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THE PHOTOSYNTHETIC ACCLIMATION OF *LOLIUM PERENNE* GROWING IN A FREE-AIR CO_2 ENRICHMENT FACE SYSTEM

by

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DISCLAIMER

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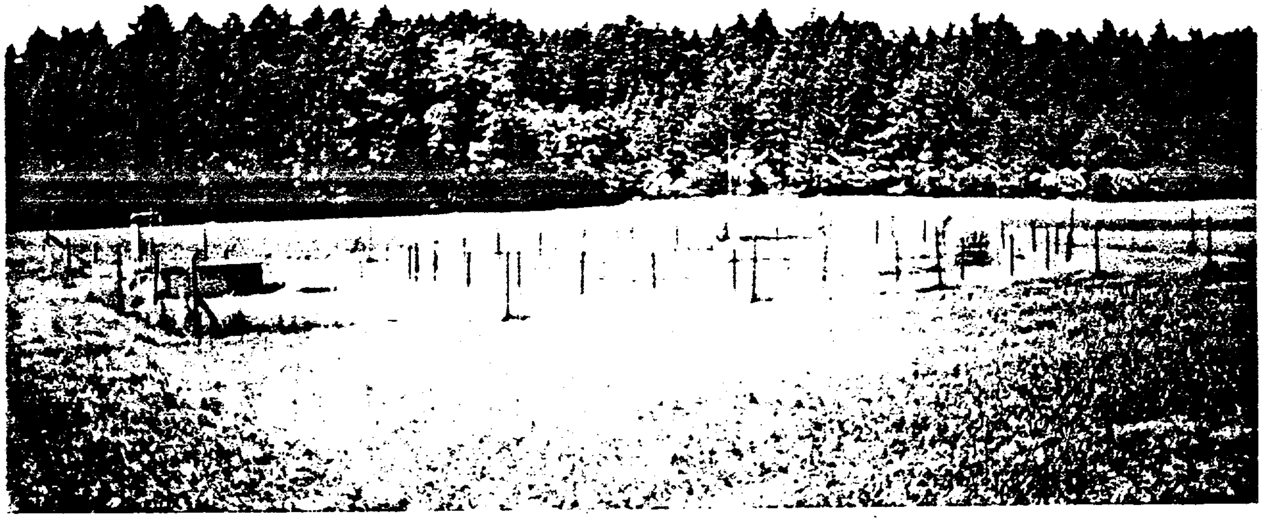

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ABSTRACT

Stands of Ryegrass (*Lolium perenne* L. cv.Bastion) were grown in the field at ambient or elevated (600µmol/mol) CO_2 concentration, high (560Kg/ha) or low (140Kg/ha) nitrogen addition and with a frequent (every 4 weeks) or infrequent (every 8 weeks) cutting regime. Plants were in the second year of a 3 year experiment. Exposure to elevated CO_2 was carried out with a Free-Air CO_2 Enrichment (FACE) system which provides the most 'realistic' system of CO_2 fumigation currently available.

Elevated CO_2 increased diurnal CO_2 assimilation by between 34 and 88% whilst reducing rates of stomatal conductance by between 1 and 42%. However, analysis of the A vs. Ci response showed considerable acclimation of the photosynthetic apparatus in response to elevated CO_2 - $Vc_{max.}$, as an in vivo measure of RubisCO activity, decreased by between 29 and 35% in high CO_2, whilst $J_{max.}$, as a measure of the RubP regeneration capacity, showed no significant change. Two out of three additional perennial grassland species studied showed similar acclimatory behaviour to Ryegrass. Diurnal assimilation rate, $J_{max.}$ and, in most cases, $Vc_{max.}$, increased significantly directly after cutting of Ryegrass stands, but nitrogen treatment had little effect on any of these parameters. Neither stomatal density, stomatal index nor stomatal pore length of Ryegrass were significantly altered by growth in elevated CO_2.

The results are discussed in terms of the limitation imposed on maximising photosynthetic and growth responses of Ryegrass at elevated CO_2, by the ability of perennial species to increase long-term sink capacity under these conditions.

Key words: *Lolium perenne*, Free-Air CO_2 Enrichment, photosynthesis, acclimation, A/Ci response, stomatal density.

ABBREVIATIONS USED IN TEXT:

A= CO_2 assimilation rate (µmol $CO_2/m^2/s$); **Ca**= ambient atmospheric CO_2 concentration (µmol CO_2/mol air); **Ci**= intercellular CO_2 concentration (µmol CO_2/mol air); **E**= H_2O transpiration rate (mmol H_2O /m^2/s); **FACE**= Free-Air CO_2 Enrichment; **g_s**= stomatal conductance (mmol/m^2/s); **$J_{max.}$**= maximum light saturated rate of electron transport (µmol/m^2/s); **Pi**= inorganic phosphate; **PPFD**= photosynthetic photon flux density (µmol/m^2/s); **RubisCO**= Ribulose-1,5 bisphosphate Carboxylase-Oxygenase; **RubP**= Ribulose-1,5 bisphosphate; **$Vc_{max.}$**= maximum rate of carboxylation (µmol/m^2/s).

INTRODUCTION:

The Earth's atmosphere is currently being flooded with CO_2 at the unprecedented rate of 8 Gt carbon per year, mainly from fossil fuel burning and changes in land use (Idso, 1989; Post *et al.*, 1990). The rate is such that an effective doubling of atmospheric CO_2 from pre-industrial levels (ca. 280 ppm) is expected by the mid - latter half of the next century (Houghton *et al.*,1990).

For plants with the C3 photosynthetic pathway such as Ryegrass, the initial rate of photosynthetic CO_2 uptake per unit leaf area (A) increases after exposure to elevated CO_2 due mainly to suppression of the competitive oxygenation of the primary carboxylation enzyme - RubisCO (Long *et al.*,1993; Stitt, 1991). Since photorespiration may depress A by up to 50% in temperate climates, substantial increases in A (Long, 1991) and plant productivity (Strain and Cure, 1985; Cure and Acock, 1986) are possible if atmospheric CO_2 concentrations continue to rise.

However, Oechel and Strain (1985) have suggested that the initial stimulatory response of A may be complicated in the long-term by physiological adjustment, or acclimation, of the photosynthetic apparatus (e.g. Besford *et al.*,1990; Rowland-Bamford *et al.*, 1991), which appears to be most marked in species where photosynthesis is limited by the capacity to generate new sinks (Long and Drake, 1992). The findings of Arp and Drake (1991) on the C3 sedge, *Scirpus olneyi*, suggest that this development of new sites of storage or utilisation may require years in perennial species.

Perennial Ryegrass (*Lolium perenne*) is just one such species which may well fall into this latter category of response. Ascertaining its physiological behaviour in elevated CO_2 is vitally important for 2 reasons:-

(i) The Ryegrasses still provide the mainstay of lowland pasture production in temperate western Europe and they or their hybrids are likely to continue to do so in the foreseeable future (Ryle *et al.*, 1992). Agricultural management practices, namely nutrient treatment and cutting regime, may well have to be modified in order to optimise any changes in productivity that may occur in Ryegrass pastures in response to high CO_2.

(ii) The response of the C3 Ryegrass could provide an indication as to the direction of future carbon storage in terrestrial ecosystems in a high CO_2 world, with all the resultant 'Gaian' overtones in terms of future planetary temperature regulation (J.E. Lovelock - personal communication).

However, in spite of their economic and ecological importance, there is precious little information on the effects of CO_2 on forage crops, the majority of literature being devoted to arable and glasshouse crops. Nijs *et al.* (1988) found that the response of *L. perenne* can deviate from some of the generalised responses of C3 plants to CO_2 enrichment (an initial enhancement of A, declining over time - Cure and Acock, 1986 - and a decline in both g_s and E - Pearcy and Bjorkman, 1983). They found that on a leaf area basis, A actually declined by 30% and although leaf E declined along with predictions, canopy E actually increased by 20%. This effect was put down to the more than doubled leaf area index of stands grown at high CO_2, causing leaves to develop under very low photosynthetic photon flux densities (PPFD). This theory is given support by Gay and Hauck (1993) on *Lolium temulentum* and by Nijs *et al.*, (1989) who found that during a diurnal cycle under elevated CO_2, A was significantly higher at high PPFD only, whilst E remained similar at both CO_2 treatments.

