Contrasting responses of photosynthesis at low temperatures in different annual legume species

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Abstract

Growth, net photosynthetic rate (P_N), chlorophyll fluorescence induction kinetics, and stromal fructose-1,6-bisphosphatase (sFBPase) in annual legumes native to the Mediterranean region, two clovers (*Trifolium subterraneum* L. ssp. *oxaloides* Nyman cv. Clare and *T. michelianum* Savi cv. Giorgia) and two *Medicago* species (*M. polymorpha* L. cv. Anglona and *M. truncatula* Gaertn. cv. Paraggio), shifted from 20 to 10 ºC for 1 d or developed at 10 ºC were compared with controls kept at 20 ºC. Cold development produced a larger stimulation of growth in the clover cv. Giorgia and the *Medicago* cv. Paraggio. Transferring plants to low temperatures affected P_N relatively less in clovers than in *Medicago* plants. Development at 10 ºC relieved the inhibition of photosynthesis in Giorgia and Paraggio, but not in Clare and Anglona, which correlated with increases in the maximum rate of carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO (V_{cmax}), and the photon-saturated rate of electron transport (J_{max}). In *Medicago*, transfer from high to low temperature inhibited photosynthesis in a lesser extent in Anglona than in Paraggio, which showed severe limitations at level of V_{cmax} and J_{max}. Development at 10 ºC in Paraggio produced an efficient photosynthetic cold acclimation, this being associated with a two-fold increase of quantum yield of photosystem 2 electron transport ($\Delta F/F'_m$) and with the activity of sFBPase. By contrast, Anglona showed an irreversible inhibition of P_N coupled with the reduction of carbon metabolism by impairment of Calvin cycle enzyme activities such as RuBPCO and sFBPase, resulting in a poor cold acclimation of photosynthesis in this cultivar.

Additional key words: chlorophyll fluorescence; cold acclimation; dry matter accumulation; leaf area ratio; net photosynthetic rate; photosystem 2; specific leaf area; ribulose-1,5-bisphosphate carboxylase/oxygenase; stromal fructose-1,6-bisphosphatase.

Introduction

Under Mediterranean-type climates, annual legumes tend to grow and reproduce during the cool and wet season because growth is seriously limited by dry summer months. As a consequence, plants adapted to Mediterranean conditions also need to grow at low temperatures to extend the growing season (Sultan *et al*. 2001). The maintenance of a high capacity for active photosynthesis during prolonged exposure to low growth temperatures may be essential for determining the successful site occupation and subsequent productivity of adapted species. The harmful effects of winter stress on the physiology of species adapted to Mediterranean climate conditions have been well documented in evergreen woody species (García-Plazaola *et al.* 1999, Oliveira and Peñuelas 2001). However, few studies have been made in herbaceous species characteristic of this zone.

Long-term acclimation to cold in herbaceous plants is strongly related to an increase of net photosynthetic rate, P_N (Huner *et al.* 1993, Hurry *et al.* 1995a,b) and carbon export at low temperatures (Leonardos *et al.* 2003). This is connected with the requirement to sustain the pool of soluble saccharides, which may act as osmotica or protect specific macromolecules during cold-induced dehydration (Guy *et al.* 1992, Larcher 2003). Thus, cold acclimation results in a reprogramming of carbon metabolism leading to a shift in partitioning of fixed carbon into sucrose rather than starch (Strand *et al.* 1999, 2003, Stitt and Hurry 2002).

In general, an increase in photosynthetic capacity following growth at low temperatures is associated with increases in orthophosphate (P_i) availability (Hurry *et al.* 1993), with activities and activation states of Calvin cycle enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and stromal fructose-1,6-bis-

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phosphatase (sFBPase) (Pérez *et al.* 2001), and with enzymes responsible for sucrose synthesis (Guy *et al.* 1992, Holaday *et al.* 1992, Hurry *et al.* 1995a,b, Savitch *et al.* 2000a). These increases probably contain three components: a specific increase in enzymatic activities *per se*, an increase in the amount of the specific enzymes, and a non-specific increase that is linked to a general increase in leaf proteins (Strand *et al.* 1999).

Therefore, the objectives of this work were: (*1*) to study the response of photosynthesis to cold by short-term exposure of four annual legumes often grown in Mediterranean region to suboptimal temperatures (10 ºC); (*2*) to understand the effects of plant development at moderately low temperatures (10 ºC) on growth, and the ability to maintain or increase its photosynthesis; and (*3*) to know the photosynthetic capacity achieved during plant development at 10 ºC (cold acclimation) measured at optimal temperatures (20 ºC). Moreover, spectrophotometric assays were used to determine the total activity of a key enzyme of Calvin's cycle such as sFBPase. We have examined photosynthesis by measuring irradiance- and CO_2-response curves of P_N as well as the chlorophyll (Chl) *a* fluorescence quenching. Care was taken to select plants of similar developmental status for the comparative study.

Materials and methods

Plants and growth: Seeds from two clover species (*Trifolium subterraneum* L. ssp. *oxaloides* Nyman cv. Clare and *T. michelianum* Savi cv. Giorgia) and two *Medicago* species (*M. polymorpha* L. cv. Anglona and *M. truncatula* Gaertn. cv. Paraggio) were surface disinfected and germinated on wet filter paper in Petri dishes. Seedlings were planted in 14×10 cm pots (750 cm^3 of volume) containing a mixture of peat : sand (1 : 1, v/v) (5 plants per pot). Moisture and nutrient levels were routinely maintained with Hoagland's nutrient solution. Plants were grown in a greenhouse with 20/15 ºC and 80/90 % relative humidity (day/night) regime and were irradiated during 11 h with natural daylight supplemented with fluorescent lamps (*Sylvania F-48T12 CW-WHO*, München, Germany), providing a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1} at canopy level.

