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Effects of long-term chilling on growth and photosynthesis of the C_4 gramineae *Paspalum dilatatum*

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Dallis grass (*Paspalum dilatatum* Poir.) is a C_4/NADP-ME gramineae, previously classified as semi-tolerant to cold, although a complete study on this species acclimation process under a long-term chilling and controlled environmental conditions has never been conducted. In the present work, plants of the variety Raki maintained at 25/18°C (day/night) (control) were compared with plants under a long-term chilling at 10/8°C (day/night) (cold-acclimated) in order to investigate how growth and carbon assimilation mechanisms are engaged in *P. dilatatum* chilling tolerance. Although whole plant mean relative growth rate (mean RGR) and leaf growth were significantly decreased by cold exposure, chilling did not impair plant development nor favour the investment in biomass below ground. Cold-acclimated *P. dilatatum* cv. Raki had a lower leaf chlorophyll content, but a higher photosynthetic capacity at optimal temperatures, its range being shifted to lower values. Associated with this higher capacity to use the reducing power in CO_2 assimilation, cold-acclimated plants further showed a higher capacity to oxidize the primary stable quinone electron acceptor of PSII, Q_A. The activity and activation of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) were not significantly affected by the long-term chilling. Cold-acclimated *P. dilatatum* cv. Raki apparently showed a lower transfer of excitation energy from the light-harvesting complex of photosystem II to the respective reaction centre and enhancement of radiationless energy-dissipating mechanisms at suboptimal temperatures. Overall, long-term chilling resulted in several effects that comprise responses with an intermediate character of both chilling-tolerant and –sensitive plants, which seem to play a significant role in the survival and acclimation of *P. dilatatum* cv. Raki at low temperature.

Introduction

Low temperature is a strong limiting factor in plant growth and distribution. Some plants, named tolerant, are able to survive under harsh environments, whereas others are sensitive to frost and chilling, exhibiting different levels of injury under these conditions (Levitt, 1980). The group of chilling-sensitive plants include many C_4 species, most of them belonging to the NADP-ME type, e.g. maize (*Zea mays*, L) and sorghum (*Sorghum* sp.). Optimal physiological temperatures of these plants are around 30°C, their photosynthetic activity decreasing dramatically below 10°C, which limits their agricultural use in extensive terms (see Long 1999 for a review). Current evidence, however, does not indicate that any step in the C_4 pathway becomes an insurmountable barrier at low temperature (Sugiyama and Hirayama 1993).

The depression of photosynthesis for short or long periods of time and even its irreversible inhibition is a

Abbreviations – A_{O2}, CO_2-dependent oxygen evolution rates; Carot, carotenoids; Chl, chlorophyll; DW, dry weight; FW, fresh weight; F_v'/F_m', trapping efficiency of PSII open centres; F_o, basal fluorescence; F_m, maximal fluorescence; F_v, variable fluorescence; IC_{50}, inhibitor concentration that reduces the enzyme activity by 50%; LA, leaf area; LHC, light harvesting complex; NADP-ME, NADP-malic enzyme; q_N, total non-photochemical quenching; q_P, photochemical quenching; PEPC, phosphoenolpyruvate carboxylase; RGR, relative growth rate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SLA, specific leaf area; W, biomass yield; ϕ_{PSII}, relative quantum efficiency of photosynthetic electron transport of PSII; RC_{II}, PSII reaction centre.

prominent and common symptom of chilling injury in all sensitive plants (see the reviews by Berry and Björkman 1980, Long et al. 1994, Allen and Ort 2001). In C_4 plants, it has been associated with direct alterations on the kinetics of CO_2 assimilation via the C_4 pathway and the irreversible damage to the chloroplast structure and development (Slack et al. 1974, Simon 1987, Nie et al. 1995, Haldimann 1996, Kingston-Smith et al. 1997).

Many temperate plant species are able to cold-acclimate upon a gradual exposure to lower temperatures. Studies on the mechanisms involved in the cold-acclimation of C_3 species, have shown that long-term chilling induces the shift of the plant optimum physiological temperature to lower temperatures and the recovery/enhancement of their photosynthetic capacity at these temperatures (Holaday et al. 1992, Hurry et al. 1994). This was shown to be paralleled by faster oxidation of the primary stable quinone electron acceptor of PSII, Q_A, higher photoinhibition resistance, changes in the status of several Calvin–Benson cycle intermediates and the up-regulated expression and/or activation of key-enzymes (Lapointe and Huner 1993, Öquist et al. 1993, Hurry et al. 1995a, b, Strand et al. 1997, 1999). Similar studies with C_4 species are scarce, but a few reports suggested the involvement of energy dissipation mechanisms and sustained activities of key-enzymes in their survival under chilling (Caldwell et al. 1977, Simon 1987, Nie and Baker 1991, Haldimann et al. 1995, Kingston-Smith et al. 1997, Fryer et al. 1998, Ribas-Carbo et al. 2000).

Dallis grass (*Paspalum dilatatum* Poiret), a C_4/NADP-ME gramineae represented worldwide, is one of the most typical and valuable grasses in the prairies of South America wet subtropical regions (Pinto da Silva 1969, Usuda et al. 1984). Previously, *P. dilatatum* was shown to behave as a species that is semi-tolerant to cold, under a short-term chilling at 10°C (Taylor and Rowley 1971, Taylor et al. 1972), and to stand freezing temperatures down to −3°C (Hacker et al. 1974, Slack et al. 1974). Furthermore, it was shown that this species is capable of cold-hardening when grown under a day/night temperature of approximately 14/5°C, improving its resistance to freezing temperatures of approximately −5 to −8°C (Rowley et al. 1975, Rowley 1976). Previous work in our laboratory, has shown that the variety Raki grown under field conditions in Portugal stands both chilling and frosts in winter (Marques da Silva et al. 1989). It was also shown that leaf content and regulation of both PEPC and Rubisco were determined by the environmental conditions in each season. A clear correlation between these enzymes activity in vivo and the plant biomass yield was observed (A. Bernardes da Silva 1996, PhD Thesis, University of Lisbon, Portugal).