Two recent studies have touched on the acclimatory gas exchange responses of Ryegrass grown at high CO_2, but the physiological basis behind these responses has largely been ignored. Ryle *et al.* (1992) found that although elevated CO_2 increased rates of leaf photosynthesis by 35 - 45%, acclimation to high CO_2 reduced rates of photosynthesis compared with plants acclimated to ambient levels of CO_2. Gay and Hauck (1994), although finding no acclimation of photosynthesis in *L. temulentum* after 5-8 weeks, have suggested that the lack of any significant growth response at high CO_2 and high light intensities may eventually lead to photosynthetic inhibition through accumulation of carbohydrates.

The importance of the effects of nitrogen supply and cutting regime on the response of *L. perenne* to elevated CO_2 is beyond doubt, but it is another largely neglected area of study. However, Newton (1991), using the data of Hardacre *et al.*, (1986), demonstrated a significantly reduced biotic growth factor - defined as the change in NPP or growth rate consequent on a doubling of CO_2 - of *L. perenne* grown at low nitrogen levels.

In addition, Nijs and Impens (1993) have suggested that more frequent cutting of Ryegrass stands could yield a higher increase in production from elevated CO_2, since higher carbon storage in the root system under high CO_2 allows faster and more effective canopy development.

Changes in stomatal characteristics could well be a contributory factor to observed gas exchange responses in plants grown at elevated CO_2. Decreases in stomatal density have been demonstrated in plants grown at high CO_2 (O'Leary and Knecht, 1981; Bristow and Looi, 1968) but the majority of studies have shown no effect of CO_2 levels above present ambient (Woodward and Bazzaz, 1988; Korner, 1988; Ferris and Taylor, 1994; Beerling and Chaloner, 1993a). Two studies on *L. perenne* fall conclusively into this latter category of response (Ryle and Stanley, 1992; Gay and Hauck, 1994).

However, confidence in all these studies is, to a large extent, limited by the lack of realism in the experimental systems, the majority of which involve various types of enclosure system. Microclimatic conditions and plant growth in these enclosures have been shown to differ markedly from those of open-field environments (Lawlor and Mitchell, 1991) and this will inevitably influence results coming from such experiments. In addition, Arp (1991) has claimed that downward regulation of photosynthetic capacity is largely an artefact caused by small experimental pots which restrict sink size, and that field-grown plants don't generally exhibit this down-regulation. However, the Free-Air CO_2 Enrichment (FACE) system developed at the Brookhaven National Laboratory (BNL), provides the realism of open-field experiments, adequate control of CO_2 concentrations and proven long-term reliability (S. Koller - personal communication; Hendrey *et al.*, 1993). It therefore has great potential as a tool for studying the effects of CO_2 enrichment on plant processes.

This study therefore aims to answer the following questions in terms of either supporting or contradicting these findings from controlled-environment studies with data from the more realistic field-situation that the FACE facility provides:

(i) Are gas exchange parameters altered to predicted levels in enhanced CO_2 and how is this affected by nitrogen supply and cutting? This question was tackled by measuring diurnal gas exchange rates of *L. perenne.*

(ii) Does acclimation of the photosynthetic apparatus really take place or is it merely an artefact of experimental conditions? If it really does take place then how far advanced is the acclimatory response? This question was confronted by the measurement and subsequent analysis of A vs. Ci sets of data on *L. perenne.* In addition, A vs. Ci measurements were taken on 2 different genotypes of 3 other monocotyledonous grassland perennials in order to highlight inter- and intra-species variations in responses to elevated CO_2.

(iii) Are leaf stomatal characteristics altered in plants growing in elevated CO_2 and does this relate to acclimatory changes in photosynthetic gas exchange?

MATERIALS AND METHODS

(a) Growth conditions

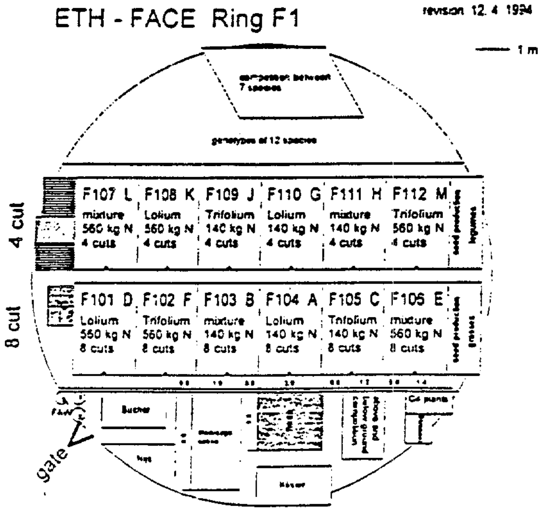
Lolium perenne L. (cv. Bastion) plants were exposed to elevated CO_2 in the field using a Free Air Carbon Dioxide Enrichment (FACE) system developed at the Brookhaven National Laboratory (BNL). For a full technical description of the FACE system see Hendrey *et al.* (1993) and Hendrey and Kimball (1990). Plants were grown in 3 FACE rings at 600 µmol/mol CO_2 and in 3 control rings at current ambient atmospheric CO_2 levels. In 1993, CO_2 concentration was maintained at 600 +/-60 µmol/mol for 91% of the total fumigation time based on 1-minute averages (ETH-FACE News, 1994). 1994 fumigation began on March 7th and thereafter was switched on if leaf temperatures were >8°C.

The plants were in the second year of a 3-year split plot (randomised within plots) experiment involving *L. perenne* and *Trifolium repens* grown in monoculture or in mixtures at 2 levels of nitrogen application (140 Kg N/ha or 560 Kg N/ha as NH_4NO_3) and at 2 cutting regimes (4 cuts or 8 cuts between April 25th and November 1st 1994) (Table 1 & Fig. 1). *L. perenne* was cut to a height of 4cm above ground level.

TABLE 1. - Dates of harvests (cutting) of *L. Perenne* in the 6 experimental rings.

Harvest	25th April	16th May	13th June	11th July	8th August
8 cuts	yes	yes	yes	yes	yes
4 cuts	no	yes	no	yes	no

FIG.1. - The layout of the various plots/treatments within a ring.



Micrometeorological data (mean day and nightime air temps.(C), mean day + nightime air temp.(C), maximum and minimum air temps.(C), mean % daily Relative Humidity, mean daily Global Radiation,(MJm^{-2}) and mean daily precipitation (mm)) was collected during the course of the experimental field-work at the I.P.W.'s own meteorological station. This is shown in Appendix. 1. and appears on graphs of diurnal sets of measurements where relevant. Unfortunately, due to a fault in the main computer, this data only runs until July 12th, and therefore misses out the 2 diurnal sets of measurements on 22nd and 28th July and the A/Ci measurements on 19th July.

(b) Gas Exchange Measurements

Leaf gas exchange measurements were made using an open, combined infra-red gas analysis system (CIRAS 1-version 1.4. - PP Systems, Hitchin, Herts., UK.) attached to a Parkinson Leaf Cuvette (Version 1.1 - PP Systems, Hitchin, Herts., UK.). The leaf chamber was 1.8cm in diameter. The CIRAS was calibrated for water vapour at 2 Pa with a water vapour generator (Type WG-600 - Analytical Development Co., Hoddesden, UK.) and for CO_2, with calibration gas at 604.7 µmol/mol (27548-Type 30L - Carbagas, Swiss Calibration). The gas exchange parameters A, Ci, g_s and E were calculated according to Von Caemmerer and Farquhar (1981). All measurements were taken, where possible, on the youngest, fully-expanded leaf of *L. perenne*, halfway down from the leaf tip, with the upper leaf surface facing upwards in the leaf chamber. Leaf area was estimated by measuring leaf width and by assuming that the blade formed a rectangle in the leaf chamber.

(i) Diurnal measurements

4 sets of diurnal gas exchange measurements were taken on the dates and at the conditions shown in Table 2.

TABLE 2 - Dates when diurnal sets of measurements were taken and the treatments sampled.

