



Research Article

Effects of Elevated Carbon Dioxide on Photosynthesis and Productivity of Alfalfa in Relation to Seasonal Changes in Temperature

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Alfalfa (*Medicago sativa* L.) was grown at ambient and elevated (ambient + 350 $\mu\text{mol mol}^{-1}$) $[\text{CO}_2]$ for four years in field plots. Periodic harvests and measurements of leaf photosynthesis were used to determine whether the stimulation of yield and photosynthesis by elevated $[\text{CO}_2]$ increased with temperature. There was no correlation between the stimulation of yield at elevated $[\text{CO}_2]$ and the temperature during the re-growth interval in any year. Short-term elevation of $[\text{CO}_2]$ for leaves of ambient-grown plants increased photosynthesis by an average of 1.5 fold, but there was no significant correlation with temperature. Leaves of plants grown at elevated $[\text{CO}_2]$ had photosynthetic rates as much as 25 % lower than for ambient-grown plants when both were measured at elevated $[\text{CO}_2]$. The down-regulation of photosynthesis was greater at cooler times of the year. The maximum carboxylation rate ($V_{c,\text{max}}$) increased more with temperature in alfalfa than the response used in standard C_3 photosynthesis models. Photosynthetic rates measured at elevated $[\text{CO}_2]$ were not limited by $V_{c,\text{max}}$ but by the maximum rate of electron transport (J_{max}). The limitation of photosynthesis by J_{max} and variation in J_{max} caused the lack of temperature effects on the short-term stimulation of photosynthesis by elevated $[\text{CO}_2]$.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most important forage species in temperate climates. In the United States it is grown on about 10 million hectares, and accounts for about half of the total hay production (USDA-NASS, 2004). However, there is very limited information concerning the response of alfalfa to projected higher atmospheric carbon dioxide concentrations ($[\text{CO}_2]$) or to global warming. Because alfalfa is a perennial crop normally harvested several times per year and is productive throughout the spring, summer and fall seasons (about 8 months) in Maryland, which encompasses a wide range of temperatures, it is well suited to test hypotheses concerning interactions between elevated $[\text{CO}_2]$ and temperature.

In the short-term, the relative stimulation of photosynthesis by elevated $[\text{CO}_2]$ usually increases with the measurement temperature. This is because, elevated $[\text{CO}_2]$ suppresses the oxygenation function of ribulose biphosphate carboxylase-oxygenase enzyme (Rubisco), and the oxygenation function increases more

strongly with temperature than does the carboxylation function (Long, 1991). The stimulation of photosynthesis by elevated $[\text{CO}_2]$ increases more strongly with temperature at high light than at limiting light, because the rate limitation for photosynthesis switches from Rubisco carboxylation at high light to electron transport and RuBP-regeneration at low light (Long, 1991). The expected increase in the stimulation of photosynthesis by elevated $[\text{CO}_2]$ with temperature assumes that the kinetic characteristics of Rubisco are unchanged by acclimation to prevailing temperatures (Bernacchi *et al.*, 2001), but recent studies indicate that this may not be the case (Borjigidai *et al.*, 2006; Bunce, 2000a; Medlyn *et al.* 2002b). Studies with species active at cool times of the year in temperate climates have sometimes indicated greater than expected stimulation of photosynthesis by elevated $[\text{CO}_2]$ at low temperatures (Bunce, 1998, 2000b, 2001; Greer *et al.*, 1995; Laing *et al.*, 2002; Tesky 1997). Furthermore, changes in the amount of down-regulation of photosynthesis at elevated $[\text{CO}_2]$ with temperature can obscure the expected pattern of stimulation of photosynthesis by elevated $[\text{CO}_2]$ and temperature (Lewis *et al.*, 2001; Tjoelker *et al.*, 1998). One purpose of this study was to examine the temperature dependence of the stimulation of photosynthesis by

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elevated $[\text{CO}_2]$ in alfalfa in relation to seasonal changes in temperature. Increased stimulation of photosynthesis at elevated $[\text{CO}_2]$ as a direct response to increased temperature could result in greater stimulation of plant growth at warm temperatures.

The stimulation of the growth of many plants by elevated $[\text{CO}_2]$ has been found to be reduced or eliminated at low temperatures well above those at which growth ceases (Idso and Idso, 1994). This is presumed to occur because low temperatures reduce the demand for the products of photosynthesis for growth. Therefore growth rates would become less responsive to increased photosynthesis, in addition to the fact that, as discussed above, photosynthesis may be less stimulated by elevated $[\text{CO}_2]$ at low temperatures. Reduced demand for photosynthesis could also make down-regulation of photosynthesis more severe at low temperatures, because down-regulation of photosynthesis is often attributed to reduced sink demand relative to source supply (Stitt, 1991). In annual plants increased rates of development at higher temperatures often negate the expected greater stimulation of final biomass by elevated $[\text{CO}_2]$ at higher temperatures (Morison and Lawlor, 1999). However, this is less likely to be the case in periodically harvested herbaceous perennial species. Experiments with young potted alfalfa plants in temperature-gradient tunnels (Aranjuelo *et al.*, 2006) indicated that elevated $[\text{CO}_2]$ did not increase growth at mean ambient temperatures of about 19 °C, but did when temperatures were increased by 4 °C. In this work we assessed whether elevated $[\text{CO}_2]$ would shift the productivity of alfalfa towards warmer times of year.