The aim of the present work was to investigate the effects on the growth and photosynthesis of *P. dilatatum cv.* Raki induced by a long-term chilling under environmental controlled conditions and therefore, to elucidate some of the primary physiological responses engaged in the apparent cold semi-tolerance of this C_4 species.

Materials and methods

Plant material

Seeds of *Paspalum dilatatum* Poiret cv. Raki, previously soaked over 90 min at room temperature, were sown in 2-l pots containing light expanded clay and a volume of distilled water up to the pot field capacity. After 1 week germination, four seedlings were kept per pot and watered, initially once and later twice a week, with Hewitt (1966) nutrient solution modified as follows: 6 m*M* KNO_3, 12 m*M* Ca $(NO_3)_2$, 1.5 m*M* $MgSO_4$, 1 m*M* NaH_2PO_4, 10 μ*M* $MnSO_4$, 1 μ*M* $CuSO_4$, 1 μ*M* $ZnSO_4$, 50 μ*M* H_3BO_3, 0.1 m*M* NaCl, 50 n*M* $(NH_4)_6Mo_7O_{24}$ and 110 μ*M*Fe-EDTA. Plants were grown for 5 weeks in a growth chamber at 25/18°C (day/night) and a photoperiod of 16/8 h (day/night). Low/moderate photosynthetic photon flux density (PPFD) 250–300 μmol m^{-2} s^{-1}, provided by Silvania L30/GRO lamps (Philips, Einhoven, the Netherlands) was used, in order to avoid eventual photoinhibitory effects resulting from the interaction between high light and low temperature (e.g. Gray et al. 1997). In all experiments but growth analysis, half of the plants with 6 weeks from sowing was maintained under these conditions for a further period of 10–15 days (control). The other half was transferred to a similar growth chamber with a day/night temperature of 10/8°C, where plants remained for a further period of 25–30 days (cold-acclimated). The time gap between the two plant groups took into account the delay in plant development (clearly expressed in the slower appearance and expansion of leaf) in cold-acclimated plants (see Results), and allowed us to compare plants at the same developmental stage. Unless stated otherwise, all experiments were carried out at least 2 h after the beginning of the light period, on the last (sixth) fully expanded leaf, which in the cold-acclimated plants was completely developed under low temperature.

Growth analysis

Plants were harvested from four pots with four plants each after 6 weeks from sowing and 20 days afterwards either at 25/18°C (control) or at 10/8°C (cold-acclimated). Shoot dead organic matter was removed prior to separation and weighing [fresh weight (FW) (g)] of leaves, stems and roots from each plant. Plant material, including the dead organic matter, was dried for 3 days in an oven at 80°C (Heraeus T 6120; Heraeus Instruments, GMBH, Hanau, Germany) and the weight of each plant part was determined [dry weight (DW) (g)]. Biomass yield (*W*) (g DW m^{-2}) and mean relative growth rate (mean RGR) (g kg^{-1} day^{-1}) were calculated according to Hunt (1978) and Long and Hallgreen (1985). The value of *W* was calculated taking into account total dry weight (g) and area (m^2) for each pot. The mean RGR was calculated taking into account each pot total dry weight (g kg^{-1}) and the growth period (20 days).

Leaf growth parameters

Leaf parameters, namely, leaf area (L_A) (cm^2), leaf fresh and dry weight (L_{Fw}, L_{Dw}; g), ratio of leaf dry weight to leaf fresh weight ($\%L_{Dw}$), leaf thickness (L_{Fw}/L_A) ($g\,FW\,m^{-2}$) and specific leaf area (SLA) ($m^2\,kg^{-1}\,DW$), were determined for the last fully expanded leaf (sixth) of both plant groups. Each leaf was weighed freshly and its area determined with an area meter (Li-3000A: Li-COR Inc., Lincoln, NE, USA). L_{Dw} (g) was determined as described in the previous section.

Measurement of CO_2-dependent oxygen evolution rates and chlorophyll *a* fluorescence

Segments cut from the middle of the last fully expanded leaf of both plant groups were used for determination of both CO_2-dependent oxygen evolution rate (A_{O2}) ($\mu mol\,O_2\,m^{-2}\,s^{-1}$ or $\mu mol\,O_2\,mg^{-1}\,Chl\,s^{-1}$) and in vivo Chl *a* fluorescence parameters. Both determinations were made at a temperature range of 5–45°C in a leaf disc O_2 electrode (Model LD2; Hansatech Instruments Ltd, King's Lynn, UK) in the presence of 5% CO_2 (1 *M* $NaHCO_3/Na_2CO_3$; pH 9). Measurements of A_{O2} were carried out under high PPFD ($800\,\mu mol\,m^{-2}\,s^{-1}$) supplied from a Hansatech light source (Model LS2H; Hansatech). In vivo Chl *a* fluorescence parameters were determined with a PAM chlorophyll fluorometer (Walz, Effeltrich, Germany). The top window of the leaf disc electrode was modified in order to accommodate the PAM optical fibre. Leaves were dark-adapted at the measuring temperature for 15 min prior to the onset of measurement. The pulse of white light (KL 1500 Scott lamp unit controlled by PAM 103 unit; Schott Glaswerke, Mainz, Germany) was adjusted to a PPFD of $6000\,\mu mol\,m^{-2}\,s^{-1}$. The duration of the flashes was 800 ms with 60 s between each flash. The PPFD of the actinic light beam obtained from the light source (KL 1500 Scott lamp unit) was $800\,\mu mol\,m^{-2}\,s^{-1}$. Basal fluorescence ($F_o$) was determined on the dark-adapted leaf sample and the maximal fluorescence (F_m) after the first saturating light pulse. The electron trapping efficiency of PSII open centres (F_v'/F_m') and the relative quantum efficiency of photosynthetic electron transport of PSII (ϕ_{PSII}), as well as photochemical (q_P) and total non-photochemical quenching (q_N) parameters were estimated under actinic light at steady-state fluorescence. The different fluorescence parameters were calculated according to Schreiber and Bilger 1987) and Genty et al. (1989), accounting for F_o quenching when necessary (Horton and Bowyer 1990).

