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THE EFFECTS ON PHOTOSYNTHETIC CO₂ ASSIMILATION TO LONG-TERM
ELEVATION OF ATMOSPHERIC CO₂ CONCENTRATION: AN ASSESSMENT OF
THE RESPONSE OF *TRIFOLIUM REPENS* L. CV. *BLANCA* GROWN AT F.A.C.E

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**THE EFFECTS ON PHOTOSYNTHETIC CO₂ ASSIMILATION
TO LONG-TERM ELEVATION OF ATMOSPHERIC CO₂
CONCENTRATION: AN ASSESSMENT OF THE RESPONSE
OF *TRIFOLIUM REPENS* L. CV. *BLANCA* GROWN AT F.A.C.E.**

by Charlotte E. Lewis.

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Keywords: *Trifolium repens*, photosynthesis, elevated CO₂, stomatal characteristics, FACE, *Chrysanthemum leucanthemum*, *Ranunculus friesianus*, *Plantago lanceolata*.

ABSTRACT

Understanding how photosynthetic capacity acclimates to elevated CO₂ concentrations is vital in predicting the response of important grassland species such as *Trifolium repens*. Previous studies of acclimatisation have been carried out in artificial experimental conditions, such as acrylic greenhouses or controlled environment chambers. The advent of FACE technology has enabled a large area of crop to be fumigated in the field, providing more realistic growing conditions. Pure stands of *Trifolium repens* L. cv. *Blanca* grown at either 355 or 600 $\mu\text{mol mol}^{-1}$ CO₂ were examined, and their photosynthetic response to elevated c_a determined via gas exchange studies. Rates of photosynthesis of young, fully expanded leaves were increased between 21 and 36% when grown and measured at elevated CO₂. This increase in A corresponded to a decrease in g_s of between 18 and 52%. No acclimation effect was observed in the most frequently cut stands, whilst the response of stands clipped only 4 times per year was more variable. When downregulation of V_{cmax} did occur, this was not nearly as marked as that which occurred in 3 other temperate species (*Chrysanthemum leucanthemum*, *Ranunculus friesianus*, *Plantago lanceolata* (L.) J. & C. Presl.), at similar growth regimes. No acclimation of stomatal frequency, SI or pore length was found to occur in the enriched clover stands.

Abbreviations: A, net rate of CO₂ uptake per unit leaf area ($\mu\text{mol m}^{-2} \text{s}^{-1}$); c, CO₂ concentration in air ($\mu\text{mol mol}^{-1}$); c_a , c in the bulk air; c_i , c in the intercellular spaces; E, transpiration per unit leaf area ($\text{mol m}^{-2} \text{s}^{-1}$); FACE, free-air CO₂ enrichment; g_s , stomatal conductance for water; J_{max} , the maximum rate of regeneration of rubP

($\mu\text{mol m}^{-2} \text{s}^{-1}$); P_i , inorganic orthophosphate; PPFD, photosynthetic photon flux density ($\mu\text{mol m}^{-2} \text{s}^{-1}$); rubisCO, ribulose 1:5 bisphosphate carboxylase/oxygenase; rubP, ribulose 1:5 bisphosphate; SI, stomatal index; V_{cmax} , maximum rate of carboxylation ($\mu\text{mol m}^{-2} \text{s}^{-1}$).

1. INTRODUCTION

The global rise in atmospheric CO₂ is a well-established phenomenon. Human activities, such as fossil fuel burning and changes in land use activities are a substantial component of this change. Given the current trend, models of global climate change predict a future doubling of c_a ; from 354 $\mu\text{mol mol}^{-1}$ in 1990, to 700 $\mu\text{mol mol}^{-1}$ in 2100 (Edwards *et al.*, 1984; Watson *et al.*, 1990).

Photosynthesis is the major physiological process by which plants sense and respond to, rising c_a (Mott, 1990). Their response is invariably complex, and dependent upon a variety of physiological and environmental variables (McKee & Woodward, 1994). Understanding this response is fundamental to the analysis of plant productivity and growth (Long *et al.*, 1993), and furthermore, is an essential component in the assessment of future vegetation-induced effects on the flux of atmospheric CO₂.

C₃ plants grown under elevated c_a often show an initial short-term enhancement of photosynthesis (for reviews see Strain & Cure, 1985; Cure & Acock, 1986), accompanied by a decrease in stomatal conductance (g_s) and of transpiration (E), (Pearcy & Bjorkman, 1983). In many instances, however, this initial photosynthetic stimulation gradually erodes with time, often resulting in similar, or even lower rates compared with plants grown in ambient CO₂ (DeLucia *et al.*, 1985; Tissue & Oechel, 1987; Sage *et al.*, 1989; Tissue *et al.*, 1993). The term 'acclimatisation' has been used to describe these effects on photosynthetic development and maintenance which, in turn determines photosynthetic capacity (Long *et al.*, 1993).

The reasons for acclimation of photosynthesis after long term exposure to elevated CO₂ are not fully understood, but several explanations have been put forward. Accumulation of carbohydrates in the leaves due to a source-sink imbalance may lead to direct inhibition as a result of mechanical damage (DeLucia *et al.*, 1985), or by

feedback inhibition through P_i -limitation (Stitt, 1991). Alternatively, a decrease in ribulose-1, 5-bisphosphate carboxylase / oxygenase (rubisCO) activity is often correlated with photosynthetic decline after prolonged exposure to high c_a levels (Vu *et al.*, 1987; Tissue *et al.*, 1993).

