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Research Article

Photosynthetic and Molecular Markers of CO_2-mediated Photosynthetic Downregulation in Nodulated Alfalfa

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Abstract

Elevated CO_2 leads to a decrease in potential net photosynthesis in long-term experiments and thus to a reduction in potential growth. This process is known as photosynthetic downregulation. There is no agreement on the definition of which parameters are the most sensitive for detecting CO_2 acclimation. In order to investigate the most sensitive photosynthetic and molecular markers of CO_2 acclimation, the effects of elevated CO_2, and associated elevated temperature were analyzed in alfalfa plants inoculated with different *Sinorhizobium meliloti* strains. Plants (*Medicago sativa* L. cv. Aragón) were grown in summer or autumn in temperature gradient greenhouses (TGG). At the end of the experiment, all plants showed acclimation in both seasons, especially under elevated summer temperatures. This was probably due to the lower nitrogen (N) availability caused by decreased N_2-fixation under higher temperatures. Photosynthesis measured at growth CO_2 concentration, rubisco *in vitro* activity and maximum rate of carboxylation were the most sensitive parameters for detecting downregulation. Severe acclimation was also related with decreases in leaf nitrogen content associated with declines in rubisco content (large and small subunits) and activity that resulted in a drop in photosynthesis. Despite the sensitivity of rubisco content as a marker of acclimation, it was not coordinated with gene expression, possibly due to a lag between gene transcription and protein translation.

Keywords: Carbon dioxide; *Medicago sativa* (alfalfa); photosynthetic downregulation; *RbcL* and *RbcS*; *Sinorhizobium meliloti*.

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Introduction

Continued human activities such as deforestation, intensive animal husbandry and fossil fuel burning are responsible for the considerable increase of atmospheric CO_2. The Intergovernmental Panel on Climatic Change (IPCC) predicts that the CO_2 concentration will be enhanced from 375 to 700 μmol/mol CO_2 by the end of the century. Due to its role as a greenhouse gas, this increase of CO_2 will lead to an increase of 4°C in atmospheric mean temperature (IPCC 2007).

Some field-based studies have found a strong and persistent stimulation of photosynthetic rates in C_3 species grown under elevated CO_2 (Ellsworth et al. 1995; Jackson et al. 1995; Curtis et al. 2000). In contrast, other studies have shown a significant decrease in photosynthetic response to elevated CO_2 (Lee et al. 2001; Rogers and Ellsworth 2002; Erice et al. 2006a; Ainsworth and Rogers 2007; Aranjuelo et al. 2009). High CO_2 concentrations enhance net photosynthesis and biomass production in short-term experiments (Lee et al. 2001; Oliveira et al. 2010). However, it has been widely reported that long-term exposures to elevated CO_2 lead to a decrease in net photosynthesis and thus a reduction in potential growth (Erice et al. 2006a; Ainsworth and Rogers 2007; Aranjuelo et al. 2009). This process, often known as photosynthetic

"downregulation" (Saralabai et al. 1997), is due to a metabolic limitation usually attributable to a reduced carboxylation activity (Erice et al. 2006b) and/or a reduced rubisco amount at elevated CO_2 (Urban 2003; Aranjuelo et al. 2005a). The photosynthetic downregulation characterized by reduced carboxylation activity has usually been related to a decrease in protein content and gene expression of rubisco under elevated CO_2 conditions (Ainsworth et al. 2002; Urban 2003; Aranjuelo et al. 2005b; Leakey et al. 2009).

One factor that could limit plant responses to elevated CO_2 is nitrogen (N) availability (Sanz-Sáez et al. 2010). Accelerated growth under elevated CO_2 (Erice et al. 2007) increases the demand for nutrients, and in soils with low N availability (such as in the Mediterranean region), N is usually the growth-limiting nutrient (Lüscher et al. 1998). This fact may lead to lower photosynthesis and therefore yield reduction (Peterson et al. 1999; Luo et al. 2004).

Species with an ability for symbiotic N_2 fixation often show a larger stimulation of photosynthesis and thus more growth under elevated CO_2 than plants unable to fix N_2 (Ainsworth et al. 2002; Lee et al. 2003). Alfalfa (*Medicago sativa* L.), an important forage crop for ecological and economical reasons, establishes a symbiotic relationship with N_2-fixing bacterium (*Sinorhizobium meliloti* L.) that provides an extra source of N to the plant in exchange of plant carbohydrates (Bergersen 1969; Bourgeois 1990). It has been suggested by Bertrand et al. (2007) that inoculation with high N_2-fixative *S. meliloti* strains avoided high CO_2-induced photosynthetic downregulation, and thus enhanced shoot dry matter (DM) in growth chamber experiments. Therefore, the selection of efficient *Sinorhizobium* strains could be of interest for improving plant performance under elevated CO_2.

It is known that plant behavior in the field frequently differs from that in growth chambers (Aranjuelo et al. 2005a). Ambient variables that influence plant growth fluctuate with circadian (Aranjuelo et al. 2005a) and seasonal rhythms, affecting plant response to rising CO_2 (Newton et al. 1994; Tissue et al. 1997). Teixeira et al. (2008) found that shoot radiation use efficiency is higher in spring than in autumn, suggesting a strong influence of season on alfalfa growth and photosynthesis. In fact, Ainsworth et al. (2003), in Swiss Free Air Concentration Enrichment (FACE) experiments with *Trifolium repens*, showed photosynthetic downregulation in autumn but not in spring. Another alternative facility to simulate the effect of projected future environmental change, including CO_2 concentration and high temperature regime under near field conditions, is the use of temperature gradient greenhouses (TGG) (Aranjuelo et al. 2005a; Pérez et al. 2005; Erice et al. 2006a,b; Sanz-Sáez et al. 2012). This facility allows the study of the effect of ambient and elevated CO_2 in combination with ambient and high temperatures (ambient $+4^{\circ}C$ in the current experiment), maintaining the daily temperature and relative humidity (RH) variation as in the field conditions. However, the FACE facility has the limitation of not providing simultaneous control of air temperature and humidity, which limits the study of combined parameters. In addition, FACE facility systems have restrictions in terms of cost, because CO_2 is emitted freely into the atmosphere. The advantage of TGG facilities are in their ability to create various CO_2 concentration and temperature regimes under field conditions at modest construction and running costs (Aranjuelo et al. 2005a,b; Erice et al. 2006a,b).

Previous studies performed on alfalfa grown in TGG under elevated CO_2 and temperature conditions showed photosynthetic downregulation, possibly caused by a reduction in N_2 fixation (Aranjuelo et al. 2005a,b; Erice et al. 2006a,b). Nevertheless, no studies have been conducted on alfalfa for determining the effect of elevated CO_2 and temperature on photosynthesis, rubisco content and gene expression in near field conditions.