Photosynthetic pigments content

Segments (0.05 g FW) used in A_{O2} assays were extracted for pigment analysis with methanol (5 ml), in the dark at 4°C, for at least 48 h. Pigments were assayed in a spectrophotometer (model Philips 8700 series UV/Vis, Unicam Ltd, Cambridge, UK). Concentration values of Chl *a*, chlorophyll *b* (Chl *b*) and carotenoids (Carot) were calculated according to Lichtenthaler (1987).

Protein content

Combined samples of frozen leaves were ground in liquid nitrogen in a mortar. Aliquots of 200 mg of ground material were extracted with 5 ml of Lowry C solution. To determine total protein content, insoluble proteins were made soluble by ultrasound treatment (30 s, 40 kHz, 80 V; Sonimass T250; Ultrasons-Anamasse SA, Hamo Branson Ultrasons, Annemasse, France) and incubation of the extract with 0.04% (w/v) deoxycholate for 5 h at 4°C. The homogenate was centrifuged at $15\,000\,g$ for 30 s at 4°C. To determine soluble protein content, aliquots of the extract were directly centrifuged at $15\,000\,g$ for 30 s at 4°C before the ultrasound treatment. In both cases, the resulting supernatant was assayed for soluble proteins spectrophotometrically at 750 nm, according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Content of soluble protein was also determined in leaf extracts used in enzyme assays by the Coomassie Blue method (Bradford 1976) at 600 nm in a microplate reader (Du Pont, MPR-A4; Tessenderlo, Belgium). BSA standard curves used in this procedure were made in each enzyme extraction media in order to exclude interfering background caused by any of their components.

Enzyme activities

PEPC and Rubisco were extracted from freshly harvested leaves (1 g) in a cold mortar containing 1% (w/v) Polyclar AT and 10 ml ice-cold extraction medium (50 m*M* HEPES-KOH, pH 7.3, 20 m*M* $MgCl_2$, 10 m*M* DTT, 6% (w/v) PVP 25, 1 m*M* PMSF and 0.1 m*M* NaF). The extract was centrifuged for 20 s at $16\,000\,g$ at room temperature and the supernatant directly assayed for both enzymes. Except for temperature response curves, both enzymes were assayed at 30°C.

Maximal activity (V_{max}) and the activity under suboptimal but physiological conditions ($V_{physiol}$) of PEPC were assayed spectrophotometrically in a continuous assay at 340 nm according to a modification of Bakrim et al. (1992) method. For V_{max} determination, the reaction mixture (1 ml) consisted of 100 m*M* HEPES-KOH, pH 8.0, 5 m*M* $MgCl_2$, 5 m*M* $NaHCO_3$, 0.2 m*M* NADH, 10 m*M* PEP and 10 U L-malate-dehydrogenase. $V_{physiol}$ was measured in a similar reaction mixture except that the buffer pH was 7.3 and PEP concentration was 2.5 m*M*. In both cases, reaction was initiated with 20 µl of extract.

Rubisco activity was assayed in the supernatant fraction by $^{14}CO_2$ incorporation into acid-stable products according to Keys and Parry (1990), immediately after extraction (initial activity; $V_{initial}$) or after 5 min activation with saturating concentrations of $^{14}CO_2$ and Mg^{2+} (total activity; V_{total}). The oxygen free reaction mixture (600 µl) contained 100 m*M* HEPES-KOH, pH 8.0, 30 m*M* $MgCl_2$, 2.5 m*M* DTT, 0.6 m*M* RuBP, 10 m*M* $NaH^{14}CO_3$ and 50 µl of extract. Assays were started either with the extract for $V_{initial}$ or with RuBP for V_{total}. Reactions were terminated after 30 or 60 s by addition of

100 µl of 2 N HCl. The mixture was dried at 60°C for 12 h and the residue re-suspended in 1 ml of distilled water. The remaining ^{14}C radioactivity of acid-stable products was measured by liquid scintillation counting (spectrometer Beckman LS 7800; Beckman Instruments, Inc., Irvine, CA, USA) (Patterson and Greene 1965).

Statistical analysis

Data were evaluated statistically, using a *t*-test ($P < 0.05$) or a two-way ANOVA ($P < 0.05$) and a post-hoc Tukey HSD test for equal/unequal N ($P < 0.05$) (STATISTICA for Windows, StatSoft, Inc. 1999, Tulsa, OK, USA). The number of replicates is indicated in every table or figure.

Results

Plant growth analysis

Exposure of *P. dilatatum* cv. Raki to a temperature of 10/8°C (day/night) for a period of 20 days, resulted in a 39% decrease of plant mean RGR (Fig. 1). The concomitant and pronounced decrease (54%) of *W* (Fig. 1) was the result of a general decrease in leaf, stem and root weight, fresh (75, 64 and 63%, respectively) or dry (of 61, 59 and 58%, respectively). No significant difference ($P < 0.05$) was found in the shoot/root (S/R) ratio between plant groups (6.99 ± 0.61 and 6.24 ± 0.48 for control and cold-acclimated plants, respectively). Furthermore, leaf was always the primary responsible for total plant weight, fresh or dry, followed by the root, and finally by the stem. Shoot longevity was high in both plant groups and it was not affected by cold as indicated by the very low percentage of dry weight of shoot dead organic matter (always less than 1%) relatively to the total plant dry weight.

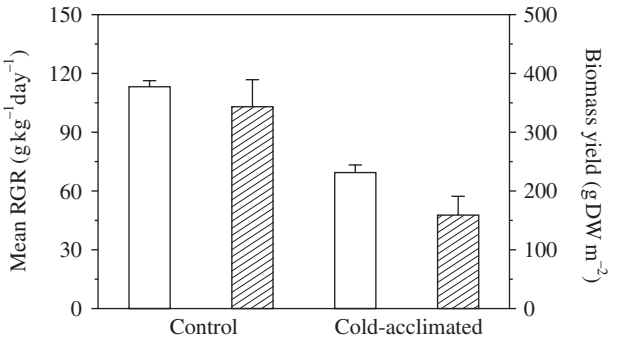

Fig. 1. Mean RGR (g kg^{-1} day^{-1}1) (□) and biomass yield (*W*) (g DW m^{-2}) (▨) of control and cold-acclimated *P. dilatatum* cv. Raki. Columns are means (+SE) of eight independent replicates obtained in two different experiments.