	DATE	NITROGEN-TREATMENT	CUTTING REGIME	DAYS FROM CUTTING
DIURNAL 1	23/6/94	High & Low	8 cuts	10 after
DIURNAL 2	25/6/94	High & Low	4 cuts	40 after
DIURNAL 3	22/7/94	High & Low	4 cuts	10 after
DIURNAL 4	28/7/94	High & Low	8 cuts	16 after

3 leaves per treatment were measured in each of the 6 rings every 2 hours from 6 am until 8 pm. Measurements were made at the appropriate growth CO_2 concentration (355 or 600 µmol/mol), and at normal daily and seasonal irradiance fluctuations. Dry days were essential pre-requisites for a diurnal set of measurements since wet leaves would prevent any estimate of stomatal conductance. For logistical reasons, the rings were always sampled in the following order - C2, F1, C1, F2, F3, C3 (for a map of the ring locations, see Appendix 2).

(ii) A vs. Ci measurements

To determine the response of leaf CO_2 uptake (A) to intercellular CO_2 concentration (Ci), the CO_2 concentration in the chamber was altered in 6 steps between 50 and 900 µmol/mol, the direction of change depending on the growth CO_2. For ambient grown plants the concentration sequence was 355, 250, 150, 50, 600, 900 and for the FACE grown plants it was 600, 250, 150, 50, 355, 900 µmol/mol CO_2. The latter sequence was employed to try and avoid unnaturally high levels of RubisCO activation by CO_2. Measurements were taken at +/- 40 µmol/mol of the target value at all concentrations apart from the growth CO_2 concentration, which was taken at +/- 15 µmol/mol.

A vs. Ci measurements were taken on 2 leaves per treatment per ring and the rings were sampled in the order stated for the diurnal sets of measurements. A stabilised quartz-iodide light source was clipped over the leaf chamber to provide uniform, near-saturating photosynthetic photon flux densities (750 µmol/m^2/s). The 12 volt power supply ensured constant illumination for up to 8 hours. When possible, measurements were taken before ca. 3pm in order to avoid the possibility of feedback inhibition of photosynthesis due to carbohydrate accumulation and cytosolic inorganic phosphate (Pi) limitation.

In the first experiment (30th May - 21st June), one set of these A/Ci curves was taken each week at the low nitrogen treatment on both the 4- and 8- cut regimes in all 6 rings. This period of time encompassed the cutting of the 8-cut regime on June 13th (Table 1). During this first experiment, measurements were taken at only 355, 250, 150 and 50 µmol/mol CO_2, because of time restrictions. However, for the second experiment, time allocation estimates were revised in order to encompass the 600 and 900 µmol/mol CO_2 measurements, thereby allowing determination of J_{max}. This second set of measurements went from June 27th - July 19th, and was made on the 4-cut regime at both nitrogen treatments. It therefore straddled the harvest on July 11th.

$Vc_{max.}$ and $J_{max.}$, as in vivo measures of RubisCO activity and maximum capacity for RubP regeneration respectively, were calculated by fitting the equations of Farquhar *et al.*, (1980), following the procedure of Wullschleger (1992). Because temperature varied significantly between measurements, all estimates of $Vc_{max.}$ and $J_{max.}$ were corrected to 25 °C following the equations of Harley *et al.*, (1992) and Evans and Farquhar, (1991). Due to this leaf temperature variability, it was not possible to make any comparisons directly from A/Ci plots, with the exception of 27th June, when average leaf temperatures during measurement of ambient grown plants happened to coincide with those of FACE plants. This will be referred to as a 'viable' A/Ci plot for the purposes of this report.

In addition to the measurements on *L. perenne*, further A/Ci readings were taken on 3 other monocotyledonous grassland species - *Holcus lanatus* L., *Dactylis glomerata* L. and *Arrhenatherum elatius* L. To complement these inter-species comparisons to long-term CO_2 enrichment, intra-species differences were looked at by sampling 2 different genotypes (V1 and B1) of *H. lanatus, D. glomerata* and *A. elatius*. V1 and B1 refer to 2 different sites, both permanent, extensively managed grassland in Kanton Zurich, Switzerland. The precise genetic make-up of the plants from the 2 sites is unknown.

Relative stomatal limitation of photosynthesis (l) was calculated from the 'viable' A/Ci curve according to Long (1985).

Two-factor Analysis of Variance was performed on $Vc_{max.}$ and $J_{max.}$ values from each individual A/Ci curve, to highlight any significant differences caused by CO_2, nitrogen treatment or, in the case of the 3 monocot. species, genotype. Additional analyses were performed in order to assess whether $Vc_{max.}$ and $J_{max.}$ varied significantly over the entire regrowth period.

(c) Stomatal Measurements

Four youngest, fully-expanded leaves were picked from both nitrogen treatments in the 4-cut treatment, from each of the 6 experimental rings - this allowed 2 replicates of both abaxial and adaxial leaf surface per treatment per ring. They were placed in moist plastic bags and immediately transported back to the laboratory.

A section of leaf in the centre of the blade was scraped with a scalpel, removing sufficient tissue so that the stomata, which on *L. perenne* are sunk in between leaf surface ridges, could be seen clearly. Leaf sections were examined under a light microscope (x 40 magnification) in order to obtain stomatal density, stomatal index (SI) and stomatal pore length. The number of stomata and the number of other epidermal cells were counted from 5 random fields of view (a rectangle of area - 0.05 mm^2) per leaf surface. Stomatal pore length was measured with an eyepiece graticule on 3 leaves per ring and 5 stomata per leaf - no nitrogen or cutting treatment effects were examined. The field of view rectangle and the eyepiece graticule were pre-calibrated with a stage micrometer. The number of stomata per field of view was converted to the number per mm^2 and from the data, the stomatal index was calculated which relates the number of stomata/unit area (S) to the number of epidermal cells per unit area (E) where $SI=(S/E+S)x100$ (Ferris and Taylor, 1994).

Data were analysed statistically using 2 factor ANOVA, to test the null-hypothesis that CO_2 and nitrogen treatment have no effect on stomatal density, index or pore size.

RESULTS

(a) Diurnal sets of measurements:

Diurnal studies of CO_2 and water vapour exchange on 4 separate days in June and July all showed a consistent increase in A and decrease in g_s for the *L. perenne* growing at elevated CO_2 (Figs. 2a-d overleaf). In addition, some basic leaf microclimatic information is provided, to which A and g_s values can be related.

Table 3 shows these changes over the whole day in A, g_s and E as percentages due to growth in elevated CO_2 for each of the 4 diurnal sets of measurements.

TABLE 3. % Change in A, g_s and E of *L.perenne* due to growth at high CO_2.

		A		**g_s**		**E**	
	Days after cutting	High N	Low N	High N	Low N	High N	Low N
23/6/94	10	+42.44	+34.36	-20.27	-29.60	-8.03	-11.29
25/6/94	40	+67.58	+47.81	-13.07	-35.08	-6.91	-16.53
22/7/94	10	+55.01	+34.15	-27.37	-42.38	+0.88	-12.01
28/7/94	16	+74.66	+88.23	-1.22	-6.60	-6.15	+2.27

At every combination of nitrogen and cutting treatment there was a considerable diurnal stimulation of A, ranging from 34.15% to 88.23%. The greatest enhancement occurred at high PPFD's although stimulation was also marked at the beginning and end of each day. The massive stimulation recorded on the 28th July was associated with the considerably reduced A in the ambient plots - which reached only 17 - 18 $\mu mol/m^2/s$ - rather than any marked extra stimulation in the FACE plots. The 28th July was also an exception in that it produced a higher % stimulation in the low nitrogen treatment (Table 3 & Fig. 2d). The other 3 dates all produced higher % stimulation in the high nitrogen treatment - the difference being greater than 20% on 22nd July. On 25th June, which was 40 days into a period of regrowth, mean A values were considerably lower than those on the other 3 dates, which were 10, 10 and 16 days respectively into

FIG. 2A Diurnal Assimilation Rates & Stomatal Conductances for *Lolium perenne* Grown in Ambient (o) and FACE (●) Plots. 8 Cuts /10 Days Post Cutting. 23/6 94.