MATERIALS AND METHODS

This study deals with four years of field experiments with alfalfa: two years of first-year crops (2003 and 2004), and one year each of second- and third-year crops (2005 and 2006). Plots were established in early spring of 2003. The first crop planted had severe mortality the first winter after planting, so a new crop was established in the spring of 2004. The experiment was terminated after the third season of the second-planted crop (in the fall of 2006), because the density of alfalfa in the stands had gradually decreased to about a quarter of the initial density. This is about the stage where alfalfa crops are terminated and replanted in normal agronomic practice in the area.

Seeds of *Medicago sativa* L. cv. Arc were planted in soil in 5 cm diameter peat pots in a glasshouse with day/night temperatures of about 20/15 °C. Pots were thinned to one plant per pot. About 3 weeks after emergence, plants were selected for uniformity, and the peat pots were dug into the recently tilled ground

in the field chambers in mid-April of 2003 and 2004. The field plot had 12 rectangular open topped chambers, each covering 1.3 m² of ground. Chamber walls were 1.8 m high and made of clear acrylic. Blowers forced outside air into perforated plastic tubes at the bottom of the chambers at a rate of 6 m³ per minute. Pure CO₂ was introduced into the inlet of the blowers of 6 of the chambers at a rate sufficient to increase the $[\text{CO}_2]$ by 350 ± 50 mmol mol⁻¹. Carbon dioxide was added to the chambers continuously except when the ground was covered with snow. The $[\text{CO}_2]$ of each chamber was sampled every 45 minutes by an absolute infrared analyzer located in an adjacent building, and CO₂ flow rates were adjusted daily as needed. The $[\text{CO}_2]$ of the ambient air averaged 378 mmol mol⁻¹ in the daytime and 455 mmol mol⁻¹ at night. Night time values ranged from 360 to 620 mmol mol⁻¹, with lower values at higher wind speeds. In each chamber, 42 alfalfa plants were planted in a square pattern with 15 cm between plants. The soil was an alluvial silt loam with a water table at about 1.9 m. The pH was about 6.5. Potassium and phosphorous were added yearly at 16 and 2.5 g m⁻², respectively, as recommended by the state of Maryland nutrient management system, based on soil tests. Plants were dependent on N₂ fixation for nitrogen. A weather station about 50 m from the plots recorded air temperature and humidity, solar radiation, wind speed and rainfall. Plots were not irrigated.

In keeping with current agronomic practice, alfalfa was harvested at the flower bud stage. This early harvest increases protein content and decreases fiber content compared to harvesting at flowering, but may reduce total yield. When alfalfa was at the bud stage, alfalfa and all weeds in each chamber were cut to 5 cm height. All plant material from the interior area of each chamber, the area which initially contained 20 alfalfa plants, was separated by species, dried at 70 °C and weighed. The same ground area in each chamber was sampled on all occasions, regardless of the number of alfalfa plants present. All sampled alfalfa plants and weeds were separated from the chamber walls by a border row of alfalfa plants. All of the alfalfa shoots in the sample area were combined and analyzed for total nitrogen content, using a CHN analyzer.

The leaf area index of each chamber was determined non-destructively using a plant canopy analyzer (LAI-2000, LiCor, Inc., Lincoln, Nebraska) twice for each re-growth period, at 8 to 12 days after cutting and at 20 to 24 days after cutting. Leaf area index measurements were made either on overcast days or shortly before dawn, to prevent interference from direct sunlight on the canopy. For each chamber, there were two above-canopy measurements and four below-canopy measurements on each occasion.

For most re-growth periods, field measurements of photosynthesis were used to determine the short-term stimulation of photosynthesis by elevated [CO₂] under the prevailing ambient temperature for plants grown at ambient [CO₂], and to determine the degree of down-regulation of photosynthesis in plants grown at elevated [CO₂]. These measurements of leaf gas exchange were made about a week before the next harvest, in full midday sunlight, using a CIRAS-1 portable photosynthesis system (PP Systems, Amesbury, MA). A terminal leaflet of a recently fully expanded upper canopy leaf from an interior plant from each ambient chamber was enclosed in a broad leaf cuvette, and the leaflet was first exposed to the air at the ambient air temperature and humidity, and 375 mmol mol⁻¹ [CO₂]. Steady-state rates of carbon dioxide assimilation, stomatal conductance and substomatal [CO₂] (C_i) were recorded, and the [CO₂] external to the leaf was then increased to 725 mmol mol⁻¹. Gas exchange parameters were recorded after stomatal conductance and photosynthesis had stabilized at the new [CO₂]. For plants grown in the elevated [CO₂] chambers, similar measurements were made, but only at the elevated [CO₂] condition. Measurements alternated between ambient and elevated [CO₂] chambers, so that environmental conditions and time of day were not confounded with [CO₂] treatments. Leaves in which gas exchange had been measured were harvested to determine the total amount of nitrogen per unit of area, using a CHN analyzer. On each measurement date, a few leaves each from ambient and elevated [CO₂] chambers were also measured at 1000 mmol mol⁻¹ [CO₂] to determine whether photosynthetic rates measured at 725 mmol mol⁻¹ [CO₂] were saturated for CO₂. Lack of increase would indicate a limitation of photosynthesis by the rate of triose phosphate utilization (Sharkey, 1985).

