% and 24% (trees), and 16% and 23% (grasses) higher at 200 ppm than at 400 ppm and 800 ppm $[CO_2]_a$. The differences in $\Delta\Psi$ between trees and grasses were driven by a combination of less negative Ψ_{PD} (+14% on average) and more negative Ψ_{MD} (-3% on average) for the trees than grasses across $[CO_2]_a$ (SI Tables S1 and S2), suggesting the trees had higher $\Delta\Psi$ than the grasses despite the soil remaining wetter for trees over the drying cycle. We hypothesised that $\Delta\Psi$ would be lower for the C_4 grasses than C_3 trees, and on the basis of these findings, we accept Hypothesis 1.

To assess the physiological implications of $\Delta\Psi$ we examined the linkage between $\ln(\Delta\Psi)$ and $\sqrt{(A_{op})}$ (Figure 3). For the trees, higher $\sqrt{(A_{op})}$ was linked with higher $\ln(\Delta\Psi)$ across all growth $[CO_2]_a$. In contrast, $\sqrt{(A_{op})}$ was independent of $\ln(\Delta\Psi)$ across $[CO_2]_a$ for the C_4

grasses (Figure 3b). Regression analysis confirmed differences in the response of $\sqrt{(A_{op})}$ to $\ln(\Delta\Psi)$ between the C_3 trees and C_4 grasses (three–way interaction effect of $\ln(\Delta\Psi) \times [CO_2]_a \times PT$ on the response of $\sqrt{(A_{op})}$: $F_{2, 427} = 3.40$; $P = 0.034$). These findings indicate that the trees could only sustain assimilation at high leaf water demand relative to supply, particularly at 200 ppm and 400 ppm $[CO_2]_a$ (Figure 3a). In other words, high photosynthesis in C_3 trees comes at the expense of a pronounced hydraulic gradient, whereas it does not in the C_4 grasses, a result that is consistent with the arguments of Osborne and Sack (2012).

Effect of Ψ_{PD} on g_S and A

The trees and grasses each had 1.4– to 2.2–fold higher g_S at 200 ppm than at 400 or 800 ppm $[CO_2]_a$; and across $[CO_2]_a$ treatments, g_S was 1.2– to 2.0–fold higher on average for the trees than grasses (Figure 4; SI Figure S4, Tables S1 and S2). Ψ_{PD} was more negative at lower growth $[CO_2]_a$ across all species, in line with higher g_S under low $[CO_2]_a$. For all the trees except *C. apiculatum*, g_S was independent of Ψ_{PD} (log–log response: $P > 0.29$ in all PT $\times [CO_2]_a$ treatments) (Figure 4a, note that dryness increases along the x–axis). In contrast, $\ln(g_S)$ decreased significantly with declining $\ln(\Psi_{PD})$ across all $[CO_2]_a$ for the C_4 grasses (Figure 4b). Three–way regression analysis showed a higher correlation between $\ln(g_S)$ and $\ln(\Psi_{PD})$ for the C_4 grasses than trees independent of $[CO_2]_a$ treatment: interaction effect of $\ln(\Delta\Psi) \times PT$ on the response of $\ln(g_S)$, $F_{1, 429} = 9.17$; $P = 0.003$.

Next we analysed the response of A_{op} to Ψ_{PD} (Figure S5). For the trees (excluding *C. apiculatum*), $\sqrt{(A_{op})}$ was not correlated with $\ln(\Psi_{PD})$ under any $[CO_2]_a$ treatment (Figure 5a). For the grasses grown at 200 ppm and 400 ppm $[CO_2]_a$, $\sqrt{(A_{op})}$ decreased significantly with $\ln(\Psi_{PD})$ (Figure 5b). At 800 ppm $[CO_2]_a$, $\sqrt{(A_{op})}$ was independent of $\ln(\Psi_{PD})$ for the C_4 grasses, indicating that higher $[CO_2]_a$ buffered the effects of drier soil on C_4 photosynthetic rates. Three–way regression analysis confirmed the higher correlation between A and Ψ_{PD} for the C_4 grasses, independent of $[CO_2]_a$ treatment: interaction effect of $\ln(\Delta\Psi) \times PT$ on the response of $\sqrt{(A_{op})}$: $F_{1, 429} = 17.4$; $P < 0.0001$.

Our second hypothesis, that C_4 grasses would maintain higher g_S and A at lower Ψ_{PD} implied the slopes of the $\ln(g_S)$–$\ln(\Psi_{PD})$ and $\sqrt{(A_{op})}$–$\ln(\Psi_{PD})$ responses would be greater for the trees than the grasses, but we found the opposite. On this basis we reject Hypotheses 2 and 3. Moreover, for the C_4 grasses, A was markedly more sensitive to Ψ_{PD} than for C_3 trees at low CO_2, and on this basis we also reject Hypothesis 4.

Discussion

We investigated photosynthesis and plant–water relations of three C_3 tree and three C_4 grass species grown at 800, 400 or 200 ppm $[CO_2]_a$. Plants were watered to maintain Ψ_{PD} within a physiological interval (-0.5 to -3.0 MPa), resembling individual days of a rainfall season within southern African dry savannas (Ripley, Frole & Gilbert 2010; Taylor *et al.* 2014). In this way we avoided confounding influences of drought stress responses (Buckley 2005), which were monitored through visual inspection (chlorosis and wilting) and instrumental measures (F_V/F_M, Figure S6).