RubisCO catalyses the fixation of CO_2 in the Calvin cycle, and is generally thought to control ('limit') the maximum rate of light-saturated photosynthesis (cf. Stitt & Schulze, 1994). Increasing atmospheric CO_2 levels will increase the CO_2 / O_2 ratio at the rubisCO fixation site, thereby favouring the carboxylation reaction and accounting for the observed initial increase in A. Sharkey (1988), has calculated that a doubling of present c_a could reduce the ratio of Photorespiration : Photosynthesis, in the absence of acclimation effects. If acclimation occurs through long-term exposure, rubisCO activity is generally observed to decrease, but this effect is not a universal one (Sage *et al.*, 1989; Rowland-Bamford *et al.*, 1991). In some species this results from a reduction in the quantity of rubisCO, in others rubisCO activity decreases, and still in some, both may change.

Perhaps a more coherent explanation can be achieved through the use of the biochemically based mechanistic models developed by Farquhar and colleagues (Farquhar & Von Caemmerer, 1982; Sharkey, 1989). These models have been widely tested and validated, the most recent of which comes from flux control coefficient studies with antisensed tobacco plants (Stitt *et al.*, 1991, Stitt & Schulze, 1994). According to Farquhar's model, photosynthesis is colimited by three processes; rubisCO activity, rubP regeneration and triose phosphate use (Long *et al.*, 1993; McKee & Woodward, 1994). In photosynthesis research, the response of A to c_i is routinely measured, providing an *in vivo* technique of separation of these effects. The A / c_i response consists of 2 phases; an initial linear response where the effect of carboxylation, V_{cmax} (i.e., the amount of active rubisCO) determines the slope, followed by an inflexion to a slower rise or plateau, determined by J_{max} , where A is

limited by the supply of rubP for carboxylation (Long *et al.*, 1993). Any induced change in the capacity for rubP carboxylation often appears to be matched by change in the capacity for rubP regeneration, so that both processes will remain co-limiting (Long & Drake, 1992). This suggests an optimisation of resource distribution, so that neither active rubisCO nor the apparatus for regeneration of rubP are in excess. In plants grown in elevated c_a , such an adjustment would also be expected, since RubisCO requires a large investment in terms of energy and N resources. Acclimation therefore may be achieved either from a down regulation of V_{cmax} or from an increase in the capacity for J_{max} (Long *et al.*, 1993).

It is necessary to reiterate the point that while acclimation is a commonly observed phenomena, it is by no means a universal effect. Acclimation appears to be most marked in species where photosynthesis is limited by the capacity to generate new sinks (Long & Drake, 1992). Field studies have, in many instances, shown that plants may not experience the downward regulation of photosynthetic capacity typically observed in long-term CO_2 enrichment experiments with pot grown plants (Arp, 1991). In conditions where the plant is grown in some type of container, photosynthesis and growth can readily be retarded by restricted development of active root sinks (Idso & Imball, 1991). Indeed, when Radin (1987) grew cotton in the field, no acclimation response was measured for most of the growing season. This is in complete contrast to the earlier findings of Delucia *et al.* (1985), who reported a strong negative correlation between leaf starch and photosynthesis in cotton grown in controlled environments.

Stomatal acclimation to long-term exposure to elevated c_a has also been observed. Because stomatal conductance affects photosynthesis by changing c_i , stomatal acclimation may have important consequences for gas exchange. It has been suggested that an inverse relationship exists between CO_2 concentration and stomatal density (Madsen, 1973; Woodward & Bazzaz, 1988). Woodward (1987), in a study of

herbarium material collected from central England over the last 200 years, found evidence of a decrease in stomatal density by 40% with increasing atmospheric CO₂. More importantly, he showed that any change in stomatal density was paralleled by a change in the SI, rather than changes in leaf cell expansion. Much of the current evidence available, however, is conflicting. Recent studies have shown that increasing CO₂ above current ambient levels had no effect on stomatal density, or SI (Woodward, 1986; Ryle & Stanley, 1992). It would appear, therefore, that predicting the effects of climate change on stomatal characteristics will be difficult, a theory echoed by Ferris and Taylor (1994) who found that component species of a plant community may differ in their response to elevated CO₂.

The effect of elevated CO₂ on photosynthesis has been studied infrequently in the pasture community of Western Europe (Ryle & Stanley, 1992). Any CO₂ induced change will have important consequences in terms of biomass production and its annual performance. The most important agricultural indigenous pasture legume is white clover, *Trifolium repens* L. cv. *Blanca* (Ryle & Stanley, 1992). At the present time, where economic and political backgrounds are progressively constraining fertiliser use, there is growing interest in the possibility of greater use of white clover in forage systems. Because of its ability to fix atmospheric nitrogen in association with *Rhizobium* in its root nodules, white clover may possess the potential to provide an alternative source of N, both for its own growth, and for that of a companion grass, *Lolium perenne* L. Set against this advantage would be any effect that management practices, such as cutting, may exert on photosynthetic responses to elevated c_a; any variable that alter the source:sink balance of a plant, will also have a profound influence on the extent of photosynthetic adjustment at high CO₂ (Arp, 1991). Temporary removal of the carbon source by trimming leaves in soybean plants has been found to alleviate the decrease in photosynthetic capacity (Peet, 1984). The effects on such management practises on *Trifolium* has been little studied in a field situation and warrants further research.