The aim of this study was to analyze the effect of elevated CO_2 and temperature on alfalfa inoculated with different *S. meliloti* strains and grown in TGG in the summer or autumn. The first objective was to find the most sensitive gas exchange parameter to detect photosynthetic downregulation under such variable ambient conditions. The second one was to define the biochemical and molecular markers of photosynthetic acclimation. The third objective was to show if photosynthetic downregulation may be altered by the *S. meliloti* strain and growth season. These objectives are in line with a wide understanding of alfalfa photosynthetic downregulation mechanisms and characteristics, and with the selection of efficient *S. meliloti* strains in order to maintain high photosynthesis under elevated CO_2, thus enabling increased production throughout the year in future climate conditions.

Results

Elevated CO_2 did not enhance net photosynthesis measured at growth CO_2 concentration (A_{growth}) in any treatment or season but increased Ci in both seasons (summer, $F = 626.9$, $P \leq 0.001$; autumn, $F = 945.87$, $P \leq 0.001$) (**Table 1**). Net photosynthesis measured at 400 μmol/mol CO_2 (A_{400}) decreased at elevated CO_2 in both seasons (summer, $F = 34.35$, $P \leq 0.001$; autumn $F = 11.77$, P also diminished photosynthesis in both seasons (summer, $F = 16.05$, $P \leq 0.001$; autumn, $F = 9.77$, $P = 0.003$) (**Figure 1**).

In summer, the maximum rate of carboxylation (Vc_{max}) increased with elevated temperature under an ambient CO_2 concentration, but decreased with CO_2 under an ambient $+$ $4^{\circ}C$ temperature regime ($F = 18.45$, $P \leq 0.001$) (**Figure 2**). In autumn, Vc_{max} decreased only in 1032GMI at elevated temperature under elevated CO_2 conditions ($F = 4.44$, $P = 0.019$) (**Figure 2**).

Total rubisco activity decreased with elevated CO_2 and temperature in plants inoculated with 102F78 or 1032GMI in

Table 1. Effect of CO_2 (ambient, around 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on net photosynthesis at growth CO_2 concentration (A_{growth},) (μmol CO_2/m^2 per s), intercellular CO_2 concentration (Ci) (μmol CO_2/mol), rubisco total activity (mmol CO_2/m^2 per s) on alfalfa grown in summer and autumn

			Summer			Autumn		
			A_{growth}	Ci	Rubisco total activity	A_{growth}	Ci	Rubisco total activity
102F78	Amb T	Ambient CO_2	28.9 ± 2.1 a	272 ± 4 bc	31.2 ± 1.68 a	20.8 ± 1.6 a	246 ± 15 c	24.36 ± 2.55 ab
		Elevated CO_2	28.35 ± 1.9 a	500 ± 25 a	18.9 ± 1.44 bc	26.0 ± 2.5 a	533 ± 10 ab	24.69 ± 1.77 a
	T + 4°C	Ambient CO_2	27.9 ± 3.2 a	223 ± 45 c	25.8 ± 4.5 a	20.3 ± 2.0 a	219 ± 13 e	15.18 ± 3.39 cd
		Elevated CO_2	27.6 ± 4.7 a	514 ± 6 a	19.5 ± 3.63 bc	24.3 ± 1.8 a	496 ± 24 b	19.92 ± 2.50 abc
102F34	Amb T	Ambient CO_2	24.2 ± 3.3 a	267 ± 5 bc	21.9 ± 2.25 b	24.8 ± 3.0 a	218 ± 10 c	25.00 ± 2.50 a
		Elevated CO_2	29.9 ± 3.4 a	505 ± 10 a	20.4 ± 3.0 bc	22.9 ± 2.2 a	522 ± 18 ab	16.40 ± 2.31 cd
	T + 4°C	Ambient CO_2	27.3 ± 1.0 a	273 ± 5 bc	18.0 ± 1.21 b	19.9 ± 0.9 a	218 ± 14 c	20.46 ± 1.92 abc
		Elevated CO_2	26.5 ± 2.1 a	520 ± 5 a	6.6 ± 1.8 cd	24.8 ± 1.1 a	520 ± 23 ab	6.69 ± 0.84 e
1032GMI	Amb T	Ambient CO_2	23.5 ± 0.9 a	286 ± 9 b	22.5 ± 3.54 b	24.3 ± 1.8 a	240 ± 12 c	16.62 ± 1.56 cd
		Elevated CO_2	29.1 ± 1.8 a	506 ± 16 a	16.8 ± 1.23 cd	25.1 ± 1.5 a	500 ± 23 b	17.97 ± 2.49 bcd
	T + 4°C	Ambient CO_2	25.6 ± 1.8 a	267 ± 7 bc	21.9 ± 1.53 bc	23.1 ± 2.8 a	221 ± 14 c	21.03 ± 0.87 abc
		Elevated CO_2	32.3 ± 4.0 a	542 ± 17 a	13.5 ± 3.03 d	25.1 ± 0.9 a	563 ± 15 a	11.85 ± 2.61 de
		CO_2	ns	***	***	ns	***	**
		T	ns	ns	*	ns	ns	***
		Strain	ns	ns	**	ns	ns	*
		CO_2 × T × strain	ns	ns	ns	ns	ns	ns

Values represent the mean ± *SE*; $n = 4$. Statistical analysis was carried out with a three factors Analysis of the Variance (ANOVA), see the results at the bottom of the table. The meanings of symbols used in ANOVA were: *, Significant difference at 5%, ** 1%, *** 0.1%. When significant differences were detected in ANOVA, Least significant difference (LSD) analysis was applied. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test parameters.

summer ($F = 5.30$, $P = 0.011$) (Table 1), whereas in autumn, the combination of elevated CO_2 and temperature diminished rubisco total activity in plants inoculated with the 102F34 and 1032GMI strains ($F = 4.42$, $P = 0.021$) (Table 1).

In summer, elevated CO_2 and temperature enhanced plant DM (Table 2). Plants inoculated with the 102F78 strain were the most productive under elevated CO_2 and temperature ($F = 14.73$, $P \leq 0.001$). In autumn, the combination of elevated CO_2 and temperature enhanced DM, especially with 102F34, which was the most efficient strain in all treatments (Table 2) ($CO_2 \times T \times strain$, $F = 11.06$, $P \leq 0.001$). In summer, elevated CO_2 reduced leaf N concentration in plants inoculated with 1032GMI, regardless of the temperature regime, and in plants inoculated with 102F78 at elevated temperature ($CO_2 \times T \times strain$, $F = 10.91$, $P \leq 0.001$). In autumn, leaf N content was not altered by elevated CO_2, temperature or inoculated strain (Table 2).

In summer, elevated CO_2 and temperature increased starch content in all strains except for 102F78 at ambient temperature ($F = 23.12$, $P \leq 0.001$) (Table 3). In autumn, elevated CO_2 increased starch content in 102F78 at elevated temperatures, and in the case of 1032GMI, at an ambient temperature ($CO_2 \times T \times strains$, $F = 3.81$, $P = 0.035$) (Table 3). In summer, total soluble protein (TSP) increased with elevated CO_2 only in plants inoculated with 102F34 (Table 3). In autumn, elevated CO_2 diminished total soluble protein (TSP) content, except at elevated temperatures in plants inoculated with the 1032GMI or 103F34 strains (Table 3).