We found that C_4 grasses had lower leaf water demand relative to supply ($\Delta\Psi$) than the C_3 trees which supports Hypothesis 1. We note that $\Delta\Psi$ can be lower for the same physical soil–to–leaf conductance, simply because C_4 photosynthesis operates at lower g_S than C_3 photosynthesis. Further, in C_4 plants, evolution doesn't seem to have led to parallel decreases in both stomatal and hydraulic conductance. The resulting lower ratio of hydraulic demand to supply may be a necessary feature, rather than an ancillary advantage of C_4 plants. In fact, for their particular physiological requirements, C_4 leaves must maintain relatively high leaf–level water potential and are inherently vulnerable to low water availability. C_4 photosynthesis is anatomically and biochemically costly compared with C_3 photosynthesis because metabolic demands are placed on both the mesophyll and bundle sheath, and rapid exchange of metabolites must be maintained between the two (Bellasio & Griffiths 2014; Bellasio & Lundgren 2016; Bellasio 2017). Transient decreases in leaf water status arising from either soil or atmospheric water deficit are known to cause severe and often permanent decreases in photosynthetic capacity (Ghannoum *et al.* 2003; Bellasio, Quirk & Beerling 2018). Consequently, when leaf water status falls, C_4 photosynthesis can become quickly inhibited. Indeed, comparative studies of related C_3 and C_4 grasses suggest that C_4 species experience greater reductions in photosynthetic rates during drought compared with C_3 species (Ripley *et al.* 2007; Ripley, Frole & Gilbert 2010; Taylor *et al.* 2011). Non–stomatal limitations, which include intercellular and intracellular CO_2 diffusion, light, metabolic and biochemical constraints, source–sink dynamics, and leaf ultrastructure (Lawlor 2002; Lawlor & Cornic 2002) are disproportionately limiting assimilation in C_4 grasses (Bellasio, Quirk & Beerling 2018). Ripley, Frole and Gilbert (2010) found that non–stomatal limitations accounted for 50% of the decline in A with declining soil moisture for C_4 grass species, compared with 25% for closely related C_3 species. In addition, the predominance of non–stomatal limitations prolonged the recovery of C_4 photosynthesis following subsequent increases in soil moisture.

Experiments with annual forbs show that this differential sensitivity of C_3 and C_4 plants to drought interacts with $[CO_2]_a$. In a pot study, the slope of the decline in A_{op} with increasingly negative Ψ_{MD} was 57%, 70%, 136% and 20% higher for C_4 *Amaranthus retroflexus* than C_3 *Abutilon theophrasti* at 180, 270, 350 and 700 ppm $[CO_2]_a$, respectively (Ward *et al.* 1999). For *A. theophrasti* (C_3) grown at low $[CO_2]_a$, g_S, A_{op} and E were not initially affected by drought exposure, whereas *A. retroflexus* (C_4) lowered A_{op} and g_S at drought onset, particularly at 180 ppm $[CO_2]_a$ (Ward *et al.* 1999). These findings demonstrate greater sensitivity of C_4 g_S and A to soil drying than C_3, and variation in this sensitivity with $[CO_2]_a$, in line with our findings.

If C_4 grasses operate at higher hydraulic conductance, it might be to preserve high leaf water status, driven by the need to avoid non–stomatal limitations, rather than simply the necessity to maintain g_S in dry environments. Because non–stomatal limitations lower the tolerance of C_4 plants to high $\Delta\Psi$ and variable leaf water potential, these species may have evolved to prioritise superior functionality when water availability is high – reflected in larger conduits and higher hydraulic conductance. Conversely, C_4 grasses may shut down leaf function (either through senescence or leaf rolling) when water availability declines, meaning there is little to be gained by C_4 grasses investing in cavitation–proof xylem with lower maximum conductance.

The stomata of the C_4 grasses were more responsive than the C_3 trees across the range of Ψ_{PD} (Figures 4 and 5) and we rejected Osborne and Sack (2012) Hypotheses 2 and 3. This result contrasts with previous findings from a comparison of closely related C_4 and C_3 grasses (Taylor *et al.* 2011), and may arise from a difference in the ecological strategies of grasses and trees, rather than an inherent physiological difference between C_3 and C_4 species. Greater stomatal sensitivity may be important in the grasses for rapidly exploiting pulses of soil water before quickly closing stomata to reduce leaf water demand as soils dry. Conversely, deeper–rooted savanna trees (Canadell *et al.* 1996) may have more sustained access to water that alleviates the requirement for stomatal control of water loss. For example, in an oak savanna, light–saturated A for the C_4 grass *Schizachyrium scoparium* increased by 90% in the days following a mid–drought rainfall event, compared with 22–26% for trees (*Quercus stellata* and *Juniperus virginiana*), despite full recovery of leaf water potential for both trees and grasses (Volder, Tjoelker & Briske 2010). This highlights markedly higher responsiveness of A to soil drying for the C_4 grass than the C_3 trees, in line with our findings.

The comparatively damped stomatal responses of C_3 plants to soil drying may

disadvantage them in periods following soil water inputs. Such adaptive responsiveness may have contributed to the evolutionary advantage of C_4 plants by facilitating their persistence in semi–arid environments, particularly under low $[CO_2]_a$. Stomatal responses to environmental drivers are also important over shorter timescales. These include responses to light– and shade–flecks that occur on the order of seconds to minutes, but when integrated over time can equate to substantial water savings (Lawson & Blatt 2014; Bellasio *et al.* 2017). Grasses have relatively fast–responding stomata, due partly to their dumbbell–shaped guard cells that facilitate rapid stomatal movements, and C_4 leaves generally have faster–responding stomata than C_3 (Knapp 1993; Raven 2002; Franks & Farquhar 2007; McAusland *et al.* 2016; Raissig *et al.* 2017). In this way, the capacity of the C_4 grasses to rapidly regulate g_S under moderately dry soil conditions relative to the C_3 trees may be linked with higher leaf level water availability.

