Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Dorsoventral variations in dark chilling effects on photosynthesis and stomatal function in *Paspalum dilatatum* leaves

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Abstract

The effects of dark chilling on the leaf-side-specific regulation of photosynthesis were characterized in the C_4 grass *Paspalum dilatatum*. CO_2- and light-response curves for photosynthesis and associated parameters were measured on whole leaves and on each leaf side independently under adaxial and abaxial illumination before and after plants were exposed to dark chilling for one or two consecutive nights. The stomata closed on the adaxial sides of the leaves under abaxial illumination and no CO_2 uptake could be detected on this surface. However, high rates of whole leaf photosynthesis were still observed because CO_2 assimilation rates were increased on the abaxial sides of the leaves under abaxial illumination. Under adaxial illumination both leaf surfaces contributed to the inhibition of whole leaf photosynthesis observed after one night of chilling. After two nights of chilling photosynthesis remained inhibited on the abaxial side of the leaf but the adaxial side had recovered, an effect related to increased maximal ribulose-1,5-bisphosphate carboxylation rates (V_{cmax}) and enhanced maximal electron transport rates (J_{max}). Under abaxial illumination, whole leaf photosynthesis was decreased only after the second night of chilling. The chilling-dependent inhibition of photosynthesis was located largely on the abaxial side of the leaf and was related to decreased V_{cmax} and J_{max}, but not to the maximal phosphoenolpyruvate carboxylase carboxylation rate (V_{pmax}). Each side of the leaf therefore exhibits a unique sensitivity to stress and recovery. Side-specific responses to stress are related to differences in the control of enzyme and photosynthetic electron transport activities.

Key words: Abaxial/adaxial leaf specification, chilling responses, C_4 photosynthetic models, *Paspalum dilatatum*, photosynthetic regulation, stomatal regulation.

Introduction

The understanding of plant responses to environmental stresses is crucial for models of plant behaviour in a changing climate. Plant growth and development are strongly influenced by the perception of signals from the environment. The perception of light quality and day length, for example, influences plant growth and architecture, flowering time, germination, and other processes. Similarly, nutrient availability, temperature, and interactions with other organisms also modify plant development. Exposure to low non-freezing (chilling) temperatures induces structural,

developmental, physiological, cellular, and molecular alterations in plants (Lyons, 1973; Smallwood and Bowles, 2002; Sung *et al.*, 2003) that are largely associated with altered membrane function (Lyons, 1973). Chilling also leads to the induction of a network of cold-responsive genes, which are considered to be responsible for the biochemical and physiological changes that allow plants to acclimate to a low temperature growth environment. Cold acclimation involves the regulated expression of effecter genes that are collectively called the *COLD-REGULATED* (*COR*) genes, whose function is to protect cell membranes and thereby prevent cellular dehydration. Low-temperature induction of the *COR* genes is regulated by *CRT/CRE BINDING FACTOR* (*CBF*) genes, whose expression is influenced by the plant circadian clock, which regulates the magnitude of the transcriptional activation of the *CBF* genes by low temperatures. For example, CBF expression is lower when chilling is experienced in the dark than in the light (Maruyama *et al.*, 2004; Fowler *et al.*, 2005). Moreover, chilling disrupts the circadian pattern of activity of some enzymes in leaves such as sucrose phosphate synthase and nitrate reductase, whose activation is delayed following exposure to chilling (Jones *et al.*, 1998).

Persistent inhibition of photosynthesis occurs when plants are exposed to chilling temperatures in a diverse group of plant species. The extent of chilling-induced inhibition depends on the species, the sensitivity to chilling, the duration of the stress, and the light intensity experienced in the photoperiod following the stress. Photosynthesis is decreased following exposure to chilling temperatures even when the stress is applied in the dark in some C_3 (e.g. Flexas *et al.*, 1999; van Heerden *et al.*, 2003a; Feng and Cao, 2005) and C_4 (e.g. Ludlow and Wilson, 1971; Pasternak and Wilson, 1972; Pittermann and Sage, 2001) species. Dark-chilling-induced limitations to stomatal function have been reported in C_3 and C_4 plants, and these contribute to the observed inhibition of photosynthesis rates at least in C_3 species (e.g. Pasternak and Wilson, 1972; Martin *et al.*, 1981; Bauer *et al.*, 1985; van Heerden *et al.*, 2003a; Feng and Cao, 2005). Non-stomatal limitations on whole leaf CO_2 assimilation have also been reported after dark chilling in both C_3 and C_4 plants. For example, direct effects on electron transport (Shen *et al.*, 1990; van Heerden *et al.*, 2003b; Bertamini *et al.*, 2006; Strauss *et al.*, 2007) and the activities of photosynthetic enzymes (Jones *et al.*, 1998; Jun *et al.*, 2001; Pittermann and Sage, 2001; van Heerden *et al.*, 2003a, b; Zhou *et al.*, 2004) have been observed.

The dorsoventral regulation of photosynthesis is well characterized in dicotyledonous C_3 plants and is related to the presence of two types of mesophyll cell and a continuous airspace system that provides gaseous continuity across the leaf blade (Terashima and Inoue, 1985a,b; Nishio *et al.*, 1993; Evans, 1995; Sun *et al.*, 1998; Sun and Nishio, 2001; Evans and Vogelmann, 2003). Unlike dicotyledonous C_3 plants, the adaxial and abaxial sides of C_4 monocotyledonous leaves like maize comprise essentially two separate compartments as there are physical dorsoventral restrictions to airspace continuity (Long *et al.*, 1989). Moreover, the

regulation of photosynthesis is different on each side of C_4 monocotyledonous leaves that belong to the NADP-malic enzyme (NADP-ME) subtype such as *Paspalum dilatatum* (Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009) and maize (Long *et al.*, 1989; Driscoll *et al.*, 2006; Soares-Cordeiro *et al.*, 2009). Marked differences in the leaf-side-specific responses of photosynthesis and stomatal closure to light orientation are observed in such C_4 monocotyledonous leaves. This led us to propose that each side of such leaves has independent signalling pathways associated with light perception, photosynthesis, and stomatal closure (Soares-Cordeiro *et al.*, 2009).

Amphistomatous leaves from dicotyledonous and mono-cotyledonous C_3 and C_4 species can exhibit differential regulation of stomatal closure on the adaxial and abaxial surfaces. Generally, the stomata on the adaxial surface are considered to be less sensitive to light intensity (Turner, 1970; Pemadasa, 1979; Travis and Mansfield, 1981; Goh *et al.*, 1995; Soares *et al.*, 2008), as well as to water stress conditions (Turner and Singh, 1984; Lu, 1988; Wang *et al.*, 1998). However, stomatal conductance was shown to be more affected on the adaxial surface in the dicotyledonous C_3 species *Rumex obtusifolius* grown under CO_2 enrichment (Pearson *et al.*, 1995). Side-specific differences in stomatal movement have been related to variations in calcium ion concentration in the guard cells (De Silva *et al.*, 1986), which are in turn regulated by the metabolic and signalling activities of the adjacent mesophyll cells in response to changes in CO_2 or light availability (Mott *et al.*, 2008). Recent evidence suggests, however, that photosynthesis does not contribute directly to the signalling network that regulates stomatal opening (von Caemmerer *et al.*, 2004; Baroli *et al.*, 2008).