The expression of rubisco large subunit (*RbcL*) was higher in plants inoculated with 102F78 or 1031GMI in summer (strains, $F = 33.57$, $P \leq 0.001$) (Figure 3). In that season, elevated CO_2 increased *RbcL* expression with all strains except 102F34 (CO_2, $F = 138.72$, $P \leq 0.001$). Nevertheless, temperature decreased *RbcL* expression with 102F34 and 1031GMI ($T \times strains$, $F = 12.66$, $P \leq 0.001$). In autumn, elevated CO_2 and temperature increased *RbcL* expression, while the interaction between elevated CO_2 and temperature decreased it in plants inoculated with 102F78 ($CO_2 \times T \times strains$, $F = 29.31$, $P \leq 0.001$) (Figure 3). In summer, elevated CO_2 enhanced rubisco small subunit *(RbcS)* expression except in plants inoculated with 1032GMI ($CO_2 \times strains$, $F = 41.98$, $P \leq 0.001$). Nevertheless, in autumn, elevated CO_2 decreased *RbcS* expression in 102F78 inoculated plants, especially in the interaction with elevated temperature ($CO_2 \times T \times strains$, $F = 11.14$, $P \leq 0.001$) (Figure 3).

In summer, rubisco small protein subunit (RSS) content was lower than rubisco large protein subunit (RLS) content in all treatments ($F = 344.292$, $P \leq 0.001$) (Figure 4). Elevated CO_2

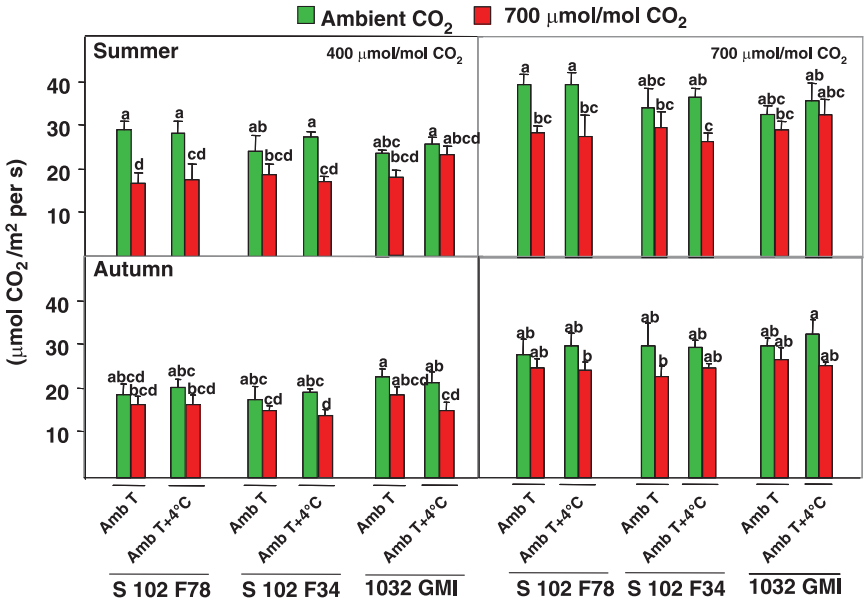

Figure 1. Effect of CO_2 (ambient, around 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on net photosynthesis (A) measured at 400 (A_{400}) and 700 (A_{700}) μmol/mol CO_2 in nodulated alfalfa grown in summer and autumn.

Bars represent the mean ± *SE*; $n = 4$. In each graph, bars with the same letter are not significantly different ($P \leq 0.05$) according to the least significant difference (LSD) test.

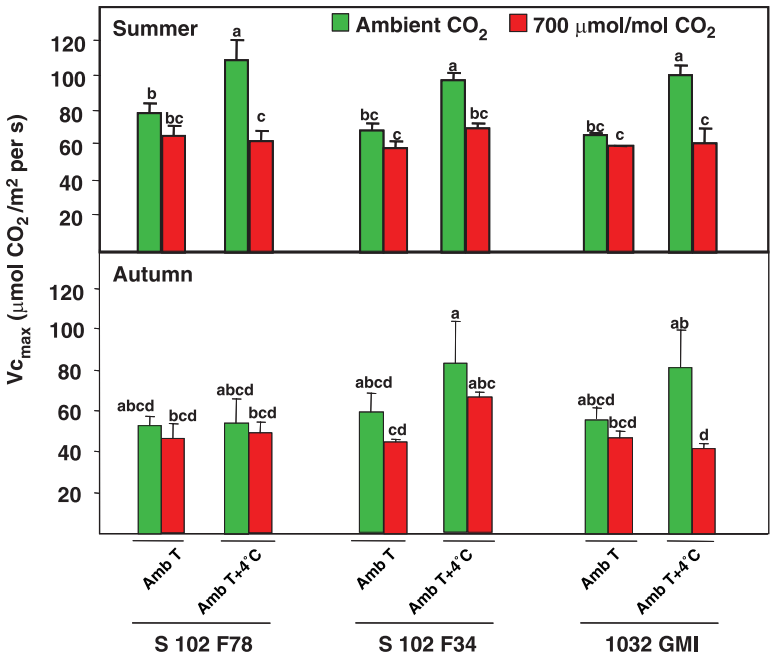

Figure 2. Effect of CO_2 (ambient around 400 and elevated 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on maximum rate of rubisco carboxylation (Vc_{max}) (μmol CO_2/m^2 per s) of nodulated alfalfa grown in summer and autumn.

Bars represent the mean ± *SE*; $n = 4$. Bars with the same letter are not significantly different ($P \leq 0.05$) according to the least significant difference (LSD) test.

Table 2. Effect of CO_2 (ambient, around 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strains (102F78, 102F34 and 1032GMI) on plant dry matter (DM) (g/plant) and leaf N content (g/m) on alfalfa grown in summer and autumn

			Summer		Autumn	
			DM	Leaf N	DM	Leaf N
102F78	Amb T	Ambient CO_2	0.51 ± 0.03 ef	1.4 ± 0.03 bcde	0.59 ± 0.08 ef	1.5 ± 0.10 a
		Elevated CO_2	1.31 ± 0.16 b	1.3 ± 0.04 def	0.93 ± 0.15 cd	1.6 ± 0.16 a
	T + 4°C	Ambient CO_2	1.01 ± 0.14 bcd	1.8 ± 0.01 a	1.46 ± 0.10 b	1.4 ± 0.10 a
		Elevated CO_2	1.70 ± 0.23 a	1.3 ± 0.07 cdef	1.44 ± 0.11 b	1.1 ± 0.21 a
102F34	Amb T	Ambient CO_2	0.48 ± 0.07 ef	1.4 ± 0.06 bcd	0.92 ± 0.11 cd	1.3 ± 0.17 a
		Elevated CO_2	0.69 ± 0.06 cdef	1.5 ± 0.02 bc	1.22 ± 0.05 bc	1.5 ± 0.05 a
	T + 4°C	Ambient CO_2	0.69 ± 0.05 def	1.6 ± 0.07 ab	0.65 ± 0.03 de	1.3 ± 0.22 a
		Elevated CO_2	1.23 ± 0.09 b	1.8 ± 0.20 a	1.99 ± 0.08 a	1.6 ± 0.17 a
1032GMI	Amb T	Ambient CO_2	0.42 ± 0.04 ef	1.4 ± 0.05 bcde	0.36 ± 0.03 f	1.2 ± 0.15 a
		Elevated CO_2	0.78 ± 0.11 cde	1.2 ± 0.04 f	0.61 ± 0.10 ef	1.5 ± 0.11 a
	T + 4°C	Ambient CO_2	0.63 ± 0.06 ef	1.5 ± 0.09 bc	0.64 ± 0.02 def	1.4 ± 0.13 a
		Elevated CO_2	1.04 ± 0.14 be	1.2 ± 0.07 ef	1.38 ± 0.21 b	1.2 ± 0.03 a
		CO_2	***	**	***	ns
		T	***	**	***	ns
		Strain	***	***	***	ns
		CO_2 × T × strain	***	***	***	ns

