New Phytologist

Adaxial/abaxial specification in the regulation of photosynthesis and stomatal opening with respect to light orientation and growth with CO_2 enrichment in the C_4 species *Paspalum dilatatum*

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Received: *17 June 2007*
Accepted: *23 July 2007*

Summary

• Whole-plant morphology, leaf structure and composition were studied together with the effects of light orientation on the dorso-ventral regulation of photosynthesis and stomatal conductance in *Paspalum dilatatum* cv. Raki plants grown for 6 wk at either 350 or 700 μl l^{-1} CO_2.

• Plant biomass was doubled as a result of growth at high CO_2 and the shoot:root ratio was decreased. Stomatal density was increased in the leaves of the high CO_2-grown plants, which had greater numbers of smaller stomata and more epidermal cells on the abaxial surface.

• An asymmetric surface-specific regulation of photosynthesis and stomatal conductance was observed with respect to light orientation. This was not caused by dorso-ventral variations in leaf structure, the distribution of phospho*enol*pyruvate carboxylase (PEPC) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) proteins or light absorptance, transmittance or reflectance.

• Adaxial/abaxial specification in the regulation of photosynthesis results from differential sensitivity of stomatal opening to light orientation and fixed gradients of enzyme activation across the leaf.

Key words: abaxial/adaxial leaf specification, CO_2 enrichment, phospho*enol*pyruvate carboxylase (PEPC), photosynthesis, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), stomatal patterning.

New Phytologist (2008) **177**: 186–198

doi: 10.1111/j.1469-8137.2007.02218.x

Introduction

Photosynthesis plays a crucial role in ecosystem sustainability (Millennium Ecosystem Assessment, 2005). In order to combat climate change and ensure the sustainability of natural and cultivated ecoystems, it is essential to understand the responses of photosynthesis to fluctuating environmental conditions, particularly atmospheric CO_2 enrichment. Surprisingly little attention has been given to the effects of increased atmospheric CO_2 on C_4 photosynthesis, despite the fact that many of the most productive bio-energy/bio-fuel crops are monocotyledonous C_4 species. It is important,

therefore, to gain a better understanding of how CO_2 enrichment will alter stomatal function and photosynthesis in such species, and particularly to elucidate any interactions with respect to light orientation, which is a crucial regulator of these processes (Poulson & DeLucia, 1993). Structure–function relationships in photosynthesis have been intensely studied in dicotyledonous leaves (Terashima & Saeki, 1983; Cui *et al.*, 1991; Vogelmann, 1993), which exhibit internal gradients in light and photosynthetic capacity (Terashima & Inoue, 1985a,b). The highest photosynthesis rates are not found near the leaf surface where the light intensity is highest, but are observed in the middle and lower palisade layers (Nishio *et al.*, 1993; Evans, 1995; Sun *et al.*, 1998; Sun & Nishio, 2001; Evans & Vogelmann, 2003), which have higher electron transport activities and greater amounts of photosynthetic proteins (Terashima & Inoue, 1985b; Terashima & Evans, 1988; Sun & Nishio, 2001). The adaxial surfaces of dicotyledonous C_3 leaves have characteristics that resemble classic 'sun' leaves, while the abaxial surfaces have properties that are consistent with 'shade' leaves (Oya & Laisk, 1976; Terashima, 1986; Terashima & Evans, 1988; Lambers *et al.*, 1998). Growth at high CO_2 alters the regulation of photosynthesis on the adaxial and abaxial leaf surfaces of maize (*Zea mays*) leaves (Domes, 1971; Driscoll *et al.*, 2006), but very little information is available on the effects of CO_2 enrichment on the surface-specific regulation of photosynthesis in other monocotyledonous C_4 species.

Atmospheric CO_2 availability exerts a strong influence on the dorso-ventral organization of leaf structure, composition and photosynthetic activity (Taylor *et al.*, 1994, 2001; Croxdale, 1998; Masle, 2000; Lake *et al.*, 2001; Poorter & Navas, 2003; Martin & Glover, 2007). Similarly, growth CO_2 concentrations exert control over stomatal density and patterning (Larkin *et al.*, 1997; Lake *et al.*, 2002). Atmospheric CO_2 enrichment decreases stomatal densities in the leaves of dicotyledonous C_3 species (Woodward *et al.*, 2002). However, the degree of this response to CO_2 enrichment differs between species and little information is available in the literature on such effects in monocotyledonous C_4 species (Woodward & Kelly, 1995; Woodward *et al.*, 2002). The CO_2-signalling pathways that orchestrate these changes in leaf structure and composition responses remain poorly characterized (Gray *et al.*, 2000; Ferris *et al.*, 2002), but signals transported from mature to developing leaves are considered to be important regulators of such responses (Coupe *et al.*, 2006; Miyazawa *et al.*, 2006).

The present study was undertaken in order to characterize the relative effects of light orientation and CO_2 availability on photosynthesis and stomatal responses in *Paspalum dilatatum*, which, like maize, is a C_4 monocotyledonous species of the NADP-malic enzyme (NADP-ME) subtype. *Paspalum dilatatum* is a common grass species of the prairies of South America (Pinto da Silva, 1969; Usuda *et al.*, 1984) and the wetter areas of Australia (Pearson *et al.*, 1985; Brown, 1999) and has already been characterized in terms of photosynthetic responses to environmental stress (e.g. Marques da Silva *et al.*, 1991; Bernardes da Silva *et al.*, 1999; Cavaco *et al.*, 2003; Carmo-Silva *et al.*, 2007) and to CO_2 enrichment (Greer *et al.*, 1995; von Caemmerer *et al.*, 2001). Here we report the influence of light orientation on the dorso-ventral regulation of photosynthesis and stomatal conductance, and the effect of growth CO_2 on the symmetry of the photosynthetic responses on the adaxial and abaxial leaf surfaces to incident light (either adaxial or abaxial), together with acclimatory responses in whole-plant morphology, biomass, whole-leaf photosynthesis, stomatal patterning and leaf composition.

Materials and Methods

All the following experiments were conducted in the laboratories at Rothamsted Research, Harpenden, UK except for the electron microscopy, which was performed at CEBAS-CSIC, Murcia, Spain on samples prepared at Rothamsted Research. Each experiment was repeated at least three times and all experiments involved a minimum of three plants.

Seeds of *Paspalum dilatatum* Poiret cv. Raki were obtained from the Margot Forage Germplasm Centre, Palmerston North, New Zealand. They were germinated on compost containing slow-release fertilizer in pots (20 cm diameter) and then grown for 6 wk in two controlled-environment cabinets (Sanyo SGC228.CFX.J; Sanyo, Osaka, Japan) in which all the growth conditions were identical except for the atmospheric CO_2 concentration, which was maintained at either 350 or 700 μl l^{-1} throughout the duration of the experiments. CO_2 was supplied from a bulk container via a Vaisala GMT220 CO_2 transmitter (Vaisala Oyj, Helsinki, Finland). The CO_2 contents within the chambers were maintained at 350 ± 20 or 700 ± 20 μl l^{-1} using a Eurotherm 2704 controller (Eurotherm Ltd, Worthing, UK). The plants were grown under a 16-h photoperiod with a 25°C (day): 19°C (night) temperature regime and 80% relative humidity. Irradiance (600–650 mmol μmol m^{-2} s^{-1} at 400–700 nm, at pot height) was provided by Philips Master TL5 HO 49w/830 fluorescent lamps (Philips Lighting UK, Guildford, UK). All plants were well watered daily and the root growth in the pots was checked periodically to ensure that an appropriate pot size was used throughout the growth period. At the harvest point the roots were growing freely.

