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Photorespiration in C_4 grasses remains slow under drought conditions

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ABSTRACT

The CO_2-concentrating mechanism present in C_4 plants decreases the oxygenase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and, consequently, photorespiratory rates in air. Under drought conditions, the intercellular CO_2 concentration may decrease and cause photorespiration to increase. The C_4 grasses *Paspalum dilatatum* Poiret, *Cynodon dactylon* (L.) Pers. and *Zoysia japonica* Steudel were grown in soil and drought was imposed by ceasing to provide water. Net CO_2 assimilation (*A*) and stomatal conductance to water vapour decreased with leaf dehydration. Decreased carbon and increased oxygen isotope composition were also observed under drought. The response of *A* to CO_2 suggested that the compensation point was zero in all species irrespective of the extent of drought stress. A slight decrease of *A* as O_2 concentration increased above 10% provided evidence for slow photorespiratory gas exchanges. Analysis of amino acids contained in the leaves, particularly the decrease of glycine after 30 s in darkness, supported the presence of slow photorespiration rates, but these were slightly faster in *Cynodon dactylon* than in *Paspalum dilatatum* and *Zoysia japonica*. Although the contents of glycine and serine increased with dehydration and mechanistic modelling of C_4 photosynthesis suggested slightly increased photorespiration rates in proportion to photosynthesis, the results provide evidence that photorespiration remained slow under drought conditions.

Key-words: amino acids; CO_2 and O_2 response curves; isotope composition; modelling C_4 photosynthesis.

INTRODUCTION

The main objective of this study was to investigate whether photorespiration was increased by drought stress in three species of C_4 grasses and could, as a consequence, contribute to metabolic factors limiting net photosynthesis. Under drought conditions, photosynthetic carbon assimilation decreases in both C_3 and C_4 plants (e.g. Chaves, Maroco & Pereira 2003). Closure of stomata is one of the major causes of the decrease in photosynthesis, but evidence has accumulated that metabolic limitations also contribute (Du *et al.* 1996; Saccardy *et al.* 1996; Ghannoum *et al.* 2003; Marques da Silva & Arrabaça 2004; Carmo-Silva *et al.* 2007). In C_4 leaves with Kranz anatomy, atmospheric CO_2 is initially fixed by phosphoenolpyruvate (PEP) carboxylase [PEPC, enzyme commission (EC) 4.1.1.31] into C_4 acids in the mesophyll (M) cells. The C_4 acids are transported to the bundle sheath (BS) cells where they undergo decarboxylation, and the released CO_2 enters the C_3 pathway via ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco; EC 4.1.1.39). Rubisco is confined to the BS cells, and the specialized leaf anatomy decreases leakage of CO_2 back to the M cells so that CO_2 accumulates.

Rubisco acts both as a carboxylase and an oxygenase. Molecules of CO_2 and O_2 are competing alternative substrates for reaction with the enediol of RuBP catalysed by Rubisco (Bowes & Ogren 1972; Laing, Ogren & Hageman 1974) and, therefore, rates of photorespiration relative to photosynthesis are determined by the relative concentrations of O_2 and CO_2 at the catalytic site of Rubisco in the chloroplast stroma. In fully hydrated leaves, the CO_2/O_2 ratio in BS cells of C_4 species is three to six times higher than in M cells under atmospheric levels of CO_2 and O_2 (Dai, Ku & Edwards 1993; Kiirats *et al.* 2002). Therefore, the oxygenase activity of Rubisco, and consequently the photorespiratory rate, is slow. The consequences are a low CO_2 compensation point in C_4 plants (Forrester, Krotkov & Nelson 1966) and the absence of an enhancement of net photosynthesis when oxygen in the gas phase is decreased from 21 to 2% (Edwards, Ku & Monson 1985). These observations have been initially interpreted as a lack of photorespiration in C_4 plants. More recent studies with several C_4 species, including the three main subtypes and both monocotyledons and dicotyledons (Dai *et al.* 1993; Maroco, Ku & Edwards 1997), have revealed that the maximum rate of net photosynthesis occurs at O_2 concentrations between 5 and 10%. The rise of C_4 photosynthesis to a maximum at 5–10%

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has been explained by an oxygen requirement for the production of extra ATP needed for the CO_2-concentrating mechanism (Maroco *et al.* 1997). The decrease in photosynthesis by C_4 species when the O_2 concentration is elevated above 10% depends on the CO_2 concentration (Dai *et al.* 1993; Maroco *et al.* 1997, 1998) and is assumed to be due to photorespiration. To the best of our knowledge, the effect of O_2 on C_4 photosynthesis by dehydrated leaves has not been recorded.

Under water deficit conditions, the CO_2 concentration in the leaves may decrease because of decreased stomatal conductance and should cause photorespiration to increase. Mechanistic modelling of C_4 photosynthesis is not used as frequently as that of C_3 photosynthesis, mostly because of the additional complexity resulting from the structural and biochemical specialization characteristic of C_4 plants (von Caemmerer 2000). The model of von Caemmerer & Furbank (1999) uses basic equations to describe the carbon fluxes in C_4 photosynthesis and, with careful assumptions being made, can be used to estimate the CO_2 concentration in the BS, the rate of oxygenation of RuBP and hence photorespiration.

Photorespiratory metabolism requires the integration of the photorespiratory carbon oxidation cycle, the photorespiratory nitrogen cycle (Keys *et al.* 1978) and photosynthetic carbon assimilation (Keys 1999); consequently, there is an interdependence of reactions in different parts of the overall process. Because photorespiration is relatively little influenced by metabolite signals, amino acids can be used as metabolite markers for this pathway (Foyer, Parry & Noctor 2003). Although de novo assimilation of nitrogen and recycling of ammonia during photorespiration interact (Stitt *et al.* 2002), the ratio glycine/serine and both aspartate and alanine levels are strongly correlated with photorespiration rates in C_3 plants (Novitskaya *et al.* 2002). In the C_4 dicotyledon *Amaranthus edulis*, increased glycine content in the leaves with increasing O_2 in the atmosphere has been taken as indicative of photorespiration (Maroco, Ku & Edwards 2000); although increased serine has also been observed in *A. edulis*, this amino acid decreased with increasing O_2 in C_3 plants and is therefore discounted as an indicator of photorespiration. Post-illumination decreases in glycine (Kumarasinghe, Keys & Whittingham 1977; Rawsthorne & Hylton 1991) are mainly responsible for the post-illumination burst of CO_2 in C_3 plants, a phenomenon leading to the early recognition of photorespiration. Post-illumination CO_2 bursts are generally not seen in C_4 plants, mostly because of the low conductance of BS cell walls to CO_2, but the post-illumination decrease in glycine should be observed if photorespiration is present.

Variations in carbon and oxygen isotope compositions ($\delta^{13}C$ and $\delta^{18}O$) reflect the influence of environmental factors on the kinetics of photosynthesis, and the two isotopes are conveniently measured on the same leaf samples. The CO_2-concentrating mechanism present in C_4 plants results in lower discrimination against ^{13}C than in C_3 plants, and hence an average $\delta^{13}C$ of –13.6‰ in C_4 as opposed to –27.8‰ in C_3 plants (Troughton 1979). Changes in $\delta^{13}C$ under stress conditions reflect mostly variations in the CO_2 concentration at the carboxylation sites and the coordination between the C_3 and C_4 cycles (Farquhar 1983). On the other hand, changes in $\delta^{18}O$ are mainly the result of changes in evapotranspiration and reflect the isotope content of the soil water as well as the fractionation during transpiration (Barbour 2007), which is likely to change in conditions affecting the evaporative demand.

The experiments described aimed to detect photorespiration in three C_4 grasses and to determine whether the rate increased under drought conditions. The indicators of photorespiration used were the response of photosynthesis to CO_2, inhibition of photosynthesis by O_2, content of amino acids, post-illumination changes in amino acids and isotope fractionation. The three C_4 species studied have been reported to belong each to a different biochemical subtype, according to the main enzyme responsible for the decarboxylation of C_4 acids in the BS: *Paspalum dilatatum* Poiret, NADP-malic enzyme (NADP-ME); *Cynodon dactylon* (L.) Pers., NAD-malic enzyme (NAD-ME); and *Zoysia japonica* Steudel, PEP carboxykinase (PEPCK).

MATERIALS AND METHODS

Plant material and drought stress induction

The C_4 grasses *Paspalum dilatatum* Poir. cv. Raki (NADP-ME), *Cynodon dactylon* (L.) Pers. var. Shangri-Lá (NAD-ME) and *Zoysia japonica* Steudel 'Jacklin Sunrise Brand' (produced by Jacklin Seed Company, Post Falls, ID, USA) (PEPCK) were grown from seeds in pots with peat-free compost, prepared to Rothamsted Research's specification by Petersfield Products (Leicester, UK) supplemented with a slow-release fertilizer (Hydro Agri Ltd, Lincolnshire, UK) in a glasshouse. Artificial light was provided whenever the natural light was below a photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1} during a 16 h photoperiod. Temperature was maintained at a minimum of 25 °C during the day and at 18 °C during the night.

Seeds of each species were washed with 10% hypochlorite and were soaked in water for 1 h before sowing. Water was supplied whenever needed during 2 weeks for *Paspalum dilatatum* and *Cynodon dactylon*, and 4–5 weeks for the slower-growing *Zoysia japonica*. The seedlings were transplanted to 1 L cylindrical pots containing equal amounts of soil. Five seedlings were used per pot. The dates of sowing and transplanting each species were adjusted, and the timing of the drought treatment was chosen in order to have plants at an adequate growth stage for taking measurements or for sampling all three grasses at the same time.