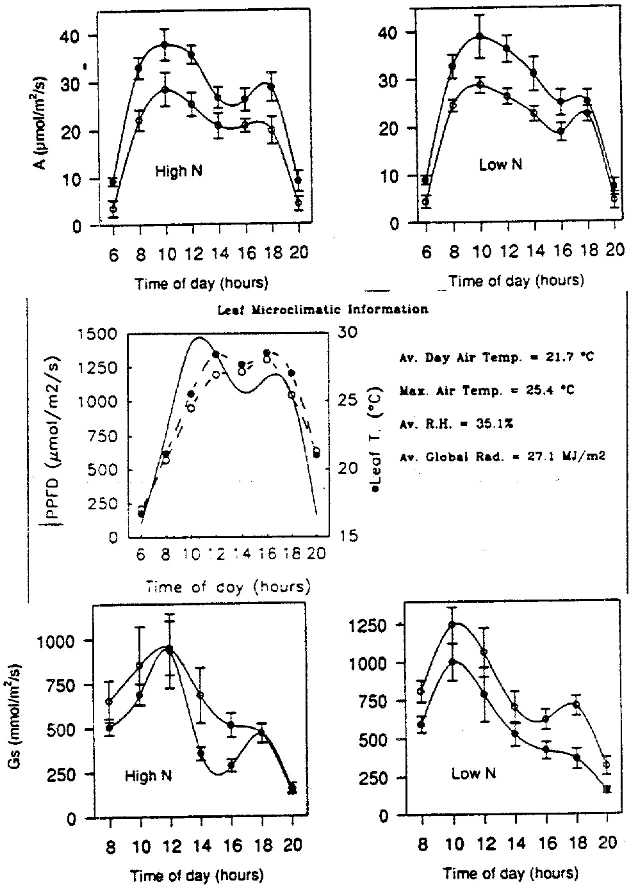


FIG 2B Diurnal Assimilation Rates & Stomatal Conductances for *L. perenne* Grown in Ambient (o) and FACE (●) Plots. 4 Cuts / 40 Days Post Cutting. 25/6/94.

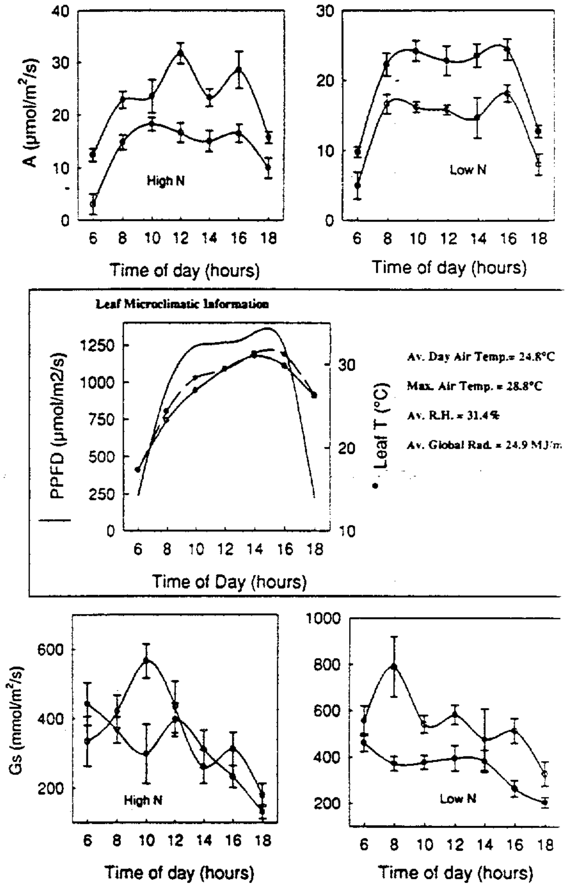


FIG 2C Diurnal Assimilation Rates & Stomatal Conductances for *L. perenne* Grown in Ambient (○) and FACE (●) Plots. 4 Cuts/10 days Post Cutting. 22/7/94

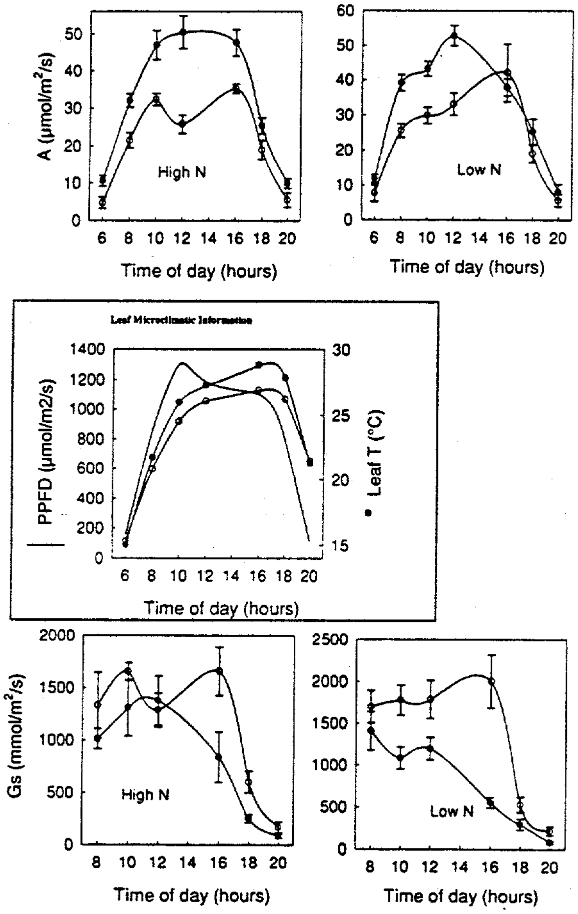
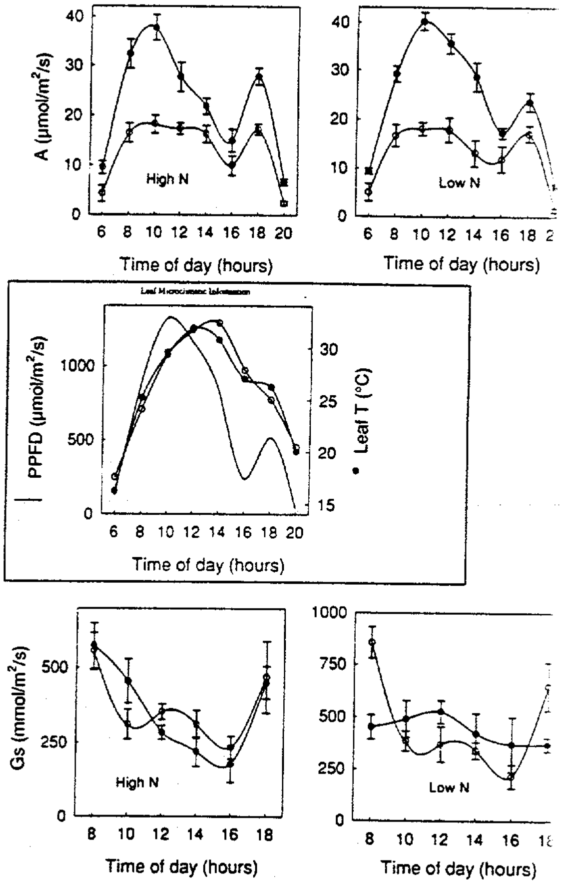


FIG 2D Diurnal Assimilation Rates & Stomatal Conductances for *L. perenne* Grow

Ambient (○) and FACE (●) Plots. 8 Cuts / 16 Days Post Cutting. 28/7/94.



the regrowth period. The peak was at just over 30 μmol/m^2/s in the high nitrogen and only 25 in the low nitrogen treatment compared to peak values of over 50 on the 22nd July (Fig.'s 2b & 2d).

At all combinations of nitrogen and cutting treatment, g_s was reduced in FACE grown plants, the range extending from 1.22% to 42.38% (Table 3). The % reduction due to growth in FACE was considerably higher at the low nitrogen treatment. However, on July 28th, g_s values at the 2 CO_2 treatments were very similar, and during the middle part of the day, in the low nitrogen treatment, the values for FACE grown plants were considerably higher than their ambient grown counterparts. The drought conditions experienced from a 4 - 6 week period up until the 28th (Appendix. 1) would appear to be bringing about more drastic water conservation measures in the ambient than the FACE grown plants, and this hypothesis is further backed up by the recorded increases in water loss (Table 3) in the FACE plants on this date.