Leaf growth parameters

Leaf growth was significantly reduced by cold, which was expressed in the parallel decrease (approximately 21%) of both L_{FW} and L_A (Table 1). In cold-acclimated plants, there was a decrease of 26% in SLA, apparently related to a higher $\%L_{DW}$ (22%). L_{FW}/L_A and L_{DW} were similar in both plant groups ($P < 0.05$) (Table 1).

Leaf content of photosynthetic pigments and protein

Long-term chilling had a clear negative impact on the leaf Chl *a* + *b* content of *P. dilatatum* cv. Raki (Table 2). The decrease was higher in the content of Chl *a* content (40%) than in Chl *b* (29%), leading to a lower Chl *a*/*b* ratio (15%) in the cold-acclimated leaf. Otherwise, the decrease in the leaf Carot content of cold-acclimated plants was not statistically significant ($P < 0.05$), leading to a 32% decline in the Chl *a* + *b*/Carot ratio (Table 2).

Leaf content of total protein increased approximately 35% in cold-acclimated plants, due to the clearly higher insoluble fraction (Table 3).

Table 1. Effect of low temperature on leaf growth parameters of *P. dilatatum* cv. Raki. Data are means (± SE) of 15–16 independent replicates.

	Leaf growth parameters					
	L_{FW} (g)	L_{DW} (g)	$\%L_{DW}$	L_A (cm^2)	L_{FW}/L_A (g FW m^{-2})	SLA (m^2 kg^{-1} DW)
Control	0.4736 (± 0.0240)	0.0722 (± 0.0034)	15.8 (± 0.7)	26.29 (± 1.21)	179.5 (± 1.6)	36.7 (± 1.2)
Cold-acclimated	0.3746 (± 0.0202)	0.0759 (± 0.0051)	20.2 (± 0.6)	20.38 (± 1.13)	184.2 (± 2.1)	27.3 (± 9.0)

Table 2. Effect of low temperature on photosynthetic pigments leaf content of *P. dilatatum* cv. Raki. Data are means (± SE) of 20 independent replicates obtained in two different experiments.

	Photosynthetic pigments content (mg g^{-1} FW)					
	Chl *a*	Chl *b*	Chl *a* + *b*	Carot	Chl *a*/*b*	Chl *a* + *b*/Carot
Control	2.21 (± 0.12)	0.48 (± 0.03)	2.69 (± 0.14)	0.74 (± 0.04)	4.62 (± 0.12)	3.63 (± 0.04)
Cold-acclimated	1.33 (± 0.09)	0.34 (± 0.03)	1.67 (± 0.11)	0.66 (± 0.03)	3.94 (± 0.09)	2.48 (± 0.07)

Table 3. Effect of cold-acclimation on the protein leaf content of *P. dilatatum* cv. Raki. Data are means (± SE) of six independent replicates.

	Content of protein (mg g^{-1} FW)		
	Soluble protein	Insoluble protein	Total protein
Control	12.9 ± 1.4	9.5 ± 1	20.8 ± 0.6
Cold-acclimated	16.4 ± 1.0	15.9 ± 2	32.3 ± 2.1

CO_2-dependent oxygen evolution rates

Long-term chilling of *P. dilatatum* cv. Raki resulted in the significant increase ($P < 0.05$) of A_{O2} on a leaf area basis, only between 20 and 30°C (Fig. 2A). On a chlorophyll content basis, the increase of A_{O2} observed in the cold-acclimated plants covered the entire range of temperatures up to 30°C ($P < 0.05$), but at 10°C (Fig. 2B). The optimum temperature range of A_{O2} was narrower in the leaf of cold-acclimated plants than in the control, which was mainly due to a steeply inhibition at the higher temperatures in the former (Fig. 2). In the high temperature range, the temperature at which the A_{O2} dropped below half its maximal value (high A_{O2} 50) (Larcher 1994) decreased approximately 7°C in cold-acclimated plants (approximately 37.5°C in cold-acclimated plants and 42.5°C in the control; Fig. 2A). Otherwise, at the low temperature range the low A_{O2} 50 was about the same in both plant groups (approximately 10°C).

Fluorescence analysis

Long-term chilling of *P. dilatatum* cv. Raki leaded to a general increase in F_o and to higher F_m at all assayed temperatures, but 30°C (Fig. 3A). Otherwise, it was shown to result in the significant ($P < 0.05$) decrease of F_v/F_m ratio along the temperature gradient (Fig. 3B). This drop observed in cold-acclimated plants of *P. dilatatum* cv. Raki, was mainly the consequence of a higher F_o, for the ratio F_m/F_o was decreased in that group (Fig. 3A). q_P was similar in both plant groups between 5 and 15°C, being higher in the cold-acclimated plants above this temperature (Fig. 4A). Otherwise, q_N was significantly enhanced ($P < 0.05$) between 5 and 20°C in the cold-acclimated *P. dilatatum* cv. Raki (Fig. 4B). In cold-acclimated plants, both quenching coefficients curves are represented only up to 35°C (Fig. 4), because above this temperature no steady-state was achieved and variable fluorescence dropped below F_o. This indicates serious damage in the membranes associated with an impaired photosynthetic function beyond that temperature (Berry and Bjorkman 1980). F_v'/F_m' and ϕ_{PSII} of PSII were both depressed in the