All plants were well watered until the beginning of the drought stress treatment. Pots were placed according to a split-plot design, where each column of pots was a main plot of a particular species, and the sampling days and the treatments (control versus drought stress) were randomized in the split plots. Each pot corresponded to an independent sample. All pots were watered in the evening and weighed on the following morning in order to ensure that all of them

had similar amounts of water (mean overall weight of 800 ± 50 g). Water deficit was then imposed on the 'stress' pots by ceasing to provide water. The 'control' pots were watered once per day.

Five-week-old plants of *P. dilatatum* and *C. dactylon* and 9-week-old plants of *Z. japonica* were analysed. Control and non-watered plants of the three species were either assayed or harvested on consecutive days, starting when the weight of the non-watered pots had been suitably decreased, in order to obtain leaf samples with different levels of dehydration, and ending after a maximum of 9–12 d without watering. The water weight in each pot (*WWP*, g) was determined as the weight of the pot at each sampling time less 400 g (the mean weight of the pots with plants and totally dried soil was 404 ± 16 g).

The youngest fully expanded leaf of each *P. dilatatum* plant and two young fully expanded leaves of each plant of *C. dactylon* or *Z. japonica* were always analysed. Samples were collected in the growth environment 4 h after the beginning of the photoperiod. *In vivo* measurements were made during the first half of the photoperiod. It was assumed that, within each pot, all the leaves used were identical in terms of developmental stage, physiological and biochemical properties, and would have experienced the same drought condition.

From each pot, a sample formed by similar leaves to those being used for gas exchanges or amino acid analysis was collected to determine the leaf relative water content (*RWC*). The fresh (*FW*), turgid (*TW*) and dry (*DW*) weights were measured and used to calculate *RWC* by the equation $RWC = 100[(FW - DW)/(TW - DW)]$ (Catsky 1960). Leaf area was determined by scanning the turgid leaves and analysing the image using the software Paint Shop Pro 9 (Jasc Software, Inc., Minneapolis, MN, USA) and the software Image J 1.33u (National Institutes of Health, Bethesda, MD, USA).

In order to facilitate the analysis of the physiological responses to CO_2 and O_2, groups of plants with different drought stress conditions were chosen for each species independently, considering both the *RWC* and the response in terms of the measured gas exchanges. Therefore, control (C) corresponds to all well-watered plants; moderately stressed (MS) to non-watered plants with *RWC* values between 96 and 80% in *P. dilatatum*, 95 and 80% in *C. dactylon*, and 95 and 85% in *Z. japonica*; and severely stressed (SS) to non-watered plants with *RWC* values lower than 80 or 85%. Unfortunately, no SS samples of *C. dactylon* were obtained in these experiments as the leaves of this species did not dehydrate as much as the other two grasses.

Gas exchange measurements (CO_2 and O_2 response curves)

Gas exchanges of carbon dioxide and water vapour by attached leaves were measured by infrared gas analysis using a six-chamber system designed and developed at Rothamsted (Lawlor, Kontturi & Young 1989). Experiments were conducted at 25 ± 2 °C, 35–40% relative humidity and a PPFD of 850 ± 50 μmol m^{-2} s^{-1}, provided by overhead lamps. The air composition was controlled by a gas mixer supplying CO_2 and O_2, the balance being made up with N_2. Two different experiments were undertaken. In the first, CO_2 response curves were determined at a constant O_2 concentration (21%) and seven CO_2 concentrations (~30, 100, 250, 360, 500, 750 and 1000 μmol mol^{-1}). In the second experiment, O_2 response curves were determined at a constant CO_2 concentration (360 μmol mol^{-1}) and six O_2 concentrations (2, 5, 10, 15, 21 and 30%). Measurements on each plant were always taken with time intervals long enough for steady-state CO_2 uptake to be attained at each CO_2 or O_2 level. Ninety pots were used per experiment, making a total of 30 samples per species (10 control and 20 non-watered) assayed over five consecutive days during the drought period (2 control and 4 non-watered per species per day). Each sample was formed by the middle part of young fully expanded leaves of two plants from one pot, but, because of their different size, a different number of leaves was used for the three species: two leaves for *P. dilatatum*, four leaves for *C. dactylon* and three leaves for *Z. japonica*. After each series of measurements, the area of the leaf inside the chamber was determined. The net CO_2 assimilation rate (A) and the stomatal conductance to water vapour (*gswa*) by the control and drought-stressed plants of each species were plotted against intercellular CO_2 concentration (C_i) or against the O_2 concentration in the gas phase.

Carbon and oxygen isotope compositions

A similar experimental design to that used to provide material for gas exchange measurements was used here. Three leaves of *P. dilatatum* and 10 leaves of *C. dactylon* or *Z. japonica* of the five plants in each pot were used for each sample. After determination of *FW* and *TW*, the leaf samples were dried at 80 °C for more than 48 h and were weighed to allow calculation of *RWC*. Three control and six drought-stressed samples from each species were selected according to their *RWC* and were sent to Unidade de Análise Instrumental, Faculdade de Ciências da Universidade de Lisboa, Portugal for the analyses of the carbon and oxygen isotope compositions ($\delta^{13}C$ and $\delta^{18}O$) in the leaf dry matter. Subsamples of ground leaf tissue were analysed, to give three technical replicates of each sample, using stable isotope ratio mass spectrometers (SIRA II; VG Isogas Limited, Manchester, UK for carbon, and IsoPrime; Micromass UK Limited, Manchester, UK for oxygen, with automatic sample preparation systems, EuroEA; EuroVector S.p.A., Milan, Italy). The Pee Dee belemnite and the Vienna standard mean oceanic water were used as standards for $\delta^{13}C$ and $\delta^{18}O$ calculations, respectively. The results were expressed as parts per thousand deviation from the standards with an analytical precision of ±0.12‰.

Amino acid analysis

Amino acids contained in leaves were determined by high-performance liquid chromatography (HPLC) of

o-pthaldialdehyde (OPA) derivatives (Noctor & Foyer 1998). Three control and five non-watered pots were used per species per day during three consecutive days, making a total of 24 samples per species (9 control and 15 non-watered pots). The five plants within each pot were used for the collection of three samples: one light sample, immediately frozen with liquid N_2 (LN_2) in fully illuminated conditions; one dark sample, identical to the light sample but submitted to a period of 30 s in darkness before freezing in LN_2; and a third sample for the *RWC* determination. Taking into account the different leaf sizes, each light or dark sample of *P. dilatatum* consisted of one leaf from one plant, whereas each sample of *C. dactylon* or *Z. japonica* consisted of three leaves from two plants.

Reversed-phase HPLC was performed using a Waters Alliance 2695 Separation Module and a 474 scanning fluorescence detector operated by the Millenium[32] software (Waters, Milford, MA, USA) with a Waters Symmetry C_{18} 4.6 × 150.0 mm column (part no. WAT 054278) protected by a 4 × 3 mm guard cartridge (Phenomenex, Torrance, CA, USA). Because the fluorescent adducts formed by reaction with OPA in the presence of 2-mercaptoethanol are unstable, the autosampler was set to mix and pre-incubate 10 μL of each sample with 15 μL of OPA reagent for 2 min before injecting the mixture onto the column. The eluent used for the amino acid separation was obtained by mixing solvents containing different proportions of methanol, sodium acetate pH 5.9 and tetrahydrofuran.

Amino acids were extracted from the frozen leaf samples stored at –80 °C. Each sample was ground in LN_2, and then 1.4 mL of 0.1 M HCl was added to the fine powder. The mixture was ground further during thawing, and the homogenate was centrifuged for 10 min at 16 000 *g* and 4 °C. Samples for HPLC were prepared by adding a subsample of each supernatant to the internal standard and pure water, and these mixtures were stored at –20 °C. On the following day, the mixtures were centrifuged for 40 min at 16 000 *g* and 4 °C, and then filtered with syringe filters (0.2 μm) into HPLC autosampler vials. Standard solutions of α-amino-n-butyric acid (internal standard), serine, glycine, glutamate, glutamine, aspartate, asparagine and alanine were prepared in 0.1 M HCl. A stock solution with all the standards was prepared and then diluted in order to have increasing concentrations for the calibration curves (0, 5, 10, 15, 20 and 25 μM).

Statistical analysis

All the analyses were made using GenStat 8.2, 2005 (Lawes Agricultural Trust, Rothamsted Research, Harpenden, UK). Non-linear modelling was used to fit an asymptotic exponential model to the variation of the leaf relative water content (*RWC*) with the amount of water in the soil. Using *F*-tests, non-significantly different ($P > 0.05$) parameters between species were amalgamated in order to have a parsimonious model of the data. The responses of the net CO_2 assimilation rate (A) and the stomatal conductance to water vapour (*gswa*) to the intercellular CO_2 concentration (C_i) and to the given concentration of O_2 were modelled similarly. Firstly, non-linear curves were fitted to the data from each individual plant. Statistically, the best models were an asymptotic exponential for the variation of A with C_i, a modified logistic for the variation of *gswa* with C_i and an 'exponential plus linear' for the variation of A with O_2. The latter consisted of an exponential-associated increase followed by a linear decrease effective after maximal A was attained. Residual maximum likelihood (REML) analysis was then used to predict mean values of estimated parameters for each species by stress level combination that would occur if the number of plants in each group (C, MS and SS) was the same (Patterson & Thompson 1971). Such means were compared using a *t*-test on the appropriate degrees of freedom (d.f.) from the REML model and the standard error of the difference.