Enrichment studies to date have almost exclusively been carried out in acrylic enclosures or controlled environments (Nijs *et al.*, 1988; Nijs *et al.*, 1989; Ryle & Stanley, 1992). Since plant productivity is not exclusively controlled by any one environmental parameter, these experimental conditions do not provide a 'true' picture of the response of clover in the field to elevated CO₂. Techniques of CO₂ enrichment through release of gas into open-field crops have been little used due to the difficulty in controlling concentrations, and the cost of CO₂ (cf. Lawlor & Mitchell, 1991). Free-air CO₂ enrichment (FACE), is a relatively new development, set up for long-term fumigation studies over a relatively large crop area. It is now being applied to stands of *T.repens* and *L.perenne* in a project at the Swiss Federal Institute of Technology (ETH), Switzerland (Hendrey & Kimball, 1990). Unlike previous work, this study has several advantages in that a large area of crop can be provided for analysis, and that environmental parameters are inevitably more realistic.

The purpose of this study was to determine, via leaf gas exchange studies, the effects of elevated atmospheric CO₂ on photosynthesis, and, through stomatal and epidermal cell counts, any changes in stomatal responses, and their consequence for gaseous exchange. In addition, the influence of grassland management practises, such as cutting and fertilisation was investigated. For direct comparison, the photosynthetic responses of three other temperate grassland species (*Chrysanthemum leucanthemum* L., *Ranunculus friesianus* (VRfri 6), *Plantago lanceolata* L.), also grown at the FACE system were determined. Three important questions were asked;

Is photosynthesis of white clover enhanced in elevated CO₂? Does acclimation result from long-term growth in these conditions, and if it does, are these responses of a similar magnitude as that observed in other comparable C₃ species. In addition, will different cutting treatments exert any effect? Finally, is there any change in stomatal characteristics of white clover in response to elevated c_a, and will this have any consequence to overall gas exchange?

2. MATERIALS AND METHODS

2.1 Plant Growth Conditions

White clover plants (*Trifolium repens* L. cv. *Blanca*), and three other temperate grassland species (*Chrysanthemum leucanthemum*, *Ranunculus friesianus* (VRfri 6), and *Plantago lanceolata*), were grown at ambient (*ca.* 355 $\mu\text{mol mol}^{-1}$), and 600 $\mu\text{mol mol}^{-1}$ CO_2 in the field using a Free-Air-Carbon Dioxide-Enrichment (FACE) technique (Fig. 1). A detailed description of FACE systems has been given earlier (Hendrey *et al.*, 1993). Pure stands of *Trifolium* established in each of 3 control and 3 FACE rings were examined between May and July 1994. These stands were established in 1993 when the CO_2 treatments were initiated. In 1994, daytime fumigation of the FACE rings started on 7 March.

During the growing period (taken as between 25 April and 1 November 1994), *Trifolium* stands were fertilised at either 560 or 140 $\text{kg N ha}^{-1} \text{ year}^{-1}$, and subjected to 8 or 4 cutting treatments. At each cut, shoots were clipped by hand to approximately 3cm above ground level.

CO_2 concentration, PPFD, air temperature, relative humidity and precipitation were continuously monitored in the rings. In 1993, CO_2 concentration (based on a 1 minute average), was maintained at $600 \pm 60 \mu\text{mol mol}^{-1}$ for approximately 91% of the total fumigation time. Meteorological data is currently unavailable.

FIG 1: A detailed site map of the FACE facility. Stands of *T.repens*, *C. leucanthemum*, *R. frisianus* (VRfri 6), and *P. lanceolata* were grown in rings at CO₂ concentrations of 600 (FACE) or ca. 355 $\mu\text{mol mol}^{-1}$ (Control). CO₂ concentrations in control rings are not affected through the proximity of FACE.

2.2 Single Leaf Gas Exchange Analysis

Leaf gas exchange was measured in an open gas analysis system (CIRAS 1 -version 1.4 -PP. Systems, Hitchin, Herts., UK.). This system incorporated a circular leaf chamber, diameter 1.8cm (Version 1.1-PP. Systems, Hitchin, Herts., UK.). The infra-red gas analyser was regularly calibrated with CO₂ calibration gas containing 604.7 $\mu\text{mol mol}^{-1}$ CO₂ (27548 -Type 30L Carbagas, Swiss Calibration), and with water vapour at 20mb generated from a water vapour generator (Type WG -600 - Analytical Development Co., Hoddesden, UK.). Records were stored in the analysis system, and later downloaded onto a P.C. for conversion into spreadsheet form (Microsoft EXCEL Version 4.0).

Whenever possible, only the youngest fully expanded leaves at the top of the canopy were selected for measurement. Leaflets were sealed into the leaf chamber with the adaxial surface pointed upwards. In all cases, the leaflets were of sufficient area to completely fill the chamber, providing a constant leaf area of 2.5 cm².

In all measurements, representative leaves were sampled from each ring in the following order C2, F1, C1, F2, F3, C3. This order was preferential due to time limitations and their relative proximity. Dates and treatments over which these measurements were made are shown (Table 1).