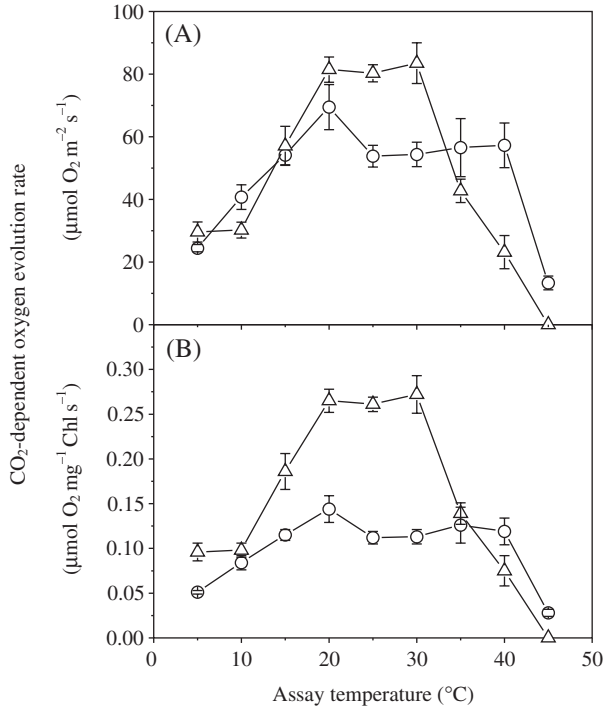

Fig. 2. Temperature response curves of CO_2-dependent oxygen evolution rates in the last fully expanded leaf of control (○) and cold-acclimated (Δ) *P. dilatatum* cv. Raki. Values are means (± SE) of 4–17 independent replicates. (A), A_{O2} (μmol O_2 m^{-2} s^{-1}) and (B), A_{O2} (μmol O_2 mg^{-1} Chl s^{-1}).

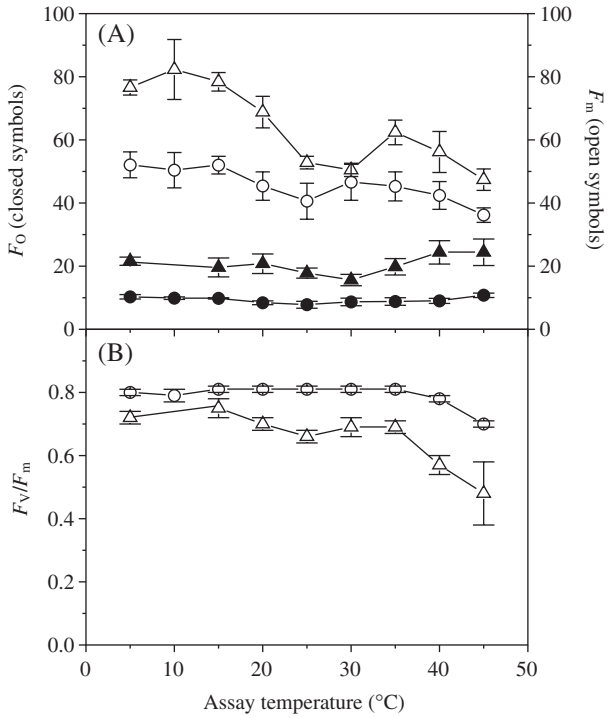

Fig. 3. Temperature response curves of basal fluorescence (F_o) (closed symbols) and maximal fluorescence (F_m) (open symbols) (A) and F_v/F_m (B) in the last fully expanded leaf of control (○/●) and cold-acclimated (Δ/▲) *P. dilatatum* cv. Raki. Values are means (± SE) of 3–8 independent replicates obtained in two different experiments.

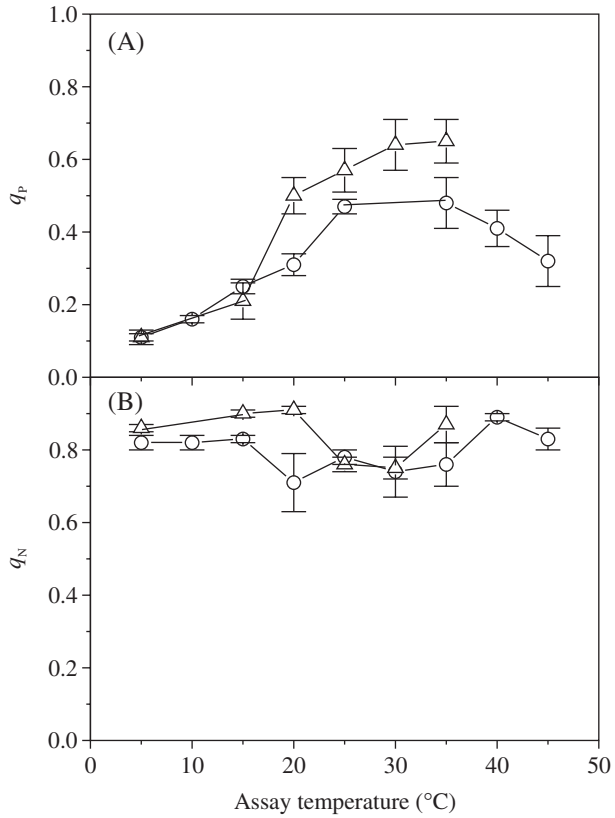

Fig. 4. Temperature response curves of q_P (A) and q_N (B) in the last fully expanded leaf of control (○) and cold-acclimated (Δ) *P. dilatatum* cv. Raki. Values are means (± SE) of 4–7 independent replicates obtained in two different experiments.