The presence of a two-compartment structure in C_4 monocotyledonous leaves with little or no apparent airspace continuity between the upper and lower sides, would allow separate dorsoventral systemic stress signalling pathways. The hypothesis that was tested in the present study was that the leaves of C_4 monocotyledonous species such as *P. dilatatum* (Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009) might exhibit differential responses and acclimation to chilling on the adaxial and abaxial sides of the leaf. The two-airspace system may thus be useful not only in the optimization of whole leaf photosynthesis but also in the regulation of surface-specific stress defence-signalling cascades. In the following experiments therefore we have explored the possibility that each side of *P. dilatatum* leaves responds independently to the stress imposed by dark chilling.

To date, relatively few studies that focus on enhancing our current understanding of how the side-specific regulation of photosynthesis and stomatal movements is altered in response to environmental stress have been performed. Studies on CO_2 enrichment in maize (Driscoll *et al.*, 2006) and *P. dilatatum* (Soares *et al.*, 2008) revealed that each surface of the leaf responded differently to altered CO_2 availability. While photosynthesis on the abaxial surfaces was largely unchanged in the leaves of these two C_4

NADP-ME species when plants were acclimated to elevated growth CO_2 levels, CO_2 assimilation rates were decreased on the adaxial surfaces. No information is available in the literature on the effects of chilling on the surface-specific regulation of photosynthesis and stomatal conductance values in monocotyledonous C_4 species. The present study was therefore undertaken to characterize the effects of dark chilling on photosynthesis and stomatal function on each side of *P. dilatatum* leaves and to determine whether chilling stress results in side-specific regulation in this C_4 grass species, which is partially tolerant to the stress imposed by dark chilling (Cavaco, 2000).

Materials and methods

Plant material

Paspalum dilatatum seeds were germinated and grown for 6 weeks in controlled environment cabinets (Sanyo SGC228.CFX.J; Sanyo, Osaka, Japan) as described previously (Driscoll *et al.*, 2006; Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009). The controlled environment cabinets provide a constant CO_2 concentration of 350 ± 20 µl l^{-1} (Eurotherm Ltd, Worthing, UK), 80% relative humidity, a temperature of 25 °C in the day and 19 °C at night, with a 16 h photoperiod at an irradiance of 600–650 µmol m^{-2} s^{-1} at pot height (400–700 nm). Irradiance was provided by Phillips Master TL5 HO 49w/830 fluorescent lamps. Plants were watered daily throughout the period of the experiments.

Dark chilling treatments and sampling

For the dark chilling treatments plants were transferred to a controlled growth chamber for one or two consecutive nights. The conditions in the controlled growth chamber were as above, except that the night temperatures were maintained at 4 °C. Leaf pigment and soluble protein determinations and gas-exchange measurements were performed on the middle (widest) part of the youngest fully expanded leaves in the subsequent light periods after one and two consecutive nights of dark chilling. Leaf harvest and measurements were performed between 1 and 5 h after the beginning of the light period.

Determination of leaf pigments and soluble protein

Leaf pigments [chlorophyll *a* (chl*a*), chl*b*, total chlorophyll, and carotenoids] and soluble protein content were determined in the middle sections of the youngest fully expanded leaves of plants that had been either maintained at optimal growth temperatures or subjected to one or two nights of dark chilling, as above. All methods used were essentially as described previously (Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009). Leaf samples were ground in liquid nitrogen and quartz sand. Pigments were determined according to the method of Lichtenhaler and Wellburn (1983) after extraction in 96% ethanol (v/v). Soluble proteins were extracted in sodium phosphate buffer as described by Soares *et al.* (2008). Total soluble protein content was determined according to the method of Bradford (1976).

Gas-exchange measurements

Photosynthetic gas-exchange measurements were performed using an infrared gas analyser (model wa-225-mk3; ADC, Hoddesdon, UK) either on whole leaves using custom-made standard Parkinson-type whole leaf chambers designed for CO_2 and water vapour analysis (Novitskaya *et al.*, 2002) or on each leaf surface separately using custom-made dual Parkinson-type chambers designed for CO_2 and water vapour analysis (Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009).

Gas-exchange measurements were performed on the middle sections of the youngest fully expanded attached leaves of plants that had been either maintained at optimal growth temperatures or subjected to one or two nights of dark chilling. Light was oriented either to the adaxial surface or to the abaxial surface, by inverting the leaves in the chambers. Since *P. dilatatum* leaves are not large enough to fully separate the two sides of the dual chamber, the leaves were expanded with gas-tight tape to seal each half of the chamber, preventing any flux of gas between the two sides as previously described by Soares *et al.* (2008) and Soares-Cordeiro *et al.* (2009).

Three types of measurement were made on the attached leaves of plants that had been either maintained at optimal growth temperatures or subjected to one or two nights of dark chilling: (i) single measurements of gas-exchange parameters on the whole leaves and on each leaf surface separately at an ambient CO_2 concentration of 350 µl l^{-1} and at an irradiance of between 900 and 1000 µmol m^{-2} s^{-1}; (ii) CO_2-response curves for photosynthesis were performed on whole leaves and on each side of the leaf separately at an irradiance between 900 and 1000 µmol m^{-2} s^{-1}. In these experiments, the CO_2 concentration within the chambers was increased in a stepwise manner from 50 to 1000 µl l^{-1}, and (iii) light-response curves for photosynthesis were performed on whole leaves and on each side of the leaf separately at an ambient CO_2 concentration of 350 µl l^{-1}. In these experiments, the light intensity was increased stepwise from darkness to 1500 µmol m^{-2} s^{-1}.

The whole leaf response curves for photosynthesis allow us to model some of the key parameters of C_4 photosynthesis, using the C_4 photosynthetic models described by von Caemmerer (2000). Such C_4 photosynthetic models have only been described for whole leaf photosynthesis. It was therefore not possible to use the leaf-surface-specific data obtained from the CO_2- and light-response curves for photosynthesis to determine the various C_4 photosynthetic parameters on each side of the leaf. In addition, we consider that the interpretation of the maximal electron transport rate and maximal photosynthetic rate values may not be trivial using values obtained for each leaf surface (S. von Caemmerer, personal communication) because we have not determined the exact amount of light that reaches the side of the leaf that is not under direct actinic illumination.

Steady-state rates for CO_2 assimilation and stomatal conductance were determined at a leaf temperature of 20 °C. This temperature was maintained constant for both whole leaf and side-specific measurements of parameters on the adaxial and abaxial surfaces, by water jackets as previously described by Soares *et al.* (2008). Measurements were conducted at 50% relative humidity and the irradiance was provided by overhead broad-spectrum lamps and supplied only from the top of the chamber both for the whole leaf measurements and for each leaf surface measurement (Soares *et al.*, 2008). Data presented for whole leaf and leaf-surface-specific parameters are the mean values obtained from leaves of nine plants per measurement that were analysed on different batches of plants grown over the whole experimental period.

Measurements of the internal resistance to gas diffusion

Ambient CO_2 concentration, intercellular CO_2 concentration, and stomatal conductance values were determined on the adaxial and abaxial surfaces of *P. dilatatum* leaves as follows. The gas flow and pressure were maintained equal and constant on each side of the leaf but a differential ambient CO_2 concentration was applied across the leaves such that one side was maintained at 2500 µl l^{-1} CO_2 while the other received 0 µl l^{-1} CO_2. Light was oriented to the adaxial leaf surface in all measurements at an intensity of 550 µmol m^{-2} s^{-1}. The leaf temperature was maintained at 20 °C with a relative humidity of 50%.