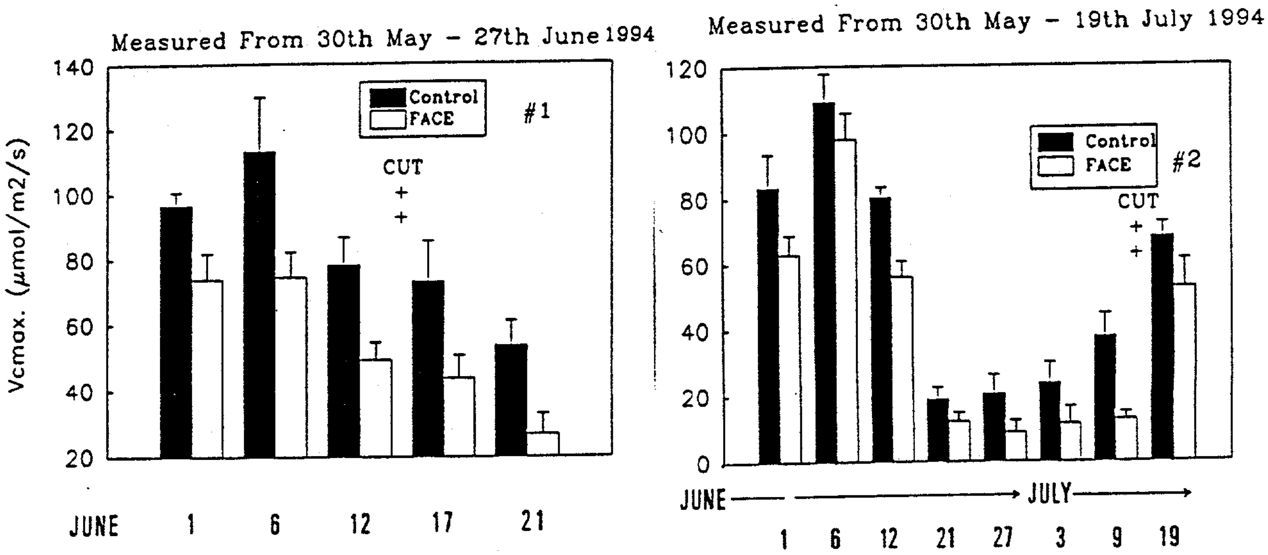
The effects of growth in FACE plots on E are more equivocal. Diurnal reductions in E occurred in all but 2 of the nitrogen/cutting treatment combinations, but the highest % reduction in FACE was only 16.53 (Table 3). The period when FACE grown plants have the greatest advantage (with the exception of 28th July) in terms of reduced water loss is during the middle of the day (10am - 4pm). As with the g_s values, reduction in E in FACE plots was generally higher in the low nitrogen treatment.

The reductions in g_s and E in FACE grown plants appear to be producing higher leaf temperatures which in turn could be having a profound effect on the rate of water loss from the plant (Fig.'s 2a-d).

(b) A vs. Ci Measurements

$Vc_{max.}$ values (and by inference RubisCO activities), calculated from the first A/Ci measurement experiment (from 30th May-21st June), for both cutting treatments at regular intervals during a period of regrowth in *L. perenne*, are, in all cases, significantly lower in FACE grown plants than in ambient grown plants (Fig. 3 & Table 4).

FIG. 3.



Variation in VCmax. Pre- and Post Cutting for Elevated (600ppm) and Ambient (355ppm) Grown *Lolium perenne* - 8 Cut Regime/Low Nitrogen(#1)
4 Cut Regime/Low Nitrogen (#2)

TABLE 4. - Significance levels of variation in Vc_{max} due to CO_2 and cutting treatment

Source of variation	30th May	6th June	12th June	21st June	Over whole regrowth
4 & 8 cuts	$P=0.12$; $F=2.70$	$P=0.39$; $F=0.76$	$P=0.48$; $F=0.53$	**$P<0.05$; $F=10.07$**	-
CO_2 treat.	**$P<0.05$; $F=8.52$**	**$P<0.05$; $F=5.28$**	**$P<0.05$; $F=21.07$**	**$P<0.05$; $F=21.76$**	-
Interaction	$P=0.88$; $F=0.02$	$P=0.22$; $F=1.60$	$P=0.70$; $F=0.15$	$P=0.07$; $F=3.61$	-
Time	-	-	-	-	**$P<0.05$; $F=65.31$**
n	6	6	6	6	24

$Vc_{max.}$ also varies significantly over the whole growth period. However, particularly in the 8 cut treatment, but also to a lesser extent in the 4 cut treatment, the advent of the cut had *little effect on $Vc_{max.}$ which contrasts sharply with the second A/Ci experiment* on the effect of nitrogen treatment (Fig. 4). In the 4 cut treatment there was a striking drop in $Vc_{max.}$ between 26 and 35 days after the cut followed by a further gradual increase up until the following cut. The difference in RubisCO down-regulation between the 2 cutting treatments is only significant on 21st June (Table 4); over the whole growth period the % decrease in inferred RubisCO activity is 35.25% in the 8-cut treatment and 28.83% in the 4-cut treatment.

In the second A/Ci experiment (27th June - 19th July), to determine any effect of nitrogen treatment, only 2 of the 4 days of measurements produced $Vc_{max.}$ values which were significantly lower in the FACE grown plants; none of the days produced any significant differences between nitrogen treatments (Fig. 4 & Table 5).

FIG. 4.

Variation in Vcmax. and Jmax. Through a Regrowth Period for *Lolium perenne* Grown in Ambient (355ppm) and Elevated (600ppm) Carbon Dioxide.

(Vcmax and Jmax values are obtained from A/Ci plots fitted to a model curve at 25 °C based on the equations of Harley et al. 1992 and Evans & Farquhar, 1991. A/Ci plots taken from June 27th - July 19th 1994.)

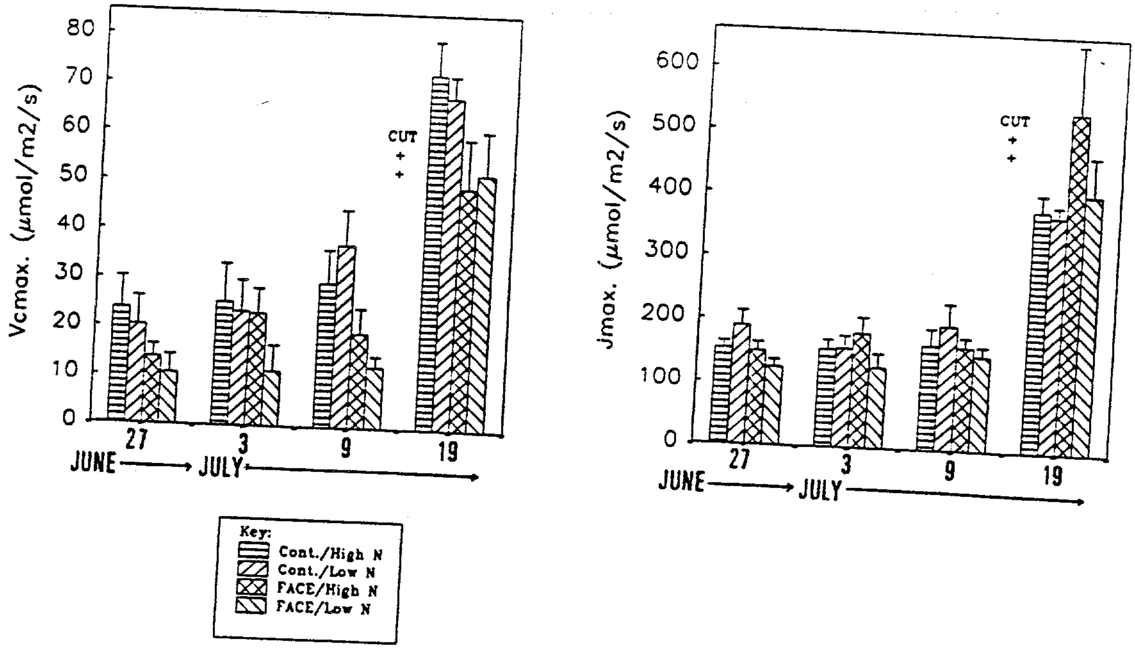


TABLE 5. - Significance levels of variation in $Vc_{max.}$ & $J_{max.}$ due to CO_2 and nitrogen.