Whole-plant growth parameters and tissue biomass

In each experiment, between eight and 14 plants were harvested at 6 wk from each CO_2 chamber, a time-point that was before anthesis in both air- and high CO_2-grown plants. Harvested plants were separated into leaves, stems and roots. The following measurements were performed: root, stem and leaf biomass, specific leaf area (SLA), leaf area ratio (LAR), leaf weight ratio (LWR), leaf density (% leaf dry weight (DW)) and leaf thickness (leaf fresh weight to leaf area ratio).

Photosynthesis measurements

Photosynthetic gas-exchange measurements were performed using infrared gas analysis (model wa-225-mk3; ADC, Hoddesdon, UK). Two different types of chamber system were used in these studies. One apparatus involves a series of standard Parkinson chambers for whole-leaf measurements (Novitskaya *et al.*, 2002). The second apparatus uses modified Parkinson chambers, as illustrated in Fig. 1. This system enables separate and simultaneous measurements of gas exchange on each leaf surface (Driscoll *et al.*, 2006).

To check the degree of CO_2 transport across the leaf, a gas stream containing 10% CO_2 was applied to each surface independently in the chamber and the concentration on the other surface was measured. In this system, we could detect no passage of CO_2 from one surface to the other through the leaf blade. While the stomata close under these conditions, this simple experiment illustrates that there is no passage of gases from one side of the chamber to the other. Each half of this dual chamber operates essentially as a whole leaf chamber as each half has a distinct gas supply and analysis unit with separate humidity, leaf temperature and individual fan controls (Fig. 1a,b,c). Flow rates were optimized on each side of the chamber to match the pressure on both leaf surfaces. Because the *P. dilatatum* leaf was not large enough to fully separate the two sides of the chamber, the leaf was expanded with gas-tight tape to seal each half of the chamber, preventing any flux of gas between the two sides (Fig. 1d,e). Irradiance was supplied only from the top of the chamber as described previously (Novitskaya *et al.*, 2002). Leaf temperatures were monitored via thermocouples attached to each surface separately (Fig. 1b) and were maintained by water jackets at 20°C on both the adaxial and abaxial surfaces to ensure that the illuminated surface had exactly the same temperature as the unilluminated surface. All experiments were conducted at 50% relative humidity. The gas composition was controlled by gas mixers supplying CO_2 at concentrations as indicated in the figures with 21% O_2 and balance N_2.

Attached last fully expanded leaves of the primary tiller of air- and CO_2-grown plants were used to obtain CO_2- and light-response curves for whole-leaf photosynthesis and photosynthesis on each leaf surface separately. Light was oriented directly to the adaxial surface or to the abaxial surface (by inverting the leaf in the chamber). For CO_2-response curves, CO_2 was increased step-wise from 50 to 1000 µl l^{-1} at an irradiance of between 900 and 1000 µmol m^{-2} s^{-1}. Light-response curves for photosynthesis were obtained via step-wise increases in irradiance from darkness to 1500 µmol m^{-2} s^{-1} at 360 µl l^{-1} CO_2. Steady-state gas-exchange measurements were obtained for each treatment after at least 10 min of incubation in each light and CO_2 condition. Vapour water deficit was kept constant throughout the assay in both types of analysis.

Leaf optical properties

Leaf reflectance and transmittance measurements were performed on each surface of *P. dilatatum* plants grown at 350 µl l^{-1}. Attached leaves of the same age and stage of development as those used for gas exchange were measured across the spectrum from 400 to 800 nm at 1-nm intervals. Five measurements were made on both sides of each leaf parallel to the middle vein and three leaves were assayed per

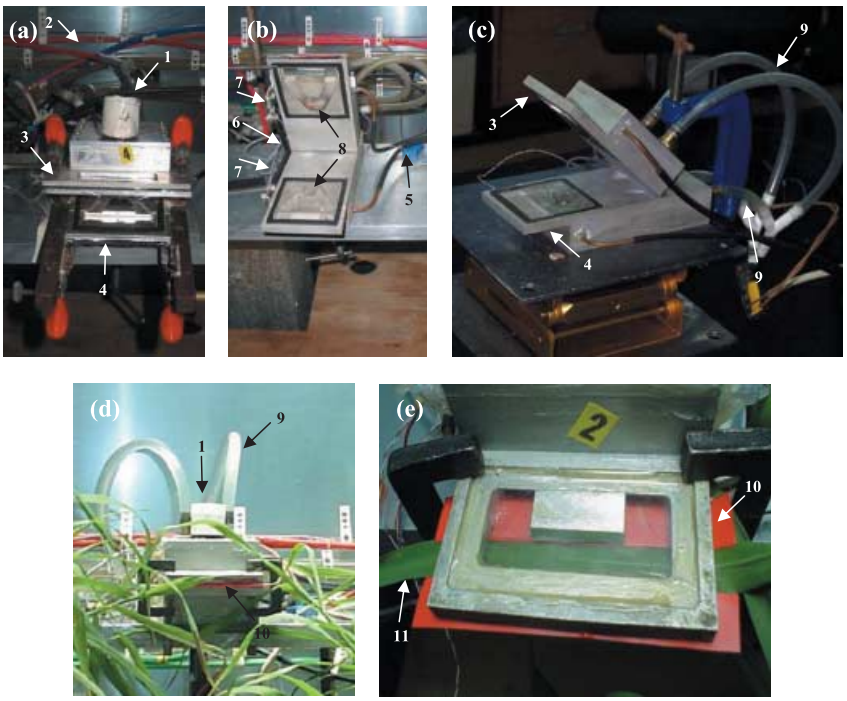


Fig. 1 The modified Parkinson leaf chamber used for separate and simultaneous gas exchange measurements on each leaf surface. Different views of the chamber are given to illustrate the following features: 1, light sensor; 2, connecting tubes to the infrared gas analyser; 3, upper side of the modified chamber with light source; 4, lower side of the modified chamber; 5, air supply in to both sides of the chamber; 6, air supply out of both sides of the chamber; 7, humidity and temperature sensors on both sides of the chamber; 8, fan on both sides of the chamber; 9, water jackets on both sides of the chamber; 10, gas-tight tape; 11, *Paspalum dilatatum* leaf.

experiment. Reflectance and transmittance were measured using reflection and irradiance integration spheres (Avasphere-30 reflection and irradiance integration spheres; Avantes, Eerbeek, the Netherlands) connected to a USB-2000 spectroradiometer (Ocean Optics, Dunedin, FL, USA). The measuring light was provided by a laboratory-built stabilized quartz-halogen source connected to a 0.6-mm optical fibre (type QP600-2-VIS-BX; Ocean Optics). In both cases the angle of incidence of the measuring beam was 8°. The 100% reflectance signal was obtained using a white reflectance standard (Spectralon; Labsphere, North Sutton, NH, USA). This was calibrated against a sheet of glass microfibre paper (Type GF/A; Whatman, Brentford, UK), which was used as a second standard. Leaf absorptance was calculated for each wavelength, as 1 – (reflectance + transmittance).