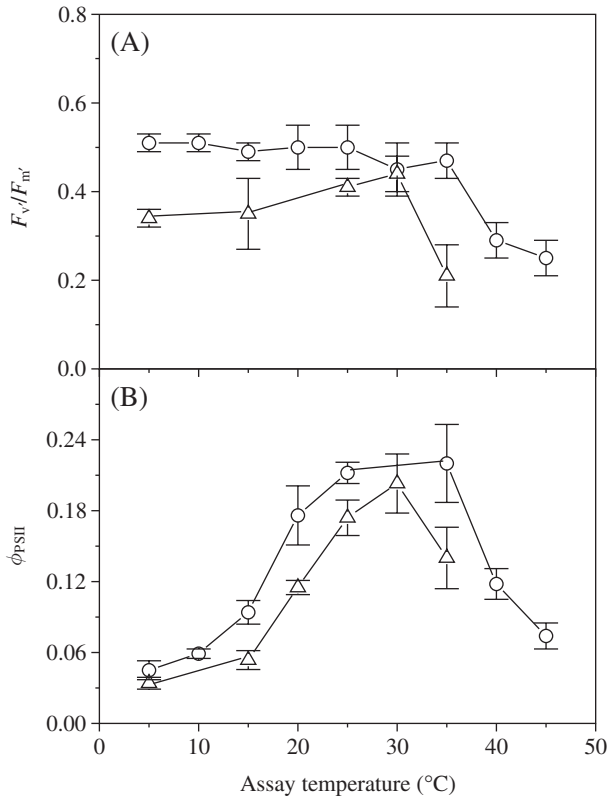

Fig. 5. Temperature response curves of F_v'/F_m' (A) and the ϕ_{PSII} (B) in the last fully expanded leaf of control (○) and cold-acclimated (Δ) *P. dilatatum* cv. Raki. Values are means (± SE) of 4–8 independent replicates obtained in two different experiments.

cold-acclimated *P. dilatatum* cv. Raki, except at 30°C (Fig. 5).

Enzyme activities

PEPC activity (V_{max} and $V_{physiol}$) in *P. dilatatum* cv. Raki, on a fresh weight basis, was not altered by the long-term-chilling although a significant increase of a 35% on a chlorophyll content basis (Fig. 6a and b). Otherwise, PEPC activity (V_{max} and $V_{physiol}$) in the cold-acclimated *P. dilatatum* cv. Raki decreased approximately 30% when expressed on a soluble protein content basis (Fig. 6C). Significant changes ($P < 0.05$) between the two plant groups were not observed either in the activation state of the enzyme (Fig. 6A) or in the IC_{50} for L-malate (Fig. 6D), suggesting that chilling had no significant effect on the enzyme phosphorylation status. With regard to Rubisco V_{total}, a significant decrease ($P < 0.05$) of 19% was observed in cold-acclimated plants on a fresh weight basis, whereas no significant change occurred in its $V_{initial}$ (Fig. 7A). However, on a chlorophyll content basis, V_{total} and $V_{initial}$ increased approximately 23 and 32%, respectively (Fig. 7B). When results were expressed on a soluble protein content basis both V_{total} and $V_{initial}$ decreased by 44 and 38%, respectively (Fig. 7C). Rubisco activation-state was not significantly altered ($P < 0.05$) by the long-term chilling (Fig. 7A).

In vitro activities of PEPC and Rubisco of *P. dilatatum* cv. Raki were highly restricted under low temperature, decreasing dramatically when they were assayed at 10°C (Fig. 8). Temperature dependency of PEPC was not significantly altered by the long-term chilling, with the activity at 10°C being approximately 15% of that observed at 30°C, in both plant groups (Fig. 8A). Otherwise, for Rubisco, this percentage was slightly decreased from 14 to 8% in the cold-acclimated *P. dilatatum* cv. Raki (Fig. 8B). Based on the respective Arrhenius plots, neither PEPC nor Rubisco exhibited an abrupt decrease in the activation energy at low temperatures that we could correlate positively with the process of cold-acclimation (data not shown).

Discussion

Long-term chilling had a clear negative impact on the mean RGR of *P. dilatatum* cv. Raki, resulting in a lower W and causing a delay in plant development (Fig. 1). This is a very common result widely reported in chilling-tolerant and chilling–sensitive species (Irigoyen et al. 1996, Wu et al. 1997). In contrast to cold-sensitive

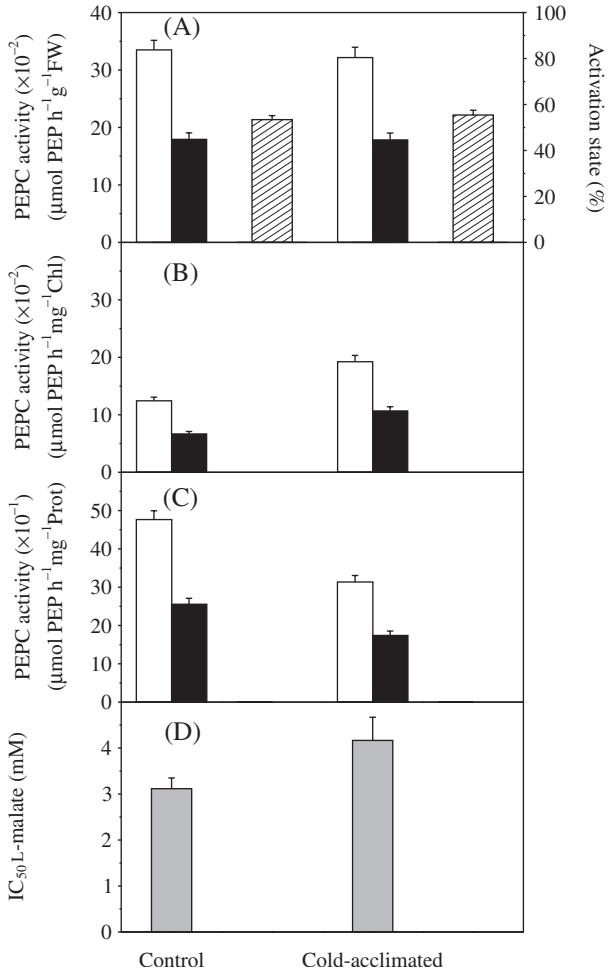

Fig. 6. PEPC activity on a fresh weight basis (A), on a chlorophyll content basis (B) and on a soluble protein content basis (C) and IC_{50} (L-malate) (D) in the last fully expanded leaf of control and cold-acclimated *P. dilatatum* cv. Raki. Columns are means (+ SE) of six independent replicates. (A)–(C) V_{max}, (□), $V_{physiol}$, (■), activation state (▨); (D), IC_{50} [L-malate] (▩).