Both C_3 and C_4 leaves increase g_S as $[CO_2]_a$ declines at similar relative rates, but C_3 leaves generally operate at higher absolute g_S (Osborne & Sack 2012). However, when we analysed the interaction with water availability we found that A in the C_4 grasses was most responsive to drying at low $[CO_2]_a$, more so than the C_3 trees, which apparently maintained A independently of Ψ_{PD}, and we rejected Hypothesis 4. Consequently, higher photosynthetic rates at low $[CO_2]_a$, especially for *E. curvula*, probably resulted from the simple fact that C_4 photosynthesis runs at lower C_i than C_3 photosynthesis, leading to higher WUE (*E. curvula* had the highest WUE of all species at 200 ppm $[CO_2]_a$) and more photosynthesis for a given water loss to transpiration. This supports recent findings with C_3, C_3–C_4 intermediate, and C_4 species of *Flaveria* showing that the initial steps towards C_4 photosynthesis characterised by C_3–C_4 intermediate species at low $[CO_2]_a$ are related to greater A and carbon gains, rather than conservative water use and improved hydraulics (Way *et al.* 2014). Our findings indicate that C_4 grasses were not more efficient in extracting water from the soil than C_3 trees under low $[CO_2]_a$. We suggest that C_4 grasses are not more competitive than trees under low water availability *per se*; instead, the interplay between the timing and duration of A, and the capacity to exploit soil water is critical. The ability of C_4 grasses to revert quickly from low to high A in response to precipitation inputs to soils would underpin higher time–integrated assimilation and seasonal growth (Ladrón de Guevara *et al.* 2015; Bellasio *et al.* 2017) and potentially minimise metabolic impairment (Ripley, Frole & Gilbert 2010). Conversely, lower A and slower stomatal responses to water inputs would disadvantage trees under low $[CO_2]_a$, making them more vulnerable to grass–fuelled fires, suppressing recruitment from saplings to trees (Bond 2008; Staver, Botha & Hedin 2017).

Further, advantages based on conservative water use of C_4 grasses, implying that soils stay wetter through water savings, may extend the duration of photosynthesis over a given time interval. In monoculture stands like our pots, higher WUE for C_4 grasses allows plants to photosynthesise for longer time because, in our cabinets where D_S was constant, the rate of soil moisture decline was proportional to g_S. This has been shown in comparative analyses, in which declines in A were slower for C_4 than C_3 plants, particularly during the initial stages (2–3 weeks) of drought (Schulze & Hall 1982; Ripley, Frole & Gilbert 2010; Taylor *et al.* 2014).

Conversely, if soil water is shared between C_3 and C_4 plants, such as in mixed stands where the majority of tree and grass roots occupy upper soil layers (February & Higgins 2010) and compete primarily for the same resources (Scholes & Archer 1997), soils are likely to be wetter on average than in C_3–only stands, providing a window of opportunity for C_3 grasses and trees to colonise stands of C_4 grasses. This is supported by theoretical analysis suggesting that increasing $[CO_2]_a$ will promote coexistence among competing species in mixed C_3 and C_4 stands (Ali *et al.* 2015). Over multi–year timescales of lower than usual precipitation, such as El Niño–La Niña climatic perturbations, the conservative water use, and more rapid responses to soil water inputs of C_4 grasses over C_3 trees could be influential in tipping the transitional balance between the alternative states of closed forest and open, fire– and, or, herbivore–controlled savanna grassland (Hirota *et al.* 2011; Staver, Archibald & Levin 2011).

Conclusion

In our experiments, C_4 grasses had lower soil–to–leaf water potential gradients than the C_3 trees, especially at 200 ppm $[CO_2]_a$, indicating reduced leaf water demand relative to supply. We hypothesise that, because C_4 photosynthesis is inherently vulnerable to low soil water potential, this is necessary to avoid non–stomatal limitations. Indeed, contrary to our expectations, we found that the dependence of stomatal conductance and CO_2 assimilation on predawn leaf water potential was greater for the C_4 grasses than C_3 trees. We therefore rejected the hypothesis that C_4 grasses would maintain CO_2 assimilation in drier soil compared with trees. Consequently, leaf–level photosynthetic advantages of C_4 grasses, particularly *Eragrostis curvula*, over C_3 trees under low CO_2 and low water availability, were not necessarily due to the maintenance of open stomata in dry soil, or the low leaf water demand relative to supply. Our findings suggest that a combination of faster stomatal regulation (in response to soil drying) and conservative water use, which increase the capacity of C_4 grasses to exploit transient water availability quickly and restore CO_2

assimilation, might be the primary, but overlooked, driver of the success of C_4 grasses over C_3 trees, particularly at low $[CO_2]_a$. However, scaling up these results to the field or ecosystem level will be a challenging task. Sub–ambient $[CO_2]_a$ treatments require enclosures or chambers, which inevitably restrict the supply of light to leaves, and limit the inferences that can be made about plant behaviour under field conditions. The best way to understand this behaviour will be a multiproxy approach whereby a range of different environment manipulation techniques are used to gain mechanistic understanding of plant–environment responses, which are then synthesized within a comprehensive mechanistic model.

Acknowledgments

Author contributions

JQ, CB and DJB designed the research. JQ, CB and DAJ performed the research and collected the data. JQ and CB analysed the data with guidance and interpretation from CPO and DJB. JQ and CB led the writing of the manuscript with critical contributions from CPO and DJB.

Data accessibility

The data included in this manuscript is made available from the Dryad Digital Repository: doi:10.5061/dryad.64797qm (Quirk *et al.* 2018).

Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Soil water retention of various substrates.

Figure S2. Dependence of assimilation on g_S.

Figure S3. Leaf–level water demand relative to supply.