Regression analysis was applied to model the variation of the isotope compositions and amino acid content with *RWC*, including a squared term in this variable to check for non-linearity. Non-significantly different ($P > 0.05$) parameters (*t*-tests) in the significant ($P < 0.05$) model terms were amalgamated in order to attain parsimony. The residuals were checked and found to generally conform to the assumptions of the analysis. All the absolute values and percentages presented in the text were calculated from the regression models pertaining to each data set. The difference between the values obtained for the content of each amino acid in the samples collected in the light and after 30 s in darkness was calculated (these being paired samples). Regression analysis revealed no significant effect of *RWC* on this difference for any of the amino acids studied, and therefore the REML method was used to output predicted mean values for the difference (dark *minus* light) for each species. Significance from zero was assessed through *t*-tests.

Modelling C_4 photosynthesis

The mechanistic model of C_4 photosynthesis of von Caemmerer & Furbank (1999), described in detail by von Caemmerer (2000) and based on the models of Berry & Farquhar (1978) and Peisker (1979), was applied to the data from the CO_2 response curves measured at high irradiance. A similar approach to that described by Massad, Tuzet & Bethenod (2007) was used on a plant-by-plant basis. Firstly, an asymptotic exponential model was found to provide the best description of the variation of net CO_2 assimilation rate (A) with the intercellular CO_2 concentration (C_i) for each plant. The equations for enzyme-limited photosynthesis (von Caemmerer 2000) were then applied to the individual plants to estimate the maximum Rubisco carboxylation activity (V_{cmax}) and the maximum PEPC carboxylation activity (V_{pmax}), as well as the CO_2 concentrations in the BS (C_s) and in the M cells (C_m), for values of C_i from 0 to 560 μmol mol^{-1} using a step size of 5 μmol mol^{-1}. Applying the method, all four parameters were primarily estimated and then, for each plant, V_{pmax} and V_{cmax} were fixed at the mean of estimated values found between 75–175 and 300–400 μmol mol^{-1} C_i, respectively, to re-estimate C_s and C_m

Table 1. Summary of C_4 photosynthesis parameters assumed as constant at 25 °C (von Caemmerer 2000) and used in the equations for enzyme-limited photosynthesis

Parameter	Value	Description
K_c	650 μbar	Michaelis–Menten constant of Rubisco for CO_2
K_o	450 mbar	Michaelis–Menten constant of Rubisco for O_2
K_p	80 μbar	Michaelis–Menten constant of PEPC for CO_2
O	210 mbar	O_2 partial pressure in the BS and mesophyll cells
R_d	0.01 V_{cmax}	Leaf mitochondrial respiration
R_m	0.5 R_d	Mesophyll mitochondrial respiration
g_{bs}	3 mmol m^{-2} s^{-1}	BS conductance to CO_2
g_i	2 mol m^{-2} s^{-1}	Mesophyll conductance to CO_2
γ^*	0.000193	Half the reciprocal of Rubisco specificity

Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PEPC, phosphoenolpyruvate carboxylase; BS, bundle sheath; V_{cmax}, maximum Rubisco carboxylation activity.

(over C_i), this time free from the instability of the numerical calculation of all four parameters simultaneously. The model parameters assumed as constant at 25 °C (von Caemmerer 2000) are listed in Table 1, and the equations applied were

$$A_n = g_i(C_i - C_m) \quad (1)$$

$$A_n = \frac{C_s V_{cmax}}{C_s + K_c\left(1 + O/K_o\right)}\left(1 - \frac{\gamma^* O}{C_s}\right) - R_d \quad (2)$$

$$A_n = \frac{C_m V_{pmax}}{C_m + K_p} - g_{bs}(C_s - C_m) - R_m. \quad (3)$$

In these equations, A_n represents the net CO_2 assimilation rate calculated from the asymptotic exponential curve. Mean values of V_{pmax} and V_{cmax} and mean curves for C_s and C_m versus C_i were then calculated for the treatment structure of the species by stress level over the plants.

The rate of PEPC carboxylation, V_p, was calculated as

$$V_p = \frac{C_m V_{pmax}}{C_m + K_p}, \quad (4)$$

and the net CO_2 assimilation rate (A_c) was calculated as the solution to the quadratic expression for enzyme-limited photosynthesis (von Caemmerer 2000), taking the fraction of O_2 evolution in the BS as zero:

$$aA_c^2 + bA_c + c = 0,$$

this being

$$A_c = \frac{-b - \sqrt{b^2 - 4ac}}{2a}, \quad (5)$$

with

$$a = 1;$$

$$b = -\{(V_p - R_m + g_{bs}C_m) + (V_{cmax} - R_d) + g_{bs}(K_c(1 + O/K_o))\};$$

$$c = (V_{cmax} - R_d)(V_p - R_m + g_{bs}C_m) - (V_{cmax}\, g_{bs}\gamma^* O + R_d g_{bs} K_c(1 + O/K_o)).$$

The rate of photorespiration (Pr) was predicted for each species by stress level combination using the equation below, which is derived from the equation of overall CO_2 assimilation that describes Rubisco carboxylation in the BS (von Caemmerer 2000):

$$A = V_c - 0.5V_o - R_d. \quad (6)$$

Considering that Pr will be half the rate of Rubisco oxygenation, V_o,

$$Pr = 0.5V_o = -(A - V_c + R_d), \quad (7)$$

where A is the overall CO_2 assimilation and the rate of Rubisco carboxylation, V_c, is calculated as

$$V_c = \frac{C_s V_{cmax}}{C_s + K_c\left(1 + O/K_o\right)}.$$

RESULTS

Water relations

The variation of the relative water content (RWC) in the leaves of *P. dilatatum*, *C. dactylon* and *Z. japonica* with the water weight in pot (WWP) was described by an asymptotic exponential model (Fig. 1). In the gas exchange experiment, RWC decreased to lower values in *P. dilatatum* (40%) than in *C. dactylon* and *Z. japonica* (75%). In the amino acid experiment, watering of the pots of *Z. japonica* and *C. dactylon* was stopped, respectively, 1 or 2 d prior to those of *P. dilatatum*. As a result, lower RWC values were observed for the most stressed leaves of *Z. japonica* (40%) than for *C. dactylon* (60%) and *P. dilatatum* (80%).

CO_2 and O_2 response curves

The net CO_2 assimilation rate (A) by the leaves of *P. dilatatum*, *C. dactylon* and *Z. japonica* increased with the intercellular CO_2 concentration (C_i), both in control and drought stress conditions, as shown in Fig. 2a–c. The mean value for the maximal net CO_2 assimilation rate (A_{max}) in fully hydrated leaves of *P. dilatatum* and *C. dactylon* plants was higher than in *Z. japonica* plants. In MS leaves of the two former species, A_{max} decreased in relation to the control, whereas no significant difference ($P > 0.05$)

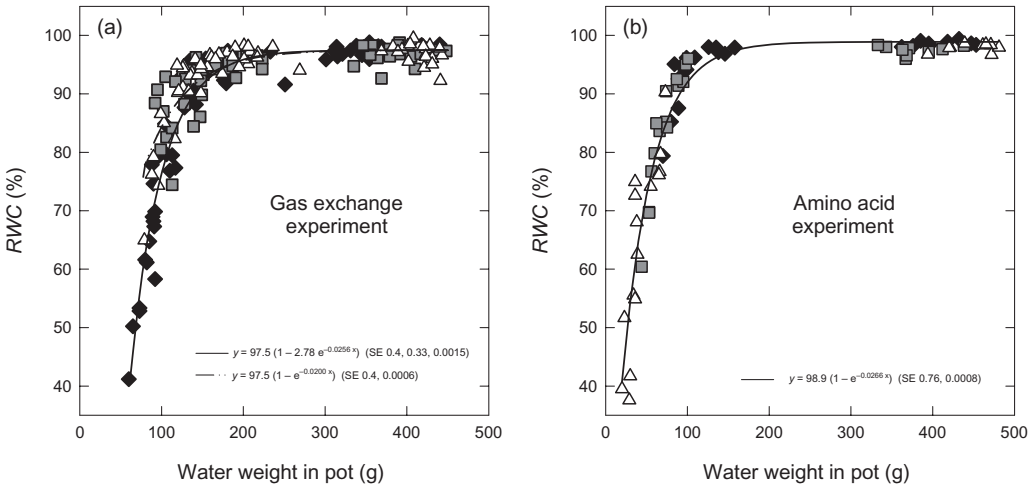

Figure 1. Leaf relative water content (*RWC*) as a function of the amount of water in the soil, measured as the water weight in pot (*WWP*), of the control and non-watered plants of *Paspalum dilatatum* (black diamonds), *Cynodon dactylon* (grey squares) and *Zoysia japonica* (open triangles). Each data point corresponds to one sample, with 30 (a) or 24 (b) samples per species. The asymptotic exponentials fitted correspond to the best models statistically significant [(a), $R^2 = 89.6\%$, $s^2 = 11.9$, d.f. = 176; (b), $R^2 = 92.3\%$, $s^2 = 19.3$, d.f. = 70]. d.f., degrees of freedom.

between MS and control leaves was observed in *Z. japonica*. The mean values of A_{max} obtained for the severely dehydrated (SS) leaves of *P. dilatatum* and *Z. japonica* were slightly less than half of the values observed for each species under control conditions.