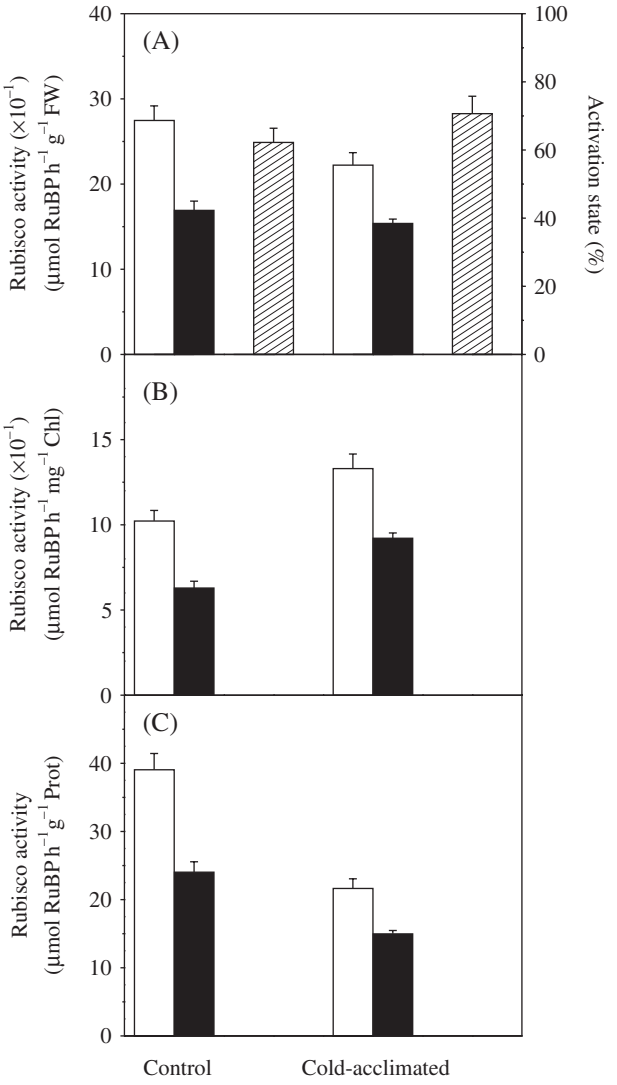

Fig. 7. Rubisco activity [V_{total} (□), $V_{initial}$ (■), activation state (▨)] on a fresh weight basis (A), on a chlorophyll content basis (B) and on a soluble protein content basis (C) in the last fully expanded leaf of control and cold-acclimated *P. dilatatum* cv. Raki. Columns are means (+ SE) of six independent replicates.

tropical or semi-tropical crops, such as maize (Irigoyen et al. 1996), *P. dilatatum* cv. Raki maintained a high shoot longevity. Furthermore, cold did not favour the investment of dry matter in the plant below ground as expected from data reported for other species (Bonicel et al. 1987). Leaf growth of *P. dilatatum cv. Raki* decreased both in terms of fresh weight and area (Table 1) under the 10/8°C regime, as previously reported for instance in white clover (Svenning et al. 1997). Long-term chilling resulted in a lower SLA, positively correlated with a higher $\%L_{DW}$ (Table 1), which may partially reflect the higher total protein content observed in the cold-acclimated plants (Table 3). The decrease of SLA represents a positive alteration usually associated with the ability to cold-acclimate reported for many chilling-tolerant species, but not in the chilling-sensitive species such as maize (Caldwell et al. 1977, Irigoyen et al. 1996, Gray et al. 1997). The association/integration of new proteins in membranes, suggested by the higher protein insoluble fraction in the cold-acclimated *P. dilatatum* cv. Raki (Table 3), could be involved in the cold-hardening process of this species (Zhou et al. 1994). However, preliminary studies on the leaf histology of *P. dilatatum* cv. Raki using light microscopy techniques suggested that cold-acclimated plants had smaller bundle sheath and vein cells with thicker cell walls (data not shown). Thus, the higher insoluble protein content (Table 3) and the higher $\%L_{DW}$ (Table 1) observed in the cold-acclimated plants can instead be ascribed to smaller cells, which would lead to more cell membranes and more cell walls. This has been frequently associated with the cold-hardiness of several species, such as Bromegrass and *Solanum acaule* (Chen et al. 1977, Tanino et al. 1991).

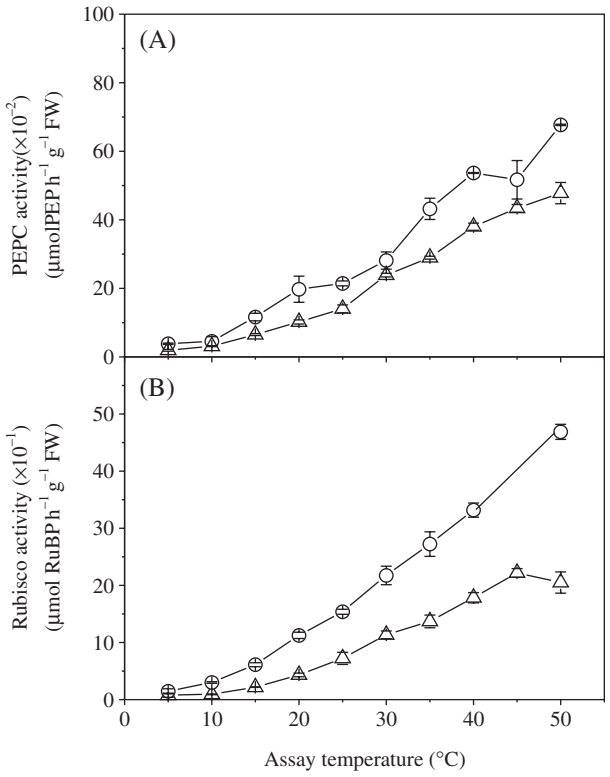

Fig. 8. Temperature curves of PEPC (V_{max}) (A) and Rubisco (V_{total}) (B) activity in the last fully expanded leaf of control (○) and cold-acclimated (Δ) *P. dilatatum* cv. Raki. Values are means (± SE) of 2–4 independent replicates.