$Vc_{max.}$	Source of variation	27th June	3rd July	9th July	19th July	Over regrowth.
	N Treat.	$P=0.52;F=0.44$	$P=0.29;F=1.18$	$P=0.92;F=0.01$	$P=0.90;F=0.02$	-
	CO_2 Treat.	$P=0.06;F=3.86$	$P=0.27;F=1.31$	**$P<0.05;F=9.49$**	**$P<0.05;F=6.12$**	-
	Interaction	$P=0.98;F=0$	$P=0.44;F=0.63$	$P=0.21;F=1.68$	$P=0.64;F=0.22$	-
	Time	-	-	-	-	**$P<0.05;F=25.8$**
$J_{max.}$	N Treat.	$P=0.69;F=0.16$	$P=0.25;F=1.42$	$P=0.68;F=0.17$	$P=0.24;F=1.49$	-
	CO_2 Treat.	$P=0.05;F=4.28$	$P=0.98;F=0$	$P=0.34;F=0.97$	$P=0.20;F=1.80$	-
	Interaction	$P=0.07;F=3.68$	$P=0.21;F=1.69$	$P=0.36;F=0.87$	$P=0.44;F=0.62$	-
	Time	-	-	-	-	**$P<0.05;F=31.8$**
	n	6	6	6	6	24

In addition, there was no significant effect of either CO_2 or nitrogen treatment on $J_{max.}$ although it did vary significantly over time. This significant variation over time is clearly the result of the large increases in both $Vc_{max.}$ and $J_{max.}$ immediately after cutting which were not so evident in the first experiment.

A scatter plot of $Vc_{max.}$ vs. $J_{max.}$ values for all the plants sampled in the second experiment, clearly illustrates the shift in plant resources away from carboxylation efficiency ($Vc_{max.}$) (Fig. 5). The upward shift in the gradients of the lines for FACE plants appears to be a result of decreased $Vc_{max.}$ rather than any increase in $J_{max.}$ (Fig. 4). The ratios of $J_{max.}$:$Vc_{max.}$ were 6.33 and 5.66 for ambient plots at high and low nitrogen respectively, the corresponding values for FACE plots being 9.8 and 8.27. This difference between FACE and ambient plots in $J_{max.}$:$Vc_{max.}$ ratio was highly significant ($P<0.05$; F=11.6; n=22).

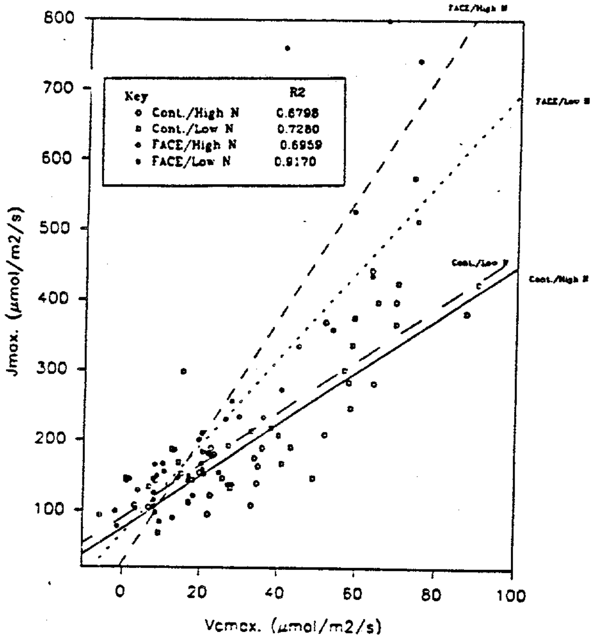


FIG. 5. - Scatter plot of $Vc_{max.}$ vs. $J_{max.}$ for *L. perenne* grown in ambient and FACE plots at high and low nitrogen-27/6-19/7/94

The results from the A/Ci curves taken on 3 additional grassland species show that there was no significant variation between the 2 genotypes in any of the species in terms of $Vc_{max.}$ but that the opposite was true with $J_{max.}$ (Fig. 6. & Table 6).

FIG. 6.

Variation in Vcmax. and Jmax. for 2 Genotypes (V1 & B1) of 3 Monocotyledenous Species (*Holcus Lanatus, Dactylis glomerata & Arrhenaterum elatius*) Grown in Ambient (■) and FACE (□) Plots.

(Vcmax. and Jmax values are obtained from A/Ci plots fitted to a model curve at 25°C Based on the equations of Harley et al. 1992 and Evans and Farquhar, 1991. A/Ci plots taken on 6th and 12th July 1994)

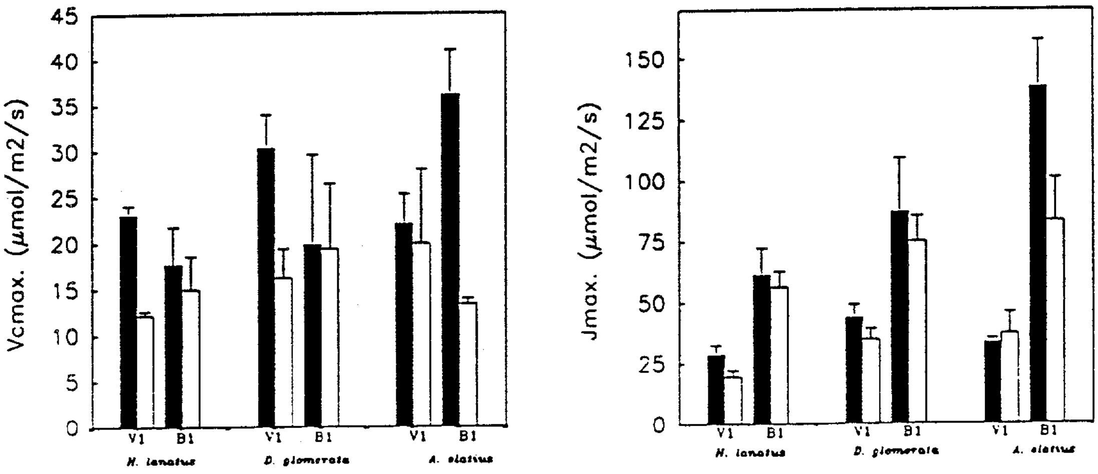


TABLE 6. - Significance levels of variation in $Vc_{max.}$ & $J_{max.}$ due to CO_2 and genotype in 3 perennial grassland species.

$Vc_{max.}$	Source of variation	*H. lanatus*	*D. glomerata*	*A. elatius*
	CO_2 Treat	**$P<0.05$; $F=5.93$**	$P=0.29$; $F=1.26$	**$P<0.05$; $F=6.09$**
	Genotype	$P=0.66$; $F=0.22$	$P=0.59$; $F=0.31$	$P=0.48$; $F=0.55$
	Interaction	$P=0.18$; $F=2.18$	$P=0.32$; $F=1.11$	$P=0.08$; $F=4.18$
$J_{max.}$	CO_2 Treat.	$P=0.33$; $F=1.08$	$P=0.43$; $F=0.68$	$P=0.06$; $F=4.78$
	Genotype	**$P<0.05$; $F=26.57$**	**$P<0.05$; $F=10.74$**	**$P<0.05$; $F=42.7$**
	Interaction	$P=0.77$; $F=0.09$	$P=0.92$; $F=0.01$	**$P<0.05$; $F=6.31$**
	n	3	3	3

FACE grown *H. lanatus* and *A. elatius* plants both showed significant reductions in inferred RubisCO activity but as in *L. perenne* there was no significant effect of CO_2 on $J_{max.}$.

(c) Stomatal Measurements

There was no significant effect of growth in elevated CO_2 on either stomatal density, stomatal index or stomatal pore length (Table 7 & Fig. 7). Although there were slightly higher stomatal densities and indices on both leaf surfaces in the low nitrogen treatment of FACE and ambient plots the effect was not strong enough to attain statistical significance.

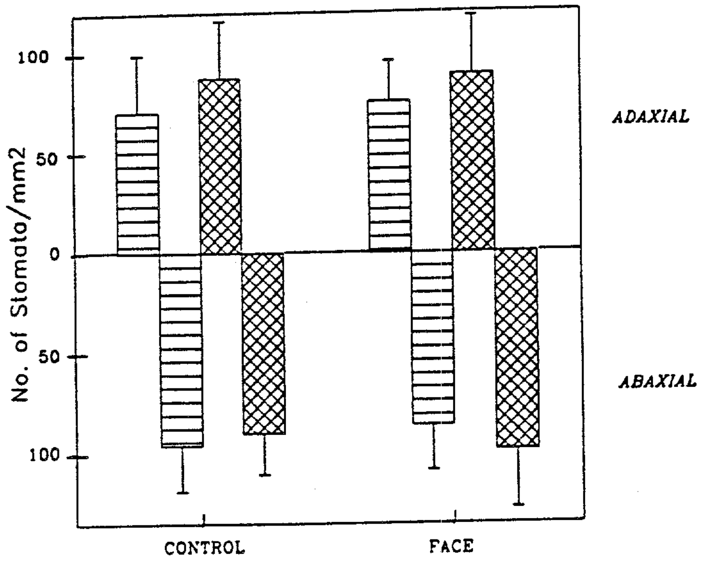
TABLE 7. - Significance levels of variation in stomatal characteristics due to CO_2 and nitrogen treatment.