TABLE 1: Timetable of gas exchange measurements for *T. repens* grown in stands at the FACE facility, Switzerland. Measurements were made using an open gas analysis system on the youngest, fully expanded leaves at the top of the canopy whenever possible. All measurements encompassed two cutting regimes (8 and 4 cuts year⁻¹) taken at low N levels (140 kg N ha⁻¹ year⁻¹), except * which denotes measurements made at the 8 cut regime at two levels of fertilisation. **represents measurements for 3 temperate grassland species; *C. leucanthemum*, *R. frisianus*, and *P. lanceolata*.

Measurement Date	Gas Exchange Analysis
May 24	A/ci response
June 1	A/ci response
June 10	A/ci response
June 13	HARVEST (8 Cut regime)
June 24	Diurnal
June 29	Diurnal*
June 30	A/ci response**
July 5	A/ci response
July 11	HARVEST (8 + 4 Cut regime)
July 17	A/ci response
July 23	Diurnal
July 24	A/ci response
July 29	Diurnal

2.2.1. Diurnal Measurements of *T. repens*

Net photosynthesis was measured on 3 representative leaves from each treatment. Each of the 6 rings were sampled every 2 hours over a diurnal period (6am - 8pm). All measurements were made at *growth* c_a and under normal daily and seasonal light fluctuations. In order to obtain comparable values for A and g_s , early morning dew was gently blotted from the leaf surface before sealing in the leaf chamber.

2.2.2. A / c_i Measurements

Using 2 representative leaves per treatment per ring, A/ c_i measurements were made over the c_a range 50 to 900 $\mu\text{mol mol}^{-1}$ after pre-equilibration in the leaf chamber at *growth* c_a . For stands grown under ambient CO_2 levels, the gas concentration passing through the leaf chamber was manipulated so that a series of measurements were made at 355, 250, 150, 50, 600 and 900 $\mu\text{mol mol}^{-1}$ CO_2 . For stands in the elevated CO_2 regime, concentrations followed 600, 250, 150, 50, 355, 900 $\mu\text{mol mol}^{-1}$ CO_2 . This was necessary in order to avoid the complications of changes in rubisCO activation which may occur in response to different c_a . However, on two of the measuring dates (1 and 10 June), due to time limitations, measurements were only made at four CO_2 concentrations, namely 350, 250, 150 and 50 $\mu\text{mol mol}^{-1}$.

A constant, saturating irradiance to the leaf surface ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) was achieved via a quartz-halide source attachment over the chamber (PP. Systems, Hitchin, Herts., UK.). A constant 12 volt power supply to the unit ensured constant illumination was maintained for approximately 8 hours.

For comparison, measurements were also made on three C₃ dicot species, *C. leucanthemum*, *R. frisianus* (VRfri6), and *P. lanceolata*. These were conducted on June 30, and one replicate from each species was sampled per ring.

Curves were fitted both for rubisCO and rubP limited photosynthesis using a maximum-likelihood method (Sigmaplot for Windows, Jandel Sci.), providing estimates of the maximum velocity of rubisCO carboxylation (V_{cmax}), and the maximum capacity for coupled electron transport (J_{max}). Because leaf temperature varied between measurements, V_{cmax} and J_{max} were extrapolated to a common 25°C for each sample from the thermal relations described by Harley *et al.* (1992).

2.3. Stomatal Measurements

In each ring, 2 replicates of abaxial and adaxial leaflet surfaces in both N treatments were sampled by a non-destructive method. A thin layer of clear nail varnish was applied to first the adaxial, and then abaxial surfaces of the leaflet. The part of the leaf used in this study was midway between the tip and the base of the leaf. Areas in the vicinity of large veins were avoided.

The nail varnish was allowed to dry before being gently peeled off using a strip of clear adhesive tape. In this way, the impression could be directly mounted onto a slide in the field. Impressions were examined under a light microscope, and the stomatal density, stomatal index (SI), and stomatal pore length determined. The number of stomata and the number of other epidermal cells were counted from five random fields of view (area = 0.05 mm²) at x40 magnification. An eyepiece graticule, pre-calibrated with a stage micrometer, was used to calculate this area. The number of stomata per field was converted to number of stomata mm⁻². From the data the stomatal index (SI) was calculated which relates the total number of stomates per field (S), to the total

number of epidermal cells (E), so that $SI = [(S/E) + S] \times 100$, (Salisbury, 1927; Ferris & Taylor, 1994). Stomatal pore length was determined from five randomly chosen stomates per field of view.

2.4. Analysis of the Data

Using EXCEL for Windows (Microsoft Version 4.0), differences between photosynthetic measurements between CO₂ treatments were compared using a two-sample t-test for means. Any differences in stomatal characteristics for the two treatments were also measured using this method. Within each CO₂ regime, the effect of cutting, or nitrogen treatment on leaf response to elevated c_a was also compared. Standard errors show any variation about the mean. In addition, individual values of J_{max} and V_{cmax} were correlated by means of a first order linear regression technique using Sigmaplot for Windows (Jandel Sci.) The probability values for the effect of CO₂ from the t-tests are indicated below.