Values represent the mean ± *SE*; $n = 4$. Statistical analysis was carried out with a three factors Analysis of the Variance (ANOVA), see the results at the bottom of the table. The meanings of symbols used in ANOVA were: *, Significant difference at 5%, ** 1%, *** 0.1%. When significant differences were detected in ANOVA, least significant difference (LSD) analysis was applied. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test parameters.

decreased RLS in all treatments, except in plants grown at an elevated temperature and inoculated with 102F34 or 1031GMI. In contrast, RSS only decreased in plants inoculated with 102F78 grown at elevated CO_2 and temperature (**Figure 4**) ($CO_2 \times T \times strains$, $F = 6.17$, $P = 0.003$). In autumn, RSS was equal or higher than RLS, except for 102F34 and 1032GMI at an elevated temperature ($F = 7.9$, $P = 0.006$). RLS was not affected by elevated CO_2 or temperature. RSS was enhanced by elevated CO_2 at an ambient temperature in the 102F78 and 102F34 strains. However, at elevated CO_2 and temperature, the RSS content was diminished in all strains ($CO_2 \times T \times strains$, $F = 3.99$, $P = 0.02$) (**Figure 4**).

In summer and autumn, A_{growth} was significantly correlated with RLS ($r = 0.472$, $P = 0.004$; $r = 0.525$, $P < 0.001$, respectively), while Vc_{max} was negatively correlated with RLS ($r = -0.417$, $P < 0.05$; $r = 0.318$, $P < 0.05$, respectively) (**Table 4**). Total N content was also correlated with RSS in summer and autumn ($r = 0.575$, $P < 0.001$; $r = 0.357$, $P = 0.033$).

Discussion

The literature available about plant responses to elevated CO_2 after long-term exposure is extensive. However, there is no consensus that defines the most suitable parameter to detect photosynthetic downregulation. The large number of papers that have reported photosynthetic downregulation has used a wide number of techniques and parameters to detect and define it. In the area of gas exchange parameters, A/Ci curves are useful tools that provide a wide range of information, but the time spent collecting the data per measurement is rather high (around 30 min per plant). In contrast, rapid measurements like net photosynthesis measured at growth CO_2 concentration (A_{growth}) are quick parameters for detecting acclimation, but they provide less information than A/Ci curves. This study attempts to find the most sensitive physiological and molecular parameters to detect photosynthetic acclimation.

Photosynthetic markers

Photosynthetic downregulation is defined as the decrease in photosynthetic capacity under elevated CO_2 conditions (Saralabai et al. 1997), and several parameters such as A_{growth}, Vc_{max}, A_{400}, A_{700} and maximum electron transport rate (J_{max}) have been used to study plant photosynthetic response to elevated CO_2 (Lee et al. 2001; Ainsworth et al. 2003; Erice et al. 2006a; Aranjuelo et al. 2008). In both the summer and autumn experiments, A_{growth} remained unchanged at elevated CO_2 concentrations when plants grown in this condition should

Table 3. Effect of CO_2 (ambient, approximately 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and inoculation with *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on starch (g/m^2) and total soluble proteins (TSP) (g/m^2) in alfalfa grown in summer and autumn

			Summer		Autumn	
			Starch	TSP	Starch	TSP
10F78	Amb T	Ambient CO_2	0.52 ± 0.06 de	3.14 ± 0.3 bc	0.29 ± 0.07 c	2.54 ± 0.13 ab
		Elevated CO_2	0.57 ± 0.08 de	3.65 ± 0.23 bc	0.57 ± 0.01 bc	1.69 ± 0.12 d
	T + 4°C	Ambient CO_2	0.41 ± 0.04 e	3.28 ± 0.23 bc	0.45 ± 0.05 bc	2.63 ± 0.19 ab
		Elevated CO_2	1.38 ± 0.13 abc	4.19 ± 0.52 b	1.60 ± 0.25 a	1.89 ± 0.06 cd
102F34	Amb T	Ambient CO_2	0.67 ± 0.12 de	3.40 ± 0.19 bc	0.24 ± 0.06 c	2.94 ± 0.24 a
		Elevated CO_2	1.47 ± 0.06 ab	5.85 ± 0.66 a	0.40 ± 0.03 bc	1.67 ± 0.04 d
	T + 4°C	Ambient CO_2	0.48 ± 0.06 de	3.57 ± 0.29 bc	1.10 ± 0.10 ab	2.31 ± 0.52 bcd
		Elevated CO_2	0.88 ± 0.15 cde	2.88 ± 0.64 c	1.60 ± 0.29 a	2.33 ± 0.18 bcd
1032GMI	Amb T	Ambient CO_2	0.97 ± 0.16 bcd	4.24 ± 0.33 b	0.25 ± 0.03 c	1.82 ± 0.19 d
		Elevated CO_2	1.88 ± 0.51 a	4.31 ± 0.50 b	0.75 ± 0.21 ab	2.25 ± 0.14 bcd
	T + 4°C	Ambient CO_2	0.67 ± 0.15 de	3.60 ± 0.23 bc	0.99 ± 0.15 b	1.96 ± 0.14 cd
		Elevated CO_2	1.40 ± 0.067 ab	4.63 ± 0.88 ab	1.51 ± 0.04 ab	2.48 ± 0.12 abc
		CO_2	*	**	***	*
		T	***	ns	ns	ns
		Strain	ns	ns	**	ns
		CO_2 × T × strain	ns	**	*	ns

Values represent the mean ± *SE*; $n = 4$. Statistical analysis was carried out with a three factors Analysis of the Variance (ANOVA), see the results at the bottom of the table. The meanings of symbols used in ANOVA were: *, Significant difference at 5%, ** 1%, *** 0.1%. When significant differences were detected in ANOVA, least significant difference (LSD) analysis was applied. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test parameters.

show higher A_{growth} than plants grown at ambient CO_2, indicating photosynthetic acclimation, as has also been reported previously with same-aged alfalfa plants and an even shorter exposure time to elevated CO_2 by Aranjuelo et al. (2005a) and Sanz-Sáez et al. (2010) (**Table 1**). This photosynthetic acclimation was not caused by lower intercellular CO_2 concentration (C_i) as a result of stomatal closure, because in previous experiments, plants grown at elevated CO_2 showed higher Ci than those grown at ambient CO_2 (data not shown), as shown by Aranjuelo et al. (2005b) and Sanz-Sáez et al. (2010). In summer, Vc_{max} dropped in elevated CO_2 and temperature treatments in all strains, while in autumn it only decreased in plants inoculated with 1032GMI (**Figure 2**). Unaltered Vc_{max} does not necessarily entail the absence of photosynthetic downregulation; Crous et al. (2010) and Sanz-Sáez et al. (2010) also showed acclimated plants without a significant reduction in Vc_{max}, but with decreased or unaltered A_{growth}.