Figure S4. Response of g_S to soil water potential.

Figure S5. Response of assimilation to soil water potential.

Figure S6. Photochemical integrity of photosystem II as indicated by F_V/F_M.

References

Ali, A.A., Medlyn, B.E., Aubier, T.G., Crous, K.Y. & Reich, P.B. (2015) Elevated carbon dioxide is predicted to promote coexistence among competing species in a trait‐based model. *Ecology and Evolution,* **5,** 4717-4733.

Anderson, L.J., Maherali, H., Johnson, H.B., Polley, H.W. & Jackson, R.B. (2001) Gas exchange and photosynthetic acclimation over subambient to elevated CO_2 in a C_3–C_4 grassland. *Global Change Biology,* **7,** 693-707.

Beerling, D.J. & Osborne, C.P. (2006) The origin of the savanna biome. *Global Change Biology,* **12,** 2023-2031.

Beerling, D.J. & Royer, D.L. (2011) Convergent Cenozoic CO_2 history. *Nature Geoscience,* **4,** 418-420.

Bellasio, C. (2017) A generalised stoichiometric model of C3, C2, C2+C4, and C4 photosynthetic metabolism. *Journal of Experimental Botany,* **68,** 269-282.

Bellasio, C., Beerling, D.J. & Griffiths, H. (2016) An Excel tool for deriving key photosynthetic parameters from combined gas exchange and chlorophyll fluorescence: theory and practice. *Plant Cell and Environment,* **39,** 1180–1197.

Bellasio, C. & Griffiths, H. (2014) The operation of two decarboxylases (NADPME and PEPCK), transamination and partitioning of C4 metabolic processes between mesophyll and bundle sheath cells allows light capture to be balanced for the maize C4 pathway. *Plant Physiology,* **164,** 466-480.

Bellasio, C. & Lundgren, M.R. (2016) Anatomical constraints to C4 evolution: light harvesting capacity in the bundle sheath. *New Phytologist,* **212,** 485–496.

Bellasio, C., Quirk, J. & Beerling, D.J. (2018) Stomatal and non-stomatal limitations in savanna trees and C4 grasses grown at low, ambient and high atmospheric CO2. *Plant Science,* **274,** 181-192.

Bellasio, C., Quirk, J., Buckley, T.N. & Beerling, D.J. (2017) A dynamic hydro-mechanical and biochemical model of stomatal conductance for C_4 photosynthesis. *Plant Physiology,* **175,** 104-119.

Bond, W.J. (2008) What limits trees in C_4 grasslands and savannas? *Annual Review of Ecology Evolution and Systematics,* **39,** 641-659.

Bond, W.J. & Midgley, G.F. (2012) Carbon dioxide and the uneasy interactions of trees and savannah grasses. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **367,** 601-612.

Buckley, T.N. (2005) The control of stomata by water balance. *New Phytologist,* **168,** 275-292.

Canadell, J., Jackson, R.B., Ehleringer, J.B., Mooney, H.A., Sala, O.E. & Schulze, E.-D. (1996) Maximum rooting depth of vegetation types at the global scale. *Oecologia,* **108,** 583-595.

Charles-Dominique, T., Davies, T.J., Hempson, G.P., Bezeng, B.S., Daru, B.H., Kabongo, R.M., Maurin, O., Muasya, A.M., van der Bank, M. & Bond, W.J. (2016) Spiny plants, mammal browsers, and the origin of African savannas. *Proceedings of the National Academy of Sciences*.

Christin, P.-A., Besnard, G., Samaritani, E., Duvall, M.R., Hodkinson, T.R., Savolainen, V. & Salamin, N. (2008) Oligocene CO_2 decline promoted C_4 Photosynthesis in grasses. *Current Biology,* **18,** 37-43.

Christin, P.A. & Osborne, C.P. (2014) The evolutionary ecology of C4 plants. *New Phytologist,* **204,** 765-781.

Edwards, E.J., Osborne, C.P., Strömberg, C.A.E., Smith, S.A. & Consortium, C.G. (2010) The origins of C_4 grasslands: Integrating evolutionary and ecosystem science. *Science,* **328,** 587-591.

Edwards, E.J. & Smith, S.A. (2010) Phylogenetic analyses reveal the shady history of C_4 grasses. *Proceedings of the National Academy of Sciences,* **107,** 2532-2537.

Ehleringer, J. & Björkman, O. (1977) Quantum yields for CO2 uptake in C3 and C4 plants: dependence on temperature, CO2, and O2 concentration. *Plant Physiology,* **59,** 86-90.

Ehleringer, J.R., Cerling, T.E. & Helliker, B.R. (1997) C4 photosynthesis, atmospheric CO2, and climate. *Oecologia,* **112,** 285-299.

Ehleringer, J.R., Sage, R.F., Flanagan, L.B. & Pearcy, R.W. (1991) Climate change and the evolution of C_4 photosynthesis. *Trends in Ecology & Evolution,* **6,** 95-99.

February, E.C. & Higgins, S.I. (2010) The distribution of tree and grass roots in savannas in relation to soil nitrogen and water. *South African Journal of Botany,* **76,** 517-523.

Franks, P.J. & Farquhar, G.D. (2007) The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiology,* **143,** 78-87.

Fynn, R.W.S. & Naiken, J. (2009) Different responses of *Eragrostis curvula* and *Themeda triandra* to rapid- and slow-release fertilisers: insights into their ecology and implications for fertiliser selection in pot experiments. *African Journal of Range & Forage Science,* **26,** 43-46.

Ghannoum, O., Conroy, J.P., Driscoll, S.P., Paul, M.J., Foyer, C.H. & Lawlor, D.W. (2003) Nonstomatal limitations are responsible for drought-induced photosynthetic inhibition in four C_4 grasses. *New Phytologist,* **159,** 599-608.