When five leaves were fully expanded (*ca*. 30 d after sowing), half of the plants were cold acclimated by shifting them to 10/5 °C day/night temperature regime leaving the rest of conditions as described previously (cold-acclimated plants, CA). Another set of plants remained at 20/15 ºC, in the same temperature conditions as the initial growth (non-acclimated plants, NA). Both groups of plants grew in their respective conditions until seven leaves were fully expanded (approximately during 14–18 d in NA and 24–28 d in CA). Three replicates from each cultivar×temperature combination were used. Growth kinetics for each species grown at 20 or 10 ºC was determined to compare leaf tissues of the same developmental stage rather than chronological age (Boese and Huner 1990). Leaf appearance rate was recorded weekly and expressed as the number of leaves produced per day. Leaf appearance rates in NA plants were: Clare 0.44, Giorgia 0.60, Anglona 0.39, and Paraggio 0.43 leaves d^{-1}. In CA plants leaf appearance rates were: Clare 0.29, Giorgia 0.41, Anglona 0.29, and Paraggio 0.32 leaves d^{-1}.

Gas exchange: Leaf P_N was measured using a *Walz* model *HCM-1000* minicuvette system (Effeltrich, Germany). Measurements were made 3 h after the onset of the photoperiod following procedures described in Antolín and Sánchez-Díaz (1993). Curves of response to incident photosynthetic photon flux density (PPFD) were measured at ambient O_2 (21 %) and CO_2 (330 μmol mol^{-1}), starting at 0 and increasing to 1 500 μmol m^{-2} s^{-1} of PPFD (10 points with 4 min of adaptation). CO_2-response curves were measured at ambient O_2 (21 %) and saturating PPFD (1 000 μmol m^{-2} s^{-1}), starting at 0 and increasing to 1 200 μmol mol^{-1} of external CO_2 concentration (10 points with 3 min of adaptation). All measurements were made in young fully expanded leaves that were completely developed during temperature treatment (20 ºC for NA or 10 ºC for CA plants). All measurements were repeated at 20 and 10 ºC in both NA and CA plants. Cold acclimated plants were kept at low temperature during 24–28 d before the beginning of measurements.

Mechanistic analyses of CO_2-response curves provide estimations of the maximum rate of carboxylation by RuBPCO (V_{cmax}) and the PPFD-saturated rate of electron transport (J_{max}). These parameters were estimated with the formulas utilized in *'Photosynthesis Assistant'* version *1.1* software (*Dundee Scientific*, Dundee, UK).

Chl *a* fluorescence induction kinetics was monitored simultaneously with leaf P_N on the leaves inserted in the leaf chamber under the same conditions as gas exchange at each temperature and at different PPFDs. Chl *a* fluorescence was measured using a portable fluorimeter (*Mini Pam*, *Walz*, Effeltrich, Germany) after leaves were dark-adapted for 30 min. The experimental protocol originally described by Genty *et al.* (1989) and recently revised by Maxwell and Johnson (2000) was basically followed. The minimal fluorescence (F_0) with all reaction centres of photosystem 2 (PS2) open was measured with modulated irradiation which was sufficiently low (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence (F_m) with all PS2 reaction centres closed was determined by a 0.8 s saturating pulse at 8 000 μmol m^{-2} s^{-1} in dark-adapted leaves. Then, leaves were continuously irradiated with "white actinic light". The steady state value of fluorescence (F_s) was thereafter ecorded and a second saturating pulse at

8 000 μmol m^{-2} s^{-1} was imposed to determine maximal fluorescence in the light-adapted state (F'_m). From these measurements, F_v was computed as $F_m - F_0$ and F_v/F_m was used to estimate the maximum photochemical efficiency of PS2. The quantum yield of PS2 electron transport ($\Delta F/F'_m$, where ΔF is equal to $F'_m - F_s$) and the non-photochemical quenching [$NPQ = (F_m/F'_m) - 1$] were also calculated. The nomenclature used was that of van Kooten and Snel (1990).

Stromal fructose-1,6-bisphosphatase (sFBPase) (EC 3.1.3.11) activity: Sub-samples of the stored leaves were ground in a mortar with liquid nitrogen and extracted in buffer for assays of sFBPase as described by Pérez *et al.* (2001). Extractions were made in a homogeniser containing 2 cm^3 ice-cold extraction medium that contained: 100 mM *Tricine*-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 % bovine serum albumin (m/v), 0.1 % *Triton X-100* (v/v), and 2 % polyvinyl pyrrolidone (m/v). Extracts were centrifuged at 200 rps. Enzyme activities were determined immediately after extraction according to Holaday *et al.* (1992) with the modifications described by Pérez *et al.* (2001). The assay medium for activity of sFBPase contained 10 mM DTT, 100 mM *Tricine*-KOH (pH 8), 10 mM $MgCl_2$, 1 mM EDTA, 0.25 mM NADP, 0.5 mM fructose 1,6-bisphosphate, 10 units of glucose phosphate isomerase (E.C. 5.3.1.9), 2.5 units of glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49), and 80 mm^3 of the extract. Enzyme was incubated during 10 min at 25 ºC and determined spectrophotometrically by measuring NADPH production at 340 nm. The soluble protein content of the whole-leaf extracts was determined by the method of Bradford (1976).