Source of variation	Stomatal Density		Stomatal Index		Stomatal Pore Length
	Abaxial	Adaxial	Abaxial	Adaxial	
CO_2 Treat.	$P=0.92$; $F=0.01$	$P=0.78$; $F=0.08$	$P=0.50$; $F=0.49$	$P=0.82$; $F=0.05$	$P=0.83$; $F=0.05$
N Treat.	$P=0.61$; $F=0.28$	$P=0.17$; $F=2.04$	$P=0.68$; $F=0.19$	$P=0.25$; $F=1.55$	-
Interaction	$P=0.16$; $F=2.16$	$P=0.88$; $F=0.03$	$P=0.70$; $F=0.16$	$P=0.92$; $F=0.01$	-
n	30	30	30	30	45

FIG.7

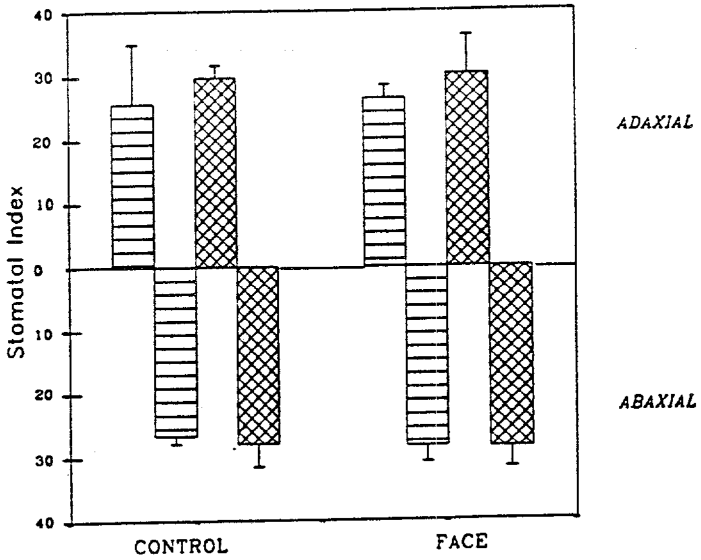
Stomatal Density and Index for *Lolium perenne* Grown in Control and FACE Plots at High (▤) and Low (▩) Nitrogen

(Measurements/Counts Taken on Fresh Tissue From 26/6/94 - 5/7/94)



AVERAGE STOMATAL PORE LENGTH (mm):

Control = 0.0322 +/- 0.0036
FACE = 0.0318 +/- 0.0004



DISCUSSION

The results from the diurnal measurements of photosynthesis in *L. perenne* were clear and unequivocal - considerable enhancement of A occurred in all plants grown in elevated CO_2, ranging from 34.15% to 88.23%. Enhancement still occurred at the beginning and end of the day when lower photon flux may have been expected to favour the ambient grown plants. This continued enhancement at low photon flux densities has been demonstrated previously in *Lolium temulentum* (Gay and Hauck, 1994) but other studies have suggested that enhancement of A at high CO_2 occurs at higher photon flux densities only (Ryle *et al.*,1992; Nijs *et al.*,1989). The present study is in direct contrast to that of Nijs *et al.* (1988) who demonstrated decreased rates of leaf A due to an increase in leaf area index caused by high CO_2 concentrations. However, there is a strong possibility that altered plant-atmosphere coupling in their growth enclosure system could at least partially explain their results.

The extremely strong enhancement of photosynthesis reported here on 28th July, and to a lesser extent on the 3 other dates, is likely to be the result of a combination of 2 factors. Firstly is the important and often neglected temperature interaction at elevated CO_2 (Long, 1991; Kirschbaum, 1994). The high leaf temperatures result in an increasing burden of photorespiration in ambient CO_2 grown plants and, hence, considerably reduced A values. The virtual elimination of RubisCO oxygenation at high CO_2 means that FACE grown plants are largely unaffected, whilst their temperature optimum for photosynthesis shifts upwards. However, in temperate ecosystems, such meteorological conditions are relatively infrequent, and further studies are required under the more predominant conditions of relatively lower light intensities and air temperatures. The second factor which is contributing to this large enhancement of A on 28th July is water stress in ambient CO_2 grown plants. Values of g_s (Fig. 2d) indicate that ambient grown plants are suffering relatively more than FACE grown plants from the long drought conditions (Appendix. 1), and the resultant partial stomatal closure is having an inhibitory effect on the supply of CO_2 to the chloroplasts. The greater water use efficiency of C3 plants growing at high CO_2 concentrations (e.g. Allen Jr., 1990) allows the stomata to remain open, even under these conditions of severe water stress. As a result, CO_2 assimilation rates are maintained at near to normal values.

Enhancement of diurnal A in elevated CO_2 still occurred at the low nitrogen treatment. This contrasts sharply with the study of Wong (1979) on another C3 plant, cotton, which suggested that low levels of nitrogen may eliminate any enhancement of photosynthesis at high CO_2. In fact, although % enhancement of diurnal A at high CO_2 generally seems to be reduced at the low nitrogen treatment, the peaks of photosynthesis show precious little difference between nitrogen treatments. The considerably denser plant canopies in the high nitrogen treatment (see Appendix. 3) could mean that many leaves are developing under low photon flux densities and are therefore not reaching their full photosynthetic potential. By contrast, the plants growing in the much less dense canopy of the low nitrogen treatment are probably operating at much closer to their full potential at both growth CO_2 concentrations. Paradoxically, this effect is normally associated with canopies developing under high and ambient CO_2 (Nijs *et al.*, 1988; Nijs and Impens, 1993; Gay and Hauck, 1993).

The effect of time after cutting treatment on diurnal A is quite evident from these results. On 25th June, which was 40 days after a cut, the average diurnal A in FACE grown plants at high nitrogen was 22.6 $\mu mol/m^2/s$ whereas on 22nd July, 10 days after a cut, the equivalent value of A was 31.8. These results support the hypothesis of Nijs and Impens (1993) that more frequent cutting of Ryegrass stands could yield a higher increase in seasonal production from elevated CO_2, since the stand would then be in the younger growth stages most of the time, where the response to high CO_2 is largest.

As expected (Pearcy and Bjorkman, 1983), rates of leaf g_s and E generally declined in high CO_2 grown plants with the obvious exception of the diurnal set of measurements taken on 28th July. The % reductions in FACE and the absolute values of g_s and E, were considerably higher in the low nitrogen treatment than the high nitrogen treatment. This effect could partially be explained by reduced growth and epidermal cell expansion in plants grown at low nitrogen, leading to higher stomatal densities per unit leaf area.

There is a suggestion of this (though not significant) in the stomatal density data that are presented here.

However, conclusions drawn from E data have to be tempered with a fair degree of caution. Humidity in the leaf chamber is likely to differ markedly from that of the ambient air, and absolute measurements of E are therefore probably unrealistic.

There is clear evidence in this study that field-grown *L. perenne* can undergo an acclimatory change in its photosynthetic apparatus in response to growth in enhanced CO_2. This throws doubt on the assertion of Arp (1991) that downward regulation of photosynthetic capacity is purely an artefact of small experimental pot size which restricts plant sink development. The significantly reduced Vc_{max} values in FACE grown plants strongly indicate a decrease in RubisCO activity which would either be in the form of a decreased amount of RubisCO per unit leaf area or a decrease in RubisCO activation state per unit leaf area (Stitt, 1991). However, under no conditions did $J_{max.}$ values, which represent a measure of the maximum capacity for regeneration of RubP, differ significantly between FACE and ambient grown plants. This disruption in the normally linear relationship between $Vc_{max.}$ and $J_{max.}$ (Wullschleger, 1993) is clearly illustrated in Fig. 5 and in the significant differences in J_{max}:$Vc_{max.}$ ratios between FACE and ambient grown plants. Similar effects, also in Ryegrass, have recently been demonstrated by Nijs (unpublished data). These findings are, broadly speaking, in agreement with those of Ryle *et al.* (1992) who recorded reduced photosynthesis in leaves of elevated CO_2 grown Ryegrass at both elevated and normal concentrations of CO_2. In *Lolium temulentum*, no such acclimatory response to elevated CO_2 has been found, although this is thought to be largely due to the fact that the plants were still growing at the end of the experiment, and were therefore at no stage sink-limited (Gay and Hauck, 1994).