On three occasions, days of year 109, 174, and 255 of 2006, the effect of short-term changes in temperature on the responses of photosynthesis to elevated [CO₂] was determined in the field, using a temperature-controlled portable photosynthesis system (CIRAS-2) equipped with a light-emitting diode light source. Steady-state rates of photosynthesis, stomatal conductance and C_i were measured in leaves of plants grown at ambient [CO₂] at 20 °C leaf temperature and 1500 mmol m⁻² s⁻¹ PPFD, first at an external [CO₂] of 375 mmol mol⁻¹, then at 725 mmol mol⁻¹. These measurements were then repeated at leaf temperatures of 25 and 30 °C. These measurements were made at nearly constant water vapor pressures, and leaf to air vapor pressure differences averaged 0.9, 1.6 and 2.5 kPa at 20, 25, and 30 °C, respectively. The complete set of measurements was made on 3 or 4 leaves on each date.

Statistics

For the testing of the effect of the [CO₂] treatments on harvested dry mass, leaf area index, and photosynthesis there were 6 replicate chambers per treatment. Within each year, repeated-measures ANOVA was used to test for effects of [CO₂] treatments, re-growth interval, and interactions between re-growth interval and the [CO₂ treatment]. Regression was used to test for effects of temperature on measured variables. A probability level of 0.05 was used as the criterion for significance.

Modeling of photosynthesis

A Farquhar-type C₃ photosynthesis model (Farquhar *et al.* 1980) was used to help interpret the observed photosynthetic rates. The temperature dependencies of the carboxylation parameters were those determined by Bernacchi *et al.* (2001) in tobacco. Estimates of V_{c,max} were taken from measurements of photosynthesis at ambient [CO₂] (Wilson *et al.*, 2000). When rates of photosynthesis of ambient-grown plants measured at elevated [CO₂] were lower than predicted from their observed V_{c,max}, the rates measured at elevated [CO₂] were used to estimate J_{max}. In analyzing short-term responses of photosynthesis to temperature, the temperature dependence of V_{c,max} was summarized by determining the "activation energy" from the exponential increase in V_{c,max} with temperature (Bernacchi *et al.* 2001).

RESULTS

The yield of alfalfa was significantly increased by the elevated [CO₂] treatment in each year except the final year (2006), when the stand was deteriorating and yield was low (Table I). Weed biomass was less than 10 % of that of alfalfa except in the first-year crop in 2003, which had a very wet spring which favored the establishment of weeds, and in 2006 when the density of alfalfa had become low. Total weed biomass was significantly higher in the elevated [CO₂] treatment only in 2006 (Table I). Total harvested biomass ranged from 21 to 42 % higher at elevated than ambient [CO₂] in the different years, with a mean increase of 28 %, while the increase in harvested alfalfa biomass ranged from 18 to 43 %, with a mean increase of 32 % (36 % excluding 2006). The percent of nitrogen in alfalfa shoots was always slightly but significantly less in plants grown at elevated than at ambient [CO₂], with mean values of 3.30 % and 3.56 %, respectively.

In 2004 and 2005, the effect of [CO₂] on alfalfa yield differed significantly with re-growth interval, while in the other two years the interaction term was not significant (Table I). Considering only the data from 2004 and 2005, the correlation coefficients between the ratio of yield at elevated [CO₂] to that at ambient [CO₂] and the mean temperature for the re-growth interval were not significant in either year (Table II). The

Table I. Harvested dry mass of alfalfa and all weeds, in grams per square meter, summed for each year of the experiment, and the probabilities associated with effects of [CO₂] treatment, re-growth interval, and their interaction. The elevated [CO₂] treatment was 350 mmol mol⁻¹ above that of the ambient outside air.

Year	2003	2004	2005	2006
Alfalfa, ambient [CO ₂]	586	1321	1406	580
Alfalfa, elevated [CO ₂]	826	1654	2007	683
P (CO ₂)	0.032	0.005	0.006	0.560
P (interval)	0.001	0.001	0.001	0.001
P (CO ₂ * interval)	0.497	0.004	0.003	0.522
Weed, ambient [CO ₂]	478	40	118	362
Weed, elevated [CO ₂]	461	53	159	465
P (CO ₂)	0.595	0.386	0.113	0.004
P (interval)	0.001	0.006	0.001	0.001
P (CO ₂ * interval)	0.077	0.823	0.764	0.100

absolute stimulation of yield at elevated [CO₂] was also not significantly correlated with temperature in either of those years (Table II).

Consistent with the lack of correlation between the stimulation of alfalfa yield by elevated [CO₂] and temperature in 2004 and 2005 and the lack of a significant interaction term on yield in 2003 and 2006, the yield data for all years show no evidence of a [CO₂] effect on the seasonal patterns of yield (Fig. 1). For established crops, yields were greatest in early spring and declined through the year for both [CO₂] treatments, while temperatures peaked in summer (Fig.

Table II. Correlation coefficients between relative and absolute differences in yield of alfalfa at ambient and elevated [CO₂] and the mean temperature for different re-growth intervals in 2004 and 2005, and the associated probability.

Year	Variable	Correlation coefficient	Probability
2004	elevated/ambient	-0.18	0.73
2004	elevated - ambient	+0.32	0.54
2005	elevated/ambient	+0.26	0.57
2005	elevated - ambient	-0.65	0.12

Table III. Activation energies of V_{c,max} obtained from measurements of short-term responses of photosynthesis to temperature of alfalfa leaves sampled on three different dates in 2006, compared to the value for tobacco obtained by Bernacchi *et al.* (2001). Activation energies were calculated as in Bernacchi *et al.* (2001). The temperature given is the mean for the 10 days prior to the measurements.