Other determinations: Leaf pigments were extracted in 80 % (v/v) methanol and then were determined spectrophotometrically using the equations of Lichtenthaler (1987). Leaf area was measured with a *Li-Cor* portable leaf area meter (model *LI-3000*, *LiCor*, USA). Dry matter was determined by drying plant samples in an oven at 80 ºC until a constant mass was obtained. Specific leaf area (SLA) was calculated as the ratio of leaf area to total leaf dry matter, and leaf area ratio (LAR) was calculated as the ratio of leaf area to total plant dry matter.

Statistical analysis: In some cases, data were submitted to a two-factor analysis of variance (ANOVA). The variance was related to the main treatments (species and growth temperature) and to the interaction between them. In other cases, data were submitted to a three-factor ANOVA to partition the variance into the main effects and the interaction between species, growth temperature, and temperature of measurement. Means ± standard errors were calculated, and when the F ratio was significant, least significant differences were evaluated by the Tukey's *t*-test as available in the *SPSS* statistical package version *9.0* programs for *Windows 98*. Correlation analyses were also performed to evaluate the degree of association and its significance.

Results

Plant morphology: Growth at low temperatures (cold acclimation) produced an increase of dry matter (DM) in Giorgia and Paraggio and there were not changes in Clare and Anglona (Table 1). This increase was especially pronounced in Giorgia, which had an increase of 4-fold in stem, 3-fold in root, and two-fold in leaf DM. Cold acclimation in Paraggio produced a two-fold increase of stem and root DM, without changes in leaf DM. There were no changes in the root to shoot ratio of any cultivars, although Giorgia showed the highest values.

CA plants of Giorgia also had a two-fold increase of leaf area, whereas it decreased in Clare and no changes were found in the *Medicago* species (Table 1). The specific leaf area (SLA) decreased in CA plants of clovers, but did not change in *Medicago*. The leaf area ratio (LAR) decreased in Clare, Giorgia, and Paraggio, but it did not change in Anglona. Although there was a general positive correlation between SLA and LAR upon cold acclimation ($r = 0.747$, $p<0.01$), Paraggio had similar SLA but decreased LAR (Table 1), which might be consequence of greater partitioning of DM to stems and roots (Table 1). There were significant interactions between species and growth temperature in most of growth parameters because each species responded in different way to growth at low temperatures (Table 1).

Chl content calculated on leaf area basis showed that CA leaves had an increase of two-fold in Clare and Paraggio, but it did not change in Giorgia and strongly decreased in Anglona (Table 2). On leaf dry matter basis, Chls (*a*+*b*), carotenoids (Cars) (x+c), and pigment ratios were similar in NA and CA leaves of Clare and Giorgia (Table 2). However, leaves of CA Anglona had lower contents of Chls and Cars and increased Chl *a/b* ratio. By contrast, cold acclimation in Paraggio resulted in an increase of pigment contents with decreased Chl *a/b* ratio. The proportional changes observed in Chls and Cars in *Medicago* species are reflected by no changes in the (*a*+*b*)/(x+c) ratio.

Short-term responses to low temperatures: To study short-term responses of photosynthesis to low temperatures, measurements in NA plants at 20 ºC and shifted to 10 ºC were compared (Fig. 1). Short-term exposure to low temperatures did not produce any change in the PPFD-response curve of P_N of Clare. This unchangeable photosynthetic capacity was associated with similar $\Delta F/F'_m$ at all PPFD (Fig. 1), but also with a decreased maximum efficiency of PS2 (F_v/F_m) (Table 3). NA leaves

had higher capacity to dissipate excess of energy (as indicated by NPQ) at 20 ºC than at 10 ºC. Analyses of CO_2 response curves provided estimations of the maximum rate of carboxylation by RuBPCO (V_{cmax}) and the photon-saturated rate of electron transport (J_{max}) (Table 3). There were not differences in V_{cmax}, but J_{max} decreased in Clare plants shifted to low temperature (Table 3). On the other hand, Giorgia had higher P_N than Clare but PPFD-saturated rates decreased after low temperature exposure, reaching 70 % of rates measured at 20 ºC (Fig. 1). This was associated with similar $\Delta F/F'_m$, NPQ, and F_v/F_m (Table 3). Calculations made from CO_2-response curve indicated that a sudden shift of Giorgia from 20 to 10 ºC did not affect either V_{cmax} or J_{max} (Table 3).

Table 1. Dry matter (DM), root to shoot ratio, total leaf area, specific leaf area (SLA), and leaf area ratio (LAR) in plants grown at 20/15 ºC (day/night) (non-acclimated, NA) or 10/5 ºC (cold-acclimated, CA) of four annual legume species (*Trifolium subterraneum* ssp. *oxaloides* cv. Clare, *T. michelianum* cv. Giorgia, *Medicago polymorpha* cv. Anglona and *M. truncatula* cv. Paraggio). Means of 15 plants. Within each column, means followed by the same letter are not significantly different (p>0.05) according to Tukey's test. *, **, ***, and ns indicate significance at 0.05, 0.01, and 0.001 probability levels, or not significant, respectively.