C_4 photosynthetic model predictions for whole leaf photosynthesis

CO_2-response curves: Whole leaf CO_2-response curves for photosynthesis were used to calculate C_4 photosynthetic parameters, according to the whole leaf C_4 photosynthetic model of von Caemmerer and Furbank (1999) and as described in detail by von Caemmerer (2000). These models are based on the earlier models of Berry and Farquhar (1978) and Peisker (1979). This model allows the prediction of the maximal phosphoenolpyruvate carboxylase (PEPC) carboxylation rate (V_{pmax}) and maximal ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate (V_{cmax}) using enzyme-limited photosynthesis equations and the maximal electron transport rate (J_{max}) using light- and electron-transport-limited photosynthesis equations. The model requires that a saturating light intensity is used to perform the measurements. We thus had to achieve a compromise with regard to the light intensity in order to avoid photoinhibition in the light period following the treatments when the plants were dark-chilled. While the light intensity (900–1000 μmol m^{-2} s^{-1}) used in the present experiments was not fully saturating for photosynthesis according to the data shown in Fig. 4, it is nearly saturating while low enough to prevent photoinhibition in plants that had experienced dark chilling. Nevertheless, it is important to note that while the values obtained through the application of the C_4 photosynthetic model in this study cannot be considered to be the absolute maximal values, they can accurately be used to compare the effects of dark chilling and light orientation on the leaf.

In addition, the C_4 photosynthetic model requires the use of some parameters measured at 25 °C (von Caemmerer, 2000). Some parameters are dependent on temperature and thus it was important to calculate the values appropriate to the temperatures at which the present curves were performed (20 °C). This is the case for the Michaelis–Menten constants of Rubisco for CO_2 (K_c) and for O_2 (K_o), and the Michaelis–Menten constant of PEPC for CO_2 (K_p). An Arrhenius function was used to transform the K_c, K_o, and K_p values to 20 °C, (Badger and Collatz, 1977):

$$\text{Parameter } (T°C) = \text{Parameter } (25°C) Q_{10}^{[(25-T°C)/10]}$$

where Q_{10} is the temperature coefficient that represents the factor by which the rate of a reaction increases for every 10 °C rise in the temperature. The tabled values of K_c and K_o at 25 °C (650 μbar and 450 mbar, respectively) and the Q_{10} values for K_c and K_o (2.24 and 1.63, respectively) (von Caemmerer, 2000) were used to calculate the K_c and K_o at 20 °C. The K_p value tabled at 25 °C (80 μbar) (von Caemmerer, 2000) and the Q_{10} value for K_p of 1.603, calculated from the PEPC activation energy value (34.8 kJ mol^{-1}) obtained for *P. dilatatum* plants at temperatures >20 °C (Cavaco, 2000), were used to calculate the K_p value at 20 °C. The parameters and the values used to apply the photosynthetic model are described in Table 1.

A similar approach to that described by Massad *et al.* (2007) was used to estimate V_{pmax}, V_{cmax}, and J_{max}. First, an asymptotic exponential model was selected as it provides the best description of the variation in CO_2 assimilation rates with intercellular CO_2 concentration (C_i) for each individual plant according to the least squares method using the program Statistical Package for Social Sciences (SPSS) (version 15.0, 2006; SPSS Inc., Chicago, IL, USA).

The equations that were used to determine enzyme-limited photosynthesis and light- and electron-transport-limited photosynthesis (as shown in Table 2) were applied using the Mathematica version 6.0 (2007) software (Wolfram Research Inc., Champaign, IL, USA). Data obtained from each individual plant were analysed in the range 0–500 μl l^{-1}, in a stepwise manner (i.e. per 5 μl l^{-1} C_i increase) in order to determine V_{pmax}, V_{cmax}, and J_{max} values at each C_i. The estimated values of V_{pmax}, V_{cmax}, and J_{max} were then plotted against C_i. The mean values were then determined for each individual plant in the ranges 50–80 μl l^{-1} C_i for V_{pmax} and 300–500 μl l^{-1} C_i for V_{cmax} and J_{max}. The values obtained for each plant were then used to determine the mean values for V_{pmax}, V_{cmax}, and J_{max} for each treatment. Values for mitochondrial

Table 1. Parameters used for modelling C_4 photosynthesis based on the CO_2-response curves for photosynthesis (von Caemmerer, 2000)

Parameter	Value	Description
K_c	0.973 mbar	Michaelis–Menten constant of Rubisco for CO_2 (variable with temperature, corrected to 20 °C)
K_o	575 mbar	Michaelis–Menten constant of Rubisco for O_2 (variable with temperature, corrected to 20 °C)
K_p	101 μmol mol^{-1}	Michaelis–Menten constant of PEPC for CO_2 (variable with temperature, corrected to 20 °C)
O	21%	O_2 partial pressure in the bundle sheath and mesophyll cells
g_{bs}	3 mmol m^{-2} s^{-1}	Bundle-sheath conductance to CO_2
g_i	2 mol m^{-2} s^{-1}	Mesophyll conductance to CO_2
R_d	0.01*V_{cmax}	Mitochondrial respiration
R_m	0.5*R_d	Mesophyll mitochondrial respiration
γ^*	0.000193	Half the reciprocal of Rubisco spefcity
X	0.4	Partitioning factor of electron transport rate
θ	0.7	Empirical curvature factor
F	0.15	Factor that corrects for spectral quality of light
abs	0.85	Absorptance of leaves
I	1000 μmol m^{-2} s^{-1}	Irradiance used
I_a	361.2 μmol m^{-2} s^{-1}	Total absorbed irradiance

respiration and mesophyll mitochondrial respiration were then calculated from the V_{pmax} and V_{cmax} data according to von Caemmerer (2000).

Light-response curves: Light-response curves for photosynthesis in whole leaves were analysed (Statistica 8.0, 2007; StatSoft, Inc., OK, USA) to determine the apparent quantum yield (ϕ), curvature degree (θ), maximal rate of photosynthesis (A_{max}), and mitochondrial respiration (R_d), using the equations below as described by von Caemmerer (2000) and Lambers *et al.* (1998):

$$A = \frac{I \times \phi + A_{max} - \sqrt{(I \times \phi + A_{max})^2 - 4 \times \theta \times I \times \phi \times A_{max}}}{2 \times \theta} - R_d$$

where, A is the CO_2 assimilation rate, and I the irradiance used.

Each parameter was calculated for each individual curve and then the mean values were obtained for each dark chilling and light orientation treatment. The C_4 photosynthetic light-response curve model requires that analysis is performed under conditions of saturating CO_2 concentrations. In the present study, the light-response curves for photosynthesis were performed at 350 μl l^{-1} CO_2 (corresponding to a C_i value of ~120–200 μl l^{-1}), which is a saturating CO_2 concentration (according to Fig. 3). The predicted ϕ, θ, A_{max}, and R_d values determined from the model are therefore considered to be accurate.