Source	Activation energy kJ mol ⁻¹	Standard error	Temperature (°C)
Day of year 109	90.0	1.5	15.1
Day of year 174	71.6	2.2	24.2
Day of year 255	81.9	3.3	19.5
Bernacchi <i>et al.</i>	65.3

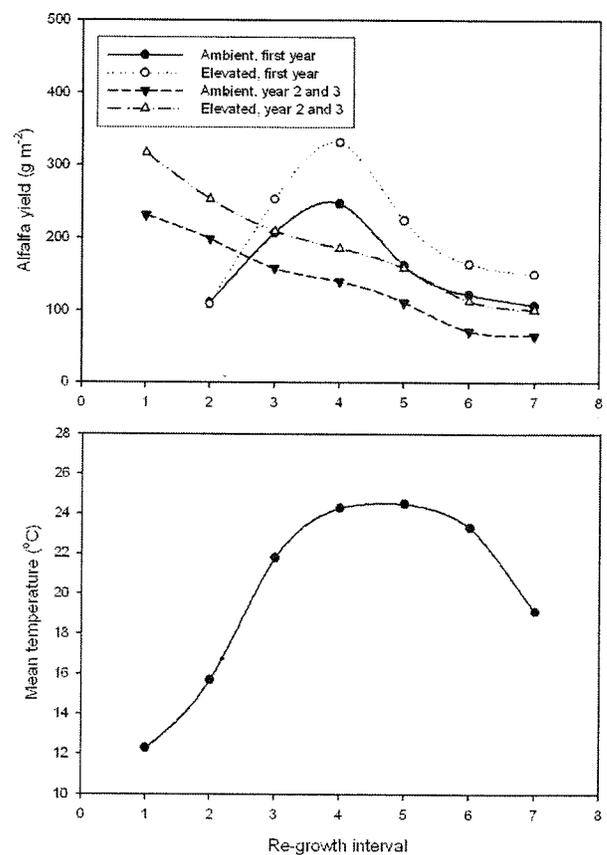


Figure 1 : Mean yield of alfalfa grown at ambient and elevated [CO₂] for each re-growth interval, and mean temperatures for the re-growth intervals. Separate yield curves are given for the first-year crops and for the older crops.

1). In first-year crops, yields were low for the first two harvests, and then followed a pattern similar to that of established crops. The average length of re-growth periods 2 through 7 was 26 ± 2 days and did not vary systematically through the season. The length of the first period is not as clearly defined, because it was difficult to determine exactly when growth resumed in early spring. The mean temperature presented in Figure 1 for the first re-growth period was for 26 days.

The overall mean leaf area index was identical for the two [CO₂] treatments, with both treatments having means of 2.46. However, when leaf index early in the re-growth periods was considered separately, the ambient [CO₂] treatment had mean values 20 % higher than the elevated [CO₂] treatment, 1.12 and 0.93, respectively, which was significant at $P = 0.05$. Leaf area index measured late in the re-growth cycle was slightly, but not significantly higher at elevated [CO₂], with values of 4.0 at elevated [CO₂] and 3.8 at ambient [CO₂]. There was no significant effect of re-growth interval on the response of leaf area index to the [CO₂] treatments (not shown).

Midday photosynthesis measured under the growth conditions of temperature, humidity, and [CO₂] in full sunlight was higher for the elevated than the ambient [CO₂] treatment on all 21 measurement occasions (Fig. 2). There were no occasions for which photosynthesis did not increase between 725 and 1000 mmol mol⁻¹, indicating that triose phosphate utilization rate did not limit photosynthetic rates measured at 725 mmol mol⁻¹. Photosynthetic rates increased somewhat with temperature at least over the lower range of temperatures for both [CO₂] treatments (Fig. 2). The difference between the [CO₂] treatments in photosynthetic rates under the midday growth conditions increased significantly with the measurement temperature (Fig. 2). However, this increase with temperature did not result from any consistent effect of temperature on the direct stimulation of photosynthesis by elevated [CO₂] (Fig. 3), but rather was caused by the fact that the amount of down-regulation of photosynthesis decreased with increasing mean temperature (Fig. 4). The r^2 value of a linear regression of the down-regulation of photosynthesis on the temperature at the time of measurement was 0.24, whereas the r^2 values using the mean temperature of the prior day or of the prior 3 days were higher, at 0.41 and 0.44, respectively (Fig. 4). The seasonal pattern of the down-regulation of photosynthesis was nearly parallel to that of seasonal temperature (Fig. 4).

The lack of temperature effect on the direct stimulation of photosynthesis by elevated [CO₂] (Fig. 3) did not result from an effect of temperature on the increase in C_i , because C_i at both the ambient and

elevated [CO₂] was nearly constant across temperatures (Fig. 3). There was no relationship between the degree of down-regulation of photosynthesis and the total solar radiation on the day prior to the photosynthetic measurements, nor for the prior 3 days (not shown). At no measurement time was there a significant difference between the [CO₂] treatments in leaf nitrogen per unit of area. The mean values were 1.82 g N m⁻² at elevated [CO₂] and 1.83 at ambient [CO₂].