Determination of pigments and protein content

Leaf pigments (chlorophyll *a* (chl*a*), chl*b*, total chlorophyll and carotenoids) and soluble protein contents were determined in the leaves of air- and high CO_2-grown plants. Leaf sections (4 cm), excised from both sides of each leaf parallel to the middle vein, were ground in liquid nitrogen and quartz sand. Pigments were determined according to the method of Lichtenhaler & Wellburn (1983) in ethanol (96%) extracts. Total leaf soluble proteins were extracted in 1 mM sodium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol (DTT), 1 mM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride (PMSF), 5 mM 2-mercaptoethanol and 1% (weight/volume (w/v)) Polyclar AT (Sigma-Aldrich, Saint Louis, MO, USA). Protein was determined according to the method of Bradford (1976).

Analysis of epidermal structure

The structure of the epidermis was examined in the leaves of air- and high CO_2-grown plants using comparable sections to those used for photosynthesis measurements. Leaf sections were painted on the adaxial and abaxial surfaces with clear nail varnish. Epidermal imprints were then stripped from both surfaces and examined by optical microscopy (Olympus BH-2; Olympus Optical Co. Ltd, Tokyo, Japan). The number of stomata was counted on 24 randomly selected digitized images from the adaxial or the abaxial epidermal imprints of eight plants. To calculate epidermal and stomatal cell areas and densities, the dimensions of at least 100 cells from the adaxial or the abaxial epidermal layers were measured per experiment using Sigma ScanPro photographic analysis software, version 5 (Sigma Chemical Co., St Louis, MO, USA). The stomatal index was calculated as the number of stomata/(number of epidermal cells + number of stomata) × 100, as defined by Salisbury (1927). The ratio of stomata on each surface was calculated from the number of stomata counted on the adaxial and abaxial surfaces.

Fixation, embedding and sectioning for optical and electron microscopy

Leaf blade samples (1 mm^2) were sectioned from the middle of the last fully expanded leaf of *P. dilatatum* plants grown at 350 µl l^{-1} CO_2. Sections were fixed at ambient temperature in 4% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) for 3.5 h. The samples were then washed in the same buffer three times for 15 min. They were then dehydrated at ambient temperatures using a graded ethanol series (35, 50, 70, 96 and twice at 100% (v/v) ethanol) with 30 min exposure at each step. The embedding was carried out in London Resin White acrylic resin (LRWhite; Electron Microscopy Sciences, Fort Washington, PA, USA) according to the following schedule: 75% (v/v) ethanol + 25% (v/v) LRWhite, 50% (v/v) ethanol + 50% (v/v) LRWhite, 25% (v/v) ethanol + 75% (v/v) LRWhite, 100% (v/v) LRWhite (1 h each series), and 100% (v/v) LRWhite overnight. The samples were transferred to tubes filled with resin and polymerized under nitrogen ambient at 55% for 24 h. Transverse semithin leaf sections (0.5 µm) for optical microscopy and transverse ultrathin leaf sections for electron microscopy (50–60 nm) were prepared using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Optical microscopy sections were stained with toluidine blue. Electron microscopy sections were mounted on nickel grids and stained with lead citrate (Reynolds, 1963) and 2% (w/v) uranyl acetate. Optical microscopy sections were observed using a Leica DMR light microscopy (Leica Microsystems, Wetzlar, Germany).

In situ immunolocalization of ribulose 1,5-bisphosphate carboxylase/oxygenase and phospho*enol*pyruvate carboxylase

The nickel grids were incubated at ambient temperature for 30 min in phosphate-buffered saline (PBS) containing 5% (w/v) bovine serum albumin (BSA). They were then incubated at room temperature for 3 h with either rabbit preimmune serum (dilution 1 : 500), rabbit anti-ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Agrisera AB, Vännäs, Sweden) (dilution 1 : 250) or rabbit anti-phospho*enol*pyruvate carboxylase (PEPC) (courtesy of Jean Vidal, Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique, Université de Paris-Sud, France) (dilution 1 : 500), in the above PBS/BSA mixture. After washing twice with PBS (5 min each wash), the sections were incubated at ambient temperature for 1.5 h with goat anti-rabbit antibodies labelled with gold 10 nm (British Biocell International, Cardiff, UK) diluted in PBS containing 1% (w/v) BSA (dilution 1 : 50). The sections were washed sequentially with PBS containing 1% (w/v) BSA and then twice with PBS alone followed by five washes with filtered (0.2 µm) ultra-pure water (5 min each wash). The grids were

dried at ambient temperature. Immunogold electron microscopy images were collected using a Philips Tecnai 12 electron microscope (Philips, The Hague, the Netherlands) operated at 80 kV. The number of gold particles on the adaxial and abaxial sides of the leaves and the parameters of ultrastructural morphology were quantified using the 3.2 image analysis software (Soft Imaging System, Münster, Germany). Quantification of gold labelling was performed on three leaf samples per experiment with a total of 45 different bundle sheath (BS) and mesophyll (M) cells measured in each analysis.

Statistical analysis

The data were statistically analysed using parametric tests at a stringency of $P < 0.05$. The significance of variation in mean values for growth parameters and pigment and protein determinations was determined using a *t*-test. The significance of the data for epidermal structure and immunological measurements was analysed using ANOVA and Tukey HSD tests.

Results

Plant phenotype, leaf protein and pigment contents

Plants grown at the higher CO_2 concentration were larger with greater numbers of larger leaves than those grown at 350 μl l^{-1} CO_2 (Fig. 2a, Table 1a). While plants grown at 700 μl l^{-1} CO_2 had a higher shoot and root biomass, CO_2 enrichment decreased the shoot to root ratio (Table 1a). In contrast to the increase in leaf thickness, the SLA, LAR, LWR and leaf density values were not affected by doubling the growth CO_2 concentration (Table 1b). The leaf soluble protein contents were similar in the two growth conditions, but the high CO_2-grown leaves generally had a slightly lower pigment content with less total carotenoids (7%) and chlorophyll (11%; Table 2).

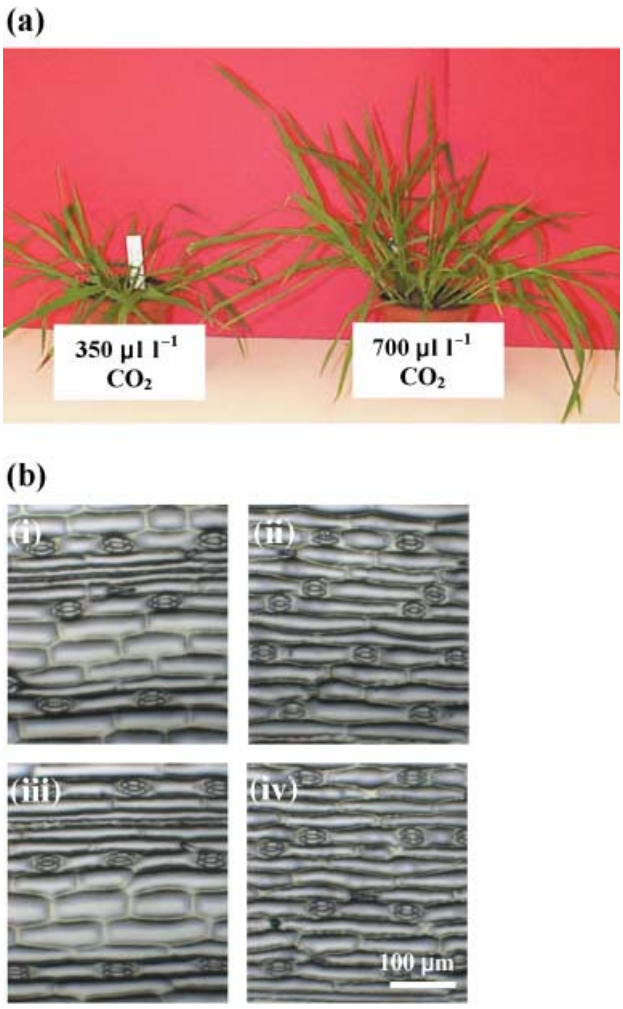


Fig. 2 A comparison of the effects of growth with CO_2 enrichment on *Paspalum dilatatum* (a) shoots and (b) leaf epidermal structure. Stomata and epidermal cells on the adaxial (i, iii) and abaxial leaf epidermis (ii, iv) of plants grown for 6 wk at either (i, ii) 350 μl l^{-1} or (iii, iv) 700 μl l^{-1} CO_2 are shown. Bar, 100 μm.