Species	Treatment	DM [g per plant] Leaf	Stem	Root	Root/shoot [kg kg^{-1}]	Leaf area [cm^2 per plant]	SLA [m^2 kg^{-1}]	LAR [m^2 kg^{-1}]
Clare	NA	0.32 a	0.16 a	0.16 a	0.33 b	107.19 a	33.82 b	16.74 a
	CA	0.33 a	0.13 a	0.18 a	0.41 ab	55.81 b	17.02 d	8.60 bc
Giorgia	NA	0.07 c	0.03 b	0.06 b	0.54 ab	29.34 cd	44.27 a	19.68 a
	CA	0.20 b	0.16 a	0.24 a	0.67 a	54.43 b	26.43 bc	9.13 bc
Anglona	NA	0.09 c	0.07 b	0.05 b	0.27 b	23.94 cd	26.63 bc	11.56 abc
	CA	0.08 c	0.04 b	0.06 b	0.45 ab	20.61 d	28.63 b	11.47 abc
Paraggio	NA	0.13 bc	0.07 b	0.07 b	0.34 b	36.55 c	22.44 c	13.41 ab
	CA	0.19 b	0.14 a	0.16 a	0.51 ab	39.65 c	23.32 c	7.95 c
Species		***	***	***	**	***	***	ns
Growth temperature		***	***	***	**	*	***	***
Interaction		**	***	***	ns	***	**	*

Table 2. Contents of total chlorophylls (*a*+*b*) and carotenoids (x+c), and pigment ratios in leaves of plants grown at 20/15 ºC (day/night) (non-acclimated, NA) or 10/5 ºC (cold-acclimated, CA) in different annual legume species. Means of 6 samples obtained from different plants. Otherwise as in Table 1.

Species	Treatment	(*a*+*b*) [mg m^{-2}]	(*a*+*b*) [g kg^{-1}(DM)]	(x+c) [g kg^{-1}(DM)]	*a*/*b*	(*a*+*b*)/(x+c)
Clare	NA	251.1 b	8.49 cd	1.83 c	3.52 ab	4.63 b
	CA	518.6 a	8.79 cd	1.60 cd	3.37 ab	5.51 ab
Giorgia	NA	239.4 b	10.40 bc	1.77 c	3.76 a	5.92 a
	CA	360.2 b	9.47 bc	1.61 cd	3.46 ab	5.88 a
Anglona	NA	559.4 a	14.50 a	2.73 a	2.77 c	5.29 b
	CA	366.6 b	10.48 bc	2.11 bc	3.44 ab	5.03 b
Paraggio	NA	276.2 b	6.23 d	1.47 d	3.70 a	4.19 b
	CA	497.8 a	11.58 b	2.51 ab	2.94 b	4.63 b
Species		***	**	**	ns	***
Growth temperature		***	*	*	*	ns
Interaction		***	***	***	**	ns

Anglona was the cultivar with the highest P_N regardless of growth temperature but it strongly decreased at 10 ºC, reaching 66 % of P_N measured at 20 ºC (Fig. 1). This decrease in photosynthetic capacity was coupled with no changes in $\Delta F/F'_m$ or NPQ, but the maximum efficiency of PS2 (F_v/F_m) was clearly reduced in this cultivar (Table 3). Estimations from the CO_2-response curves showed that Anglona had the highest PPFD-saturated rate of electron transport (J_{max}) under warm temperatures but, when they were shifted to 10 ºC, there was a strong decrease in V_{cmax} and J_{max}, which reached 46 and 42 % of the values obtained at 20 ºC, respectively (Table 3). In the cv. Paraggio, PPFD-saturated P_N also decreased at 10 ºC, reaching 54 % of rates measured at 20 ºC (Fig. 1). This was associated with no differences in $\Delta F/F'_m$ or F_v/F_m (Table 3), but it showed a very strong increase in NPQ from a PPFD of 400 µmol m^{-2} s^{-1} to the end of the curve. On the other hand, the CO_2-response curves showed a strong decrease both in V_{cmax} and J_{max} (9 and 24 % with respect to activity at 20 ºC) reaching the lowest values of all cultivars assayed (Table 3). Thus, Paraggio appears to be the cultivar most affected by moderately low temperatures.

Table 3. Maximal photochemical efficiency (F_v/F_m), maximum rate of carboxylation by RuBPCO (V_{cmax}), and electron transport capacity (J_{max}) estimated from CO_2-response curves of photosynthesis in different annual legume species. Measurements were made on the youngest fully expanded leaves of non-acclimated (NA) and cold-acclimated (CA) plants. Means of 6 determinations. Otherwise as in Table 1.

Species	Treatment	Temperature [°C]	F_v/F_m	V_{cmax} [µmol m^{-2} s^{-1}]	J_{max} [µmol m^{-2} s^{-1}]
Clare	NA	20	0.84 ab	55.0 d	130.2 b
		10	0.77 c	24.8 de	83.5 c
	CA	20	0.83 abc	164.9 a	144.3 b
		10	0.82 abc	35.0 de	119.3 bc
Giorgia	NA	20	0.83 abc	43.0 d	128.9 bc
		10	0.82 abc	32.4 de	83.5 c
	CA	20	0.80 abc	106.4 ab	343.5 a
		10	0.81 abc	95.9 bc	328.8 a
Anglona	NA	20	0.85 a	95.8 bc	296.5 a
		10	0.78 bc	44.5 d	123.8 b
	CA	20	0.84 ab	80.1 c	171.0 b
		10	0.80 abc	29.6 de	110.8 bc
Paraggio	NA	20	0.81 abc	137.8 ab	172.7 b
		10	0.80 abc	12.9 e	41.7 d
	CA	20	0.83 abc	129.1 ab	116.4 bc
		10	0.80 abc	23.2 de	64.3 cd
Species (A)			ns	ns	***
Treatment (B)			ns	**	***
Temperature (C)			***	***	***
A×B			ns	**	***
A×C			*	**	*
B×C			*	ns	*
A×B×C			ns	ns	ns