The photosynthetic rates of the plants grown at ambient [CO₂] presented in Figure 2 were fit with a quadratic response to temperature to provide an estimate of $V_{c,max}$, which was then used to calculate a predicted photosynthetic response to short-term

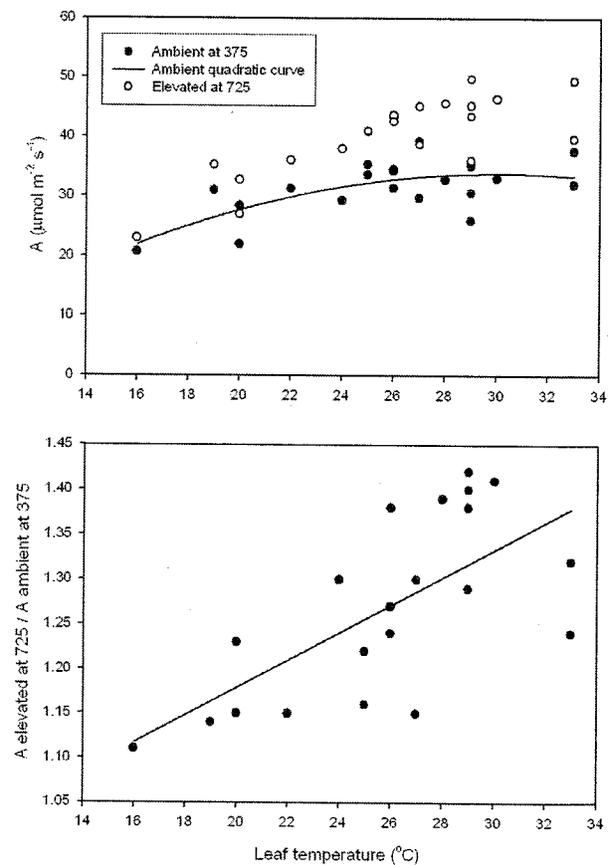


Figure 2 : Photosynthetic rates measured at the current ambient temperature, humidity, and [CO₂] for leaves of alfalfa grown at ambient and elevated [CO₂], and the ratio of rates of leaves at elevated [CO₂] to those at ambient [CO₂] as a function of the mean leaf temperature on that measurement date. Each point represents at mean of six leaves, each from a different open topped chamber. A quadratic curve was fit to the data for the ambient [CO₂] treatment in order to estimate a mean value at different temperatures. The straight line is a linear regression with a correlation coefficient of 0.624, $P = 0.004$.

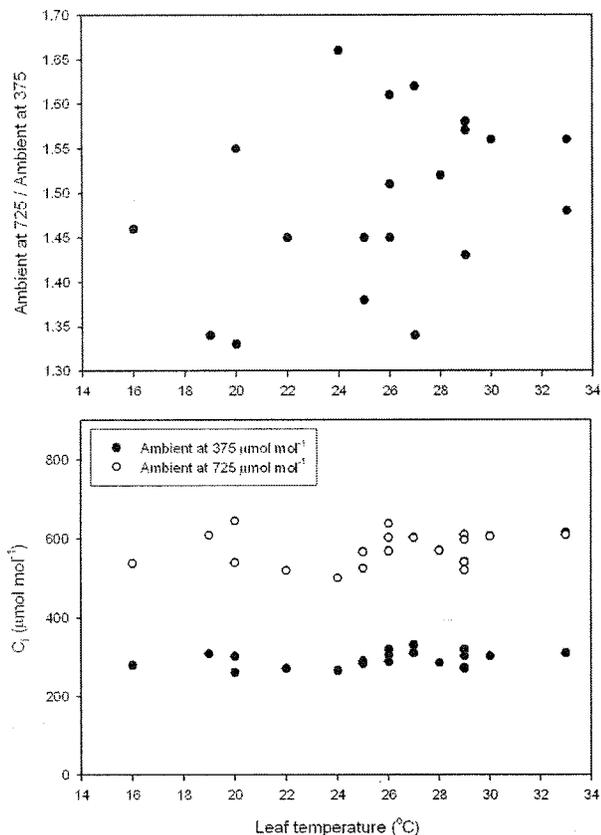


Figure 3 : The ratio of photosynthetic rates of alfalfa leaves grown at ambient [CO₂] when measured at 725 and 375 mmol mol⁻¹ at the current ambient temperature and humidity at different times of year, and the internal [CO₂] (C_i) values at the two measurement [CO₂]. Each point represents at mean of six leaves, each from a different open topped chamber.

elevation of [CO₂] under the assumption that photosynthesis at elevated [CO₂] remained limited by V_{c,max}. The predicted rates of photosynthesis at elevated [CO₂] formed an upper envelope over the observed rates (Fig. 5), with most observed rates well below this limit. This means that in almost all cases the rates of photosynthesis of the ambient-grown plants measured at elevated [CO₂] were not limited by V_{c,max}.