Statistical analysis

Statistical analysis of the data was performed using parametric tests at a stringency of $P<0.05$. The significance of variation for mean values regarding pigment, protein, single gas-exchange measurements obtained for whole leaves and for each leaf side separately and for whole leaf photosynthetic model determinations was analysed using an ANOVA followed by a Tukey HSD test. All the analyses were performed separately for each parameter.

Table 2. Equations for enzyme-limited photosynthesis and for light- and electron-transport-limited photosynthesis used to predict the maximal PEPC carboxylation rate (V_{pmax}), maximal Rubisco carboxylation rate (V_{cmax}), and the maximal electron transport rate (J_{max}) from the CO_2-response curves of whole leaf photosynthesis according to von Caemmerer (2000)

See Table 1 for all abbreviations except A (CO_2 assimilation rate), C_i (intercellular CO_2 concentration), C_m (mesophyll cells CO_2 concentration), C_s (bundle sheath cells CO_2 concentration), and J_t (predicted electron transport rate).

Parameter	Equations
V_{pmax} and V_{cmax}	Equations for enzyme-limited photosynthesis: $A = g_i \times (C_i - C_m)$ $A = \frac{C_s \times V_{cmax}}{C_s + K_c \times (1 + O/K_o)}\left(1 - \frac{\gamma^* \times O}{C_s}\right) - R_d$ $A = \frac{C_m \times V_{pmax}}{C_m + K_p} - g_{bs} \times (C_s - C_m) - R_m$
J_{max}	Equations for light- and electron-transported-limited photosynthesis: $A = g_i \times (C_i - C_m)$ $A = \frac{(1 - \gamma^* \times O/C_s) \times (1-x) \times J_t}{3 \times \left(1 + 7 \times \gamma^* \times O/C_s\right)} - R_d$ $A = \frac{x \times J_t}{2} - g_{bs} \times (C_s - C_m) - R_m$
	Equation characterizing the relationship between electron transport rates and absorbed irradiance: $I_a = \frac{I \times a_{bs} \times (1 - f)}{}$ $J_t = \frac{I_a + J_{max} - \sqrt{(I_a + J_{max})^2 - 4 \times \theta \times I_a \times J_{max}}}{2 \times \theta}$

Results

Effects of dark chilling stress on leaf pigments and protein

The leaves of plants that had been subjected to a single night of dark chilling showed a decrease in the content of both chl*a* and chl*b*. However, the chl*a*/chl*b* ratio was increased because the chilling-induced decrease (19%) in chl*b* was higher than that (9%) in chl*a* (Table 3). After a second night of chilling the decrease in chl*a* and chl*b* was of a similar magnitude (13% and 14%, respectively). Thus, the ratio chl*a*/chl*b* was not significantly different from that of the control plants that had been maintained under optimal temperatures. Leaf carotenoid content decreased significantly after the second consecutive night of chilling (Table 3) but the ratio of chl*a* plus chl*b* to carotenoids was unaffected by dark chilling. The amount of soluble protein in the leaves was not affected by the chilling treatments (Table 3).

Effects of dark chilling on whole leaf photosynthesis and stomatal conductance

Whole leaf photosynthesis rates at ambient CO_2 and a light intensity of 900–1000 $\mu mol\ m^{-2}\ s^{-1}$ were 14% lower when CO_2 assimilation was measured with light oriented to the abaxial surface (Fig. 1A). Whole leaf CO_2 assimilation rates measured under adaxial illumination were 32% and 14%

Table 3. Effects of dark chilling on leaf pigment and protein contents. Plants were either maintained at optimal growth temperatures (controls) or were subjected to dark chilling for either one or two consecutive nights

Data represent the mean values ± SE of seven leaves *per* treatment. Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05. Chl, chlorophyll.

	Control	1 Night Chilling	2 Nights Chilling
Chl*a* (mg m^{-2})	572 ± 15.7 a	518 ± 14.7 b	499 ± 4.9 b
Chl*b* (mg m^{-2})	156 ± 4.9 a	127 ± 4.3 b	135 ± 2.0 b
Chl*a+b* (mg m^{-2})	727 ± 2.0 a	645 ± 18.9 b	634 ± 5.7 b
Carotenoids (mg m^{-2})	114 ± 3.3 a	108 ± 2.6 b,a	100 ± 1.4 b
Chl*a*/Chl *b* ratio	3.7 ± 0.06 a	4.1 ± 0.04 b	3.7 ± 0.05 a,b
Chl*a+b*/Carotenoid ratio	6.4 ± 0.07 a,b	6.0 ± 0.06 a	6.3 ± 0.10 a,b
Soluble proteins (mg m^{-2})	3084 ± 148.9 a	2773 ± 65.3 a	2797 ± 78.8 a

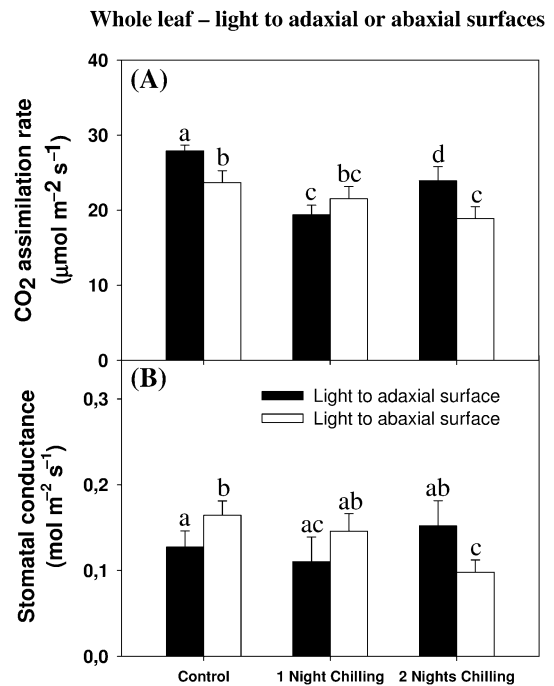**Whole leaf – light to adaxial or abaxial surfaces**

Fig. 1. The effect of light orientation and dark chilling on whole leaf photosynthesis (A) and stomatal conductance (B) in *P. dilatatum* leaves. Plants were either maintained at optimal growth temperature (controls), or subjected to dark chilling for either one or two nights. Light was oriented to either the adaxial (closed bars) or the abaxial (open bars) leaf surface. Data are the mean±SE of nine plants per treatment with each gas-exchange parameter measured separately. The different letters represent significant differences (*P*<0.05).

lower after, respectively, one and two nights exposure to dark chilling compared with controls that had been maintained under optimal temperatures. Photosynthesis was only decreased under abaxial illumination after two nights of chilling and then only by 20%. Stomatal conductance values were higher in control plants under abaxial

illumination than under adaxial illumination (Fig. 1B). No effects of dark chilling on the stomatal conductance were detected either under adaxial illumination after one or two nights of dark chilling or under abaxial illumination after one night of chilling. However, a 40% decrease in stomatal conductance was observed under abaxial illumination after two nights of stress (Fig. 1B).

Effects of dark chilling on photosynthesis and stomatal conductance on each leaf surface

The dark chilling treatments affected CO_2 assimilation differently on each leaf surface under adaxial illumination. The CO_2 assimilation rates on each leaf surface were similar under adaxial illumination in control plants (Fig. 2A). Under the same light condition, a single night of exposure to the chilling stress was sufficient to decrease the photosynthetic rates by half. However, photosynthesis rates recovered on the adaxial surface, but not on the abaxial surface after two nights of chilling in these conditions.