Other parameters used to detect photosynthetic downregulation are A_{400} and A_{700}. In summer, similar responses to elevated CO_2 were obtained for A_{400} and A_{700}, with significant decreases with 102F78, regardless of temperature regime, and with 102F34 at elevated temperature. Nevertheless, in autumn, A_{400} only showed significant decreases with 102F34 and 1032GMI at elevated temperature, whereas A_{700} was not affected by elevated CO_2. This result showed that A_{400} is more effective than A_{700} for detecting photosynthetic downregulation, as has been suggested by Ellsworth et al. (2004).

Summer appears as the season when the clearest acclimation occurs to elevated CO_2, according to the results of A_{400}, A_{700} and Vc_{max}. Downregulation was detected in 50% of summer comparisons (**Table 5**) but only in 16% of those during autumn. A_{growth} has been revealed to be the most sensitive photosynthetic parameter for detecting acclimation to elevated CO_2, because as we have observed before, A_{growth} did not increase with elevated CO_2 under studied conditions.

Biochemical and molecular markers

Rubisco total activity is an *in vitro* parameter that is well correlated with CO_2 assimilation (Caemmerer and Edmondson 1986). In summer, rubisco activity decreased in 83% of cases at elevated CO_2 (**Table 5**). Nevertheless, in autumn, the elevated CO_2 only decreased it in 50% of the comparisons (**Table 5**), confirming the greater acclimation under warmer temperatures (summer *vs* autumn) as we had observed for photosynthetic markers. This result agrees with the findings of Galmés et al. (2006) who considered rubisco *in vitro* activity as a more reliable parameter than Vc_{max}, particularly under drought and high

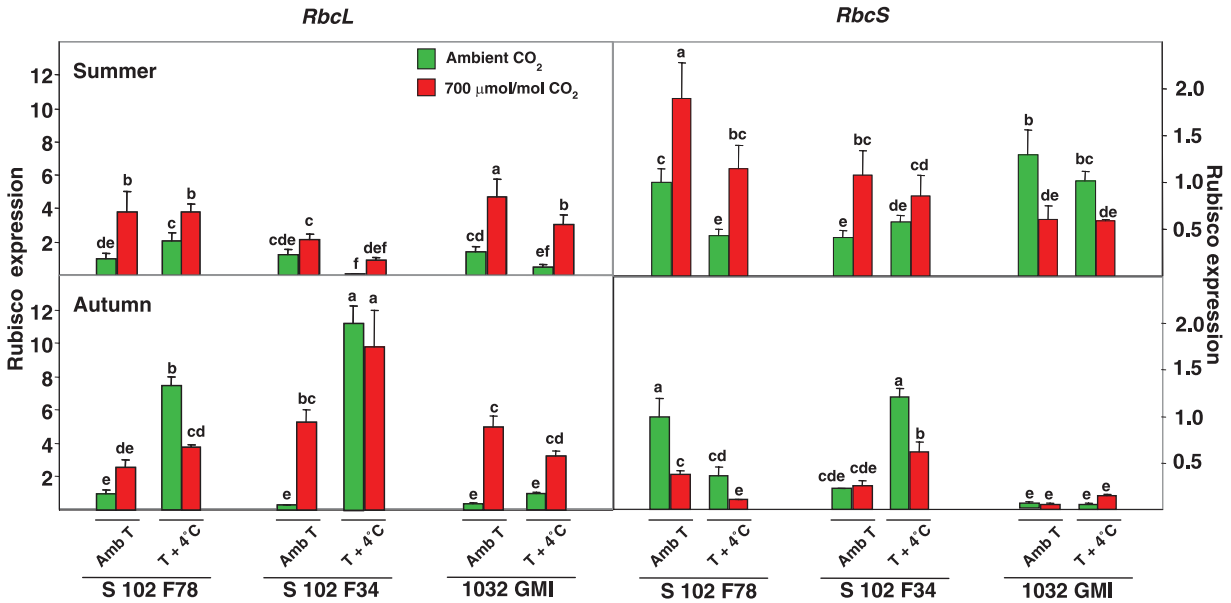

Figure 3. Effect of CO_2 (ambient, around 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on relative expression of the RbcL and RbcS genes in alfalfa plants grown in summer and autumn.

Bars represent the mean ± *SE*; $n = 4$. Bars with the same letter are not significantly different ($P \leq 0.05$) according to the least significant difference (LSD) test.

temperature. The rubisco enzyme catalyzes two pathways: (i) photosynthesis, which is initiated by carboxylase reaction, and photorespiration, which is initiated by oxygenase. The balance between these two competitive reactions is determined by the properties of rubisco, CO_2 and O_2 concentrations (Laing et al. 1974).

$$V_c/V_o = (V_cK_o/V_oK_c)([CO_2]/[O_2]$$

Where (V_cK_o/V_oK_c) is the substrate specificity factor (τ) that determines the relative rates of the two reactions at any given CO_2 and O_2 concentrations (Galmés et al. 2006). Current models to calculate the *in vivo* photosynthesis through IRGA's facilities assume that τ is a constant value among C_3 plants (Long and Bernacchi 2003). However, according to Galmés et al. (2006), τ may change under varying environmental conditions, such drought and/or high temperature. In addition, Roy and Andrews (2000) and Parry et al. (2003) have hypothesized that a change in *RbcS* expression could induce a change in rubisco τ. In the current study, environmental conditions were different between treatments (CO_2, temperature and seasons); therefore, τ may change among the different treatments. In addition, elevated CO_2 and temperature changed *RbcS* expression, and this change may also vary rubisco τ. These variations could be the explanation for the differences observed between Vc_{max} (calculated with a constant τ) and rubisco activity (calculated with the natural τ) to detect the photosynthetic downregulation at elevated CO_2.