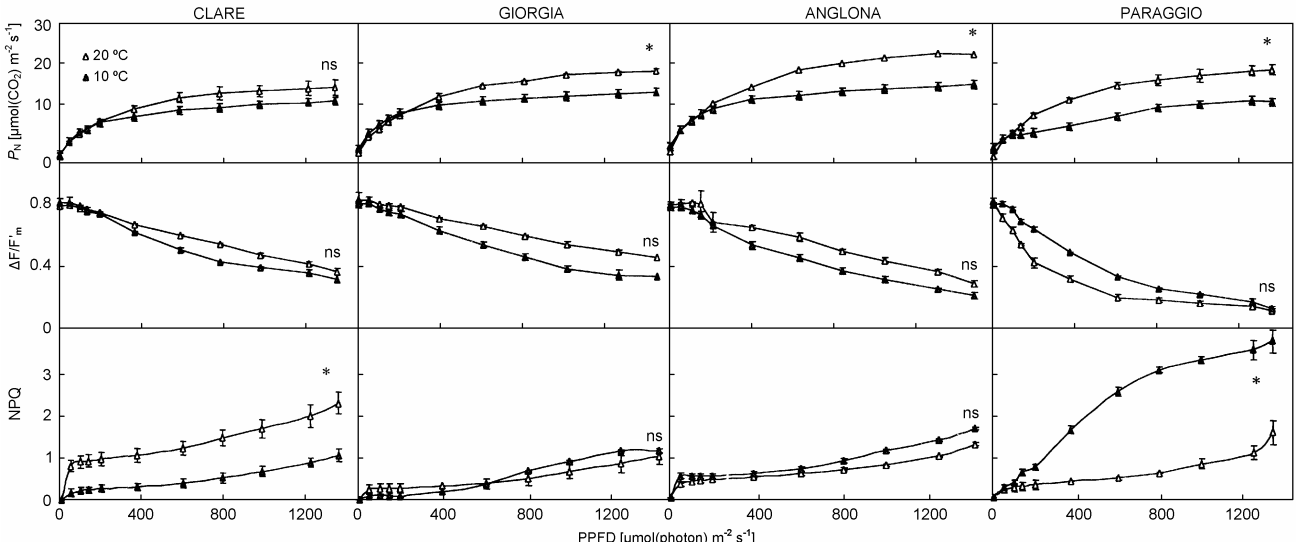

Fig. 1. PPFD response curves of the net photosynthetic rate (P_N), photochemical efficiency of photosystem 2 ($\Delta F/F'_m$), and non-photochemical quenching (NPQ) in plants grown at 20/15 ºC (day/night) (non-acclimated, NA) of four annual legume species (*Trifolium subterraneum* ssp. *oxaloides* cv. Clare, *T. michelianum* cv. Giorgia, *Medicago polymorpha* cv. Anglona, and *M. truncatula* cv. Paraggio). Measurements were made at ambient CO_2 (330 µmol mol^{-1}) and 20 ºC (*open symbols*) or 10 ºC (*filled symbols*). Means ± SE of 6 measurements made in different plants. *Asterisks* indicate significant differences ($p \leq 0.05$) according to Tukey's test.

Long-term responses to low temperatures (cold acclimation): To study the ability of acclimation at low temperatures, measurements made in NA and CA plants at the same low temperature (10 ºC) were compared

(Fig. 2). In Clare, long-term growth at low temperature (10 ºC) did not influence the PPFD-response curve of P_N, $\Delta F/F'_m$, NPQ (Fig. 2), or F_v/F_m (Table 3). Moreover, CA leaves exhibited similar V_{cmax} and J_{max} as NA leaves (Table 3). CA leaves of Giorgia showed the highest P_N when compared to the other cultivars. Thus, PPFD-saturated P_N under ambient CO_2 increased by 63 % from 12.4 to 20.3 µmol m^{-2} s^{-1}, when NA and CA leaves were compared at 10 ºC. This coincided with a slight increase of NPQ (Fig. 2). The highest P_N observed in Giorgia during cold acclimation was reflected by increased values of V_{cmax} and J_{max}, which increased 3–4-fold (Table 3).

Distinctly, growth at low temperature in the *Medicago* cv. Anglona did not influence PPFD-saturated rates and parameters estimated from CO_2-response curves (Fig. 2, Table 3) although V_{cmax} remained significantly low in both NA and CA at 10 ºC when compared to the respective values obtained at 20 ºC. However, CA leaves in