Photosynthesis on the adaxial surface was below the level of detection under abaxial illumination. However, the abaxial surface had increased CO_2 assimilation rates compared with the values obtained under adaxial illumination (Fig. 2A, C). In contrast to photosynthesis under adaxial illumination, CO_2 assimilation rates on the abaxial surface were unaffected by one night of chilling, but they

were decreased (21%) after two consecutive nights of chilling (Fig. 2C). Dark chilling had similar effects on stomatal conductance under adaxial (Fig. 2B) and abaxial illumination (Fig. 2D).

Effects of dark chilling on the whole leaf CO_2-response curves for photosynthesis

Whole leaf CO_2 assimilation rates increased with C_i under all conditions irrespective of the light orientation (Fig. 3). The steady-state rates of photosynthesis were similar after one night of chilling when light was oriented either to the adaxial or the abaxial surface of the leaves. However, steady-state rates of photosynthesis in the control leaves maintained under optimal growth temperatures and those of plants that had experienced two nights of dark chilling were highest when light was oriented to the adaxial surface (Fig. 3A). The initial slopes of the CO_2-response curves for photosynthesis were similar in control and chilled leaves regardless of light orientation. However, chilling had different effects on the steady-state rates of photosynthesis measured under adaxial or abaxial illumination. Photosynthesis rates were decreased under adaxial illumination after the plants had been chilled for one night (Fig. 3A). However, a partial recovery was observed after the second chilling treatment (Fig. 3A). In contrast, a second night of chilling led to a greater inhibition of photosynthesis when
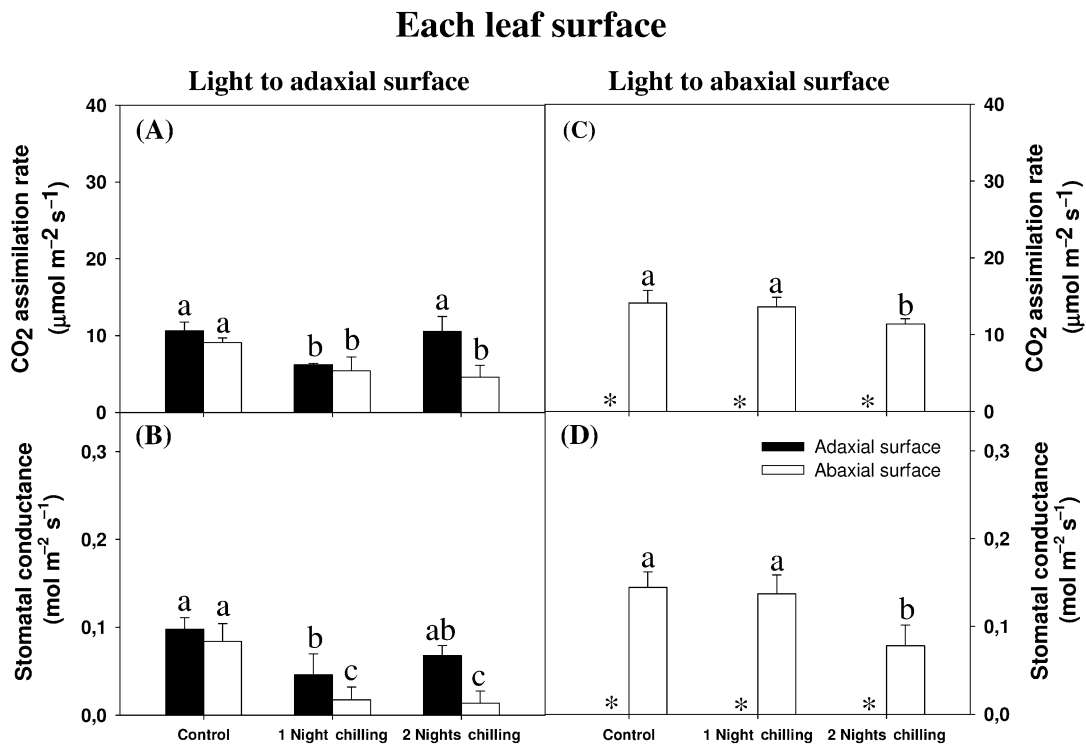
Each leaf surface

Fig. 2. The effect of light orientation and dark chilling on leaf-side-specific rates of photosynthesis (A) and stomatal conductance (B) in *P. dilatatum* leaves. Plants were either maintained at optimal growth temperatures (controls), or subjected to dark chilling for either one or two nights. CO_2-assimilation rates (A, C) and stomatal conductance (B, D) were determined on the adaxial (closed bars) and the abaxial (open bars) leaf surfaces. Light was oriented to either the adaxial (A, B) or the abaxial (C, D) leaf surface. Data are the mean values±SE of nine plants per treatment with each gas-exchange parameter measured separately. The different letters represent significant differences ($P<0.05$) and * indicates values below the levels of detection.

measured under abaxial illumination (Fig. 3B). These data indicate that the adaxial sides of the leaves are better able to recover from chilling-induced inhibition of photosynthesis than the abaxial sides.

Effects of dark chilling on the whole leaf light-response curves for photosynthesis

Under saturating light intensities (900–1000 µmol m^{-2} s^{-1}) photosynthesis rates were significantly lower when light was oriented to the abaxial surface (Fig. 1). However, this inhibition was not observed under lower light intensities (Fig. 4). Thus, the kinetics of the light-response curves for photosynthesis were largely unaffected by dark chilling under either adaxial or abaxial illumination (Fig. 4). Only the light-saturated rate was decreased (Figs 1, 4).

Effects of dark chilling on calculated photosynthetic response curve parameters

The orientation of the leaf to light has effects on whole leaf V_{pmax}, V_{cmax}, and J_{max} values. In plants maintained at optimal growth temperatures, these parameters were 48%, 19%, and 22% lower, respectively, under abaxial relative to adaxial illumination (Table 4). However, the estimated values for the whole leaf A_{max}, ϕ, θ, and R_d were not affected by light orientation at optimal growth temperatures (Table 5).

One night of chilling decreased the V_{pmax}, V_{cmax}, J_{max}, and A_{max} values by between 30 and 50% when light was oriented to the adaxial leaf surface (Tables 4, 5). These parameters largely recovered to control values after the second night of chilling, except for V_{pmax}, which showed values similar to those measured after one night of dark chilling (~45% decrease) (Table 4). The V_{pmax} and A_{max} values were similar in control and dark-chilled plants when the light was oriented to the abaxial surface (Tables 4, 5). Under abaxial illumination the estimated values for V_{cmax} and J_{max} decreased by 15% and 11%, respectively, but only after two nights of chilling (Table 4). Dark chilling and light orientation had no effects on ϕ, θ, and R_d (Table 5).
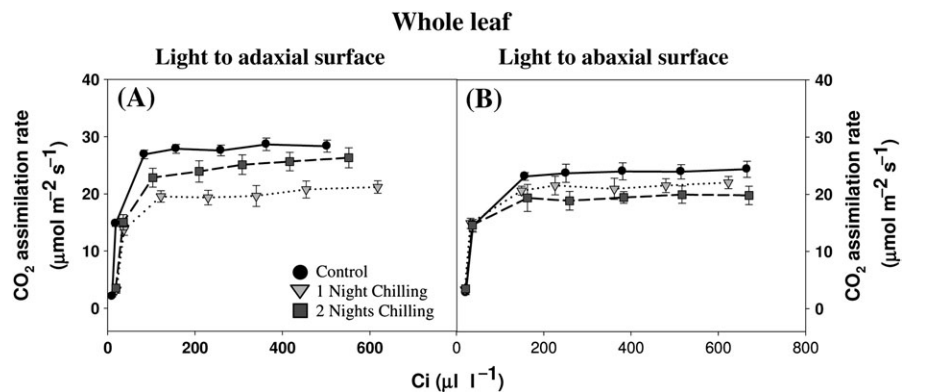