Some authors have associated the photosynthetic downregulation with decreases in leaf N in forbs, grasses and trees (Ellsworth et al. 2004; Crous et al. 2010). In our summer experiment, plants grown at elevated CO_2 and temperature showed decreased leaf N; however, in autumn, with less marked downregulation, this parameter remained unchanged (**Table 2**). The greater decrease in leaf N in summer could be due to the negative effect of high temperatures decreasing N_2 fixation and thus N availability (Aranjuelo et al. 2007; Sanz-Sáez et al. 2012). However, in autumn, the mild temperatures allowed a better N_2 fixation, remaining leaf N was unchanged, and this thus resulted in a less severe downregulation (Sanz-Sáez et al. 2012). The decrease in leaf N was accompanied by generally enhanced summer starch content (**Table 3**). It has already been stated that under elevated CO_2, the decrease in leaf N is a consequence of dilution by carbohydrate accumulation, the formation of leaf structural material, and increases in plant internal demand for N (Ellsworth et al. 2004). In a low N availability situation as observed in the summer experiment, elevated CO_2 may partition resources away from leaves and, through increased production, result in sequestration of nutrients (like N) into organic matter, causing deficiencies that indirectly result in decreased photosynthetic capacity (Rogers and Ellsworth 2002; Ainsworth et al. 2003). Our study revealed that the leaf N

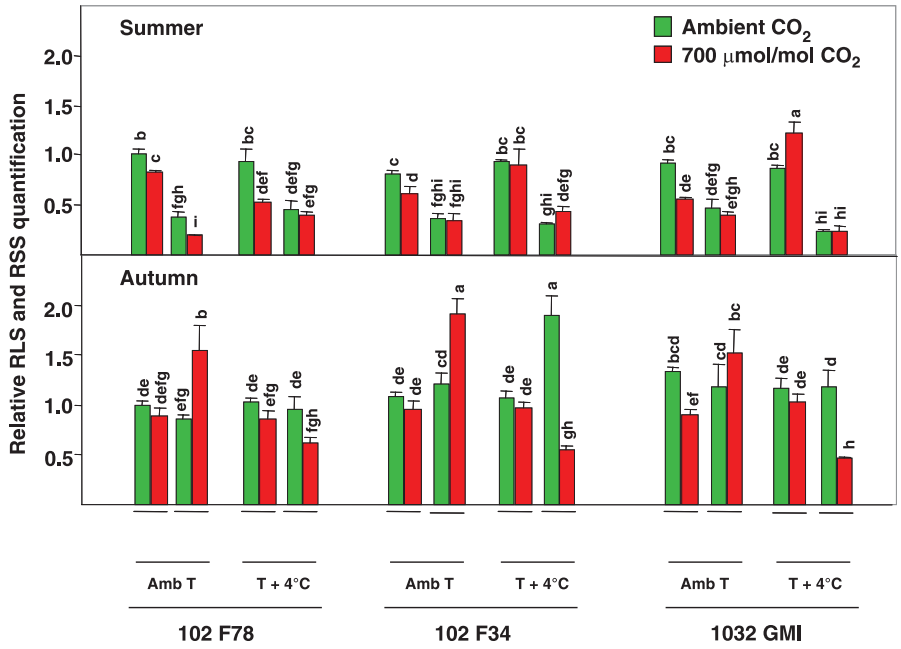

Figure 4. Effect of CO_2 (ambient, around 400 and elevated, 700 μmol/mol), temperature (ambient and ambient +4°C) and *Sinorhizobium meliloti* L. strain (102F78, 102F34 and 1032GMI) on semi-quantification of the rubisco large (RLS) and small subunits (RSS) in alfalfa plants grown in summer and autumn.

The relative proportion of RLS and RSS was calculated in reference to the abundance value of RLS observed in plants inoculated with 102F78 and grown at ambient temperature and CO_2 concentration. Bars represent the mean ± *SE*; $n = 4$. Bars with the same letter are not significantly different ($P \leq 0.05$) according to the least significant difference (LSD) test.

Table 4. Correlation coefficients among A_{growth}, Vc_{max} and leaf nitrogen (N) with rubisco large and small subunit contents (RLS and RSS, respectively) and rubisco large and small subunit gene expression (RbcL and RbcS, respectively)

	Summer			Autumn		
	A_{growth}	Vc_{max}	leaf N	A_{growth}	Vc_{max}	leaf N
RLS	0.472**	−0.417*	0.224	0.525***	−0.318*	0.224
RSS	0.230	−0.109	0.575***	0.133	0.147	0.575***
RbcL	0.120	0.396*	0.208	0.177	0.278	0.208
RbcS	0.124	0.255	0.131	0.172	0.341*	0.131

*Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$; ***Significant at $P \leq 0.001$.

content was not directly associated with downregulation in all treatments; however, in treatments in which leaf N decreased under elevated temperature, the photosynthetic acclimation was also associated with reduced Vc_{max} (**Table 5**).

In general, the photosynthetic response to elevated CO_2 is enhanced with increasing temperatures within the optimum specific temperature limits for the studied species (Long 1991; Sage et al. 1995; Jifon and Wolfe 2005). For example, it has been described that sour orange tree grown at elevated CO_2 showed a linear increase in photosynthesis from 75% to 200% with increasing day temperatures from 31°C to 42°C, because the summer daytime temperature range where the plants were grown was within the optimum temperature for photosynthesis of this citrus cultivar (Idso et al. 1995). However, rice photosynthesis enhancement stayed relatively constant at 60% when the plants were grown in a temperature range of 32–38°C (Vu et al. 1997). In N_2 fixing plants, this enhancement of photosynthesis is usually observed in short-term expositions to elevated CO_2 and increasing temperature, as has been described by Jifon and Wolfe (2005) with *Phaseolus vulgaris* plants grown in such conditions during 24 d. However, the same plants after long-term exposition to elevated CO_2 and high temperature showed downregulation. In other experiments conducted in alfalfa by Aranjuelo et al. (2005b) and Erice et al. (2006a), the plants exposed to elevated CO_2 and temperature showed higher downregulation than plants grown at elevated CO_2 and ambient temperature. In the present study, alfalfa grown in summer under elevated CO_2 and temperature featured more symptoms of photosynthetic downregulation, revealed as decreases of Vc_{max}, A400, A700 and leaf N, and accumulation of starch content, than plants grown in autumn under elevated CO_2 and ambient temperature (**Table 5**). This negative effect observed on

Table 5. Schematic summary of the effect of CO_2, 700 μmol/mol CO_2 versus ambient CO_2 in all studied parameters in summer and autumn experiments

Summer		A_{growth}	A_{400}	A_{700}	V_{cmax}	Rubisco activity	Leaf N	Starch	TSP	RLS	RSS	RbcL	RbcS
102F78	Amb T	*	*	*		*				*	*		
	Amb T + 4°C	*	*	*	*	*	*	*		*			
102F34	Amb T	*						*		*			
	Amb T + 4°C	*	*	*	*	*							
1032GMI	Amb T	*				*	*	*		*			*
	Amb T + 4°C	*			*	*	*	*					*

Autumn		A_{growth}	A_{400}	A_{700}	V_{cmax}	Rubisco activity	leaf N	Starch	TSP	RLS	RSS	RbcL	RbcS
102F78	Amb T	*							*				*
	Amb T + 4°C	*						*	*		*	*	*
102F34	Amb T	*				*			*				
	Amb T + 4°C	*	*			*					*		*
1032GMI	Amb T	*						*		*			
	Amb T + 4°C	*	*		*	*					*		

The symbol (*) indicates that the considered parameter is downregulated.

photosynthesis and growth of elevated temperature treatment in plants grown at elevated CO_2 mostly in summer was possibly due to exceeding the optimum temperature limit for alfalfa N_2-fixation and photosynthesis (25–33°C) (Pankhurst and Sprent 1976). This could decrease photosynthetic activity and the acquisition of N through N_2-fixation, which may induce a marked photosynthetic acclimation as has been demonstrated recently by Sanz-Sáez et al. (2012).