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

3. RESULTS

3.1. Leaf Diurnal Measurements

On the four days examined (24, 29 June and 23, 29 July, 1994), photosynthetic rates were consistently higher, and stomatal conductance's consistently lower, over the diurnal course for Trifolium grown and measured in the elevated CO₂ concentration treatment (Figs. 2 & 3). Rates of assimilation, when averaged over the entire day, were between 21 and 36% higher for white clover at 600 $\mu\text{mol mol}^{-1}$ CO₂ than those at ambient CO₂ concentrations. The higher values of A in FACE grown plants corresponded with a decrease in stomatal conductance of between 18 and 52%. This stimulation of A, and parallel decline in g_s appeared completely negated at very low light intensities (<300 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

The response of A to elevated CO₂ was not found to vary with cutting treatment. Although at ambient c_a photosynthetic rates were intrinsically higher and g_s intrinsically lower in the 8 cut regime, the degree of stimulation in the elevated rings was not statistically different from that which occurred in the 4 cut treatment ($t_{0.5[2]} = 0.839^{\text{n.s.}}$). Similarly, stomatal conductance showed little variation between cutting treatments at high CO₂ levels.

In June, an additional set of diurnal measurements were made for Trifolium at two levels of N fertilisation in the 8 cut regime (figs. 2c and 2d). The frequently cut treatment was chosen due to its relatively higher photosynthetic rate at ambient CO₂ levels ($t_{0.5[2]} = 9.34^{**}$). Again, no statistically significant differences were observed in either fertilisation regime in the magnitude of response to elevated c_a.

A comparison of mean assimilation rates showed little variation between growth periods. In general, July was a drier month than June, (data not currently available).

Any variation in the response of A to elevated c_a , and hence g_s , could therefore be attributed to growth treatments and parameters such as PPFD.

FIG 2: Mean diurnal time course at growth c_a of CO₂ uptake (A), and stomatal conductance (g_s), in leaves of *Trifolium repens* grown at the FACE facility, Switzerland. Solid symbols denote FACE grown plants (600 $\mu\text{mol mol}^{-1}$), open symbols denote their controls (355 $\mu\text{mol mol}^{-1}$). a and b represent cutting regimes at low N levels (140 Kg ha⁻¹ year⁻¹), c and d denote two N treatments (560 and 140 kg ha⁻¹ year⁻¹) at low cutting frequency. Measurements are for June 24, 1994; 11 and 39 days post cutting in the first instance, and 29 June, 1994; 16 days post cutting for the N regimes.

FIG 3: Mean diurnal time course at growth c_a of CO_2 uptake (A), and stomatal conductance (g_s), in leaves of *Trifolium repens* grown at the FACE facility, Switzerland. Solid symbols denote FACE grown plants ($600\mu\text{mol mol}^{-1}$), open symbols denote their controls ($355\mu\text{mol mol}^{-1}$). **a** and **b** represent intensive cutting treatments, **c** and **d** denote the extensive, 4 cut regime. All measurements were taken at low N treatments ($140\text{ kg ha}^{-1}\text{ year}^{-1}$). Measurements are for July 23; 12 days post cutting in the first instance, and 29 July, 1994; 18 days post cutting.

3.2. V_{cmax} and J_{max} Measurements

In elevated CO_2 , a common acclimatisation response of plants after long-term exposure is a lower photosynthetic rate when *measured* at ambient c_a compared with plants *grown* at ambient c_a . According to the Farquhar *et al.* model, this could be achieved from a down-regulation of V_{cmax} . Examination of both cutting regimes revealed that differences in V_{cmax} between elevated and ambient grown *Trifolium* were most marked in the 4 cut treatment (Fig. 4). A one-way analysis of variance (ANOVA) conducted between means showed values of V_{cmax} to be significantly decreased for three sampling dates in the extensive cutting treatment (8 and 25 days post cutting in the first re-growth period, 13 days post cutting in the second, $F_{1,6}=10.2^{**}$, $F_{1,6} = 7.01^*$, $F_{1,8} = 15.0^{**}$ respectively). V_{cmax} measurements at all other dates were not significantly different between CO_2 treatments. Differences between values for J_{max} at both CO_2 treatments were not found to follow any definite trend throughout the re-growth periods. In both cutting regimes, J_{max} for FACE grown plants either decreased, showed no difference, or in the 8 cut regime increased with respect to controls. In all cases, however, differences were not significant when tested statistically (e.g. $F_{1,8} = 2.04^{n.s.}$).

In the first measuring period (May 24 to June 10), V_{cmax} (8 cuts) increased with the number of days after cutting. A similar trend was observed in the 4 cut treatment over the same time period. Measurements taken on 5 July, 51 days post cutting in the extensive treatment, showed a sharp drop in V_{cmax} values. At this point in the growth period, the canopy was mature and dense, and a relatively large proportion of individual leaves had begun to senesce. However, in the 8 cut treatment, corresponding measurements taken on the same day (23 days post cutting in this regime), showed similar values for V_{cmax} . Such a decrease is therefore likely to correspond to the meteorological conditions at that time (little rainfall, and high mean

daily temperature), and subsequent low values for both treatments may also reflect the environmental conditions during the remaining growth periods. J_{max} values appear to show a reverse relationship, and decrease with the number of days after cutting in both regimes. Again, this decline in control and face measured plants was not significant.

The relationship between these two component processes of CO_2 assimilation was investigated further by plotting values of J_{max} against those obtained for V_{cmax} in both cutting treatments, and under both CO_2 regimes (Fig. 4). A strong correlation between V_{cmax} and J_{max} was observed in the 4 cut treatment (r^2 values >0.7), whilst the 8 cut treatment showed considerably more scatter (r^2 values <0.45). A comparison between CO_2 regimes showed that acclimation (i.e. J_{max}/V_{cmax}) appeared to be most marked in the 8 cut treatment, with a slope of 3.01 at high c_a , compared with a slope of 1.56 for ambient grown *Trifolium*.