Fig. 3. The effect of light orientation and dark chilling on the CO_2-response curves for photosynthesis in whole *P. dilatatum* leaves. The light-response curves for photosynthesis were performed at 350 µl l^{-1} CO_2 (corresponding to a C_i value of ~120–200 µl l^{-1}), which is a saturating CO_2 concentration. Plants were either maintained at optimal growth temperatures (black circles), or subjected to dark chilling for either one (light grey inverted triangles) or two (dark grey squares) nights. The light intensity was orientated either to the adaxial (A) or the abaxial (B) leaf surface. Data are the mean±SE of nine plants per treatment.
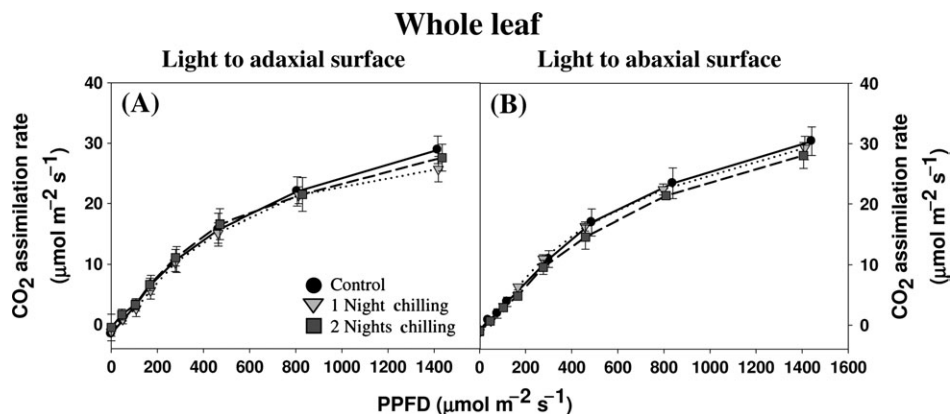

Fig. 4. The effect of light orientation and dark chilling on the light-response curves for photosynthesis in whole *P. dilatatum* leaves. Plants were either maintained at optimal growth temperatures (black circles) or subjected to dark chilling for either one (light grey inverted triangles) or two (dark grey squares) nights. Light intensity was increased in a stepwise manner from 0 to 1500 µmol m^{-2} s^{-1} with orientation to either the adaxial (A) or the abaxial (B) leaf surface. Data are the mean values±SE of nine plants per treatment.

Table 4. The effects of light orientation and dark chilling on whole leaf maximal phospho*enol*pyruvate carboxylase (PEPC) carboxylation rates (*Vpmax*), maximal ribulose-1,5-bisphosphate (Rubisco) carboxylation rates (*Vcmax*) and maximal electron transport rates (*Jmax*). Plants were either maintained at optimal growth temperatures (controls) or subjected to dark chilling for either one or two consecutive nights

Data represent the mean values ± SE of nine plants *per* treatment and were calculated following the application of the C_4 photosynthetic model of von Caemmerer (2000) to the whole leaf CO_2-response curves (See Fig. 3). Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05.

	*V*pmax (μmol m^{-2} s^{-1})	*V*cmax (μmol m^{-2} s^{-1})	*J*max (μmol m^{-2} s^{-1})
Light Adaxial			
Control	111 ± 4.3 a	32 ± 1.0 a	214 ± 15.6 a
1 Night Chilling	52 ± 1.4 c	22 ± 0.7 c	144 ± 11.3 b
2 Nights Chilling	63 ± 1.0 b,c	28 ± 1.1 a,b	183 ± 10.2 a,b
Light Abaxial			
Control	58 ± 2.9 b,c	26 ± 0.5 b	167 ± 1.6 b
1 Night Chilling	67 ± 4.8 b	24 ± 0.8 b,c	152 ± 6.4 b,c
2 Nights Chilling	66 ± 2.5 b,c	22 ± 1.1 c	148 ± 3.5 c

Table 5. The effect of light orientation and dark chilling on whole leaf maximal rate of photosynthesis (*Amax*), apparent quantum yield (φ), curvature degree (θ) and mitochondrial respiration (R_d) in plants that had been maintained at optimal growth temperatures (controls) or subjected to dark chilling for either one or two consecutive nights

Data represent the mean values ± SE of nine plants *per* treatment and was calculated accordingly to von Caemmerer (2000) and Lambers *et al.* (1998) from the whole leaf light-response curves (See Fig. 4). Statistical analysis was performed separately for each parameter. The different letters represent statistical differences at P<0.05.

	*A*max (μmol m^{-2} s^{-1})	ϕ (*10^2 μmol μmol^{-1})	θ(relative units)	R_d(μmol m^{-2} s^{-1})
Light Adaxial				
Control	41 ± 2.4 a	5.7 ± 0.58 a	0.64 ± 0.045 a	1.9 ± 0.06 a
1 Night Chilling	26 ± 0.6 b	4.6 ± 0.66 a	0.82 ± 0.044 a	1.8 ± 0.08 a
2 Nights Chilling	39 ± 2.7 a	6.5 ± 0.23 a	0.69 ± 0.090 a	1.9 ± 0.13 a
Light Abaxial				
Control	43 ± 1.7 a	4.2 ± 0.40 a	0.64 ± 0.073 a	1.6 ± 0.05 a
1 Night Chilling	37 ± 1.0 a	5.4 ± 0.12 a	0.61 ± 0.035 a	1.6 ± 0.03 a
2 Nights Chilling	37 ± 1.0 a	4.6 ± 0.50 a	0.64 ± 0.073 a	1.7 ± 0.07 a

CO_2 diffusion between the adaxial and abaxial sides of the leaf

The diffusion of CO_2 between the adaxial and abaxial sides of the *P. dilatatum* leaves was measured in plants maintained at optimal growth temperatures (Fig. 5). When the adaxial side of the leaf was exposed to high atmospheric

CO_2 levels with zero amounts on the abaxial side of the leaf (Fig. 5A), C_i values were below the level of detection on the abaxial surface (Fig. 5B) despite the stomata being fully open on that surface (Fig. 5C). Conversely, when the abaxial side of the leaf was exposed to high atmospheric CO_2 levels with zero amounts on the adaxial side of the leaf (Fig. 5D), C_i values were below the level of detection on the adaxial side (Fig. 5E) despite the stoma being fully open on that surface (Fig. 5F).