Measurements of the effect of short-term changes in temperature on the stimulation of photosynthesis by elevated [CO₂] also indicated that the stimulation of photosynthesis by elevated [CO₂] did not change substantially with temperature (Fig. 6). These experiments also indicated that photosynthetic rates measured at elevated [CO₂] were below those predicted from a limitation of photosynthesis by V_{c,max} at all temperatures (Fig. 6). The responses of photosynthetic rates measured at ambient [CO₂] to temperature were used to determine the temperature dependence of

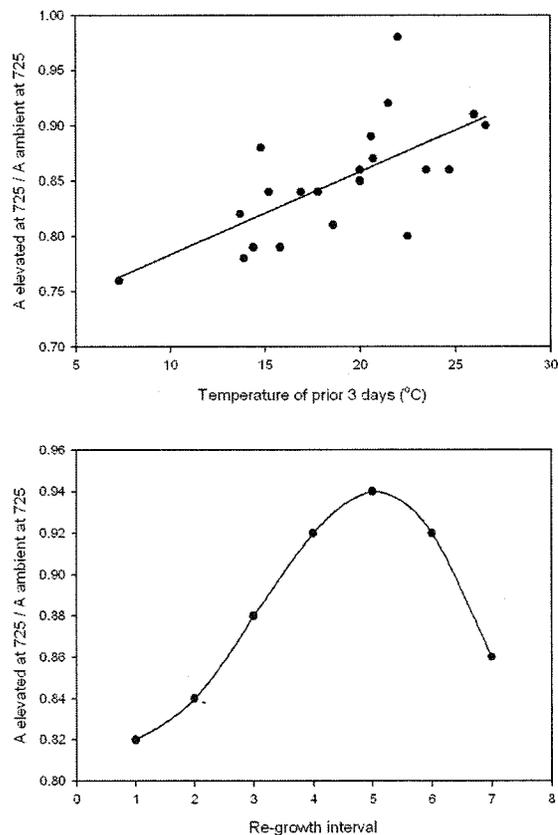


Figure 4 : The ratio of photosynthetic rates of leaves grown at elevated [CO₂] to those grown at ambient [CO₂] when both were measured at 725 mmol mol⁻¹ [CO₂], as a function of the mean temperature for the three days prior to the measurements (A), and as a function of the re-growth interval (B). Photosynthetic measurements were made at the current temperature and humidity at different times of year. Each point in A) represents a mean ratio based on six leaves per [CO₂] treatment. The linear regression had a correlation coefficient of 0.660, P = 0.001. Each point in B) represents a mean of two to four measurement dates, with six leaves measured per [CO₂] treatment on each date.

V_{c,max} for the three times of year when these measurements were made. As expected, V_{c,max} increased exponentially with temperature. However, the response to temperature, as quantified by the activation energy was steeper in spring than fall or summer, and was related to the prevailing temperatures (Table 3). At all three times of year, the activation energy of V_{c,max} in alfalfa was significantly larger than found in Rubisco from tobacco (Table III). Photosynthetic rates measured at elevated [CO₂] were used to estimate the response of J_{max} to temperature. J_{max} increased relatively less between 25 and 30 °C for plants measured in summer than in spring or fall (Fig. 6).

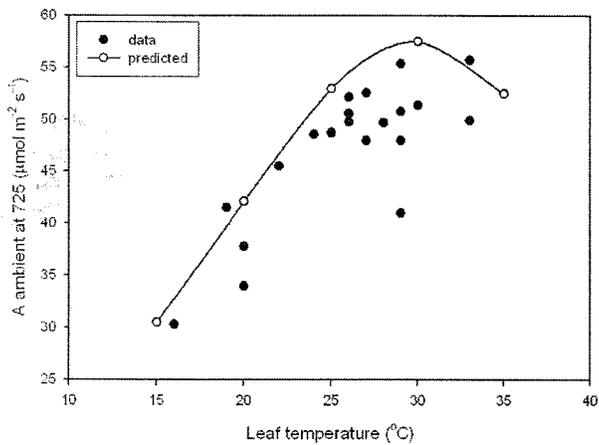


Figure 5 : Photosynthetic rates of leaves of alfalfa plants grown at ambient [CO₂], measured at the current temperature and humidity, at 725 mmol mol⁻¹ [CO₂], and the rates predicted from measurements made on the same leaves at lower [CO₂] and extrapolated to rates at higher [CO₂] using a model of photosynthesis with the assumption that V_{c,max} was always limiting to photosynthesis, as a function of leaf temperature. Each data point represents a mean of six leaves measured on a given date.

DISCUSSION

The data on yield of alfalfa in this study showed that there was no increase in the [CO₂] effect on yield at higher seasonal temperatures and that elevated [CO₂] did not cause a shift in the seasonal pattern of production (Fig. 1). Luscher *et al.* (2000) reported that elevated [CO₂] increased harvested alfalfa plant mass by about 50 % over 2 seasons, which is a somewhat larger increase than found here, but they provided no information on seasonal patterns of the response. The lack of an effect of the season of the year on the relative yield response to elevated [CO₂] found here in alfalfa is similar to the results reported for white clover (Hebeisen *et al.*, 1997). Results for these two species contrast with the response of ryegrass (Casella *et al.*, 1996), where the growth stimulation by elevated [CO₂] was larger in summer than in spring or fall. However, even in ryegrass a 3 °C temperature increase reduced the effect of elevated [CO₂] on yield in summer (Casella *et al.* 1996), indicating that elevated [CO₂] also did not improve the tolerance of the yield of that crop to high temperatures. There are clearly exceptions to the idea that higher temperatures will increase the stimulation of growth by elevated [CO₂] in herbaceous perennial crops.