Leaf epidermal structure

The epidermal cells on the adaxial leaf surface were arranged in parallel rows with stomata in every third or fourth row (Fig. 2b(i,iii)). In contrast, while the epidermal cells on the abaxial surface were similarly arranged in parallel rows, the stomata were located in every second or third row (Fig. 2b(ii,iv)). Moreover, the average stomatal area on the abaxial surface was lower than on the adaxial surface (Table 3). The average epidermal cell area was comparable on both leaf surfaces whether plants were grown at 350 or 700 μl l^{-1} CO_2 (Table 3). The ratio of stomata on the adaxial compared with the abaxial surface was *c.* 0.7 at both growth CO_2 conditions (Fig. 2b, Table 3). However, the stomata were smaller in size and greater in number in plants grown at the high CO_2 concentration (Table 3). The lowest stomatal density was observed on the adaxial surface of leaves grown at 350 μl l^{-1} CO_2 while the highest stomatal density was found on the abaxial surface of leaves grown at 700 μl l^{-1} CO_2 (Table 3).

Whole-leaf photosynthesis, absorptance, transmittance and reflectance

Photosynthetic CO_2 assimilation rates increased as atmospheric CO_2 was increased over a range of low intercellular CO_2 concentrations (C_i), in a similar manner regardless of the growth CO_2 environment or light orientation (Fig. 3a(i,ii)). However, maximal steady-state rates of photosynthesis were slightly higher in air-grown plants when light was oriented to the adaxial surface (Fig. 3a(i)). The maximal steady-state rates of photosynthesis were similar in plants grown at the higher CO_2 concentration, whether the leaf received adaxial or abaxial illumination (Fig. 3a(ii)). Stomatal conductance decreased

Table 1 The effects of CO_2 enrichment on plant biomass (a) and leaf parameters (b) in *Paspalum dilatatum* plants grown at either 350 or 750 μl l^{-1} CO_2

(a)

Parameter	350 μl l^{-1} CO_2	700 μl l^{-1} CO_2
Total leaf number	112 ± 15.39 a	136 ± 23.65 b
Total leaf area (m^2)	55.38 ± 10.19 a	91.83 ± 18.76 b
Leaf dry weight (g)	1.89 ± 0.315 a	3.29 ± 0.842 b
Stem dry weight (g)	3.42 ± 0.655 a	5.37 ± 1.436 b
Root dry weight (g)	1.93 ± 0.616 a	5.37 ± 1.436 b
Total plant dry weight (g)	9.36 ± 1.913 a	18.29 ± 6.768 b
Shoot::root ratio (g g^{-1} DW)	1.36 ± 0.233 a	0.98 ± 0.203 b

Data represent the average ± SD for 12–14 plants at each CO_2 concentration. The different letters represent statistical differences at $P < 0.05$.

(b)

Parameter	350 μl l^{-1} CO_2	700 μl l^{-1} CO_2
SLA (m^2 kg^{-1})	29.35 ± 1.899 a	28.34 ± 2.322 a
LAR (m^2 kg^{-1})	5.98 ± 0.533 a	5.31 ± 0.922 a
LWR (kg kg^{-1})	0.20 ± 0.017 a	0.19 ± 0.026 a
Leaf density (%)	19.39 ± 0.785 a	19.11 ± 0.786 a
Leaf thickness (g m^{-2})	176.44 ± 10.142 a	185.68 ± 10.657 b

Data represent the average ± SD for 12–14 plants at each CO_2 concentration. The different letters represent statistical differences at $P < 0.05$.
SLA, specific leaf area; LAR, leaf area ratio; LWR, leaf weight ratio.

Table 2 The effects of CO_2 enrichment on leaf pigment and protein content in *Paspalum dilatatum* plants grown at either 350 or 750 μl l^{-1} CO_2

Parameter	350 μl l^{-1} CO_2	700 μl l^{-1} CO_2
Chl*a* (mg m^{-2})	677 ± 50.3 a	597 ± 39.5 b
Chl*b* (mg m^{-2})	177 ± 14.6 a	158 ± 10.0 b
Chl*a* + *b* (mg m^{-2})	854 ± 64.1 a	755 ± 48.7 b
Carotenoids (mg m^{-2})	135 ± 8.6 a	125 ± 6.2 b
Soluble proteins (g m^{-2})	3604 ± 226.1 a	3571 ± 37.1 a

Data represent the mean values ± SD for eight plants at each CO_2 concentration. The different letters represent statistical differences at $P < 0.05$.
Chl, chlorophyll.

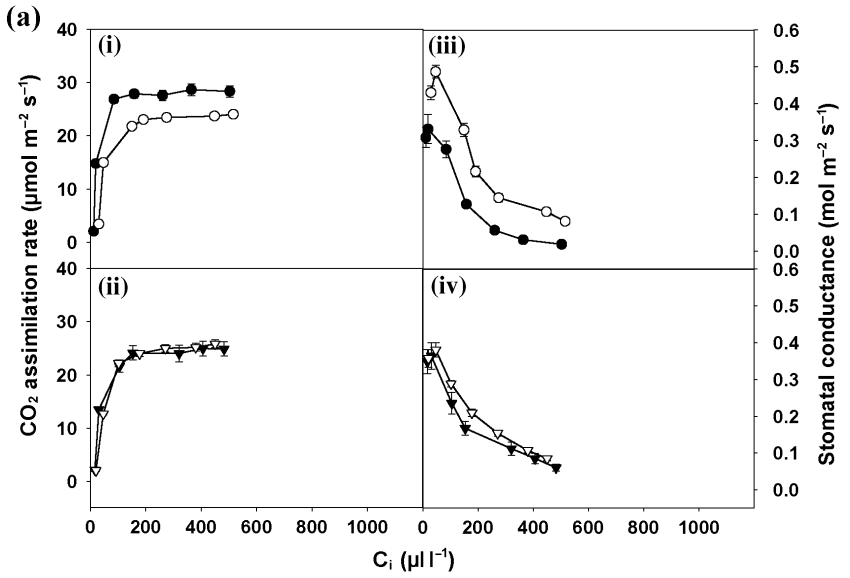
with increasing C_i and showed similar trends whether light was supplied via the adaxial or abaxial surface of the leaves. While the trends were consistent in plants from both growth CO_2 conditions (Fig. 3a(iii,iv)), the overall values were lower in plants grown in air when irradiance entered the leaf via the adaxial surface (86%; Fig. 3a(iii)).