Discussion

Plant responses to environmental stresses are important in determining overall productivity and crop yields. The decreases in leaf protein and chlorophyll following short-term exposure to low temperatures observed in the leaves of *P. dilatatum*, which is nominally regarded as semi-tolerant to dark chilling (Cavaco *et al.*, 2003), are consistent with previous observations in chilling-sensitive plants (Lyons, 1973; Slack *et al.*, 1974; Forde *et al.*, 1975; Haldimann *et al.*, 1995; Nie *et al.*, 1995; Haldimann, 1996; Cavaco *et al.*, 2003; Naidu *et al.*, 2003; Strauss *et al.*, 2007). More cold-tolerant C_4 species such as *Miscanthus* show a greater degree of cold acclimation capacity (Naidu *et al.*, 2003). The information provided here on the chilling-dependent inhibition of photosynthesis in *P. dilatatum* leaves, together with observations concerning the differential sensitivities of each leaf surface to chilling-induced inhibition, advances our current understanding of how exposure to stress influences the dorsoventral regulation of photosynthesis and allows us to draw the following conclusions.

(i) Light orientation modifies whole leaf responses to dark chilling: Under optimal growth conditions whole leaf photosynthesis was decreased under abaxial illumination compared with adaxial illumination (Fig. 1A). Moreover, differences in the calculated V_{pmax}, V_{cmax}, and J_{max} values (Table 4) are related to the lower photosynthesis rates on the adaxial surface under abaxial illumination (Fig. 2C), in agreement with our earlier observations on this and other monocotyledonous C_4 NADP-ME species (Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009). Under abaxial illumination, the stomata on the adaxial surface of the leaf were tightly closed and thus it is not possible to determine CO_2 exchange for the adaxial surface. Under these conditions the abaxial surface alone contributes to detectable CO_2 assimilation rates. One possible reason for these differences is that the abaxial surface is more limited by light absorption under adaxial illumination. Under most circumstances the lower side of the leaf would experience much lower light levels than the upper surface (Soares *et al.*, 2008). While side-specific differences in the capacity for light absorption have not been detected to date (Soares *et al.*, 2008), there may be subtle variations that cannot be determined by simple whole leaf measurements. Moreover, the sensitivities of enzymes such as PEPC might be different on the two leaf surfaces.
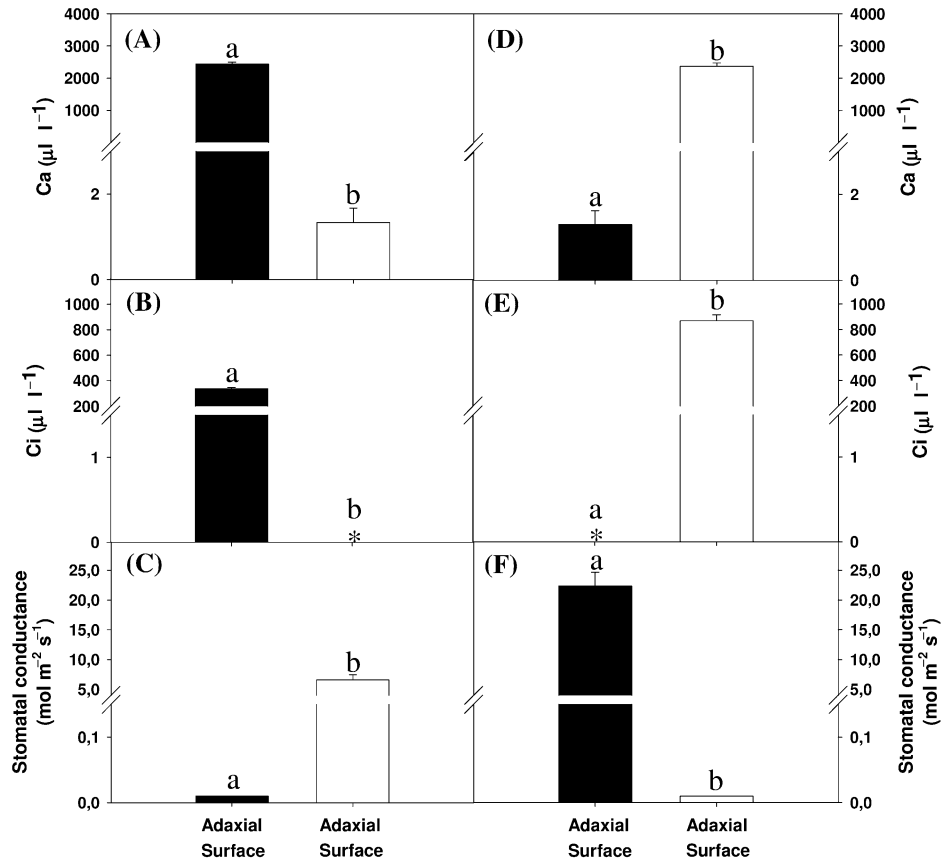
Fig. 5. Internal resistance to CO_2 diffusion across *P. dilatatum* leaves. Ambient CO_2 concentrations (C_a; A, D), intercellular CO_2 concentrations (C_i; B, E), and stomatal conductance values (C, F) were determined on the adaxial (dark bars) and abaxial (white bars) surfaces of attached *P. dilatatum* leaves, on plants that were maintained at optimal growth temperatures. The ambient CO_2 concentration was maintained either at 2500 µl l^{-1} on the adaxial surface and 0 µl l^{-1} on the abaxial surface (A–C) or 2500 µl l^{-1} on the abaxial surface and 0 µl l^{-1} on the adaxial surface (D–F). Light was oriented to the adaxial leaf surface. Data are the mean values±SE of four plants per treatment. The different letters represent significant differences ($P<0.05$) and * indicates values below the level of detection.

When the abaxial surface was directly illuminated, the limitation by light availability was diminished and thus there was a marked increase in CO_2 assimilation rates. The rates of photosynthesis on the abaxial surface under abaxial illumination were much higher than those obtained on the adaxial surface under adaxial illumination. This is presumably because the abaxial surface has a higher capacity to absorb light and to assimilate CO_2.

The results presented here show that the responses of whole leaf photosynthesis to dark chilling were modified by light orientation (Fig. 1A, B). This result is similar to that obtained previously with *P. dilatatum* plants, in response to another environmental stress, i.e. growth with CO_2 enrichment (Soares *et al.*, 2008). It has long been known that dark chilling decreases photosynthesis under adaxial illumination in chilling-sensitive species (e.g. Ludlow and Wilson, 1971; Ivory and Whiteman, 1978; van Heerden *et al.*, 2003a; Feng and Cao, 2005). Chilling-induced inhibition of photosynthesis observed here in *P. dilatatum* leaves under adaxial illumination after one night of chilling is thus in agreement with previous observations. However, in photosynthesis recovered following a second chilling treatment (Fig. 1A), reflecting the modest chilling sensitivity of this species. The

recovery of photosynthesis after the second night of stress, demonstrated by the values for V_{cmax} and J_{max} (Table 4), is not observed in many chilling-sensitive C_3 species (van Heerden *et al.*, 2003a; Feng and Cao, 2005). Moreover, photosynthesis was only decreased after two nights of chilling under abaxial illumination (Fig. 1A). The results presented in this study (Fig. 1A, B; Tables 4, 5) together with the data that we have obtained previously in plants grown with CO_2 enrichment (Soares *et al.*, 2008) demonstrate that these stresses initially exert their greatest effects on photosynthesis under adaxial illumination but the resultant inhibition is followed by a rapid recovery upon a second stress exposure. In contrast, under abaxial illumination the *P. dilatatum* leaf is initially more resistant to dark chilling. This evidence demonstrates that each side of the leaf has a unique sensitivity to dark chilling.