Some authors have reported that leaves grown under elevated CO_2 concentrations show higher carbohydrate content than those grown under ambient CO_2 (Geiger et al. 1999; Mishra et al. 2008). In our study, all plants grown at elevated CO_2 showed a slight increase in leaf starch content at ambient temperature, but the starch accumulation was more significant at elevated temperature (**Table 3**). It is generally assumed that the enhancement of non-structural carbohydrates and the inhibition of the expression of genes that encode for different photosynthetic apparatus compounds are suppressed through a possible increase of hexose cycling within the leaf, resulting in decreased photosynthetic capacity and a notable decrease in the amount of rubisco (Drake et al. 1997; Moore et al. 1999). That enhancement of non-structural carbohydrates under elevated CO_2 conditions in alfalfa was caused by the increase in starch content without the increase in total soluble sugars, as has been shown previously by Erice et al. (2006a) and Sanz-Sáez et al. (2010). As mentioned above, when plants are N-limited, downregulation has been related to reallocation of N away from the photosynthetic apparatus (Aranjuelo et al. 2005b) or from particular photosynthetic enzymes (Moore et al. 1999; Rogers and Ellsworth 2002). In this context, in the summer experiment, TSP generally remained unaffected at elevated CO_2 (**Table 3**); however, a selective decrease in RLS content was detected (**Figure 4**). This could be due to a selective drop in rubisco concentration under elevated CO_2 (Aranjuelo et al. 2009). In addition, in both seasons, Vc_{max} was negatively correlated with RLS content (**Table 4**). This could mean that the reduction of RLS caused by elevated CO_2 may increase the rubisco activation state or activity to balance the loss of rubisco content. The differences observed between treatments in A_{growth} and Vc_{max} and the absence of correlation between these parameters could be caused by the presence or absence of rubisco inhibitors such as 2-Carboxy-D-arabinitol 1-phosphate (CA1P) (Gutteridge et al. 1986; Berry 1987). CA1P is an analog of the transition-state of the carboxylase reaction, which binds tightly to the active site of RLS, and thus inhibits catalytic activity under different ambient conditions (Berry 1987; Gutteridge et al. 1989; Andralojc et al. 2002). It is considered that, due to the relative quantity of rubisco large (RLS) and small subunits (RSS) and the lowest specificity of RSS (Jordan and Ogren 1981), rubisco enzymes lack RSS, which may suggest that RSS contribute substantially to the differences in kinetic properties of rubisco (Andersson and Backlund 2008). In summer, RLS showed significant decreases in 66% of CO_2 comparisons, whereas RSS only decreased in 16% (**Table 5**). In this season, a strong correlation between A_{growth} and RLS was found (**Table 4**). Nevertheless, in autumn, RSS was specifically decreased by a combination of elevated CO_2 and temperature (**Figure 4**). This decrease in both rubisco subunits could be due to N leakage (Ainsworth et al. 2003) away from the photosynthetic apparatus, caused by higher DM production (**Tables 2**, **5**). Increase in plant DM at elevated CO_2 is the consequence of total photosynthesis enhancement as a result of increased area in these treatments (data not shown) (Craine et al. 2003; Luo et al. 2004; Aranjuelo et al. 2008; Sanz-Sáez

et al. 2010). This drop in RLS and RSS and the associated reduced rubisco activity may be responsible for photosynthetic downregulation (Urban 2003; Aranjuelo et al. 2005b; Erice et al. 2006b). In summer, expression corresponding to the *RbcL* and RbcS genes that encode the RLS and RSS subunits of rubisco expression was generally increased at elevated CO_2, depending on the inoculated *S. meliloti* strain, as was also reported by Bertrand et al. (2007) (**Figure 3**). Despite the general decrease in rubisco protein under elevated CO_2, *RbcL* was enhanced in autumn (**Figure 3**; **Table 5**). A_{growth} was directly related to rubisco content but was not coordinated with rubisco mRNA expression (**Table 4**), contrary to some species like pea, soybean, wheat and tomato (Webber et al. 1994; Majeau and Coleman 1996; Moore et al. 1998). This divergence between rubisco content and expression could be due to a temporal control of photosynthetic gene expression that can oscillate in a circadian way, as pointed out by Moore et al. (1999).

In this study, parameters to detect photosynthetic downregulation were analyzed. All plants grown at elevated CO_2 presented downregulation in both seasons, especially when combined with high temperatures. This could be due to a low N availability caused by a decrease in N_2 fixation induced by higher summer temperatures (Sanz-Sáez et al. 2012). The most sensitive photosynthetic parameters for detecting acclimation were A_{growth} and A_{400}. Within biochemical parameters, rubisco *in vitro* activity was suitable and even more sensitive than Vc_{max}. The decrease in leaf N was useful for detecting severe acclimation in summer. Elevated CO_2 did not decrease leaf TSP but specifically affected rubisco protein (RLS and RSS), resulting in decreased rubisco activity *in vitro* and hence reduced photosynthesis rates. Opposite to observations in other species, rubisco content and expression in alfalfa were not directly correlated, which may be due to a temporal (circadian) control of both processes.

Materials and Methods

Plant Materials

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, seedlings were planted in pots (20 plants per pot) containing a mixture of perlite–vermiculite (2/1; v/v). Pots with a capacity of 13 L were used to avoid the possibility of them becoming pot-bound. Plants were grown in two temperature gradient greenhouses (TGG) and irrigated alternatively with Evans N-free solution (Evans 1974) and distilled water to avoid salt accumulation in the substrate during the experiment. The only nitrogen (N) source for the plants was the N_2-fixed by nodules. Plants were inoculated immediately after planting with different *Sinorhizobium meliloti* L. strains: 102F78, 102F34 (Nitragin, Milwaukee, WI, USA), or 1032GMI (Biotechnology Department, Polytechnic University of Madrid, Spain). In previous studies conducted with 102F78, the tolerance of this strain to elevated temperatures has been demonstrated (Aranjuelo et al. 2005a,b; Erice et al. 2006a,b). In contrast, there was less information available about 102F34 and 1032GMI, although previous studies have shown that 102F34 was more efficient than 1032GMI (Muro 2009).