However, when the relationship of J_{max} to V_{cmax} was considered as a mean ratio, no significant difference was apparent ($F_{1,38} = 1.45^{n.s.}$). Likewise, no differences in this ratio between control and FACE grown plants was found to occur in the 4-cut treatment ($F_{1,30} = 2.20^{n.s.}$).

FIG 4: Average V_{cmax} and J_{max} measurements in two cutting regimes for leaves of *Trifolium repens* grown at the FACE facility, Switzerland. Measurements were taken at low N levels ($140 \text{ kg ha}^{-1} \text{ year}^{-1}$) over a 2 month period. Arrows indicate cutting dates. V_{cmax} and J_{max} values were estimated from the relationship of A to c_i using a maximum-likelihood method. Because leaf temperature varied within and between each sample date, V_{cmax} and J_{max} were extrapolated to a common 25°C (Harley *et al.*, 1992).

FIG 5: Relationship between the maximum rate of carboxylation, $V_{c_{max}}$, and the maximum rate of electron transport, J_{max} , as calculated from the A/C_i curves of *Trifolium repens* grown at the FACE facility, Switzerland. Solid symbols denote FACE grown plants (600 $\mu\text{mol mol}^{-1} \text{CO}_2$), open symbols represent controls (355 $\mu\text{mol mol}^{-1} \text{CO}_2$). Measurements were taken at two cutting regimes (8 and 4 cuts year $^{-1}$) and at four sampling dates over a two month period (24 May - 24 July).

3.3. Species measurements of V_{cmax} and J_{max}

Species specific estimates of V_{cmax} and J_{max} were also estimated for 3 temperate grassland species, *C. leucanthemum*, *R. frisianus*, and *P. lanceolata* measured on 30 June (Fig. 5). The response of white clover has been included for comparison (measured 5 July). In each species, V_{cmax} and J_{max} decreased in FACE grown plants when compared with those plants grown at ambient c_a . All differences were significant, with the greatest decrease in both parameters found in *Plantago* at elevated and ambient CO_2 ($t_{0.5[3]} = 2.35^{**}$, $t_{0.5[3]} = 4.99^{**}$). An analysis between the maximum rate of carboxylation and the minimum rate of electron transport indicated that the ratio of J_{max} / V_{cmax} did not differ significantly in the first three species, but significant differences were observed in *Trifolium* (Table 2), perhaps suggesting a much tighter coupling between both processes for leaves of *Chrysanthemum*, *Ranunculus* and *Plantago*.

TABLE 2. Comparison of mean ratios of J_{max} and V_{cmax} for *C. leucanthemum*, *P. lanceolata*, *R. frisianus*, and *T. repens*, grown at the FACE facility, Switzerland. Measurements were taken on June 29 for the first three species and 5 Jul for *Trifolium*. Values are means ($n = 4$) $\pm$ s.e.m.

	<i>C. leucanthemum</i>		<i>P. lanceolata</i>		<i>R. frisianus</i>		<i>T. repens</i> [#]	
CO ₂ concentration ($\mu\text{mol mol}^{-1}$)	355	600	355	600	355	600	355	600
J_{max}	147 $\pm$ 7	122 $\pm$ 11	139 $\pm$ 9	85 $\pm$ 6	125 $\pm$ 16	78 $\pm$ 5	192 $\pm$ 7	171 $\pm$ 15
V_{cmax}	30 $\pm$ 1	20 $\pm$ 3	25 $\pm$ 2	12 $\pm$ 1	30 $\pm$ 7	14 $\pm$ 1	40 $\pm$ 2	33 $\pm$ 4
J_{max} / V_{cmax}	5.0	6.5	8.0	5.8	5.6	1.8	5.7	4.6
Variation between groups	(F _{1,6} =3.52 ^{n.s})		(F _{1,4} =1.95 ^{n.s})		(F _{1,4} =2.15 ^{n.s})		(F _{1,4} =21.9 ^{**})	

Statistical analysis was carried out using an analysis of variance.

FIG 6: A comparison between V_{cmax} (a) and J_{max} (b) values derived from A versus c_i measurements for 4 temperate grassland species; *Crysanthemum leucanthemum*, *Plantago lanceolata*, *Ranunculus frisianus* (RfriV6), and *Trifolium repens*, grown at the FACE facility, Switzerland. Measurements were taken on 29 June for the first three species, and at 5 July for *Trifolium*. Data was extrapolated to a common 25°C using thermal relations described by Harley *et al.*, (1992).

3.4. Stomatal Characteristics.

Exposure to elevated CO₂ had no significant effects on SI, stomatal density or pore length on either the adaxial or the abaxial leaf surfaces of *T. repens* (Table 3). Leaflets were sampled on 20 July from the 4 cut regime at both N treatments.

TABLE 3. Mean stomatal density, stomatal index (SI), and stoma pore length of both the adaxial and abaxial leaf surfaces of *Trifolium repens* grown at 2 different N regimes in ambient (c_a, 355 μmol mol⁻¹ CO₂) and elevated growth c_a (600 μmol mol⁻¹ CO₂). Values are means ± s.e.m. Stomatal index = (stomatal density)/(stomatal density + epidermal cell density) × 100. Statistical analysis showed no significant difference between means for all ambient and elevated CO₂ grown plants.