Griffiths, H., Weller, G., Toy, L.F.M. & Dennis, R.J. (2013) You're so vein: bundle sheath physiology, phylogeny and evolution in C3 and C4 plants. *Plant, Cell & Environment,* **36,** 249-261.

Hirota, M., Holmgren, M., Van Nes, E.H. & Scheffer, M. (2011) Global resilience of tropical forest and savanna to critical transitions. *Science,* **334,** 232-235.

Hoetzel, S., Dupont, L., Schefusz, E., Rommerskirchen, F. & Wefer, G. (2013) The role of fire in Miocene to Pliocene C_4

grassland and ecosystem evolution. *Nature Geoscience,* **6,** 1027-1030.

Kgope, B.S., Bond, W.J. & Midgley, G.F. (2010) Growth responses of African savanna trees implicate atmospheric [CO$_2$] as a driver of past and current changes in savanna tree cover. *Austral Ecology,* **35,** 451-463.

Knapp, A.K. (1993) Gas-Exchange Dynamics in C3 and C4 Grasses - Consequences of Differences in Stomatal Conductance. *Ecology,* **74,** 113-123.

Ladrón de Guevara, M., Lázaro, R., Arnau-Rosalén, E., Domingo, F., Molina-Sanchis, I. & Mora, J.L. (2015) Climate change effects in a semiarid grassland: Physiological responses to shifts in rain patterns. *Acta Oecologica,* **69,** 9-20.

Lawlor, D.W. (2002) Limitation to photosynthesis in water - stressed leaves: stomata vs. metabolism and the role of ATP. *Annals of Botany,* **89,** 871-885.

Lawlor, D.W. & Cornic, G. (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant, Cell & Environment,* **25,** 275-294.

Lawson, T. & Blatt, M.R. (2014) Stomatal Size, Speed, and Responsiveness Impact on Photosynthesis and Water Use Efficiency. *Plant Physiology,* **164,** 1556-1570.

McAusland, L., Vialet-Chabrand, S., Davey, P., Baker, N.R., Brendel, O. & Lawson, T. (2016) Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologist,* **211,** 1209-1220.

O'Connor, T.G. (1994) Composition and population responses of an African savanna grassland to rainfall and grazing. *Journal of Applied Ecology,* **31,** 155-171.

Osborne, C.P. & Beerling, D.J. (2006) Nature's green revolution: the remarkable evolutionary rise of C$_4$ plants. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **361,** 173-194.

Osborne, C.P. & Freckleton, R.P. (2009) Ecological selection pressures for C$_4$ photosynthesis in the grasses. *Proceedings of the Royal Society B: Biological Sciences,* **276,** 1753-1760.

Osborne, C.P. & Sack, L. (2012) Evolution of C$_4$ plants: a new hypothesis for an interaction of CO$_2$ and water relations mediated by plant hydraulics. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **367,** 583-600.

Pinto, H., Sharwood, R.E., Tissue, D.T. & Ghannoum, O. (2014) Photosynthesis of C3, C3–C4, and C4 grasses at glacial CO2. *Journal of Experimental Botany,* **65,** 3669-3681.

Polley, H.W., Johnson, H.B. & Mayeux, H.S. (1992) Carbon Dioxide and Water Fluxes of C$_3$ Annuals and C$_3$ and C$_4$ Perennials at Subambient CO$_2$ Concentrations. *Functional Ecology,* **6,** 693-703.

Quirk, J., Bellasio, C., Johnson, D.A., Osborne, C.P. & Beerling, D.J. (2018) Data from: C4 savanna grasses fail to maintain assimilation in drying soil under low CO2 compared with C3 trees despite lower leaf water demand. Dryad digital repository.

Raissig, M.T., Matos, J.L., Anleu Gil, M.X., Kornfeld, A., Bettadapur, A., Abrash, E., Allison, H.R., Badgley, G., Vogel, J.P., Berry, J.A. & Bergmann, D.C. (2017) Mobile MUTE specifies subsidiary cells to build physiologically improved grass stomata. *Science,* **355,** 1215-1218.

Raven, J.A. (2002) Selection pressures on stomatal evolution. *New Phytologist,* **153,** 371-386.

Ripley, B., Frole, K. & Gilbert, M. (2010) Differences in drought sensitivities and photosynthetic limitations between co-occurring C$_3$ and C$_4$ (NADP-ME) Panicoid grasses. *Annals of Botany,* **105,** 493-503.

Ripley, B.S., Cunniff, J. & Osborne, C.P. (2013) Photosynthetic acclimation and resource use by the C3 and C4 subspecies of Alloteropsis semialata in low CO2 atmospheres. *Global Change Biology,* **19,** 900-910.

Ripley, B.S., Gilbert, M.E., Ibrahim, D.G. & Osborne, C.P. (2007) Drought constraints on C4 photosynthesis: stomatal and metabolic limitations in C3 and C4 subspecies of Alloteropsis semialata. *Journal of Experimental Botany,* **58,** 1351-1363.

Sage, R.F. (2004) The evolution of C$_4$ photosynthesis. *New Phytologist,* **161,** 341-370.

Scholes, R.J. & Archer, S.R. (1997) Tree-grass interactions in savannas. *Annual Review of Ecology and Systematics,* **28,** 517-544.

Schulze, E.-D. & Hall, A.E. (1982) Stomatal responses, water loss and CO$_2$ assimilation rates of plants in contrasting environments. *Physiological plant ecology II: Water relations and carbon assimilation* (eds O.L. Lange, P.S. Nobel, C.B. Osmond & H. Ziegler), pp. 181-230. Springer Berlin Heidelberg, Berlin, Heidelberg.