The light-response curves for photosynthesis of plants grown at the lower CO_2 concentration were similar regardless of the orientation of the leaves towards the light (Fig. 3b(i)). Maximal photosynthetic rates were slightly lower in plants grown at the higher CO_2 concentration when the light entered the leaf via the adaxial surface, a feature not observed when the light was oriented to the abaxial surface (Fig. 3b(ii)). Stomatal conductance increased with increasing irradiance in plants grown at both CO_2 concentrations whether light was

Table 3 Effects of CO_2 enrichment on leaf epidermal structure in *Paspalum dilatatum p*lants grown at either 350 μL l^{-1} or 750 μL l^{-1} CO_2

	350 μl l^{-1} CO_2		700 μl l^{-1} CO_2	
Parameter	Adaxial	Abaxial	Adaxial	Abaxial
Epidermal cell area (μm^2)	2762 ± 843 a	2704 ± 717 a	2613 ± 861 a	2585 ± 641 a
Epidermal cells (number mm^{-2})	283 ± 39 b	294 ± 30 b	303 ± 16 b	372 ± 20 a
Stomatal area (μm^{-2})	743 ± 101 a	696 ± 84 b	699 ± 101 b	627 ± 88 c
Stomatal density (number mm^{-2})	62 ± 12 a	89 ± 5 c	75 ± 7 b	104 ± 11 d
Stomatal index	18 ± 1.6 c	23.3 ± 2.1 a	19.8 ± 1.7b c	21.8 ± 1.8 ab
Ratio of stomata (adaxial/abaxial)	0.70		0.72	

Data represent the mean values ± SD for eight plants at each CO_2 concentration. The different letters represent statistical differences at $P < 0.05$.



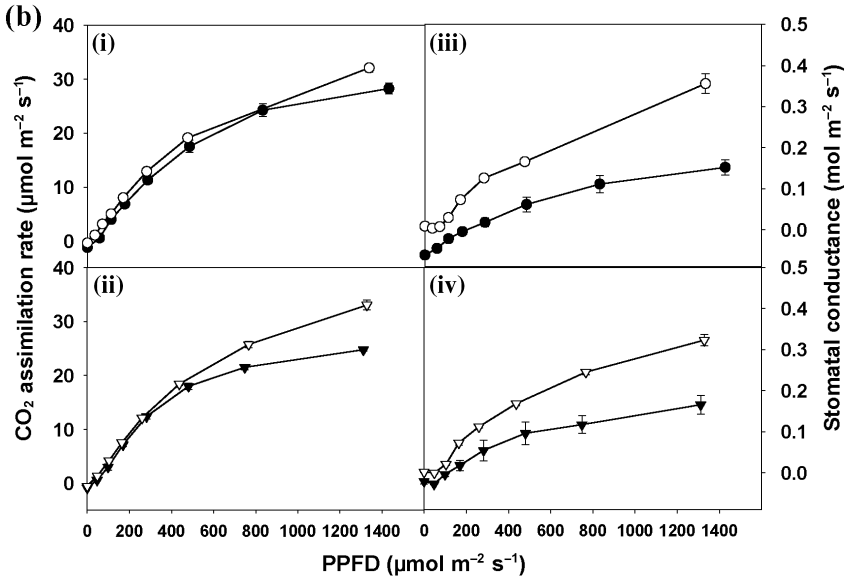


Fig. 3 The effect of light orientation on whole-leaf photosynthesis and stomatal conductance in *Paspalum dilatatum* plants that had been grown for 6 wk at either 350 µl l^{-1} (i, iii) or 700 µl l^{-1} CO_2 (ii, iv). (a) The CO_2-response curves for photosynthesis (i, ii) and stomatal conductance (iii, iv) and (b) the light-response curves for whole-leaf photosynthesis (i, ii) and stomatal conductance (iii, iv) are presented. The light source was oriented either to the adaxial surface (closed circles and triangles) or to the abaxial surface (open circles and triangles). Data are the mean values ± SE of three plants at each CO_2 concentration. PPFD, photosynthetic photon flux density.

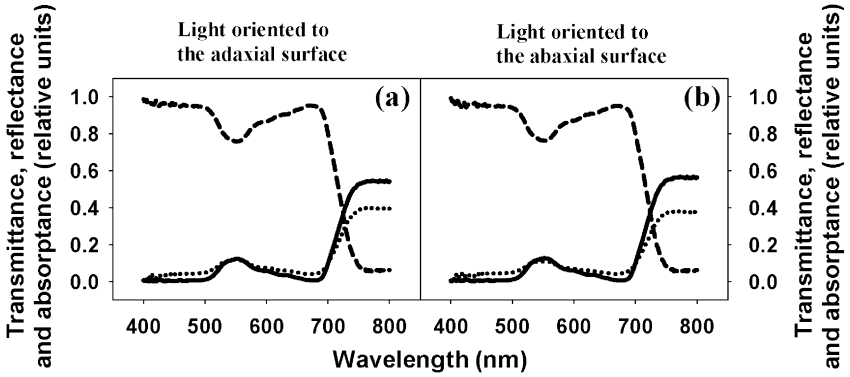


Fig. 4 The effect of light orientation on the transmittance (solid line), reflectance (dotted line) and absorption (dashed line) spectra of *Paspalum dilatatum* leaves. Measurements were made on plants that had been grown for 6 wk at 350 µl l^{-1} CO_2. The light source was oriented to either (a) the adaxial surface or (b) the abaxial surface. A single representative data set is shown.

supplied via the adaxial or abaxial surface (Fig. 3b(iii,iv)). However, values were higher when the light entered the leaf via the abaxial surface (Fig. 3b(iii,iv)).

Leaf absorptance, transmittance and reflectance profiles were similar on the adaxial and abaxial surfaces in plants grown at the lower CO_2 concentration across the light spectrum from 400 to 800 nm, whether light was supplied via the adaxial (Fig. 4a) or abaxial (Fig. 4b) surface.