CO ₂ concentration (μmol mol ⁻¹)				
	355		600	
High N	Adaxial	Abaxial	Adaxial	Abaxial
Stomatal Density (mm ⁻²)	338 ± 27.2	214.3 ± 19.3	369.3 ± 31.5	200.6 ± 16.2
Stomatal Index	15.4 ± 0.79	12.9 ± 1.37	14.9 ± 0.9	11.9 ± 1.2
Stoma pore length (mm)	0.0025 ± 6.3 × 10 ⁻⁵	0.0041 ± 0.0001	0.0027 ± 7.6 × 10 ⁻⁵	0.0047 ± 0.0001
Low N				
Stomatal Density (mm ⁻²)	425.3 ± 51.1	206 ± 24.7	350 ± 30.7	184.6 ± 14.7
Stomatal Index	15.17 ± 0.2	12.9 ± 1.5	16.7 ± 0.7	11.8 ± 1.6
Stoma pore length (mm)	0.0024 ± 9.5 × 10 ⁻⁵	0.0042 ± 7.5 × 10 ⁻⁵	0.0027 ± 9.5 × 10 ⁻⁵	0.0047 ± 0.0002

Statistical analysis was carried out using a Two-sample *t*-test.

(mean values were obtained from 6 leaf replicates for each CO₂ concentration, and from 5 random fields of view per leaf).

4. DISCUSSION

Growth of *Trifolium* at elevated c_a is associated with consistently higher rates of A , and considerably lower rates of g_s , when measured over a diurnal time period (cf. Figs. 2 & 3). The increase in leaf A , of between 21 and 36% for plants grown and measured at high CO_2 concentrations, is in close accordance with previous published data for this species (Ryle & Stanley, 1992). Equally, the measured reduction in stomatal conductance is similar to values for whole canopy transpiration given by Nijs *et al.*, (1988) for small artificial swards of clover.

Increased availability of carbohydrates could have important consequences for grassland management practices if this is associated with enhanced development of N-fixing nodules. At a time when both economic and environmental pressures are progressively constraining fertiliser usage, an increase in the supply of organic N through increased nodule development, will have a profound influence on management strategies. Recent analysis of clover root growth at the FACE site has suggested that higher CO_2 concentrations lead to enhanced root growth (ETH FACE News, 1994). Similar results have also been obtained from the analysis of soil cores (ETH FACE News, 1994). Since no figures are currently available for this data, and since overall root / shoot ratios were not considered in this study, any advantage in terms of N-fixing capacity can not be speculated. Previous studies, such as that by Ryle *et al.*, (1992), have considered this, and have persistently lower root / shoot ratios for clover grown in controlled environments at $680\mu\text{mol mol}^{-1}CO_2$ relative to controls. Similar N contents were observed in all plant tissues, irrespective of growth regime.

It is interesting to note that, in terms of A , leaves of *Trifolium* did not appear to respond to N-concentration, possibly reflecting their ability to fix atmospheric N_2 (cf. Figs 2c and 2d). These results are similar to those found in other reports (Dennis &

Woledge, 1985), and may indicate increased nodule development arising as a result of the increased assimilate supply associated with elevated c_a , is sufficient to meet increased growth demands of the plant. However, these inferences have been drawn from a single set of diurnal measurements, and since overall biomass measurements were not made, data should not be extrapolated beyond the scope of this experiment.

When averaged over the measuring period (Fig. 4), estimates of V_{cmax} and J_{max} for white clover indicate no acclimation in leaves from the 8-cut regime. This parallels the response shown by soybean, where a reduction of the C source by trimming leaves has been found to alleviate the reduction in photosynthetic capacity of plants grown in elevated- CO_2 (Peet, 1984). If the source-sink balance is a major factor in determining photosynthetic acclimation to high CO_2 in clover, then variables affecting this balance, such as cutting frequency, may explain the differential responses observed between treatments over part of the growing season. Interaction between the number of potential sinks, and the response to enhanced CO_2 has also been noted by Kramer (1981). Several mechanisms have been proposed by which photosynthesis may be affected, if available sinks are becoming saturated, as indicated in the 4 cut regime. Firstly, if sucrose utilisation in the sink is lower than the rate of synthesis, sucrose concentration at the source may increase, resulting in a negative feedback on the enzymes involved. This, in turn, would lead to lower concentrations of inorganic orthophosphate (P_i), which induces higher rates of starch synthesis (Herold, 1980; Arp, 1991). Additionally, if photosynthesis exceeds maximum rate of starch and sucrose synthesis, P_i can decline to levels such that photophosphorylation is affected. If this occurs, the rate of photosynthesis is limited by the rates of assimilate use, and becomes insensitive to the concentration of O_2 and CO_2 (Sharkey, 1985). Accumulation of starch grains in the leaf can cause direct mechanical damage to the photosynthetic apparatus; a response well documented in leaves of *Trifolium subterraneum* (Cave *et al.*, 1981). However, it must be pointed out that the Farquhar *et al.* model of CO_2 assimilation, on which these estimates of V_{cmax} and J_{max} are

based, only describes the initial fixation of CO₂ in the chloroplast by rubisCO. The subsequent translocation and allocation of this carbon to different plant organs, and the translation of this into growth are very much separate questions (Wullschleger, 1993).