Experimental design and description of TGG

Alfalfa seedlings were placed into two TGG located at the Pamplona campus of the University of Navarra, (42.80N, 1.66W; Spain). The design of the TGGs was similar to that described by Aranjuelo et al. (2005a) and Pérez et al. (2005). CO_2 concentration, temperature, relative humidity and solar radiation levels inside and outside the greenhouses were continuously monitored and controlled by a computerized system. Plants were divided into 12 treatments comprising the combination of two CO_2 levels (ambient, around 387 and elevated, 700 μmol/mol), two temperature regimens (ambient and ambient + 4°C) and three *S. meliloti* strains (102F78, 102F34 and 1032GMI). The experiment was carried out in two different seasons, summer and autumn, with similar degree-day accumulation (around 750), which means 8 weeks of growth during summer and 9 weeks of growth in autumn. According to McMaster and Wilhelm (1997), accumulated degree-days over the experiment were calculated with a base temperature of 5°C (Confalioneri and Bechini 2004). One greenhouse was maintained at ambient CO_2 concentration (approximately 392 μmol/mol CO_2), and the other at elevated CO_2 (700 μmol/mol CO_2). Each greenhouse was divided into three modules, thereby providing different temperature values. The middle module was considered as a transition module, and no experimental plants were included. In each greenhouse, the inlet module was maintained at ambient temperature (medium temperature was 18°C in summer and 14°C in autumn) and the outlet module at ambient temperature + 4°C. The CO_2 concentration was monitored continuously using a Guardian Plus (Edinburg Instruments, Livingston, UK) at the outlet module. Its signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Pots were placed at inlet and outlet modules, and rotated daily in each module to avoid edge effects. The harvest was carried out before flowering, 60-d-old plants were sampled in summer and 67-d-old plants in autumn.

Plant growth parameters

Harvested plants were separated into leaves, stems, roots and nodules. Plant dry matter (DM) was obtained after drying at 60°C for 48 h and calculated as the sum of all organs' DM.

Gas exchange

Gas exchange parameters were measured in fully expanded young leaves in 60-d-old plants in summer and 67-d-old plants in autumn using a portable gas exchange system GFS-3000 (Walz, Effeltrich, Germany). The gas exchange response to CO_2 was measured from 60 to 1 400 μmol/mol CO_2 at 1 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPDF) provided by LED light. Measurements started at 400 μmol/mol of CO_2, decreased stepwise to 250, 100, 0 μmol/mol, and restarted at 400 and increased stepwise to 700, 850, 1,000 and 1,400 μmol/mol. Net photosynthesis (A) and leaf conductance (g) were calculated as described by Long and Hallgreen (1985). Net photosynthesis measured either at 400 (A_{400}) and 700 μmol/mol (A_{700}) or at growth conditions (A_{growth}, ambient ones at 400 μmol/mol and elevated ones at 700 μmol/mol) were compared. The A/Ci curve measurements were used to assess the maximum rate of carboxylation (Vc_{max}), using the mathematical model developed by Ethier and Livingston (2004) and Sharkey et al. (2007).

N content

Leaf, stem, root and nodule samples were collected corresponding to 60-d-old plants in summer and 67-d-old plants in autumn. Samples were dried at 60°C for 48 h and analyzed for %N of total organic matter (TOM). One milligram of ground sample was used for each determination, and eight replicates were analyzed for each treatment (four experimental and two laboratory replicates). Leaf N was determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy). Total N content was calculated as the sum of N content of all organs and expressed as mg of N per plant. Leaf N content was calculated as g/m^2.

Leaf total soluble proteins and starch concentration

Leaf TSP and starch concentration were quantified by grinding and filtering 200 mg of leaf fresh weight of 60 and 67-d-old plants in summer and autumn, respectively, in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28 710 g for 15 min at 4°C. The TSP quantification was performed in supernatant, whereas starch was measured using the pellet as described by Jarvis and Walker (1993). TSP levels were measured using the methods of Bradford (1976). All measured parameters are expressed on a per area basis.

Rubisco activity and semi-quantification

Rubisco (E.C. 4.1.1.39) activity was measured by grinding and filtering 250 mg of leaf fresh weight in a cold mortar using an extraction buffer containing 100 mM bicine (pH 7.8), 10 mM $MgCl_2$, 10 mM, 2-β-mercaptoethanol and 2% PVPP. The extract was clarified by centrifugation at 26,850 *g* for 10 min at 4°C. Enzyme activity was determined by measuring the absorbance at 340 nm, as described by Lilley and Walker (1974) using a U-2001 spectrophotometer (Hitachi Instruments, San Jose, CA, USA). Rubisco total activity was calculated after incubating the leaf extract in 10 mM $NaCO_3H$ over 10 min in order to activate all rubisco protein.

For rubisco semi-quantification, extracts from protein quantification were precipitated using the sodium deoxycholate-trichloroacetic acid protocol described by Peterson (1983). The resulting pellets were air dried and resuspended in Laemmli lysis buffer (Laemmli 1970) and boiled for 10 min to denature proteins. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 4 μg of soluble proteins were prepared and electrophoresis was performed using a 150 g/L acrylamide separation gel and stained with silver nitrate (Blum et al. 1987). Gel images were scanned and analyzed using ImageQuant TL 7.0 software (GE Amersham Biosciences, UK). The relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated in reference to the abundance value of RLS observed in plants inoculated with 102F78 and grown at an ambient temperature and CO_2 concentration.

RNA isolation, synthesis of cDNA and quantitative real-time RT-PCR

Total RNA was isolated from alfalfa leaves with a phenol/chloroform extraction method (Kay et al. 1987). Rubisco large and small subunits gene expression (*RbcL* and *RbcS,* respectively*)* was studied by real-time quantitative PCR using iCycler (Bio-Rad, Hercules, CA, USA). cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20 μL reaction containing 500 ng of random hexamer primers, 0.5 mM of each dNTP, 10 mM dithiothreitol (DTT), 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, CA, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify *RbcL* were: 5′-GAGTAGCTCTGGAAGCATGTG-3′ as forward and 5′-GACTCCATTTGGTAGCCTCAC-3′ as reverse; to amplify *RbcS* the primers used were forward 5′-TTCGGAGCCACTGATTCTTCTC-3′ and reverse 5′-ACTGCACTTGACGAACATTGTC-3′. Each 25 μL q-PCR reaction contained 1 μL of a 1:10 dilution of the cDNA, 200 nM of the dNTPs, 400 nM of each primer, 3 mM $MgCl_2$, 2.5 μL of 1x SyBR Green (Molecular Probes, Eugene, OR, USA), and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted of a 4 min incubation at 95°C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 94°C, 45 s at 69°C, and 50 s at 72°C, where the fluorescence signal was

measured. The results obtained on the different treatments were standardized according to the β-tubulin gene expression levels, which were analyzed with primer forward 5′-GAAGCAAGCGGTGGAAGATATG-3′ and primer reverse 5′-CCAAATGGACCAGAACGCAAAC-3′, which showed stable expression under the conditions tested in this study.

Real-time PCR experiments were carried out with at least four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Negative controls without cDNA were used in all PCR reactions. The relative gene expression of rubisco large (*RbcL*) and small (*RbcS*) subunits was calculated in reference to the abundance value of *RbcL* and *RbcS* observed in plants inoculated with *S. meliloti* 102F78 and growth at ambient temperature and CO_2 concentration.

Statistical analysis

Statistical analysis was undertaken with three factor ANOVA (factorial 2 × 2 × 3) (SPSS v.15.0), taking CO_2 as the first factor, temperature as the second factor and *S. meliloti* strain as the third factor. In total, 12 treatments were performed, with four experimental replicates. Significant differences between factors and interactions were calculated at 5%, 1% and 0.1% levels of significance. When differences between treatments were significant according to ANOVA, least significant differences were evaluated post-hoc using the least significant difference test ($P \leq 0.05$).

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