Spriggs, E.L., Christin, P.-A. & Edwards, E.J. (2014) C$_4$ photosynthesis promoted species diversification during the Miocene grassland expansion. *Plos ONE,* **9,** e97722.

Staver, A.C., Archibald, S. & Levin, S.A. (2011) The global extent and determinants of savanna and forest as alternative biome states. *Science,* **334,** 230-232.

Staver, A.C., Botha, J. & Hedin, L. (2017) Soils and fire jointly determine vegetation structure in an African savanna. *New Phytologist,* **216,** 1151-1160.

Strömberg, C.A.E. (2011) Evolution of grasses and grassland ecosystems. *Annual Review of Earth and Planetary Sciences,* **39,** 517-544.

Taylor, S.H., Hulme, S.P., Rees, M., Ripley, B.S., Ian Woodward, F. & Osborne, C.P. (2010) Ecophysiological traits in C3 and C4 grasses: a phylogenetically controlled screening experiment. *New Phytologist,* **185,** 780-791.

Taylor, S.H., Ripley, B.S., Martin, T., De-Wet, L.-A., Woodward, F.I. & Osborne, C.P. (2014) Physiological advantages of C4 grasses in the field: a comparative experiment demonstrating the importance of drought. *Global Change Biology,* **20,** 1992-2003.

Taylor, S.H., Ripley, B.S., Woodward, F.I. & Osborne, C.P. (2011) Drought limitation of photosynthesis differs between C$_3$ and C$_4$ grass species in a comparative experiment. *Plant, Cell & Environment,* **34,** 65-75.

Temme, A.A., Cornwell, W.K., Cornelissen, J.H.C. & Aerts, R. (2013) Meta-analysis reveals profound responses of plant traits to glacial CO$_2$ levels. *Ecology and Evolution,* **3,** 4525-4535.

Volder, A., Tjoelker, M.G. & Briske, D.D. (2010) Contrasting physiological responsiveness of establishing trees and a C$_4$ grass to rainfall events, intensified summer drought, and warming in oak savanna. *Global Change Biology,* **16,** 3349-3362.

von Caemmerer, S. & Furbank, R.T. (2003) The C4 pathway: an efficient CO2 pump. *Photosynthesis Research,* **77,** 191-207.

Ward, J.K., Tissue, D.T., Thomas, R.B. & Strain, B.R. (1999) Comparative responses of model C$_3$ and C$_4$ plants to drought in low and elevated CO$_2$. *Global Change Biology,* **5,** 857-867.

Way, D.A., Katul, G.G., Manzoni, S. & Vico, G. (2014) Increasing water use efficiency along the C$_3$ to C$_4$ evolutionary pathway: a stomatal optimization perspective. *Journal of Experimental Botany,* **65,** 3683-3693.