It is evident that *Trifolium* plants from the extensively managed regime (4 cuts), maintain a much closer functional balance in their allocation of resources to these two component processes. This is representative of the linear relationship between V_{cmax} and J_{max} (cf. Fig 5). It has been suggested, that across a wide range of conditions, plants are able to optimise their allocation of resources, particularly N, in order to conserve a balance between enzymatic (i.e. rubisCO) and light-harvesting (i.e. chlorophyll) capabilities (Wullschleger, 1993). Any acclimation response might therefore be characterised by an increase in the J_{max} / V_{cmax} ratio. When the relationship between the maximum rate of carboxylation and the maximum rate of electron transport was analysed, virtually no differences were observed between CO₂ concentrations in the 4- and in the 8-cut regime over the measuring period.

A predominant finding from this study was that C₃ species differ in their estimates of V_{cmax} and J_{max} and hence in their rates of CO₂ assimilation (Fig. 6). Down-regulation of rubisCO appears most marked in species that do not have the ability to fix their own N. This may echo observations made by Farquhar *et al.* (1980) who suggested that the proportion of leaf N in rubisCO and chlorophyll were important determinants of carboxylation- and rubP regeneration- limited rates of assimilation, respectively. The strong linearity between maximum rates of CO₂ assimilation and leaf N (Field & Mooney 1986, Evans 1989) points to the importance of N, and its allocation, in optimising CO₂ exchange processes in C₃ plant species (Wullschleger 1993). The intrinsically lower N-use efficiencies of these non-leguminous species may result in smaller investments of N into photosynthetic efficiency at elevated Ca. This in turn, could account for the observed decrease in V_{cmax} but may not for the parallel

decrease in J_{max} . A probable explanation is achieved when source / sink relationships are considered. Considerable variation in the regulation of enzymes involved in sucrose synthesis and partitioning exists between species, and this is also true for sensitivity to increases in carbohydrate concentration (Stitt, 1991). If end-product inhibition of rubP regeneration did not occur for these species in elevated c_a , this would therefore account for accompanied decreases in the rate of carboxylation and regeneration of rubP. These variations in sensitivity to increases in carbohydrate concentrations, may also explain any differences in the response observed between species. A parallel decline in both component processes is reflected by the relatively constant ratio of J_{max} to V_{cmax} at elevated and ambient levels of CO_2 .

Estimates of J_{max} and V_{cmax} for clover taken within a week of the species measurements show marked differences. V_{cmax} for *Trifolium* appeared to decrease at this date, corresponding to an increase in the J_{max} / V_{cmax} ratio. The reasons for this differential response are unclear, compared with estimates for the grassland species. This is enforced by the absence of corroborative evidence over the entire measuring period. A possible explanation that may be considered is the relative stage in the growing period at which estimates for J_{max} and V_{cmax} for *Trifolium* leaves were made. Measurements were taken 51 days after cutting, when stands were composed of large proportions of old or senescing leaves, and canopy density was high. This would contribute to a considerable degree of self shading experienced by leaves lower down in the canopy, and may impose an overall reduction in plant photosynthesis. If photosynthesis is reduced, an effect would be exerted through a reduction in the availability of organic N to the plant due to decreases N_2 -fixation in the nodules. This, in turn, could account for the observed decrease in V_{cmax} , and parallel increase in the J_{max} / V_{cmax} ratio, so that investment of resources (in particular N), in different parts of the photosynthetic machinery would be optimised. The relative time scales over which this could occur warrants future research.

Both adaxial and abaxial SI of *T. repens* were insensitive to a change in CO₂ concentration (Table 3). These results agree with that of Woodward (1986) who found that increasing CO₂ above current ambient levels had no effect on stomatal density, or SI in the shrub *Vaccinium myrtillus* L.. Given this insensitivity to rising CO₂ concentrations, no affect on the efficiency of photosynthesis in this species is expected.

The results of this study show that long-term growth at elevated c_a greatly increases the supply of carbohydrates in *T. repens*, and does not appear to affect photosynthetic capacity for most of the measuring period. Since, in general, photosynthetic capacity did not decline, N and sink limitations are unlikely. Additionally, since stomatal characteristics (as characterised by pore length, density and SI), are relatively unresponsive to enhanced c_a , no direct limitation to photosynthesis would be expected to occur.

Non-leguminous species such as *C. leucanthemum*, *R. frisianus* and *P. lanceolata* do however show a much greater degree of acclimation under similar experimental conditions (extensive regime). This may be linked to low N availability and subsequent re-allocation of N away from rubisCO, or by sink regulation of photosynthesis.

Responses of white clover to elevated CO₂, will have important consequences for future management practices. Clover is an important forage crop, and, according to these results may be expected to increase its competitive ability with regard to the other members of the grassland community. This is hypothesised from this study on pure and not mixed stands of clover, and therefore requires additional verification. Since FACE provides such a useful tool in terms of CO₂ field studies, and since mixed stands of *T. repens* and its companion grass *L. perenne* are already established at the site, future research could be directed towards the investigation of the relative competitive abilities of both species. Previous studies have indicated that clover can derive a sustained competitive advantage over ryegrass at high CO₂ (Overdieck *et al.*,

1984). However, these experiments have been conducted using small glasshouse grown stands and do not take account of the additional and complex interactions that occur in the field. It seems therefore, that there is a real need for long-term exhaustive studies, where plant responses to a range of environmental conditions and species communities are examined. Only then will the future implications of climate change on agricultural systems become apparent. This may have wide ranging implications for the management strategies of future grassland communities.

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