The stomatal conductance to water vapour (*gswa*) decreased with increasing C_i for both fully hydrated and drought-stressed plants of the three species (Fig. 2d–f). The mean values of *gswa* in fully hydrated leaves of *C. dactylon* were generally higher than in the other two species, especially at low CO_2 concentrations, and were decreased by drought stress in all species. In MS leaves of *C. dactylon* and in SS leaves of *P. dilatatum* and *Z. japonica*, the maximal value of *gswa* was lower than in control or less dehydrated leaves, but no significant differences ($P > 0.05$) were observed between the control and MS plants of *P. dilatatum* and *Z. japonica*.

Photosynthesis by *P. dilatatum*, *C. dactylon* and *Z. japonica* plants under control or drought stress conditions was not dramatically affected by the O_2 concentration

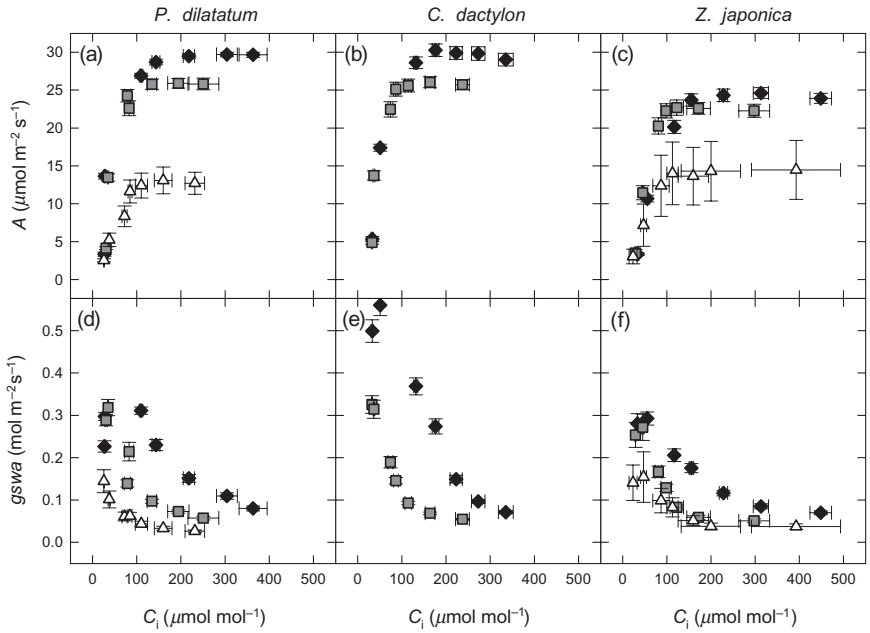

Figure 2. Mean values of net CO_2 assimilation rate (A, a–c) and stomatal conductance to water vapour (*gswa*, d–f) in response to the intercellular CO_2 concentration (C_i) in the control (black diamonds), moderately stressed (grey squares) and severely stressed (open triangles) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. The bars correspond to the standard errors of each mean value. Measurements were taken at ambient O_2 (21%), a photosynthetic photon flux density of 850 μmol m^{-2} s^{-1} and at 25 °C.

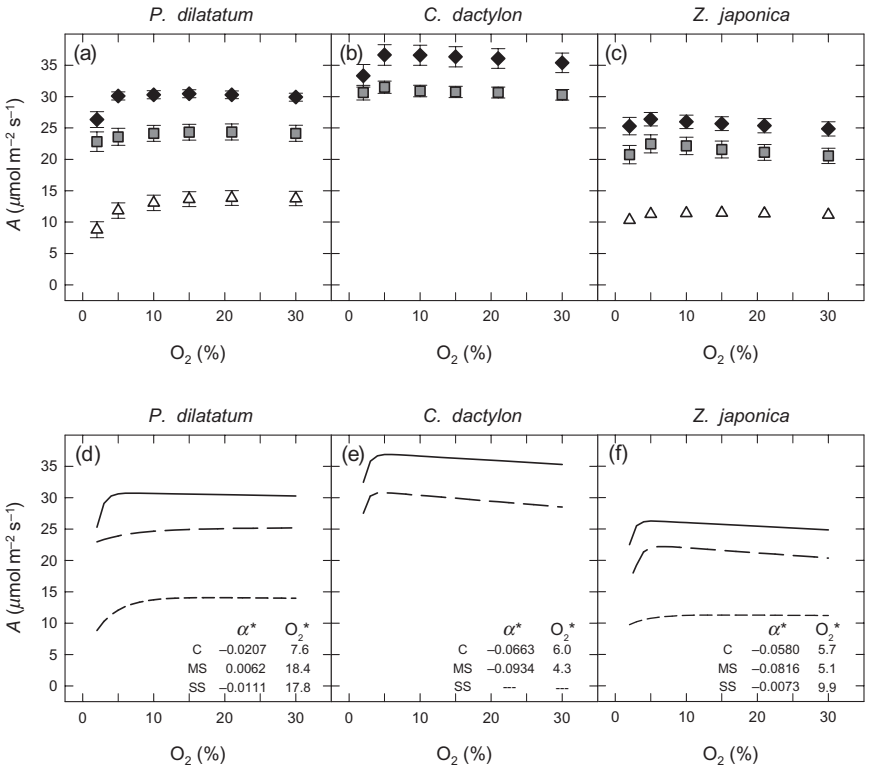

Figure 3. (a–c) Mean values of net CO_2 assimilation rate (A) at different O_2 concentrations in the control (C, black diamonds), moderately stressed (MS, grey squares) and severely stressed (SS, open triangles) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. The bars correspond to the SEs of each mean value. Measurements were taken at ambient CO_2 (360 μmol mol^{-1}), a photosynthetic photon flux density of 850 μmol m^{-2} s^{-1} and at 25 °C. (d–f) Representation of the 'exponential plus linear' model fitted to the variation of A with O_2 in the C (solid lines), MS (long-dashed lines) and SS (short-dashed lines) plants of each species. The lines correspond to the curves obtained by plotting the best model statistically significant. Also shown are the mean values estimated for the slope α*, representing the linear decrease of A with O_2 after maximal net CO_2 assimilation rate (A_{max}) was attained, and the O_2 concentration corresponding to A_{max} (O_2*). The 'average' standard error of difference considering all data was 0.0683 for α* and 2.5 for O_2* (with 55 degrees of freedom).

(Fig. 3a–c). However, lower values of A were generally observed at 2% compared with higher O_2 concentrations, and an 'exponential plus linear' model was successfully applied to the variation of A with O_2 (Fig. 3d–f), showing that after the maximal value of net photosynthesis (A_{max}) was attained, at O_2 concentrations generally between 4 and 10% (O_2*), a slight decrease of A with increasing O_2 tends to occur. Note that the predictions of the O_2* were poor because there was no clear definition of the point corresponding to A_{max}. Apart from an increase of O_2* to values closer to the atmospheric concentration in the dehydrated leaves of *P. dilatatum* (MS and SS), no other significant differences ($P > 0.05$) were observed.

Carbon and oxygen isotope compositions

The carbon isotope composition ($\delta^{13}C$) in fully hydrated leaves of *P. dilatatum* (–15.2‰) was less negative than in *C. dactylon* (–16.3‰) and *Z. japonica* (–16.6‰). Drought stress had an identical effect on $\delta^{13}C$ for the three species, $\delta^{13}C$ being decreased by –0.5‰ when *RWC* decreased to 60% (Fig. 4a). No significant differences in the oxygen isotope composition ($\delta^{18}O$) were found between *C. dactylon* and *Z. japonica* ($P > 0.05$). Higher values were generally observed for these two species than for *P. dilatatum* (Fig. 4b), and an increase of ca. 4‰ with dehydration (to 60% *RWC*) was observed for all species.

Amino acid analysis

The variation of the amino acid content in illuminated leaves of the three C_4 grasses with leaf dehydration is shown in Fig. 5. The fully hydrated leaves of *P. dilatatum* had generally more serine (Ser) than the other two species. This amino acid increased steeply in all three species when *RWC* decreased from 98 to 60% (*C. dactylon* and *Z. japonica*) or only down to 80% (*P. dilatatum*). Below 60% *RWC*, a slight decrease was observed for the leaves of *Z. japonica*. Glycine (Gly) had a quadratic variation with *RWC*. In *C. dactylon*, there was only a slight increase when *RWC* started to decrease and then glycine was kept nearly constant. *Z. japonica* had less glycine than the other two species in fully hydrated conditions, and the amount increased as the *RWC* decreased from 98 to 60%, but was not so strongly affected