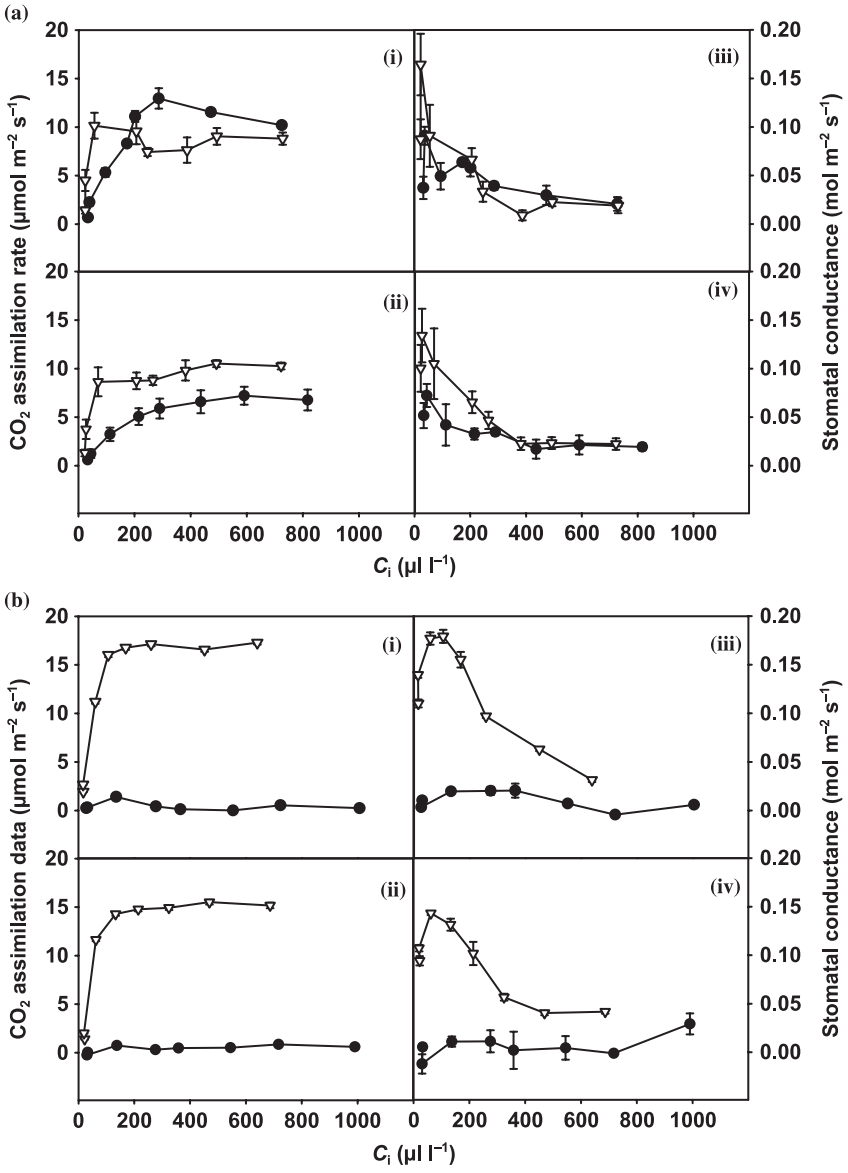


Fig. 5 The effect of light orientation on the CO_2-response curves (a, b) for photosynthetic rate (i, ii) and stomatal conductance (iii, iv) on the adaxial (closed circles) and abaxial surfaces (open triangles) of *Paspalum dilatatum* plants that had been grown for 6 wk at either 350 μl l^{-1} CO_2 (i, iii) or 700 μl l^{-1} CO_2 (ii, iv). The light source was oriented to either (a) the adaxial surface or (b) the abaxial surface. Data are the mean values ± SE of three plants from each CO_2 concentration.

Adaxial and abaxial photosynthesis

The adaxial surface had much lower rates of CO_2 assimilation than the abaxial leaf surface at low C_i values, regardless of the growth CO_2 concentration (Fig. 5a(i,ii),b(i,ii)). Maximal CO_2 assimilation rates in plants grown in air were similar on the two leaf surfaces when light was oriented to the adaxial surface (Fig. 5a(i)). However, when light was oriented directly to the abaxial surface, maximal photosynthesis rates were higher on this surface (Fig. 5a(ii)). In marked contrast, no CO_2 assimilation was detected on the adaxial surface under these conditions (Fig. 5a(ii)). Similar trends were observed in plants grown with CO_2 enrichment (Fig. 5b(i,ii)) except that photosynthetic rates on the abaxial surface were always higher than those on the adaxial surface when the light was oriented to the adaxial surface (Fig. 5b(i)). When light was oriented to the adaxial surface, stomatal conductance patterns decreased with increasing C_i on both leaf surfaces (Fig. 5a(iii,iv)). When the light was oriented to the abaxial surface, higher values of stomatal conductance were found on this side of the leaf (Fig. 5b(iii,iv)). In contrast, no stomatal conductance was detected on the adaxial surface in these conditions regardless of the growth CO_2 concentration (Fig. 5b(iii,iv)).

When light was oriented to the adaxial surface, the photosynthesis rates on the two leaf surfaces increased with irradiance in a similar manner in the air-grown plants (Fig. 6a(i)). In contrast, when light was oriented to the abaxial surface, photosynthetic rates on the adaxial surface remained close to the compensation point regardless of the light intensity applied (Fig. 6b(i)). In marked contrast, the abaxial surface showed even higher CO_2 assimilation rates than when light was oriented to the adaxial surface (Fig. 6a(i),b(i)).

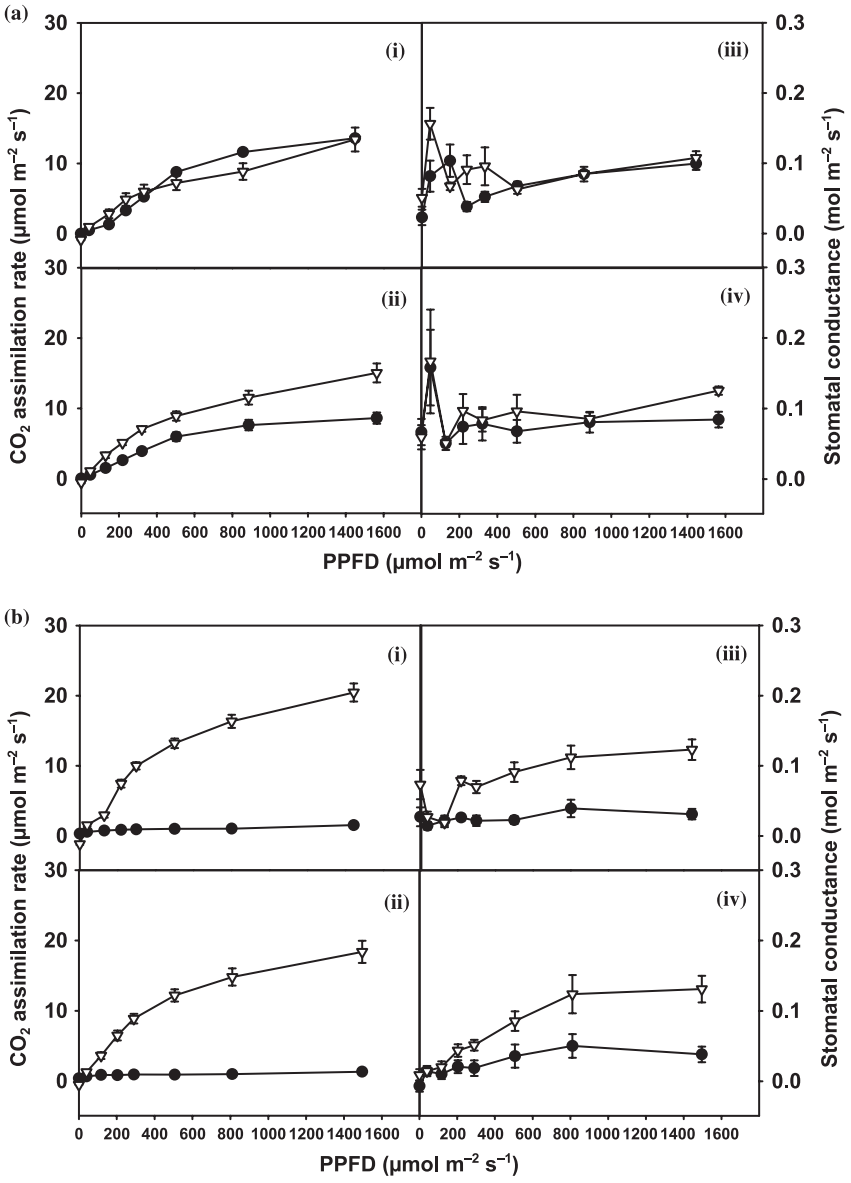


Fig. 6 The effect of light orientation on the light-response curves (a, b) for photosynthetic rate (i, ii) and stomatal conductance (iii, iv) on the adaxial (closed circles) and abaxial surfaces (open triangles) of *Paspalum dilatatum* plants that had been grown for 6 wk at either 350 μl l^{-1} CO_2 (i, iii) or 700 μl l^{-1} CO_2 (ii, iv). The light source was oriented to either (a) the adaxial surface or (b) the abaxial surface. Data are the mean values ± SE of three plants from each CO_2 concentration. PPFD, photosynthetic photon flux density.