The lack of an effect on temperature on the increase in yield of alfalfa due to elevated [CO₂] in this study occurred despite a larger CO₂-induced increase in leaf photosynthesis measured under the midday growth

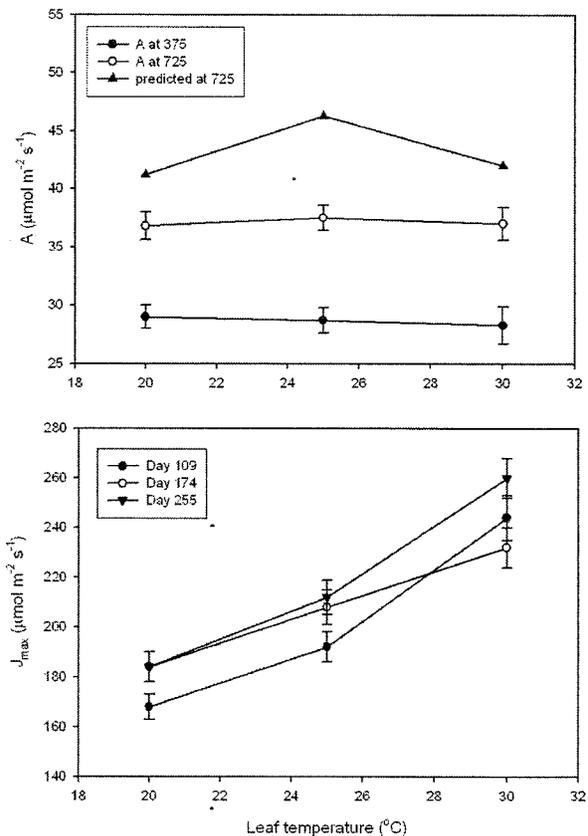


Figure 6 : Responses of photosynthesis of alfalfa leaves grown at ambient [CO₂] to short-term changes in [CO₂] and temperature, and the rates at the higher [CO₂] predicted from measurements made on the same leaves at lower [CO₂] and extrapolated to rates at higher [CO₂] using a model of photosynthesis with the assumption that V_{c,max} was always limiting to photosynthesis (A), and J_{max} values determined from photosynthetic rates measured at high [CO₂] at three times of year (B). Vertical bars indicate SE for 10 leaves in A), and for 3 or 4 leaves in B).

conditions at warmer temperatures (Fig. 2). It is, of course, not uncommon for the responses of plant growth and leaf photosynthesis to differ in their response to environmental changes. Such divergence implies environmental effects on the utilization of the products of photosynthesis. The smaller leaf area index at elevated [CO₂] early during the re-growth periods suggests that elevated [CO₂] reduced the efficiency of production of leaf area. This could simply result from lower specific leaf area. The decline in yield of alfalfa from spring through summer and into fall is typical for this species in temperate climates, but no firm explanation has been established. A simple negative response to temperature would not explain why yield remains low in the fall after temperatures decline. However, solar radiation is lower in fall than spring at

the same mean air temperature, and soil temperatures are higher. The plants could also be responding to the short photoperiods by altering resource allocation in preparation for winter. The decline in yield with the increase in temperature from spring to summer for both [CO₂] treatments can not be explained by single leaf photosynthetic rates, as air temperatures did not exceed the optimum temperature for leaf photosynthesis at either [CO₂] level (Fig. 2).

The greater down-regulation of photosynthesis of plants grown at elevated [CO₂] at low temperatures observed here in alfalfa (Fig. 4) was responsible for the temperature dependence of the difference between the [CO₂] treatments in midday photosynthesis (Fig 2). Despite its importance to understanding plant carbon balances at elevated [CO₂], there are very few species in which variation in the amount of down-regulation of photosynthesis at elevated [CO₂] is yet predictable from weather variables. In soybeans, the amount of down-regulation was related to the water vapor pressure deficits on the day prior to the measurements (Bunce and Sicher, 2001). In collards, it was related to the amount of solar radiation experienced by the plants the day before the measurements (Bunce and Sicher, 2003). Evidence of down-regulation of photosynthesis at elevated [CO₂] in strawberry disappeared during water stress and freezing stress, probably because these stresses reduced rates of photosynthesis of the plants grown at ambient [CO₂] more than at elevated [CO₂] (Bunce, 2001). In alfalfa, the fact that the correlation was much higher with the mean temperature of the prior days than with the current temperature indicates that it was not the assay temperature that was most important to down-regulation of photosynthesis at elevated [CO₂], but more likely related to source-sink balance, with low temperatures reducing sink strength. On the other hand, no effect of prior solar radiation on the amount of down-regulation was detected in alfalfa, and further studies would be required to establish source-sink balance as the cause of the down-regulation of photosynthesis. A short-term regulatory response is however suggested by the fact that down-regulation of photosynthesis occurred without any decrease in leaf nitrogen per unit of leaf area such as occurs with sugar repression of gene expression (Moore *et al.*, 1999) or nitrogen deficiency (Stitt and Krapp, 1999) induced by elevated [CO₂].

The seasonal pattern of down-regulation of photosynthesis in alfalfa grown at elevated [CO₂] indicated more down-regulation in spring and fall than in mid-summer (Fig. 4), consistent with the idea that cool temperatures limited sink activity. Seasonal patterns of down-regulation of photosynthesis at elevated [CO₂] in trees (Hymus *et al.*, 2001; Lewis *et*

al., 2001) and clover (Ainsworth *et al.*, 2003) have also been attributed to changes in sink activity, but in all of these studies, including alfalfa, the evidence of sink limitation is only circumstantial. In annual crops, changes in the extent of down-regulation of photosynthesis at elevated [CO₂] through the season may occur and may be related to plant ontogeny and nitrogen requirements for reproduction (e.g. Adam *et al.*, 2000; Seneweera *et al.* 2002).