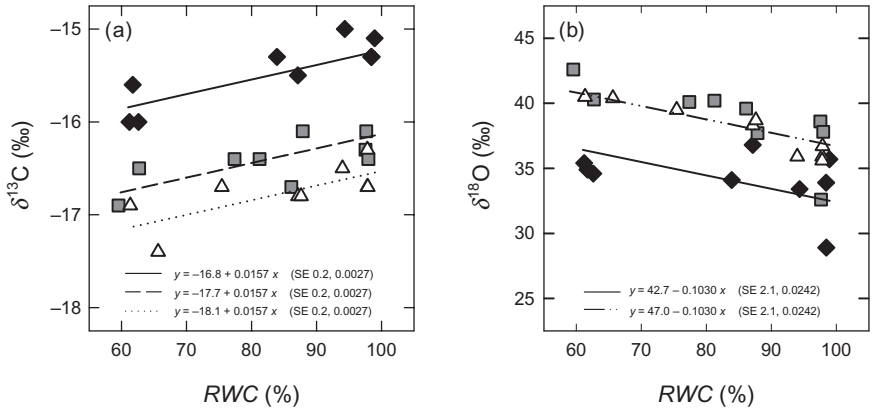

Figure 4. Carbon ($\delta^{13}C$, a) and oxygen ($\delta^{18}O$, b) isotope compositions as a function of the relative water content (*RWC*) in the leaves of *Paspalum dilatatum* (black diamonds), *Cynodon dactylon* (grey squares) and *Zoysia japonica* (open triangles). Each data point corresponds to one sample, with 9 samples per species. The regression lines correspond to the best models statistically significant [*P. dilatatum*, solid lines; *C. dactylon*, dashed lines; and *Z. japonica*, dotted lines; (a), $R^2 = 90.1\%$, $s^2 = 0.40$, d.f. = 23; (b), $R^2 = 65.3\%$, $s^2 = 3.23$, d.f. = 24]. d.f., degrees of freedom.

by further decreases in *RWC*. In *P. dilatatum*, although high variability was observed, the regression showed increased glycine when *RWC* decreased from 99 to 80%. The ratio Gly/Ser was only decreased with *RWC* in *C. dactylon*. Glutamate (Glu) decreased with decreasing *RWC* only in *C. dactylon*, and glutamine (Gln) was not significantly affected by leaf dehydration ($P > 0.05$), the same being observed for the ratio Gln/Glu. Aspartate (Asp) decreased linearly with *RWC* in *P. dilatatum* and *Z. japonica*, but no significant variation with *RWC* was observed for *C. dactylon* ($P > 0.05$). Alanine (Ala) was not significantly affected by leaf dehydration ($P > 0.05$) in the three species, showing high variability, especially in *C. dactylon*. Conversely, asparagine (Asn) increased linearly with decreasing *RWC*, but only in *C. dactylon* and *Z. japonica*.

The content of some amino acids in the leaves collected after a period of 30 s in darkness changed significantly compared with corresponding leaves collected in the light (Table 2), but the difference 'dark *minus* light' was not affected by leaf dehydration ($P > 0.05$). In the three species, a decrease in glycine content after 30 s in darkness was observed. The ratio between glycine and serine also decreased, mostly because of the decreased glycine, as no significant differences were observed for serine. Both glycine and the ratio Gly/Ser showed a larger decrease in *C. dactylon* than in *P. dilatatum* or *Z. japonica*. Glutamate decreased in the dark compared with the light samples, but only for *P. dilatatum* and *C. dactylon*. Conversely, glutamine content was not significantly changed ($P > 0.05$), and the ratio Gln/Glu increased only in *P. dilatatum*, with no significant changes in *C. dactylon* or *Z. japonica* ($P > 0.05$). Aspartate decreased after 30 s in darkness in *C. dactylon* and *Z. japonica*. Concomitantly, in these two species, a substantial increase in alanine was observed. The content in asparagine was not significantly changed in 30 s of darkness ($P > 0.05$).

Mechanistic modelling of C_4 photosynthesis

The variation of the enzyme-limited net CO_2 assimilation rate (A_c) with the CO_2 concentration in the M cells (C_m) for each of the three C_4 grasses was affected by drought stress as shown in Fig. 6. The mean values estimated for the maximum PEPC carboxylation activity (V_{pmax}) and maximum Rubisco carboxylation activity (V_{cmax}) were higher in *C. dactylon* than in *P. dilatatum* and lower in *Z. japonica*, and were significantly affected ($P < 0.01$) by the drought stress level.

By application of the model equations, the concentration of CO_2 in the BS (C_s) is predicted to increase steeply with the increase in C_i, reaching values above 1000 μmol mol^{-1} at low C_i (Fig. 7a–c). Accordingly, the rate of photorespiration (*Pr*) for each of the three C_4 grasses at the different stress levels was estimated to be always lower than 1.5 μmol m^{-2} s^{-1} and decreased with increasing C_i (Fig. 7d–f). The values predicted for *Pr* at ambient levels of CO_2 (360–390 μmol mol^{-1}) were slightly higher for the MS plants of each species than in the corresponding controls, whereas slightly lower values were obtained for the SS relative to the MS plants of *P. dilatatum* and *Z. japonica*.

DISCUSSION

Drought stress condition and photosynthetic responses to CO_2

The relative water content (*RWC*) in the leaves of *P. dilatatum*, *C. dactylon* and *Z. japonica* decreased with the decrease in water available in the soil (Fig. 1) and was used as a reference to analyse the effects of drought. The three species showed some differences in the extent of leaf dehydration. In the gas exchange experiment, *P. dilatatum* leaves were more severely dehydrated than the other two species. In the amino acid experiment, the plants of *C. dactylon* and *Z. japonica* were deprived of water before *P. dilatatum* in an attempt to get a similar range of *RWC* values in the samples of the three species. However, *C. dactylon* and (more dramatically) *Z. japonica* plants were more dehydrated in this experiment. The difficulty in obtaining similar dehydration levels for the three species was a result of the faster loss of water by the leaves of *P. dilatatum* than by those of *C. dactylon* and *Z. japonica* in response to the drying soil. When water deficit was rapidly imposed in these C_4 grasses by the addition of polyethylene glycol to the nutrient solution (Carmo-Silva *et al.* 2007), *C. dactylon* showed a faster decrease in *RWC* than *P. dilatatum* and *Z. japonica*. The

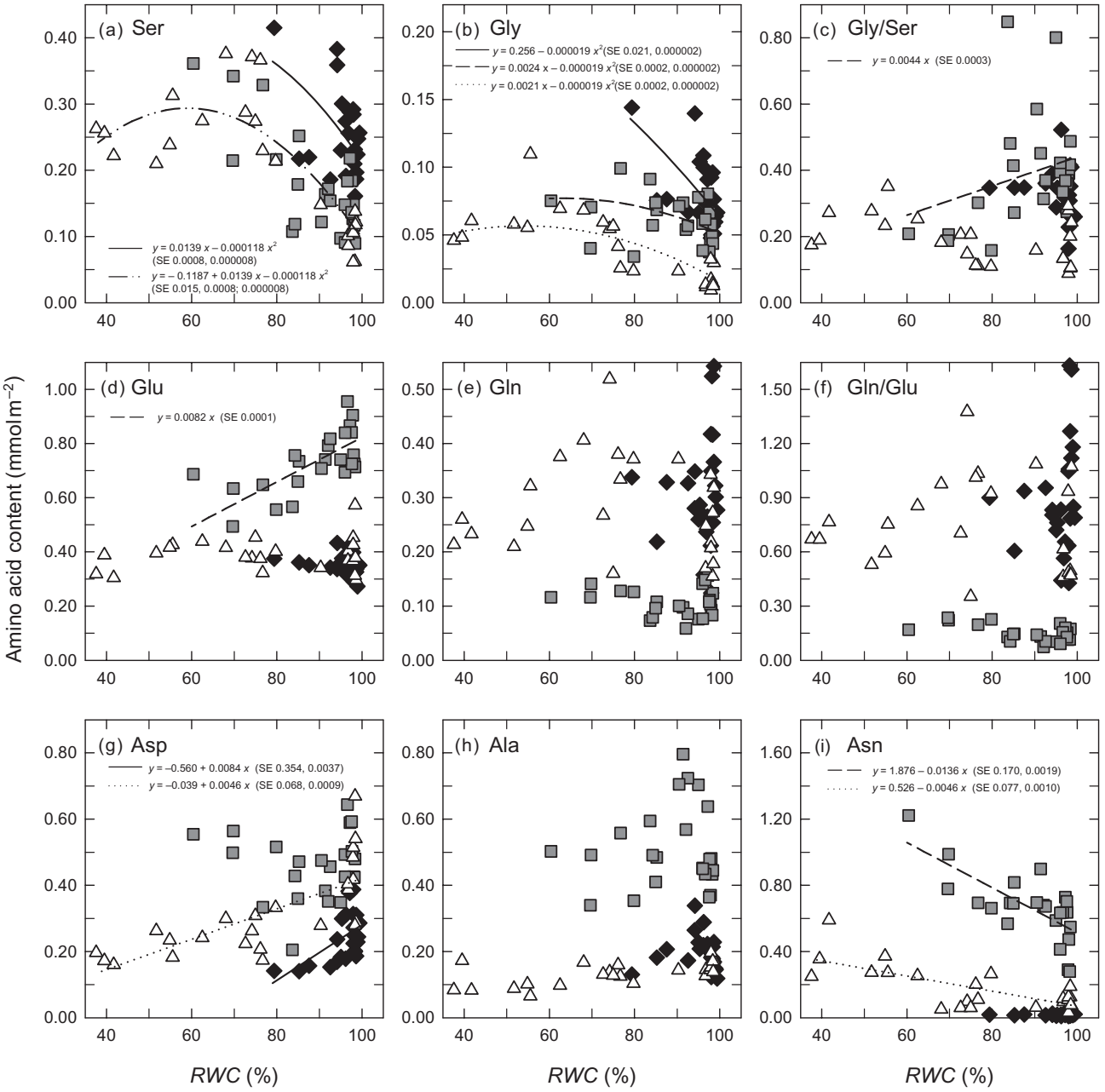

Figure 5. Variation of the amino acid content with the relative water content (*RWC*) in the leaves of *Paspalum dilatatum* (black diamonds), *Cynodon dactylon* (grey squares) and *Zoysia japonica* (open triangles) collected in the light: serine (Ser), glycine (Gly), Gly/Ser ratio, glutamate (Glu), glutamine (Gln), Gln/Glu ratio, aspartate (Asp), alanine (Ala), asparagine (Asn) (a–i, respectively). Each data point corresponds to one sample, with 24 samples per species. The regression lines and curves, when applied, correspond to the best models statistically significant [*P. dilatatum*, solid lines; *C. dactylon*, dashed lines; and *Z. japonica*, dotted lines; (a) $R^2 = 59.8\%$, $s^2 = 0.297$, d.f. = 69; (b) $R^2 = 56.1\%$, $s^2 = 0.035$, d.f. = 67; (c) $R^2 = 40.5\%$, $s^2 = 0.012$, d.f. = 68; (d) $R^2 = 88.5\%$, $s^2 = 0.406$, d.f. = 69; (e) $P > 0.05$; (f) $P > 0.05$; (g) $R^2 = 56.1\%$, $s^2 = 0.035$, d.f. = 67; (h) $P > 0.05$; (i) $R^2 = 56.1\%$, $s^2 = 0.035$, d.f. = 67]. d.f., degrees of freedom.

differences are likely to result from the different methods used for drought stress induction (Chaves *et al.* 2003).