Similar trends were observed with plants grown at high CO_2 (Fig. 6a(ii),b(ii)) except that photosynthetic rates on the abaxial surface were always higher than those on the adaxial surface, independent of light orientation to the leaf. Stomatal conductance values were similar on the two leaf surfaces when light was oriented to the adaxial leaf surface, whether plants were grown in air or high CO_2 concentrations (Fig. 6a(iii,iv)). When light was oriented to the adaxial surface, stomatal conductance patterns were similar with respect to increasing light on the two leaf surfaces (Fig. 6a(iii,iv)). When the light was oriented to the abaxial surface, however, higher values of stomatal conductance were found on this side of the leaf regardless of the growth CO_2 concentration (Fig. 6a(iii,iv)). In contrast, stomatal conductance values were very low on the adaxial surface in these conditions regardless of the growth CO_2 concentration (Fig. 6b(iii,iv)).

Structure of the leaf vascular and mesophyll tissues

The air-grown *P. dilatatum* leaves have a single layer of BS cells surrounded by the M cells (Fig. 7a). The BS was composed of three to five larger cells on the adaxial side with between two and four smaller cells on the abaxial side (Fig. 7a). The relative variation in BS cell numbers was always consistent. For example, if the bottom side of the BS had four cells then the top side of the BS had three cells and if the bottom had three BS cells then the top had two BS cells. The mean value obtained for 50 different vascular bundles was 4.1 BS cells on the abaxial side and 3.0 cells on the adaxial side. The number of chloroplasts per cell ranged from three to eight in the BS and from four to seven in the M, regardless of the position in the leaf. The area occupied by chloroplast area was much higher in the BS cells (27.9 ± 7.5 μm^2; ± standard deviation) than in the M cells (7.6 ± 1.8 μm^2).

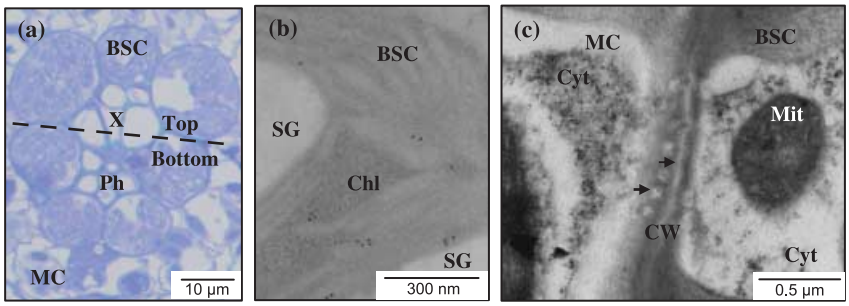


Fig. 7 (a) The structure of the *Paspalum dilatatum* vascular bundle sheath and (b) immunogold labelling of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and (c) phospho*eno*l/pyruvate carboxylase (PEPC) proteins in the leaves of 6-wk-old *P. dilatatum* plants grown at 350 μl l^{-1} CO_2. BSC, bundle sheath cell; MC, mesophyll cell; X, xylem; Ph, phloem; SG, starch grain; Chl, chloroplast; Cyt, cytoplasm; CW, cell wall; Mit, mitochondria. Dashed line, plane of the leaf, indicates the zone of separation between the top and the bottom of the BS. Arrows indicate plasmodesmata.

Table 4 Quantification of immunogold labelling for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*eno*l/pyruvate carboxylase (PEPC) proteins in the adaxial and abaxial sides of *Paspalum dilatatum* leaves

	Rubisco in the chloroplasts (gold particles μm^{-2})	PEPC in the cytosol (gold particles μm^{-2})
Bundle sheath cells		
Adaxial side	14.6 ± 3.0 a	7.0 ± 3.1 a
Abaxial side	15.6 ± 3.2 a	7.5 ± 3.5 a
Mesophyll cells		
Adaxial side	3.44 ± 1.1 b	306 ± 52 b
Abaxial side	3.53 ± 1.1 b	333 ± 50 b

Plants were grown at 350 μl l^{-1} CO_2.
Data represent the mean values ± SD for 15 cells of three plants (n = 45). Independent analyses for Rubisco and PEPC were performed. The different letters represent statistical differences at $P < 0.05$.

In situ distribution of ribulose 1,5-bisphosphate carboxylase/oxygenase and phospho*eno*l/pyruvate carboxylase

In situ immunolocalization studies were performed on the leaves of *P. dilatatum* plants grown at 350 μl l^{-1} CO_2 using specific antibodies against either the Rubisco large subunit (Fig. 7b) or the PEPC protein (Fig. 7c). This analysis revealed that these enzyme proteins were uniformly distributed across the leaf. No significant differences ($P > 0.05$) in the amounts of Rubisco or PEPC proteins were found in the adaxial and abaxial cells of the BS or M tissues across the leaf blade (Table 4).

Discussion

An accurate knowledge of the impacts of the environment on photosynthesis in pasture grasses such as *P. dilatatum* is crucial in any prediction of ecosystem viability and sustainability in the face of climate change. Here we show that *P. dilatatum* plants grew better and had double the total biomass under the high CO_2 growth conditions. *Paspalum dilatatum* benefits from CO_2 enrichment in terms of growth and biomass production, as observed in other C_4 species (e.g. Ghannoum *et al.*, 1997, 2001; Wand *et al.*, 1999, 2001). These results are perhaps surprising given the widely accepted view that C_4 plants might benefit less than their C_3 counterparts from growth in a high CO_2 world because of their intrinsic CO_2-concentrating mechanisms (Bowes, 1993). However, the presence of the ATP-dependent CO_2-concentrating systems in C_4 species has probably already resulted in a substantial degree of acclimation of photosynthesis to a CO_2-enriched state, at least in the BS cells. C_4 species may therefore accommodate the large increases in carbon gain that result from CO_2 enrichment better than C_3 species. In the present study, the *P. dilatatum* plants grown at the higher CO_2 concentration had decreased shoot:root ratios and showed other changes in whole-plant morphology parameters that are consistent with the acclimation of source–sink processes to CO_2 enrichment and increased carbon gain (Ghannoum *et al.*, 1997, 2001; Walting & Press, 1997).