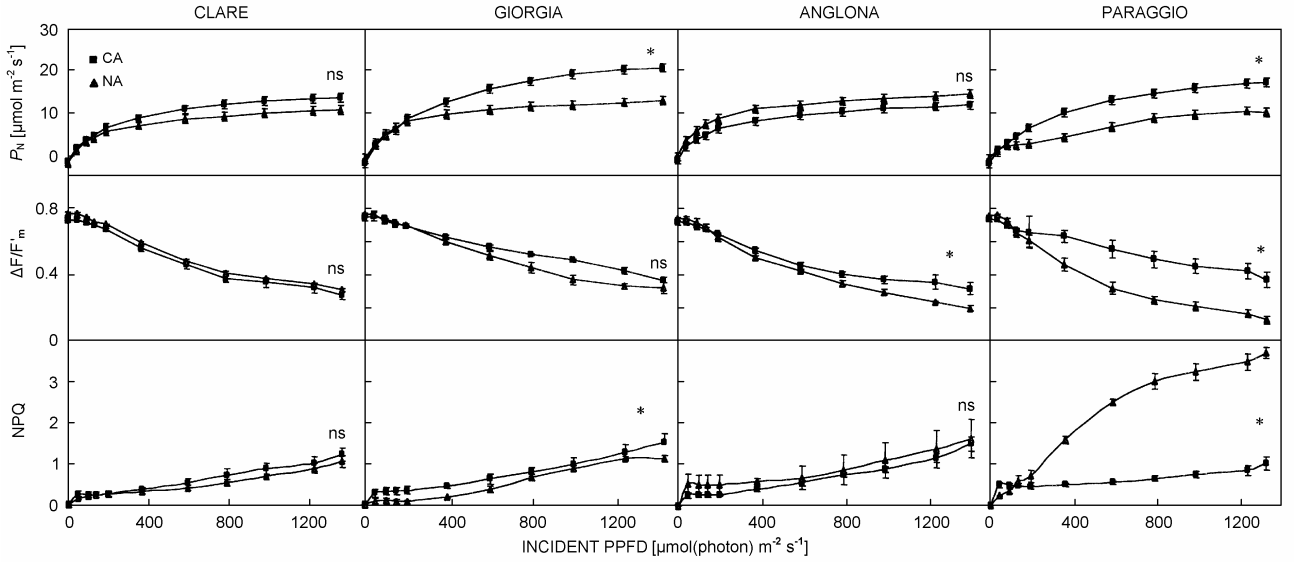

Fig. 2. PPFD response curves of the net photosynthetic rate (P_N), photochemical efficiency of photosystem 2 ($\Delta F/F'_m$), and non-photochemical quenching (NPQ) in leaves of different annual legume species. Measurements were made at ambient CO_2 (330 µmol mol^{-1}) and 10 ºC on non-acclimated (*triangles*) and cold-acclimated (*squares*) leaves. Otherwise as for Fig. 1.

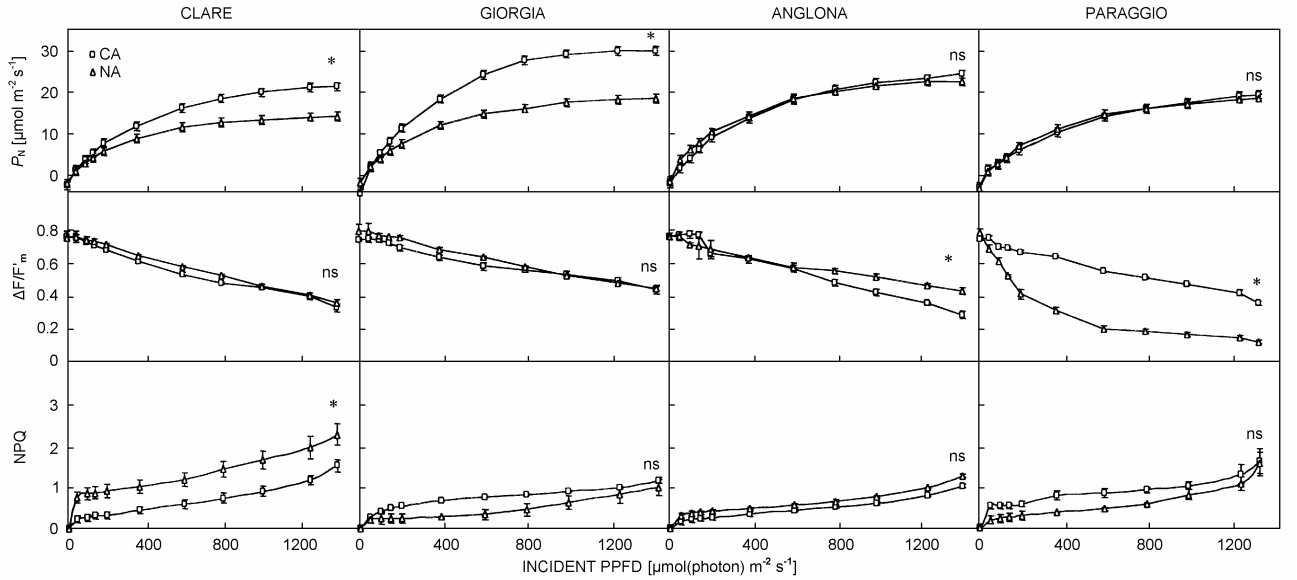

Fig. 3. PPFD response curves of the net photosynthetic rate (P_N), photochemical efficiency of photosystem 2 ($\Delta F/F'_m$), and non-photochemical quenching (NPQ) in leaves of different annual legume species. Measurements were made at ambient CO_2 (330 µmol mol^{-1}) and 20 ºC on non-acclimated (*triangles*) and cold-acclimated (*squares*) leaves. Otherwise as for Fig. 1.

Anglona had higher $\Delta F/F'_m$ at high PPFD than NA leaves. In Paraggio, long-term growth at low temperatures resulted in an increase in PPFD-saturated rates from 9.4 to 16.6 μmol m^{-2} s^{-1} (*ca*. 77 %), which was coupled with increased $\Delta F/F'_m$ and low NPQ (Fig. 2). As described for Anglona, V_{cmax} and J_{max} remained similarly low in NA and CA leaves (Table 3).

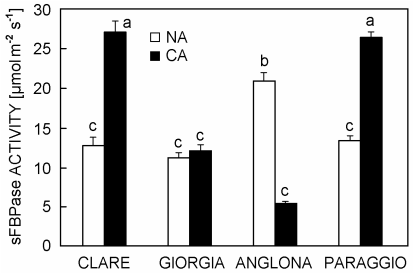

Fig. 4. Stromal fructose-1,6-bisphosphatase activity in non-acclimated (NA) and cold-acclimated (CA) leaves of different legume species. Means ± SE of 6 samples obtained from different plants. *Different letters* indicate significant differences (p<0.05) according to Tukey's test.