Stomatal closure and decreased photosynthesis are generally accepted as early consequences of leaf dehydration (e.g. Chaves *et al.* 2003), and this was observed among several C_4 grasses (Ghannoum *et al.* 2003; Marques da Silva & Arrabaça 2004; Carmo-Silva *et al.* 2007). The net CO_2 assimilation rate (*A*) decreased in the MS leaves of *P. dilatatum* and *C. dactylon* but not in *Z. japonica* (Fig. 2) compared with the controls, suggesting that photosynthesis by the latter species might be more resistant to moderate drought conditions. In SS leaves of *P. dilatatum* and *Z. japonica*, *A* decreased to about half of the values observed in control conditions. Stomatal closure was also observed in response to drought stress, as shown by the decrease in the stomatal conductance to water vapour (*gswa*) in the MS and

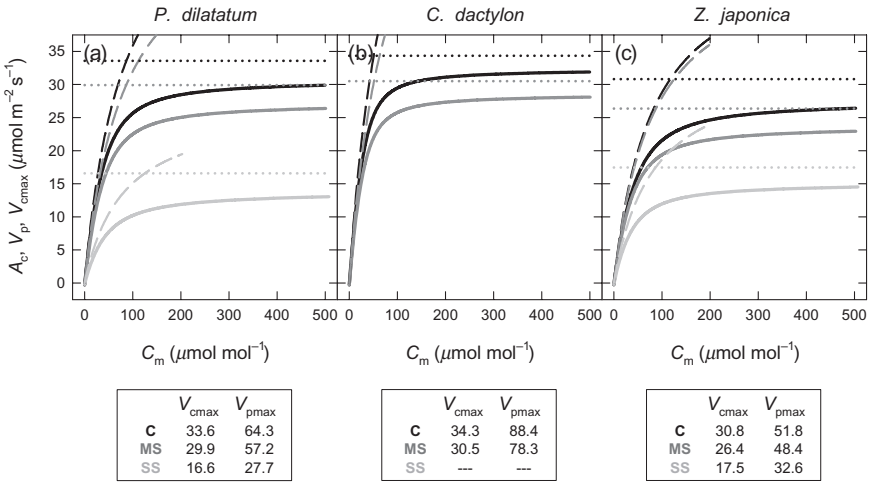

Figure 6. Enzyme-limited net CO_2 assimilation rate (A_c) as a function of the mesophyll CO_2 concentration (C_m) in the control (C, black lines), moderately stressed (MS, dark-grey lines) and severely stressed (SS, light-grey lines) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Also shown are the rates of phosphoenolpyruvate (PEP) carboxylation (V_p, dashed lines) and the maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation activity (V_{cmax}, dotted lines). The mean values of V_{cmax} and maximum PEP carboxylase carboxylation activity (V_{pmax}) are presented below each graph. All parameters were calculated by applying a mechanistic model of C_4 photosynthesis (von Caemmerer & Furbank 1999). For simplification, assumption was made that the fraction of O_2 evolution in the bundle sheath is zero.

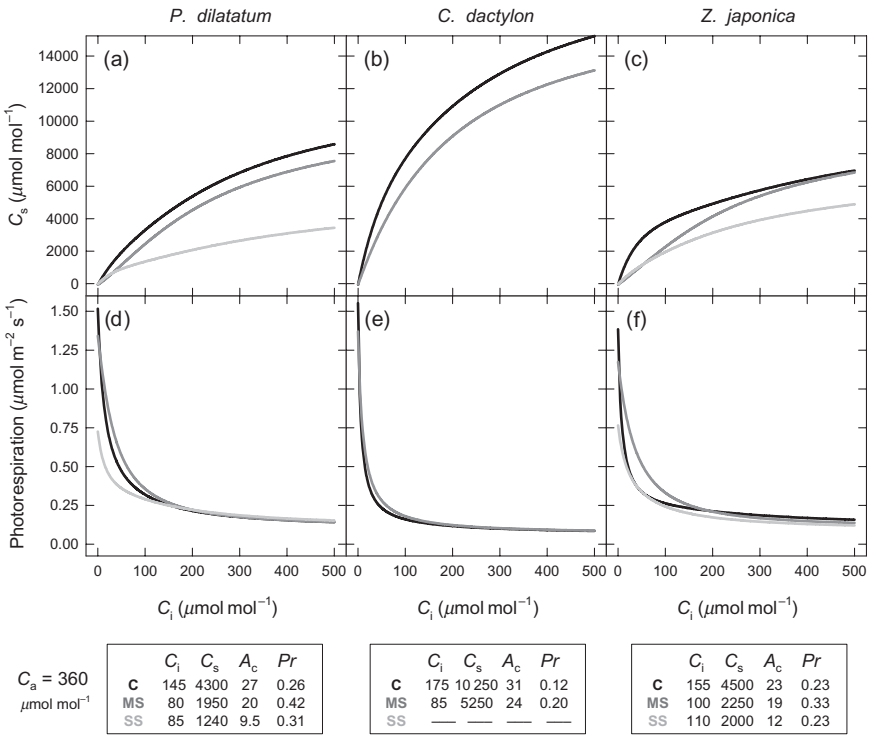

Figure 7. Representation of the predicted CO_2 concentration in the bundle sheath (BS) cells (C_s) and rate of photorespiration (Pr) as a function of the intercellular CO_2 concentration (C_i) in the control (C, black lines), moderately stressed (MS, dark-grey lines) and severely stressed (SS, light-grey lines) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. For the estimation of C_s and Pr, using the equations for enzyme-limited C_4 photosynthesis (von Caemmerer & Furbank 1999), maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation activity (V_{cmax}) and maximum phosphoenolpyruvate carboxylase carboxylation activity (V_{pmax}) were fixed at the mean values obtained for each group of plants (see Fig. 6), and other model parameters, namely, the O_2 concentration in the BS, were assumed to be constant at 25 °C. Also shown are the mean values of C_i, C_s, net CO_2 assimilation rate (A_c) and Pr, estimated through the modelling approach, at ambient concentrations of CO_2 (360–390 μmol mol^{-1}) and O_2 (21%).

Species	Ser	Gly	Gly/Ser	Glu	Gln	Gln/Glu	Asp	Ala	Asn
P. dilatatum	NS	–0.007 (0.004)	–0.025 (0.019)	–0.029 (0.020)	NS	0.125 (0.056)	NS	NS	NS
C. dactylon	NS	–0.019 (0.004)	–0.145 (0.019)	–0.059 (0.020)	NS	NS	–0.055 (0.019)	0.115 (0.023)	NS
Z. japonica	NS	–0.007 (0.004)	–0.043 (0.019)	NS	NS	NS	–0.031 (0.019)	0.085 (0.023)	NS

Table 2. Estimated mean values of the difference between the amino acid content (mmol m^{-2}) in the leaves of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica* collected in the light and after 30 s in darkness ('dark *minus* light') and respective SE of differences (SEDs) (in brackets)

There was no significant variation with relative water content (*RWC*) ($P > 0.05$) and, therefore, the overall mean values obtained for the samples of each species (considering all control and non-watered plants, in a total of 24 samples per species) were analysed by the residual maximum likelihood (REML) method.
NS, not significantly different from zero ($P > 0.05$); Ser, serine; Gly, glycine; Glu, glutamate; Gln, glutamine; Asp, aspartate; Ala, alanine; Asn, asparagine.

SS plants compared with the controls (Fig. 2). The maximal values of *gswa* in the MS leaves of *P. dilatatum* and *Z. japonica* were not affected compared with the control, which demonstrates that the effect of the low CO_2 concentrations promoting the opening of stomata was stronger than the drought-induced stomatal closure at that stage. However, for higher CO_2 concentrations, *gswa* was always lower in dehydrated leaves compared with the control plants of each species, resulting in generally lower values of C_i for a given CO_2 concentration. The decrease of A_{max} in the most dehydrated leaves of each grass species compared with their controls suggests that metabolic limitations of photosynthesis are also present. The same conclusion has been reached for other C_4 grasses (Ghannoum *et al.* 2003). Net CO_2 assimilation consistently increased with increasing C_i and the CO_2 concentration corresponding to zero A (the CO_2 compensation point) was not significantly different from zero ($P > 0.05$) for either control or dehydrated leaves of any of the three species, suggesting little photorespiration.