The absence of effects on stomatal conductance after one night of chilling under adaxial illumination (Fig. 1B) suggests that the decrease in the photosynthetic rate is associated with non-stomatal limitations to photosynthesis, as also indicated by the results in Table 4. Moreover, after one night of chilling, the dorsoventral differences in the C_i values on each side of the leaf disappeared. Under optimal conditions of

irradiance and temperature, the mean C_i values were 157 ± 15.2 when light was oriented to the adaxial surface, and 251 ± 22.3 when light was oriented to the abaxial surface. After one night of chilling the mean C_i values were similar on both sides of the leaf, being 230 ± 20.1 and 226 ± 19.8, respectively. However, after the second night of chilling the dorsoventral asymmetry in mean C_i values was partially restored; the mean C_i values were then 209 ± 19.8 when light was oriented to the adaxial surface, and 259 ± 23.5 when light was oriented to the abaxial surface.

Dark chilling is known to decrease the activity of several photosynthetic enzymes in C_3 and C_4 plants (e.g. Jones *et al.*, 1998; Pittermann and Sage, 2001; van Heerden *et al.*, 2003a) and also cause decreased electron transport capacity in C_3 plants (e.g. Bertamini *et al.*, 2005; Strauss *et al.*, 2007). The higher decrease in V_{pmax} observed in *P. dilatatum* leaves after one night of chilling compared with V_{cmax}, J_{max}, and A_{max}, together with the failure of this parameter to recover after two nights of chilling stress under adaxial illumination (Tables 4, 5), suggests that the maximal activity of the first carboxylation step in C_4 plants has a higher sensitivity to dark chilling. Moreover, the decrease in V_{cmax} and J_{max} values under abaxial illumination together with the absence of an effect on V_{pmax} after the second night of chilling (Table 4) suggest that the maximal activity of the first carboxylation enzyme of C_4 plants on the abaxial surface is less sensitive to dark chilling. It is important to note that while photosynthesis on both sides of the leaf is determined under adaxial illumination, this is not the case under abaxial illumination where photosynthesis on the abaxial side of the leaf alone can be detected. These results strongly suggest that PEPC activity on each side of the *P. dilatatum* leaf has a different sensitivity to dark chilling.

(ii) Dark chilling effects on photosynthesis are leaf-side specific, the abaxial side being more sensitive to inhibition: Dark chilling had a greater impact on photosynthesis on the abaxial sides of the leaves under adaxial illumination. In marked contrast, under abaxial illumination the dark chilling effect on the abaxial side of the leaf was observed only on the second consecutive night of chilling (Fig. 2C). These results demonstrate that the acclimation of photosynthesis to dark chilling is most rapid on the surface that is directly exposed to light. Although the effects of dark chilling on metabolism, physiology, and membrane fluidity are expected to be similar on each side of the leaf irrespective of the light orientation, the higher light intensity arriving on the abaxial surface under abaxial illumination may increase the activities of photosynthetic enzymes in such a way as to overcome any metabolic limitations incurred during the chilling treatment. The activation of photosynthetic activity may be insufficient to overcome metabolic constraints resulting from the second chilling treatment. Taken together, these results suggest that the orientation of light is an important determinant of the ability of each side of the *P. dilatatum* leaf to respond to the stress imposed by dark chilling.

Growth with CO_2 enrichment decreased the photosynthetic rates only on the adaxial sides of the *P. dilatatum*

leaves under adaxial illumination (Soares *et al.*, 2008). A similar response was observed in maize plants grown with CO_2 enrichment (Driscoll *et al.*, 2006). In both studies, photosynthesis fell below the levels of detection on the adaxial sides of the leaves under abaxial illumination, while the abaxial sides of the leaves have rates similar to controls. These results presented on this study and those presented by Driscoll *et al.* (2006) and Soares *et al.* (2008) suggest that the effects of environmental stress are different on each leaf surface, revealing the importance of these studies in our current understanding of the dorsoventral regulation of photosynthesis in monocotyledonous C_4 species.

(iii) Differential sensitivity of stomatal conductance to dark chilling suggests leaf-side-specific stress signalling: The data presented here demonstrate that the two sides of *P. dilatatum* leaves are not functionally symmetrical, as the C_4 leaf structure might suggest (Cavaco, 2000). Moreover, we show here that each side of the leaf can respond differently to environmental stress. In agreement with this view, stomatal conductance is less sensitive to the effects of dark chilling on the adaxial side of the leaf under adaxial illumination (Fig. 2B). This observation is similar to that obtained in plants subjected to water deficit, a stress that, like chilling requires cellular osmotic adjustment (Turner and Singh, 1984; Lu, 1988; Wang *et al.*, 1998). The lower sensitivity of the adaxial stomata in leaves exposed to water deficit has been explained by a lower sensitivity of the guard cells on this leaf surface to abscisic acid and to calcium ions (De Silva *et al.*, 1986; Wang *et al.*, 1998). While we have no direct evidence for a specific effect of dark chilling on the stomata *per se*, the different responses of stomata on the adaxial and abaxial surfaces are intriguing, particularly as it may be a common feature of the response of C_4 cereal leaves to stress situations.

The significance of independent, dorsoventral systemic stress signalling pathways

We have previously described the dorsoventral regulation of photosynthesis with respect to light orientation under optimal growth conditions in two monocotyledonous species that belong to the NADP-ME subtype, *P. dilatatum* and maize, and we have discussed the practical relevance of the findings at a whole-plant level within the context of the natural diurnal light environment that C_4 plants face every day (Driscoll *et al.*, 2006; Soares *et al.*, 2008; Soares-Cordeiro *et al.*, 2009). The data presented here show that each side of the leaf responds differently in terms of inhibition by dark chilling and the ability to recover from the stress. We consider that these differential responses are possible because of the structure of the *P. dilatatum* leaves, which restricts gas flow. The data presented here confirm the two-compartment model for monocotyledonous C_4 leaves developed in order to explain the dorso-ventral differnces in the regulation of photosynthesis (Long *et al.*, 1989). The finding that dark chilling, like growth with CO_2

enrichment (Soares *et al.*, 2008), modifies not only the dorsoventral regulation of photosynthesis with respect to light orientation but also the acclimation ability of each side of the leaf to the stress, is intriguing. While more studies are required to unravel the physiological relevance of these findings at the whole plant level, it is apparent that the two-compartment, two-airspace system of C_4 cereal leaves would enable leaf-side-specific CO_2-signalling and defence-signalling pathways. This type of compartmentation would also facilitate the independent regulation of stomatal move-ments on each leaf surface, in a manner that may not primarily be related to photosynthesis (von Caemmerer *et al.*, 2004; Baroli *et al.*, 2008). Moreover, differential dorsoventral stress signalling pathways would allow in-dependent acclimation processes to occur on each side of the leaf. While the significance of leaf-side-specific stress responses remains to be elucidated, the leaf-side-specific regulation of photosynthesis may be controlled by the developmental genetic programme that governs the adax-ial–abaxial patterning of leaves (Chitwood *et al.*, 2009).

Acknowledgements

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