Effects of cold acclimation on photosynthetic capacity: To examine the photosynthetic capacity of different plant species, measurements made in NA and CA plants at optimal temperature (20 ºC) were compared (Fig. 3). Further, the total *in vitro*-activated activity of sFBPase was assayed also under optimal temperature (Fig. 4). PPFD-saturated P_N increased in Clare and Giorgia by 52 and 65 %, respectively (Fig. 3). This was associated with a strong rise in the maximum rate of carboxylation by RuBPCO (V_{cmax}), which increased by almost two times in Clare and by 150 % in Giorgia (Table 3). However, there were some differences between both clovers; firstly, CA leaves of Clare showed decreased NPQ (Fig. 3) but increased sFBPase activity, which increased by approximately 110 % following cold acclimation (Fig. 4). Secondly, there were no changes in the sFBPase activity.

In both *Medicago* cultivars Anglona and Paraggio, growth at low temperatures did not change PPFD-response curves of P_N (Table 3). Further, there were not any changes in V_{cmax}, which reached similar high values as in NA leaves (Table 4). In Anglona, cold acclimation resulted in a clear decrease of $\Delta F/F'_m$, but NPQ did not change. Estimations from CO_2-response curves indicated that J_{max} decreased by approximately 58 % in this cultivar. Further, sFPBase is very sensitive to growth at low temperatures because its activity decreased by 75 % following cold acclimation (Fig. 4). In Paraggio, J_{max} in CA leaves reached similar high values as in NA (Table 3) whereas sFBPase activity increased two times (Fig. 4). In this cultivar, cold acclimation also resulted in increased $\Delta F/F'_m$, which increased two-fold (Table 3).

Discussion

Short-term exposure of plants to low temperature usually causes inhibition of P_N. This is due to the accumulation of soluble saccharides and to reduced orthophosphate (P_i) cycling from the cytosol back to the chloroplast (Hurry *et al.* 1998). Decreasing temperature leads to a decrease in the PPFD required to saturate photosynthesis, which has been interpreted to reflect the accumulation of photosynthates as a result of the restriction of sucrose synthesis at low temperatures (Stitt and Grosse 1988). Our results showed that shifting plants from 20 to 10 ºC generally decreased P_N (Fig. 1); this was coupled with the decrease in the maximum rate of carboxylation by RuBPCO (V_{cmax}) and PPFD-saturated rate of electron transport (J_{max}) in *Medicago* plants but not in clovers (Table 3). In addition, in Anglona the decrease in P_N was accompanied by a clear decrease of F_v/F_m (Table 2). A chronic decrease in the efficiency of photosynthetic electron transport through PS2 (as indicated by F_v/F_m) can indicate photoinhibition, which may be result from impaired development of NPQ (Fig. 1) (Maxwell and Johnson 2000).

Long-term acclimation allowing new growth and development at low temperatures results not only in the accumulation of soluble saccharides but also in an increase in the capacity for photosynthesis (Hurry *et al.* 1998). We found that development at suboptimal temperatures produced different responses of growth in the four cultivars of annual legumes studied. Thus, there was a decrease of LAR in cold-acclimated plants, which is associated with higher DM rather than with decreased leaf area, in Giorgia and Paraggio (Table 1) but not in Clare and Anglona. This response may reflect the fact that the former cultivars store amino acids and sugars at low temperature and allocate less assimilate to the growth of new tissues, facilitating the accumulation of solutes with possible cryoprotective functions (Griffith and McIntyre 1993).

Regarding the two clovers, *T. subterraneum* cv. Clare and *T. michelianum* cv. Giorgia, the latter performed better than Clare during growth at low temperatures. Thus, CA plants of Clare had decreased leaf area without changes in DM (Table 1), which was accompanied by a decrease in SLA and LAR (Table 1) without any change in pigment contents per unit of DM (Table 2). By contrast, CA plants of Giorgia showed an increase of DM and leaf area that was two to four times higher than in NA plants (Table 1). Moreover, Giorgia always had higher $(a+b)/(x+c)$ ratios than the other cultivars (Table 2). This ratio is an indicator of the greenness of plants and normally lies between 4.2 and 5.0 in sun-exposed plants, and between 5.5 and 7.0 in shade-exposed plants (Lichtenthaler and Buschmann 2001). Since all cultivars were exposed to the same PPFD, the high values of $(a+b)/(x+c)$ in Giorgia might be a result of a proportional higher content of Chls than Cars per unit of DM. In

general, results of Giorgia agree with others that showed a link between anatomical changes in leaf development at low temperatures and metabolic adjustments under cold conditions (Strand *et al*. 1999). Thus, these authors observed an increase in the volume of the cytoplasm, which may provide an important mechanism for increasing the contents of enzymes and metabolites in cold-acclimated leaves.

Acclimation of Giorgia to low growth temperatures is linked with an increase in photosynthetic capacity both at 10 and 20 ºC (Figs. 2 and 3), coupled with higher V_{cmax} and J_{max} than in NA leaves (Table 3). However, a large portion of the high P_N of the CA Giorgia leaves can be attributed to their thickness (low SLA) and the resulting high amount of photosynthetically active mass per leaf area (Table 1) (Boese and Huner 1990). The high P_N of this cultivar was associated with similar $\Delta F/F'_m$ (Figs. 2 and 3) and F_v/F_m (Table 3), although CA leaves exhibited higher NPQ at high PPFD than NA leaves when measured at 10 ºC (Fig. 2). Higher values of NPQ reflect greater extent of thermal dissipation of excitation energy, and may be an important mechanism to avoid photoinhibitory damages under long-term exposure to low temperatures (Germino and Smith 2000). In general, responses observed in Giorgia agree with those observed previously in cold-tolerant winter cereals (Huner *et al.* 1993, Hurry *et al.* 1995a). However, its higher photosynthetic capacity is probably more associated with improved RuBPCO activity (as indicated by V_{cmax}) than with enhanced sFBPase activity (Fig. 4). Another factor such as larger accumulation of phosphorylated intermediates and P_i pools cannot be excluded (Hurry *et al*. 2000, Pérez *et al.* 2001). The primary effect of leaf development at low temperature is to restore a high overall P_N, but there is also a shift in carbon partitioning, away from starch and toward soluble compounds including sugars (Strand *et al.* 1999). The most important pathway for synthesis involves the export of triose phosphates to the cytosol, where they are converted to sucrose. Thus, it is possible that CA leaves of Giorgia have high capacity for sucrose synthesis (Guy *et al.* 1992, Savitch *et al.* 2000a, Stitt and Hurry 2002), which might be related with a greater freezing tolerance exhibited in this cultivar (data not shown) (Strand *et al*. 2003).