Figures

Figure 1. Dependence of assimilation on stomatal conductance. Log–square root plots showing the response $\sqrt{A_{op}}$ to $\ln(g_S)$ under operational growing conditions for (**a**) C_3 trees and (**b**) C_4 grasses grown at 200 ppm (left panels), 400 ppm (centre panels) and 800 ppm (right panels) $[CO_2]_a$. C_3 trees are *Vachellia karroo*, *Celtis africana*, and *Combretum apiculatum*; and C_4 grasses are *Eragrostis curvula*, *Heteropogon contortus*, and *Themeda triandra*. Stomatal conductance increases along the *x*–axis. GLM functions and ANOVA statistics are given for each photosynthesis type $\times$ $[CO_2]_a$ treatment.
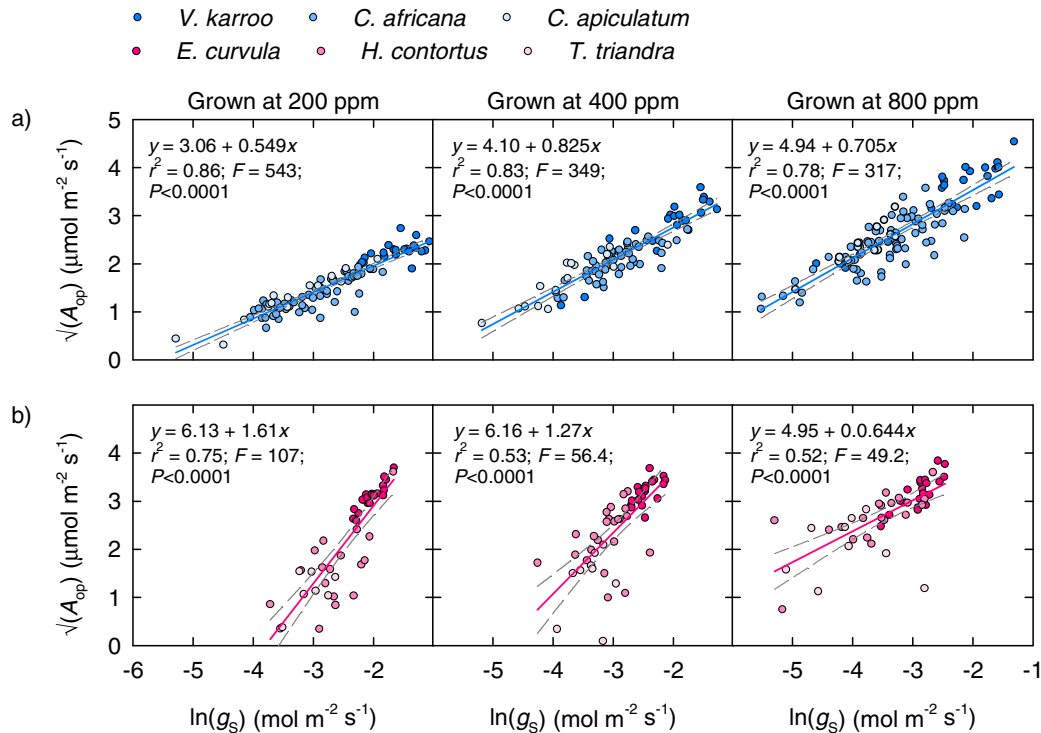
Figure 2. Soil to leaf gradient in water potential, ΔΨ. Box plots showing median ΔΨ and interquartile range with whiskers showing 10th and 90th percentiles (n=4–10) for C_3 tree species and C_4 grass species grown at 200 ppm (**a**), 400 ppm (**b**) or 800 ppm (**c**) $[CO_2]_a$. C_3 trees are *Vachellia karroo*, *Celtis africana*, and *Combretum apiculatum*; and C_4 grasses are *Eragrostis curvula*, *Heteropogon contortus*, and *Themeda triandra*. Boxes sharing the same letter range across all treatments are not statistically different at $\alpha = 0.05$, and the dotted lines with grey shading behind groups of boxes denote the mean ± S.E. (n=3) for the C_3 trees and C_4 grasses at each $[CO_2]_a$.
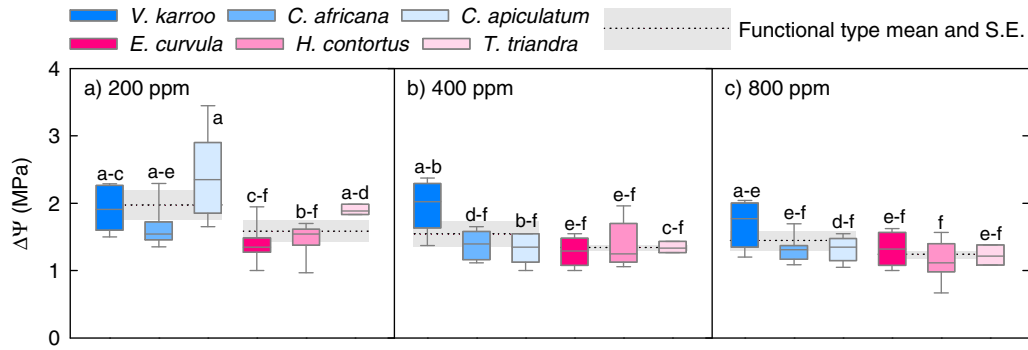
Figure 3. Response of assimilation to the soil–to–leaf gradient in water potential, ΔΨ. Log–square root plots showing the response $\sqrt{(A_{op})}$ to $\ln(\Delta\Psi)$ under operational growing conditions for (**a**) C_3 trees and (**b**) C_4 grasses grown at 200 ppm (left panels), 400 ppm (centre panels) and 800 ppm (right panels) $[CO_2]_a$. C_3 trees are *Vachellia karroo*, *Celtis africana*, and *Combretum apiculatum*; and C_4 grasses are *Eragrostis curvula*, *Heteropogon contortus*, and *Themeda triandra*. Increasing ΔΨ along the *x*–axis indicates larger leaf hydraulic demand relative to soil and root hydraulic supply and implies increasing hydraulic strain on operational leaves. GLM functions and ANOVA statistics are given for each photosynthesis type × $[CO_2]_a$ treatment.
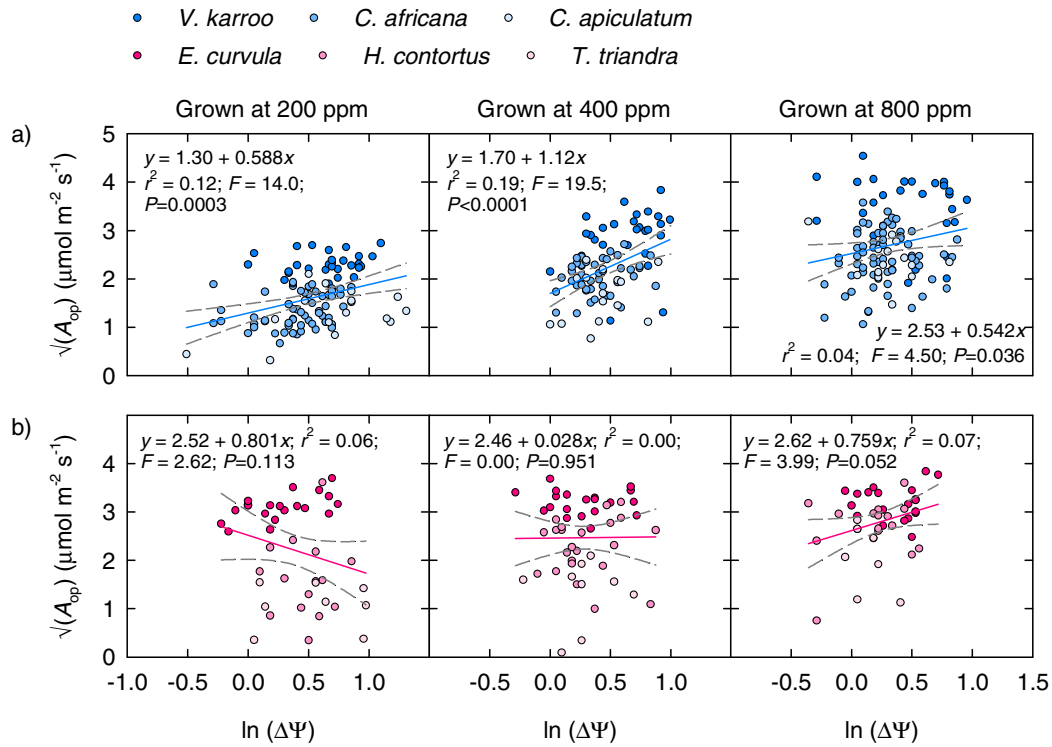