O_2-sensitivity of C_4 photosynthesis

Net CO_2 assimilation rates (A) at ambient CO_2 (360 μmol mol^{-1}) were little affected by the O_2 concentration in the gas phase, but A was lowest at 2% O_2 and decreased significantly with O_2 after the maximal value (A_{max}) was attained (Fig. 3). Little sensitivity of C_4 photosynthesis to O_2 was found in two other grasses, *Panicum antidotale* (NADP-ME) and *Panicum coloratum* (NAD-ME), at low C_i (Ghannoum *et al.* 1998). The estimation of the O_2 concentration corresponding to A_{max} in the three C_4 grasses of the different metabolic subtypes (O_2*, Fig. 3) agrees with previously reported results (Maroco *et al.* 1997). The inhibition of A at low O_2 concentrations is probably due to an extra energy requirement for the regeneration of PEP and the proper functioning of the C_4 cycle (Ku *et al.* 1991; Dai *et al.* 1993; Maroco *et al.* 2000). A reduced ATP production, by the O_2-dependent photochemical reactions or even by mitochondrial respiration (Maroco *et al.* 1997), might lead to a deficient function of the C_4 pathway. The slight decrease of A at O_2 concentrations higher than 10% is likely to be due to the competing oxygenation of RuBP that initiates the photorespiratory carbon oxidative cycle.

The rate of decreasing A with increasing O_2 (Fig. 3d–f), partly caused by photorespiration, suggests that rates in ambient air would be in the range 0–2 μmol m^{-2} s^{-1} (representing 0–6% of A_{max}) and would be slightly faster in *C. dactylon* than in the other two C_4 grasses, but the variability is large and there is no clear trend for its variation with leaf dehydration. Because A was affected by drought (Figs 2 & 3), there is a tendency for increased photorespiration in proportion to photosynthesis when leaf dehydration increases. Photorespiration in *A. edulis* (C_4 dicotyledon), estimated by the release of NH_3, represented 6% of the rate of CO_2 assimilation (Lacuesta *et al.* 1997). In two C_4 grasses, a sharp increase of the CO_2 compensation point when the *RWC* fell below 60%, compared with very low values in fully hydrated leaves, led to the conclusion that photorespiration was greatly enhanced under drought conditions (Ghannoum *et al.* 2003). The results presented here show that photorespiration by the three C_4 grasses studied is slow and not sufficient to explain the decrease observed in A under the drought conditions attained (Fig. 1).

Effects of dehydration on carbon and oxygen isotope compositions

Drought stress affected leaf carbon and oxygen isotope compositions ($\delta^{13}C$ and $\delta^{18}O$) similarly in the three C_4 grasses (Fig. 4), with a decrease of 0.5‰ in $\delta^{13}C$ and an increase of 4‰ in $\delta^{18}O$ when the *RWC* decreased down to 60%. In cotton leaves (C_3 plant), stomatal closure after abscisic acid treatment resulted in ^{13}C and ^{18}O enrichment (Barbour & Farquhar 2000). Conversely, a study with several C_4 grasses revealed a decrease of $\delta^{13}C$ under drought but no consistent variation in $\delta^{18}O$ (Ghannoum, von Caemmerer & Conroy 2002). The less negative values of $\delta^{13}C$ in *P. dilatatum* (NADP-ME) than in *C. dactylon* (NAD-ME) and *Z. japonica* (PEPCK) agree with previously reported differences among C_4 grasses from the different subtypes (Hattersley 1982). On the other hand, differences in the leaf length and inter-veinal distance among grasses (Helliker & Ehleringer 2000) might explain the higher values of $\delta^{18}O$ in *C. dactylon* and *Z. japonica* than in *P. dilatatum*.

Variations in $\delta^{13}C$ are mostly due to changes in the ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a) and/or in changes in the fraction of CO_2 fixed by PEPC that subsequently leaks out from the BS without being assimilated by Rubisco (leakiness, ϕ) (Farquhar 1983). Variation in ϕ can result either from alterations in the physical conductance of BS cells to CO_2 or from alterations in the balance between PEPC and Rubisco activities (Peisker & Henderson 1992). In *Sorghum bicolor* (Williams *et al.* 2001) and in sugarcane (Saliendra *et al.* 1996), both carbon isotope discrimination and ϕ increased under drought conditions, suggesting that the coordination between the C_4 and C_3 cycles was affected. Conversely, Buchmann *et al.* (1996) related the decrease of $\delta^{13}C$ in several C_4 grasses (including *P. dilatatum*, *C. dactylon* and *Z. japonica*) under drought with decreased stomatal conductance, which affected the intercellular CO_2 concentration. Decreased *gswa* observed in the dehydrated leaves of each species (Fig. 2) resulted in decreased C_i/C_a compared with the control plants of each species (data not shown) and could contribute to the decrease in $\delta^{13}C$ (Fig. 4a). However, the data obtained do not exclude the possibility that impairment of photosynthetic metabolism increased ϕ and contributed to the decrease in $\delta^{13}C$ with drought in the three C_4 grasses.

The increase of leaf $\delta^{18}O$ with *RWC* (Fig. 4b) reflects the variation of $\delta^{18}O$ in the soil water, which becomes enriched in the heavy isotope under drought conditions because of evaporation, and variation because of evaporative and diffusional effects during transpiration (Barbour 2007). Decreased *gswa* in the dehydrated leaves (Fig. 2) probably contributed to the leaf ^{18}O enrichment because $H_2{}^{18}O$ diffuses more slowly and has lower vapour pressure than $H_2{}^{16}O$, causing the water in the leaf to become enriched in ^{18}O during transpiration. Variations in evaporation during plant growth are integrated in the oxygen isotope composition of the leaf material (Barbour & Farquhar 2000). Although fractionation of oxygen isotopes occurs during exchanges of CO_2 and O_2 with the atmosphere resulting from the sum of photosynthesis, photorespiration and mitochondrial respiration, these effects are quickly buffered in the leaf because of rapid isotopic exchange between the carbonyl oxygen in organic molecules and leaf water (Barbour 2007).

Although some suggestions have been made that photorespiration should be considered when interpreting the $\delta^{13}C$ (Gillon & Griffiths 1997) and $\delta^{18}O$ (Farquhar, Barbour & Henry 1998) of the leaf material, the rate of photorespiration present in C_4 plants is not likely to be of reasonable size to make considerable contribution to the variations in isotope ratios of leaf dry matter. In the present study, drought did not cause changes in $\delta^{13}C$ in the same direction as observed for C_3 species (Cerling 1999), where photorespiration is rapid.

Effects of dehydration on steady-state contents of amino acids in illuminated leaves

The increased amounts of glycine and serine in the dehydrated leaves of all three species, especially with decreases in *RWC* down to 80 and 60% (Fig. 5a,b), might be interpreted as an increase in photorespiratory metabolism and flux of glyoxylate into the pathway because of increased oxygenation of RuBP. Alternatively, these changes may reflect an increase in pool sizes of glycine and serine because of slower transfer to the mitochondria and peroxisome. Increased glycine and serine contents were also observed in droughted maize leaves (Foyer *et al.* 1998).

In leaves of *C. dactylon*, when *RWC* decreased from 98 to 60%, asparagine increased from 0.5 to 1.1 mmol m^{-2} (Fig. 5i) and glutamate decreased from 0.8 to 0.5 mmol m^{-2} (Fig. 5d). An increase of two- to sixfold in the amount of asparagine was previously reported in *C. dactylon* plants submitted to drought stress (Barnett & Naylor 1966), and the same authors reported a concomitant decrease in glutamate and alanine. In *Z. japonica*, the content of asparagine increased by fourfold and aspartate decreased when *RWC* decreased to 40% (Fig. 5i,g), but glutamate amounts were not affected. No significant effect of *RWC* on the alanine content was observed for any of the three C_4 grasses (Fig. 5h). The contents of glutamate, asparagine and alanine were generally higher in *C. dactylon* than in *P. dilatatum* and *Z. japonica*, whereas the content of glutamine was lower in the first species.