Cold acclimation of Clare produced an increase in photosynthetic capacity only at 20 ºC (Fig. 3) that was coupled with high V_{cmax} (Table 3). However, CA leaves developed lower capacity to dissipate excess of energy (as indicated by NPQ) than NA leaves (Fig. 3). These low NPQ values suggest that this cultivar might dissipate the excess of PPFD through enhanced photochemical mechanisms as increasing Calvin cycle's enzyme activities such as sFBPase (Fig. 4) and/or by other dissipating mechanisms such as photorespiration (Streb *et al.* 1998, Savitch *et al.* 2001) or Mehler reaction (Savitch *et al.* 2000b). The enhancement of enzyme activities involved in CO_2 fixation and in sucrose synthesis depends, at least in part, on increased protein synthesis (Strand *et al.* 1999, Hurry *et al.* 2000) but it is not our case because no changes in protein content have been detected in any case (data not shown).

Comparing the two *Medicago* species, CA plants of Paraggio performed better than Anglona, as shown by a two-fold increase of DM that can be attributed to higher production of stems and roots (Table 1). However, there were no differences in CA plants concerning leaf area, leaf DM, or SLA in none of these cultivars (Table 1). Relative to warm controls, cold development in Paraggio produced a two-fold increase in leaf pigments, especially Chl *a* (as indicated by the decreased *a/b* ratio) (Table 2), which may be indicative of a change in the internal structure of the thylakoid membrane induced during growth at low temperature (Huner *et al*. 1984).

Cold acclimation of Paraggio increased its photosynthetic capacity only at 10 ºC that was coupled with high $\Delta F/F'_m$ and low NPQ (Fig. 2). This suggests that Paraggio might dissipate the excess of PPFD through enhanced photochemical mechanisms such as increasing activity of sFBPase (Fig. 4). However, CA leaves did not improve RuBPCO activity (low V_{cmax}) (Table 3). Comparing measurements at 20 ºC, higher $\Delta F/F'_m$ in CA leaves does not correspond with higher P_N, which suggests a significant contribution of alternative electron sinks such as photorespiration (Streb *et al.* 1998) or Mehler reaction (Savitch *et al.* 2000b) to dissipate excessive photon energy. Further, other processes as decreases in the pool sizes of phosphorylated intermediates (Stitt and Grosse 1988, Holaday *et al.* 1992, Pérez *et al.* 2001) or changes in P_i availability (Strand *et al.* 1999, Hurry *et al.* 2000) may be affected by growth at low temperature.

Cold acclimation in Anglona did not result in changes of PPFD-saturated P_N (Figs. 2 and 3) that was accompanied by decrease of V_{cmax} and J_{max} (Table 3) and leaf pigment contents, especially Chl *a* (increased *a/b* ratio) (Table 2). In addition, the activity of a key enzyme for RuBP regeneration such as sFBPase was strongly decreased (Fig. 4). The apparent general inhibition of carbon metabolism in this *Medicago* was not related to decreases in protein synthesis (data not shown) but other factors cannot be excluded. In this sense, photosynthetic capacity during cold acclimation is altered by limitations at the level of consumption of reducing power in carbon metabolism (Savitch *et al.* 2001).

In addition, Anglona had some injuries that might result from impaired development of NPQ (Figs. 2 and 3). The NPQ is linearly related to the excessive radiation over a wide range of incident PPFDs and the extent of NPQ development in leaves is correlated with Car content ($r = 0.59$, $p<0.01$) and more exactly, with zeaxanthin formation (revised in Roháček and Barták 1999). Since development at low temperatures in Anglona leaves resulted in decreased Car content (Table 2), it could contribute to its inability to develop NPQ, which mediates

the non-radiative energy dissipation of excess excitation energy (Verhoeven *et al.* 1996). This pattern was completely different to that in other cultivars reported here, which corroborates that, as indicated by Huner *et al.* (1993) and Savitch *et al.* (2001), the response of photosynthesis to low growth temperature is clearly species dependent.

In summary, transferring plants to low temperatures affected P_N relatively less in clovers than in *Medicago* plants, which had significant limitations at the level of V_{cmax} and J_{max}. In clovers, growth at low temperature produced an acclimation enhancement of photosynthesis in Giorgia that was not observed in Clare. As for *Medicago* plants, Paraggio performed better than Anglona, which suffered a reduction of carbon metabolism by impairment of sFBPase activity. This could contribute to the poor cold acclimation of photosynthesis in this cultivar. In general, cultivars with high PPFD-saturated P_N exhibited high rates of carboxylation by RuBPCO (V_{cmax}) ($r = 0.47$, $p<0.01$), PPFD-saturated rate of electron transport (J_{max}) ($r = 0.68$, $p<0.01$), and low NPQ ($r = –0.59$, $p<0.01$).

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