Acclimation to CO_2 enrichment often involves changes in leaf morphology, stomatal patterning and the structure of the leaf epidermis (Lake *et al.*, 2001; Martin & Glover, 2007). Growth at the higher CO_2 concentration increased the density of stomata in *P. dilatatum* leaves, particularly on the abaxial surface, as has been reported in *Panicum antidotale* (Tipping & Murray, 1999). However, the changes observed here in *P. dilatatum* were somewhat different from those observed previously in maize, which is also a C_4 monocotyledonous NADP-malic enzyme species (Driscoll *et al.*, 2006). Maize

leaves grown with CO_2 enrichment had fewer, much larger cells on both surfaces. *Paspalum dilatatum* leaves grown under similar conditions had a greater number of smaller stomata on both surfaces with a significant increase in epidermal cell numbers only on the abaxial surface. The nature of the responses of epidermal structure to CO_2 enrichment is therefore species-specific. This is perhaps not surprising given the complex repertoire of environmental and developmental signals that influence cell size and cell number (Taylor *et al.*, 1994, 2001; Masle, 2000; Ferris *et al.*, 2001, 2002) as well as stomatal density and patterning (Larkin *et al.*, 1997; Lake *et al.*, 2002). However, stomatal densities were increased by high CO_2 in the present study with *P. dilatatum* as in *P. antidotale* (Tipping & Murray, 1999), suggesting that CO_2 is a less powerful negative regulator of stomatal density in *Paspalum* and *Panicum* than in *Arabidopsis* (Gray *et al.*, 2000).

Like maize leaves (Domes, 1971; Driscoll *et al.*, 2006), *P. dilatatum* leaves display a pronounced dorso-ventral asymmetry in the regulation of photosynthesis, each surface showing unique characteristic responses to available CO_2 and light. The specific dorso-ventral regulation of photosynthesis with respect to light orientation to the adaxial or abaxial surface was observed at both growth CO_2 concentrations. This regulation is not related to surface-dependent differences in light absorption, reflectance or transmission as the two leaf surfaces have the same optical properties. Light-harvesting complexes and photosystems are therefore equally distributed on the adaxial and abaxial sides of the leaf. While it is logical to assume that gradients in CO_2 fixation rates that have been documented in dicotyledonous C_3 leaves (Oya & Laisk, 1976; Terashima, 1986; DeLucia *et al.*, 1991; Sun & Nishio, 2001; Evans & Vogelmann, 2003) are also present in monocotyledonous C_4 leaves, there have been no comparative studies on C_4 leaves that enable deductions to be made regarding the nature of possible mechanisms of acclimation to growth at high CO_2. However, it is clear that the dorso-ventral gradients in photosynthetic machinery that determine adaxial/abaxial light absorption and CO_2 fixation characteristics in C_3 leaves must be entrained early in leaf development (Smith & Ullberg, 1989; Evans *et al.*, 1993; Poulson & DeLucia, 1993; James & Bell, 2000; Ustin *et al.*, 2001) as they can only be changed by inversion of the leaves at the onset of development (Terashima *et al.*, 1986; Smith *et al.*, 1997). Similar to the leaves of other monocotyledonous species, *P. dilatatum* leaves grow vertically from the sheath and then bend over so that the adaxial surface tends to be uppermost with the abaxial surface beneath. However, the leaves tend to curl as they expand so that the adaxial and abaxial surfaces can both experience orientation to the light in different sections of the same leaf.

The degree of stomatal opening was markedly affected by light orientation in *P. dilatatum* leaves. Abaxial illumination resulted in the closure of stomata on the adaxial surface, a response that was accompanied by a complete suppression of photosynthesis. The differential sensitivity of adaxial and abaxial stomata to light and the absence of CO_2 transport between the two surfaces are important factors governing overall photosynthesis rates (Turner, 1970; Pospíšilová & Solárová, 1980). However, the complete suppression of photosynthesis on the adaxial surface when the stomata are closed is surprising given that this is a C_4 species able to fix CO_2 even with closed stomata, as a result of the presence of a high-affinity PEPC (Bauwe, 1986). Furthermore, these data show that there is little transfer of CO_2 across the leaf as the abaxial stomata were open in these conditions.

Photosynthesis on the adaxial surface was much less responsive to low C_i values than that on the abaxial surface. The initial slopes of the CO_2-response curves, which represent the maximal PEPC rate according to von Caemmerer (2000), indicate a higher maximal PEPC activity on the abaxial surface. The immunolocalization studies reported here show that there is a more or less uniform distribution of the carboxylating enzyme (Rubisco and PEPC) proteins across the *P. dilatatum* leaf. PEPC activation in response to increasing light intensities and other factors occurs via post-translational regulation involving an increase in the phosphorylation state of the enzyme protein (Chollet *et al.*, 1996). Our data therefore strongly suggest that dorso-ventral asymmetric gradients operate in *P. dilatatum* leaves and that these greatly affect the photosynthetic capacity on each surface. We are drawn to the conclusion that the dorso-ventral gradient in photosynthesis in *P. dilatatum* leaves results from asymmetric enzyme activation across the leaf, which allows much higher activation on the abaxial surface.

Whole-leaf photosynthesis displayed a higher degree of flexibility with regard to light orientation when the *P. dilatatum* plants were grown at the higher CO_2 concentration. The characteristic decreases in maximal CO_2 assimilation rates observed with respect to C_i when the light was oriented to the abaxial surface were absent in plants grown at the higher CO_2 concentration. Hence, CO_2 enrichment caused a small but important adjustment in the dorso-ventral specification of photosynthesis. Light absorption profiles in *Flaveria bidentis* and maize have shown that, while green light is absorbed throughout the leaf, blue light is only strongly absorbed near the surface with little light penetrating the BS cells (J.R. Evans, T.C. Vogelmann & S. von Caemmerer, pers. comm.). However, given the similarity of internal structure on the adaxial and abaxial sides of monocotyledonous leaves, which have a bilateral symmetry, one would predict that light orientation would have similar levels of penetration from each surface (Moss, 1964; Syvertsen & Cunningham, 1979). Growth at the higher CO_2 concentration had little effect on the overall photosynthetic capacity of *P. dilatatum* leaves, as observed previously (von Caemmerer *et al.*, 2001). However, other studies using CO_2 enrichment have yielded rather mixed results in different C_4 species. In some studies, enhancement of photosynthesis was reported (LeCain & Morgan, 1998; Wand *et al.*, 2001) but in others down-regulation of photosynthesis was observed (Greer *et al.*, 1995; Ghannoum *et al.*, 1997; Walting

& Press, 1997). Here we show that growth at the higher CO_2 concentration caused a small but important adjustment in the dorso-ventral specification of photosynthesis, an effect that was most apparent when light was oriented to the adaxial surface. These results indicate that metabolic and/or structural adjustments to CO_2 enrichment have occurred within the leaves and that these facilitate the altered response to light orientation.

We conclude that the dorso-ventral specification in the regulation of photosynthesis and stomatal conductance in *P. dilatatum* arises from genetically programmed differences in stomatal sensitivity to light orientation coupled to a fixed gradient in enzyme activation, probably PEPC activation state. The results presented here together with those reported previously in maize (Driscoll *et al.*, 2006) suggest that dorso-ventral asymmetry in the regulation of photosynthesis and stomatal conductance may be a common feature of monocotyledonous C_4 species. The dorso-ventral asymmetry may have functional significance in situations that favour leaf rolling, for example drought avoidance, where such mechanisms would help to prevent water deficits while allowing high rates of photosynthesis.

Acknowledgements

ASS was supported by the Fundação para a Ciência e a Tecnologia (PhD grant no. SFRH/13728/2003), the Fundação Calouste Gulbenkian and the Society for Experimental Biology. Rothamsted Research receives grant-aided support from the UK Biotechnology and Biological Sciences Research Council. The authors thank Dr G. L. Lockett, Margot Forage Germplasm Centre, New Zealand for providing the *P. dilatatum* cv. Raki seeds. We are deeply indebted to Bob Furbank and Susanne von Caemmerer for critical reading of the manuscript and also to Till Pellny for the photographs in Fig. 1.

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