Amino acid changes in 30 s of darkness

In illuminated leaves, carboxylation and oxygenation of RuBP will produce P-glycerate and P-glycolate. The latter enters the photorespiratory carbon oxidative cycle and, as a result, glycine will be converted into serine in the mitochondria. In darkness, the regeneration of RuBP and the production of both P-glycerate and P-glycolate will stop. The glycine pool will then decrease because the amino acid is no longer being formed, but it is still converted into serine. Therefore, the decrease in the amount of glycine in the leaves after 30 s in darkness compared with the amounts in fully illuminated leaves during steady-state photosynthesis will be related to the rate of photorespiratory production of P-glycolate through the oxygenation of RuBP. The pools of glycine and serine in the leaves are thought to reflect the photorespiratory fixation of O_2 quite accurately, given that the synthesis of these two amino acids occurs mostly through this process (Jolivet-Tournier & Gerster 1984). Earlier studies reveal that in C_3 plants, steady-state photosynthesis followed by 1 or 2 min in darkness results in a considerable decrease in glycine and an increase in serine (Roberts, Keys & Whittingham 1970; Kumarasinghe *et al.* 1977). The rate of incorporation of ^{18}O into glycolate and glycine in maize (C_4 monocotyledon, NADP-ME) leaves increased with increasing O_2 concentrations, providing direct evidence for photorespiratory O_2 uptake, albeit at a much lower rate than in wheat (de Veau & Burris 1989). In *A. edulis* (C_4 dicotyledon, NAD-ME), the decrease of both glycine and serine contents at very low O_2 was consistent with photorespiratory production of both amino acids at atmospheric O_2 levels (Maroco *et al.* 2000). In the C_4 grasses *P. dilatatum*, *C. dactylon* and *Z. japonica*, there was no effect of decreased *RWC* ($P > 0.05$)

on the difference between the content of amino acids in the light and dark samples, and therefore, the mean changes after 30 s in darkness for each species, presented in Table 2, were estimated considering all the data for the control and non-watered plants together. A decrease in the glycine content was observed after 30 s in darkness, but serine content was not affected in any of the three species. The changes in glycine suggest that photorespiration was faster in *C. dactylon* (0.32 ± 0.07 μmol m^{-2} s^{-1}) than in *P. dilatatum* and *Z. japonica* (0.12 ± 0.07 µmol m^{-2} s^{-1}). Maroco *et al.* (2000) proposed that changes in the glycine pool would be a better indicator of the occurrence of photorespiration in maize (C_4) than changes in the serine pool. On the other hand, Novitskaya *et al.* (2002) demonstrated that the ratio between the two amino acids is strongly correlated with the photorespiration in C_3 plants. The decrease in Gly/Ser ratio in the dark was notably higher in *C. dactylon* than in *P. dilatatum* and *Z. japonica* (Table 2), suggesting that photorespiration in the NAD-ME grass might be faster than in the other two species, although still much slower than the values reported for C_3 species (Keys 1986; Novitskaya *et al.* 2002). Photorespiratory CO_2 evolution rates at atmospheric O_2 (21%) and CO_2 (350 μmol mol^{-1}) for wheat (C_3) and maize (C_4) were estimated to be 27 and 2%, respectively, of net photosynthetic CO_2 assimilation (de Veau & Burris 1989). More extensive studies on isotopic oxygen uptake in the light found slightly faster photorespiration rates in NAD-ME compared with NADP-ME monocotyledon species (Furbank & Badger 1982; Siebke *et al.* 2003).

Glutamine synthetase is responsible for the re-assimilation of NH_3 produced in the photorespiratory carbon oxidation cycle (Keys *et al.* 1978), and the glutamate produced by the glutamine:2-oxoglutarate aminotransferase (GOGAT) complex during the photorespiratory nitrogen cycle acts as a donor of amino groups needed for the photorespiratory carbon oxidation cycle (Keys 1999). The GOGAT reaction would be expected to stop quickly in the dark, justifying the decrease in glutamate content in the darkened leaves of *P. dilatatum* and *C. dactylon*, which was not evident in *Z. japonica* (Table 2). As suggested by Novitskaya *et al.* (2002), the trends in glutamate and glutamine might well reflect processes linked to photosynthesis other than photorespiration, including primary N assimilation, and cannot be interpreted as a result of altered photorespiratory NH_3 flux.

The decrease in aspartate, the primary C_4 acid formed in the M cells of NAD-ME and PEPCK species, in the darkened leaves of *C. dactylon* and *Z. japonica* (Table 2), is a clear consequence of stopping the primary fixation of CO_2 by PEPC. Accordingly, the increase in alanine in the same two species is consistent with continuing amination of pyruvate in the C_4 cycle. In wheat and potato (C_3 plants), decreased contents in both aspartate and alanine were found under photorespiratory conditions (Novitskaya *et al.* 2002). However, in durum wheat, a period of 30 s in darkness induced no changes in aspartate or alanine, although a clear decrease in glycine and serine was observed (data not shown). Moreover, the post-illumination changes in aspartate and alanine were restricted to *C. dactylon* and *Z. japonica*, with no changes being observed in *P. dilatatum* (NADP-ME), suggesting their association with the C_4 photosynthetic pathway rather than with the photorespiratory metabolism.

Modelling the CO_2 response of C_4 photosynthesis under drought conditions

A mechanistic model of C_4 photosynthesis (von Caemmerer & Furbank 1999) was applied to the photosynthetic response of the three C_4 species to the intercellular CO_2 concentration at high irradiance shown in Fig. 2. This approach allowed the simulation of the effect of moderate and severe leaf dehydration on the rates of photosynthesis and photorespiration by the three grasses. At high irradiance, photosynthesis is assumed to be enzyme limited and mostly determined by the rates of PEP and RuBP carboxylation by PEPC and Rubisco, respectively, and by the regeneration of PEP (von Caemmerer 2000). The variation of the enzyme-limited net CO_2 assimilation rate (A_c) with the CO_2 concentration in the M cells (C_m) is represented in Fig. 6. The values predicted seem to agree fairly well with the experimental results (Fig. 2). However, there might be some overestimation of the modelled values of A_c for *C. dactylon* and *Z. japonica*. For simplification, it was assumed that the fraction of O_2 evolution in the BS is zero for all three species but this is not likely to be correct for the NAD-ME or PEPCK species, in which photosystem II (PSII) activity will probably increase the O_2 concentration in the BS, decreasing the actual value of photosynthesis. The mean values estimated for the maximum PEPC carboxylation activity (V_{pmax}) and maximum Rubisco carboxylation activity (V_{cmax}) were higher in *C. dactylon* than in *P. dilatatum* and were lower in *Z. japonica*, and decreased with leaf dehydration (Fig. 6). These results suggest that both enzymes responsible for the carboxylation of PEP and RuBP are down-regulated under drought conditions. The negative effect of drought on the photosynthesis response to CO_2 is more notable in the SS plants of *P. dilatatum* and *Z. japonica*, but a clear effect of leaf dehydration on the two enzymes and on A_c was simulated for the MS plants of each of the three species.

The application of the model equations to the experimental data from the CO_2 response curves allowed the simulation of a rate of photorespiration (*Pr*) for each of the three C_4 grasses at the different stress levels (Fig. 7). Even at the lowest C_i values, the predicted *Pr* was never higher than 1.5 μmol m^{-2} s^{-1}, and at ambient levels of CO_2 (360–390 μmol mol^{-1}), *Pr* was estimated to be less than 3.5% of A_c for all species and stress levels. The modelled data suggest that photorespiration is present at higher rates in the MS relative to the well-watered plants of each species, especially in *Z. japonica*. The lower values of *Pr* estimated for the SS compared with the MS plants of *P. dilatatum* and *Z. japonica* indicate that metabolic inhibition of photosynthesis occurs at the level of Rubisco, affecting both the rates of photosynthesis and photorespiration. *Pr* was estimated to be lower in *C. dactylon* than in the other two species.

However, higher values could have been predicted for either *C. dactylon* (NAD-ME) or *Z. japonica* (PEPCK) if a more realistic O_2 concentration in the BS, likely to be higher than in *P. dilatatum* (NADP-ME), had been considered.

Stomatal closure causes decreased C_i under drought conditions (Fig. 2). Concomitantly, lower values of C_s for a given CO_2 concentration are estimated for the dehydrated leaves compared with the controls (Fig. 7). The slower response of increasing C_s with increasing C_i under drought stress suggests that metabolic impairment might be present, affecting the effectiveness of the C_4 cycle. As a result, A_c is also decreased and *Pr* increases, that is, the predicted rates of photorespiration increase as a proportion to the modelled net photosynthesis when leaf dehydration increases for all three species. Many assumptions have been made to allow the simulation of plant photosynthetic and photorespiratory responses by the application of a mechanistic model and, consequently, much uncertainty is involved. It was assumed that stomata apertures decreased uniformly and that g_m and g_{bs} were not changed with stress. Furthermore, it was accepted that the estimated values of V_{cmax} and V_{pmax} changed with stress. More accurate predictions will be possible when more measurements of the assumed parameters are available as, for example, the kinetic constants of Rubisco from each of the C_4 species.

CONCLUSIONS

Drought stress induced decreased leaf water contents, stomatal closure and decreased net CO_2 assimilation in the leaves of *P. dilatatum*, *C. dactylon* and *Z. japonica*. The response of net photosynthesis to CO_2 was typical of C_4 leaves, and extrapolation to zero *A* gave no evidence of a significant CO_2 compensation point even in droughted plants. Net CO_2 assimilation was decreased only slightly with increasing O_2 concentrations above 10% in the atmosphere around the leaves, but this trend was not increased by drought stress. Leaves of both control and droughted plants showed $\delta^{13}C$ and $\delta^{18}O$ typical of C_4 plants, and changes with *RWC* were not indicative of changes in photorespiration. Increased contents of glycine and serine in the dehydrated leaves of the three species provided the only suggestion for increased photorespiratory metabolism by water deficit. Changes in amino acid content after 30 s in darkness, especially the decrease in glycine, were consistent with slow photorespiration rates in all three species, but were slightly faster in *C. dactylon* (NAD-ME) than in *P. dilatatum* (NADP-ME) and *Z. japonica* (PEPCK), and not changed by drought stress in either species. Mechanistic modelling of CO_2 response curves as well as the O_2 sensitivity of C_4 photosynthesis by the three grasses suggested slightly increased photorespiration rates in proportion to photosynthesis. However, the overall results presented here suggest that the C_4 grasses of the three different metabolic subtypes are able to maintain levels of CO_2 at the Rubisco site sufficiently high to limit oxygenase activity under drought stress.

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