Figure 4. Response of stomatal conductance to soil water potential. Log–log plots showing the response of $\ln(g_S)$ to $\ln(\Psi_{PD})$, a proxy of soil water potential, under operational growing conditions for (**a**) C_3 trees and (**b**) C_4 grasses at 200 ppm (left), 400 ppm (centre), or 800 ppm (right) $[CO_2]_a$. C_3 trees are *Vachellia karroo*, *Celtis africana*, and *Combretum apiculatum*; and C_4 grasses are *Eragrostis curvula*, *Heteropogon contortus*, and *Themeda triandra*. Increasing $\ln(\Psi_{PD})$ along the *x*–axis indicates increasing soil water limitation with soil drying. GLM functions and ANOVA statistics are given for each photosynthesis type × $[CO_2]_a$ treatment. There were significant main–effect responses of $\ln(g_S)$ to $\ln(\Psi_{PD})$ ($F = 14.9$; $P < 0.0001$), $[CO_2]_a$ ($F = 72.7$; $P < 0.0001$) and species ($F = 70.5$; $P < 0.0001$). There is no correlation between $\ln(\Psi_{PD})$ and $\ln(g_S)$ across the C_3 trees in general, but $\ln(g_S)$ for *Combretum apiculatum* (lightest blue circles) decreases significantly with drying soil at 200 ppm $[CO_2]_a$ ($y = -3.21-1.08x$; $r^2 = 0.45$; $F = 10.6$; $P = 0.006$) and 400 ppm ($y = -3.60-0.956x$; $r^2 = 0.36$; $F = 7.4$; $P = 0.017$).
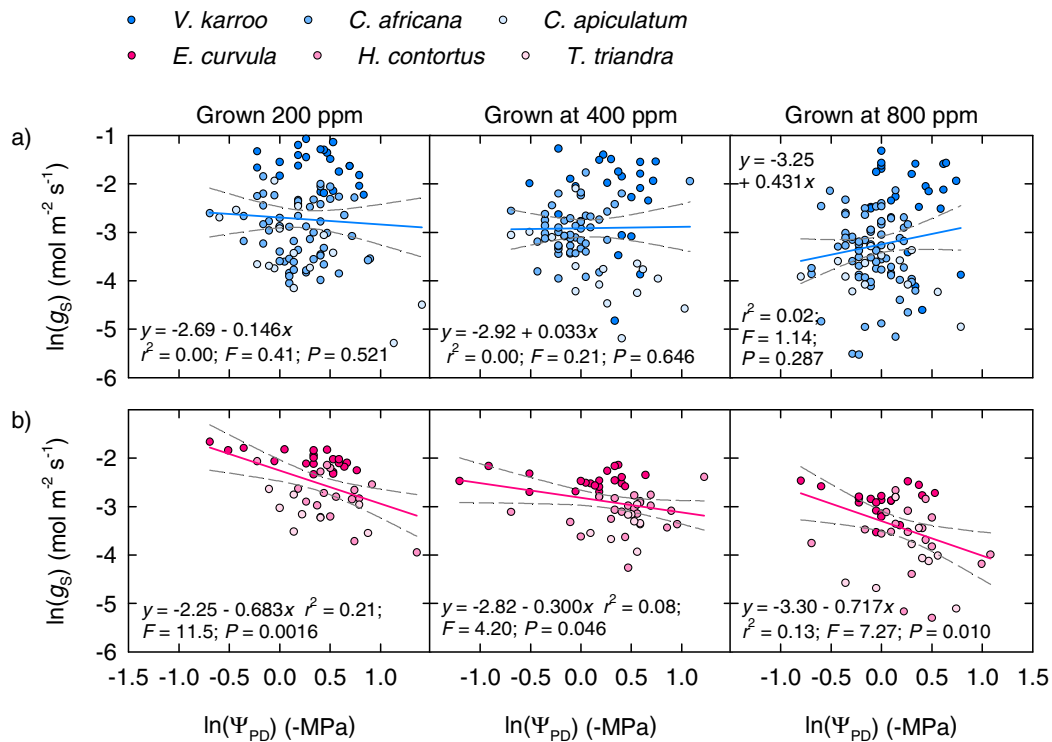
Figure 5. Response of assimilation to soil water potential. Log–square root plots defining the relationship between $\ln(\Psi_{PD})$, a proxy of soil water potential, and $\sqrt{(A_{op})}$, for (**a**) C_3 trees and (**b**) C_4 grasses at 200 ppm (left), 400 ppm (centre), or 800 ppm (right) $[CO_2]_a$. C_3 trees are *Vachellia karroo*, *Celtis africana*, and *Combretum apiculatum*; and C_4 grasses are *Eragrostis curvula*, *Heteropogon contortus*, and *Themeda triandra*. Increasing $\ln(\Psi_{PD})$ along the *x*–axis indicates increasing soil water limitation with soil drying. GLM functions and associated ANOVA statistics are given for each photosynthesis type × $[CO_2]_a$ treatment. There is no correlation between $\ln(\Psi_{PD})$ and $\sqrt{(A_{op})}$ across the C_3 trees in general, but $\sqrt{(A_{op})}$ for *Combretum apiculatum* (lightest blue circles) does decrease significantly with drying soil at 200 ppm $[CO_2]_a$ ($y = 1.41\text{-}0.667x$; $r^2 = 0.51$; $F = 13.7$; $P = 0.003$) and 400 ppm ($y = 1.77\text{-}0.640x$; $r^2 = 0.34$; $F = 6.67$; $P = 0.023$).