The maximum carboxylation rate ($V_{c,max}$) is a key parameter in biochemically-based models of C₃ photosynthesis (Farquhar *et al.*, 1980). When calculated from photosynthetic rates and C_i , the apparent $V_{c,max}$ incorporates the resistance to CO₂ movement from outside cell walls to the site of fixation, and there are other uncertainties in its estimate (Bunce 2000b). However, Bernacchi *et al.* (2002) concluded that until this resistance and its temperature dependence are better known, the simplifications are acceptable for modeling photosynthesis. Some have argued that $V_{c,max}$ should have the same dependence on temperature in all C₃ plants because of the conservative properties of the Rubisco enzyme (Bernacchi *et al.*, 2001). However, the values of the activation energy of $V_{c,max}$ found here in alfalfa were always higher than the value for tobacco obtained by Bernacchi *et al.* (2001), which is used in many photosynthesis models. In alfalfa, increased values of the activation energy occurred when prior temperatures were low (Table 3), which is opposite to the pattern in some other species (Hikosaka *et al.*, 2006). Medlyn *et al.* (2002b) also found a large variation in activation energy of $V_{c,max}$ in pine at different times of year, but relationships with prior temperature were unclear. Bunce (2000a), Hikosaka *et al.* (1999) and Yamori *et al.* (2005) also found significant variation in the temperature dependence of apparent $V_{c,max}$ in plants acclimated to different temperatures and among species. A review by Leuning (2002) also pointed out the wide variation the temperature dependence of apparent $V_{c,max}$ at high temperatures. Yamori *et al.* (2005) suggested that there may be populations of Rubisco with different thermal properties within a given leaf. Variation in the temperature dependence of $V_{c,max}$ deserves more attention, as it may contribute to the acclimation of photosynthesis to temperature similarly to the better known variation in absolute value of $V_{c,max}$ (Bunce, 2000a; Hikosaka *et al.* 2006).

The maximum rate of electron transport (J_{max}) is another key parameter of photosynthesis models. Several studies have shown that both the absolute magnitude of J_{max} and its dependence on temperature may vary with growth conditions (Bunce, 2000a; Hikosaka *et al.*, 1999; Medlyn *et al.*, 2002b; Yamasaki *et al.*, 2002; Ziska, 2001). June *et al.* (2004) suggested that variation in the temperature dependence of J_{max} was

primarily due to shifts in the optimum temperature and to a lesser extent in the breadth of the optimum. The data obtained here for alfalfa did not include high enough temperatures to estimate the optimum temperature of J_{\max} , but the curve obtained in summer was clearly of a different shape than those obtained in spring and fall (Fig. 7).

In addition to changes in the temperature response of J_{\max} , its magnitude has also been found to vary with growth conditions (Bunce, 2000a; Hikosaka *et al.*, 1999; Onoda *et al.*, 2005a; Onoda *et al.*, 2005 b; Ziska, 2001). At 25 °C, the ratio of J_{\max} to $V_{c,\max}$ averages about 1.7 to 1.9 across species and growth conditions (Bunce, 2000a; Medlyn *et al.*, 2002a; Leuning, 2002), but can range from about 1 to 2.5. In alfalfa, the measurements of short-term responses to temperature and [CO₂] indicated ratios of J_{\max} to $V_{c,\max}$ at 25 °C of 1.26 to 1.40. These unusually low ratios may explain why photosynthetic rates measured at elevated [CO₂] were generally limited by J_{\max} rather than $V_{c,\max}$ in alfalfa. Similarly to the data reported here for alfalfa, the limitation of rates of photosynthesis at elevated [CO₂] by J_{\max} in *Abutilon* was responsible for the lack of increase with temperature in the short-term stimulation of photosynthesis by elevated [CO₂] (Ziska, 2001). In dandelion, photosynthetic rates measured at elevated [CO₂] at high temperatures in the field were also limited by J_{\max} (Bunce, 2000b), but $V_{c,\max}$ was always limiting in wheat and barley (Bunce, 1998) and strawberry (Bunce, 2001). Photosynthesis at elevated [CO₂] has often been discussed in terms of limitations of photosynthesis by $V_{c,\max}$, reductions in Rubisco, and changes in the ratio of J_{\max} to $V_{c,\max}$ and its effect on the efficiency of the use of nitrogen (e.g. Ainsworth *et al.*, 2007; Sage, 2004.). However, papers presenting field data on photosynthesis at elevated [CO₂] have seldom documented whether $V_{c,\max}$ or J_{\max} limits photosynthesis. In rice, Borjigidai *et al.* (2006) reported that photosynthesis at elevated [CO₂] at the optimum temperature was sometimes limited by $V_{c,\max}$ and sometimes by J_{\max} , but provided no information on which was limiting at the prevailing temperature. In perennial ryegrass, $V_{c,\max}$ limited photosynthesis measured at 25 °C at elevated [CO₂] (Ainsworth *et al.*, 2003; Davey *et al.*, 1999), but J_{\max} limited photosynthesis in white clover grown with moderate nutrients (Davey *et al.*, 1999). The possibility that J_{\max} rather than $V_{c,\max}$ may limit photosynthesis at elevated [CO₂] even at high light should be investigated, because, as evident here in alfalfa, which component limits photosynthesis can greatly affect interactions between elevated [CO₂] and temperature.

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