The photosynthetic capacity of cold-acclimated *P. dilatatum* cv. Raki was shifted to lower temperatures but not enhanced at the acclimating temperature (Fig. 2), as expected in a cold-tolerant species (Pearcy 1977, Hurry et al. 1995a, b, Strand et al. 1999). Otherwise, long-term chilling did not result in the depression of *P. dilatatum* cv. Raki A_{O2} at all assay temperatures (Fig. 2), as typically observed in chilling-sensitive species under chilling (Holaday et al. 1992, Nie et al. 1995). Overall, this set of data confirms that *P. dilatatum* cv. Raki has a low but steady ability to cold-acclimate, this involving a photosynthetic response with a clear intermediate character between the typical response of cold-tolerant and that of a chilling-sensitive species.

Long-term chilling did not significantly affect the activity, activation or temperature dependency of PEPC and Rubisco, despite the slight depression in Rubisco V_{total} of cold-acclimated plants (Figs 6A, 7A and 8). Furthermore, maximal activity of both enzymes expressed per unit of soluble protein content further suggest that they were not included in the de novo synthesis of proteins induced under chilling (Figs 6C and 7C). On the whole, the response of carboxylating-enzymes sustains the intermediate chilling semi-tolerant character of *P. dilatatum* cv. Raki, being consistent with the partial reversal of PEPC and Rubisco inhibition in the cold-acclimated plants (Pearcy 1977, Simon 1987). Indeed, following the shift of tolerant/sensitive species to cold or the long-term chilling exposure of sensitive species, the activity/activation and protein amount of these enzymes were shown to decrease considerably (Slack et al. 1974, Krapp and Stitt 1995, Kingston-Smith et al. 1997). The opposite has been reported in the cold-acclimated chilling-tolerant species (Holaday et al. 1992, Hurry et al. 1994, 1995a, b, Strand et al. 1999). The A_{O2} temperature response curve further suggests at least a similar capacity of RuBP and/or PEP regeneration in the cold-acclimated leaf, most probably due to an increased capacity of the enzymes involved (Hatch 1979, Simon 1987, Holaday et al. 1992, Hurry et al. 1994, Du et al. 1999). This would sustain photosynthesis in the cold-acclimated plants at similar rates to those of control under suboptimal temperatures, which could be readily over-expressed when leaves were assayed at optimal temperatures.

The value of q_P followed a similar pattern to photosynthetic capacity, being maintained beyond the temperature at which A_{O2} was found to decrease (Figs 2 and 4A). This indicates an increased capacity to re-oxidize Q_A in the cold-acclimated leaf of *P. dilatatum* cv. Raki, partially associated to a higher consumption rate of NADPH and ATP in the photosynthetic assimilation of CO_2 (Lapointe and Huner 1993, Öquist et al. 1993). In contrast to chilling-tolerant species, such as winter wheat (Hurry et al. 1993), the enhancement of q_P in the cold-acclimated leaf of *P. dilatatum* cv. Raki, was associated with a slightly higher photoinhibition susceptibility at a wide temperatures range (Fig. 3B). Otherwise, this increased susceptibility has been reported for many chilling-sensitive species under chilling, for example, maize (Ribas-Carbo et al. 2000). In the cold-acclimated *P. dilatatum* cv. Raki, it was mainly caused by the relative increase of F_o (Fig. 3A), which was clearly unrelated to the respective chlorophyll content (Table 2). Indeed, long-term chilling negatively affected the content of Chl $a + b$ in *P. dilatatum* cv. Raki (Table 2), resembling many chilling-sensitive species under low temperature conditions (Nie et al. 1995, Venema et al. 2000). Furthermore, long-term chilling resulted in lower F_v'/F_m' and ϕ_{PSII} at all assayed temperatures, except at 30°C (Fig. 5). This data set is consistent with a decrease in the excitation energy transfer from the light-harvesting complex (LHC) of PSII to the respective reaction centre (RC_{II}), preventing the over-excitation of the RC_{II} as previously reported in a chilling-tolerant cultivar of maize (Ribas-Carbo et al. 2000 and references therein). The reasons leading to this mechanism are not clear. Anyhow, it was shown that cold-acclimated plants had a lower Chl a/b ratio caused by the relative decrease in the Chl a fraction (Table 2), which can be a consequence of photo-oxidative degradation induced by chilling (see the review by Niyogi 1999). Otherwise, long-term chilling had only a slight negative effect in the contents of Chl b and Carot, the Chl $a + b$/Carot ratio being higher in the cold-acclimated plants

(Table 2). Apparently, the LHC of *P. dilatatum* cv. Raki, and thus the dissipation and the antenna systems were relatively less affected than the absorption core pigments under chilling (Nie and Baker 1991, Haldimann 1996). In fact, the lower maximum quantum efficiency of PSII observed in the cold- acclimated *P. dilatatum* cv. Raki was further associated with the enhancement of the radiationless dissipation of excess energy (higher q_N) at the suboptimal temperature range, when photosynthesis decreased (Figs 2,3B and 4B). Whether this involved the zeaxanthin epoxidation reactions or the activation of repair mechanisms (Krause 1988, Bilger and Björkman 1991, Haldimann et al. 1995, Verhoeven et al. 1996, Venema et al. 2000) is not known.

In conclusion, the present study has identified the main effects on growth and photosynthesis of *P. dilatatum cv*. Raki induced by a long-term chilling, some representing positive responses in this species cold-acclimation. These responses seem to involve mechanisms that go from excess energy dissipation up to improved photosynthetic biochemical performance under chilling. Data obtained further revealed that the chilling-tolerant/sensitive intermediate character of those responses underline this species semi-tolerance to cold. The moderate/intermediate acclimation response of *P. dilatatum* cv. Raki would be an advantage in temperate areas where frosts alternate with warm periods for it would allow the survival of the species during the chilling days and its fast growth upon re-warming.

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