The theory that stomatal limitation to photosynthesis may partially cause this negative acclimatory response to growth at enhanced CO_2, can be ruled out in this case.

Using the viable A/Ci plot, where leaf temperatures during measurement were very similar in FACE and ambient plots, relative stomatal limitation to photosynthesis was calculated to be 23.6% in ambient grown plants and only 9% in FACE grown plants.

It is also highly unlikely that reduced stomatal numbers are one of the causes of the reported acclimatory changes in photosynthetic capacity. There were no significant differences between CO_2 treatments in any of the stomatal parameters measured, which backs up the controlled-environment studies of Ryle and Stanley (1992) and Gay and Hauck (1994). The hypothesis that leaves grown at elevated CO_2 may become hypostomatous (M. Frehner - personal communication) would also have to be rejected on this evidence.

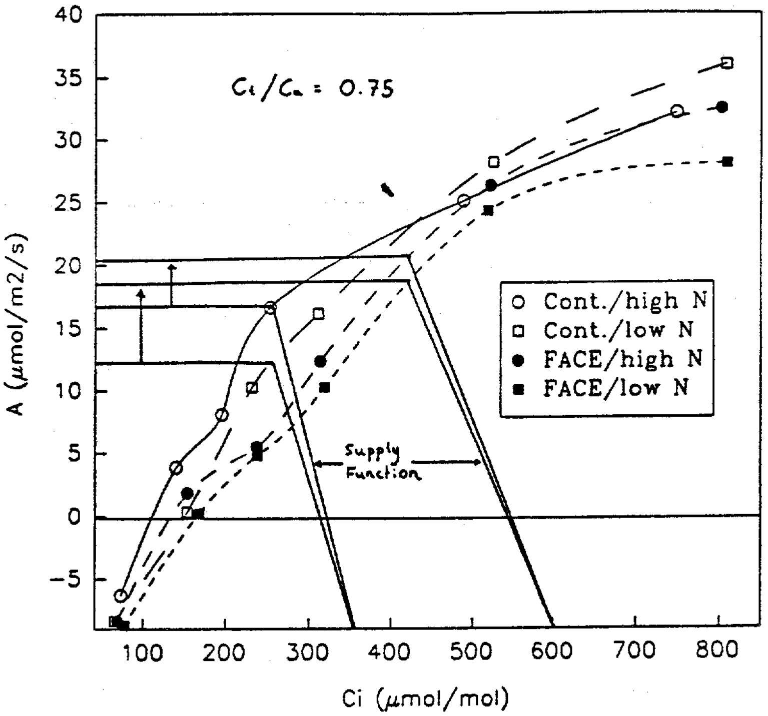
How do the management practices of nitrogen addition and cutting regime affect the acclimatory response of photosynthesis in *L. perenne* grown at high CO_2? Fig. 4 clearly shows the lack of sensitivity of response of both $Vc_{max.}$ and $J_{max.}$ to nitrogen supply which runs contrary to the findings of Wong (1979), who showed a highly significant effect of nitrogen supply on the acclimation of cotton grown at elevated CO_2. This is somewhat surprising in view of the sparse canopy of the low nitrogen grown *L. perenne* (see Appendix. 3) - restricted sink size may have been expected to lead to feedback inhibition of photosynthesis (Arp, 1991). However, a small effect of nitrogen supply can be detected from the $J_{max.} : Vc_{max.}$ ratios, which are lower at the low nitrogen treatment in both ambient and FACE grown plants. This suggests that nitrogen availability may be limiting the plant in its ability to redistribute resources away from RubisCO at high CO_2. Analysis of leaf nitrogen contents would provide more clues to this possible effect, and would be necessary before any definite conclusions could be drawn.

The response to cutting regime is somewhat more straightforward. Significant increases in both $Vc_{max.}$ and $J_{max.}$ occurred directly after a cut in all but the cutting of the 8-cut treatment on 13th June. This temporary increase in photosynthetic capacity would appear to be a measure taken to ensure rapid canopy re-establishment directly after the cut. In addition, there were no significant intrinsic differences in downregulation of RubisCO activity between the 2 cutting frequencies.

The question now arises as to how the apparent lack of any increase in the capacity for RubP regeneration is consistent with the considerable stimulation of photosynthesis at high CO_2 recorded in the diurnal sets of measurements. From the 'viable' A/Ci curve (Fig. 8) it is shown that even with the decreased carboxylation efficiencies found in FACE grown plants, the Ci's obtained at Ca 600 are sufficiently high to produce higher A values than those produced at the Ci corresponding to Ca 355, assuming a constant Ci:Ca ratio for *L.perenne* of 0.75 (Nijs *et al.*, 1989).

FIG. 8 - Viable A/Ci plot for *L. perenne.* 4 cut treatment / 42 days post cutting. 27/6/94.

(points are means of 6 A Vs. Ci values plotted at leaf temp.'s)



All these different lines of evidence develop a picture of where *L. perenne* lies in relation to the 'perfect' acclimatory response outlined by Sage *et al.*, (1989). Clearly, the capacity of the process which is non-limiting at high CO_2 (i.e. RubP carboxylation) has been regulated downwards in order to create a balance with the newly limiting processes (either Pi or RubP regeneration). It has been suggested that the next stage, whereby resources are reallocated away from the down-regulated, non-limiting components to the limiting components, may take years in perennial species (Long and Drake, 1992) and this would appear to be the case in *L. perenne.* The lack of any significant increase in $J_{max.}$ indicates that upgrading of RubP regeneration capacity is not yet complete, and there are 4 lines of evidence from other sources which suggest that the ability of *L. perenne* to generate new sinks may be limited. Firstly, it has been shown that at high light intensities (low red:far red radiation ratio), tillering ability of *L. perenne* is severely restricted, which may well result in saturation of sink capacity and a subsequent lack of growth response at high CO_2 (Deregibus *et al.*, 1983; Gay and Hauck, 1994). Secondly, *L. perenne* is known to partition relatively more of additional photosynthate produced at high CO_2 into storage rather than translocation and growth of new sinks (Wardlaw, 1982). Thirdly, the major respiratory efflux from *L. perenne* which is associated with the biosynthesis of new tissue (Sheehy *et al.*, 1979) has been shown to decrease by 30-40% per unit biomass in elevated CO_2 grown Ryegrass (Bunce and Caulfield, 1991). Finally, the results from the first year of the FACE experiment showed that the yield increase in *L. perenne* grown at 600 µmol/mol CO_2 was only 6.2% (c.f. 18.6% for *T. repens* - E.T.H.-FACE News, 1994).

It may therefore take some time for the realignment of the capacities of the limiting and non-limiting processes such that long-term photosynthetic capacity is enhanced to its maximum potential in Ryegrass grown at high CO_2. The results of Arp and Drake (1991) on the C3 sedge *Scirpus olneyi* hint at a period of up to 4 years for the realisation of the perfect acclimatory response in field-grown perennials.

This theory is given support by the results from the other 3 grassland species sampled, all of which are perennials like *L. perenne. H. lanatus* and *A. elatius* show similar behaviour to *L. perenne* in terms of significantly reduced $Vc_{max.}$ values at high CO_2 whilst all 3 species exhibited no significant change in $J_{max.}$. However, $J_{max.}$ appears to provide the major source of intra-species variation in response to elevated CO_2, with values for the B1 plants all being higher than their V1 counterparts.

In conclusion, although photosynthesis was enhanced considerably throughout the day in this study, it is still not clear whether seasonal productivity and carbon storage in Ryegrass will be increased in a future high CO_2 world. The strong acclimatory response that was observed, even in this realistic open-field situation, suggests that after almost 2 years growth at high CO_2, *L. perenne* is still some way from achieving the appropriate morphological adjustments which would allow the attainment of a significantly higher photosynthetic potential. Clearly, more frequent cutting of Ryegrass stands temporarily helps to alleviate such sink saturation but much research is still required into the more fundamental question of just how much increased rates of photosynthesis have the potential to increase plant growth at elevated atmospheric levels of CO_2.

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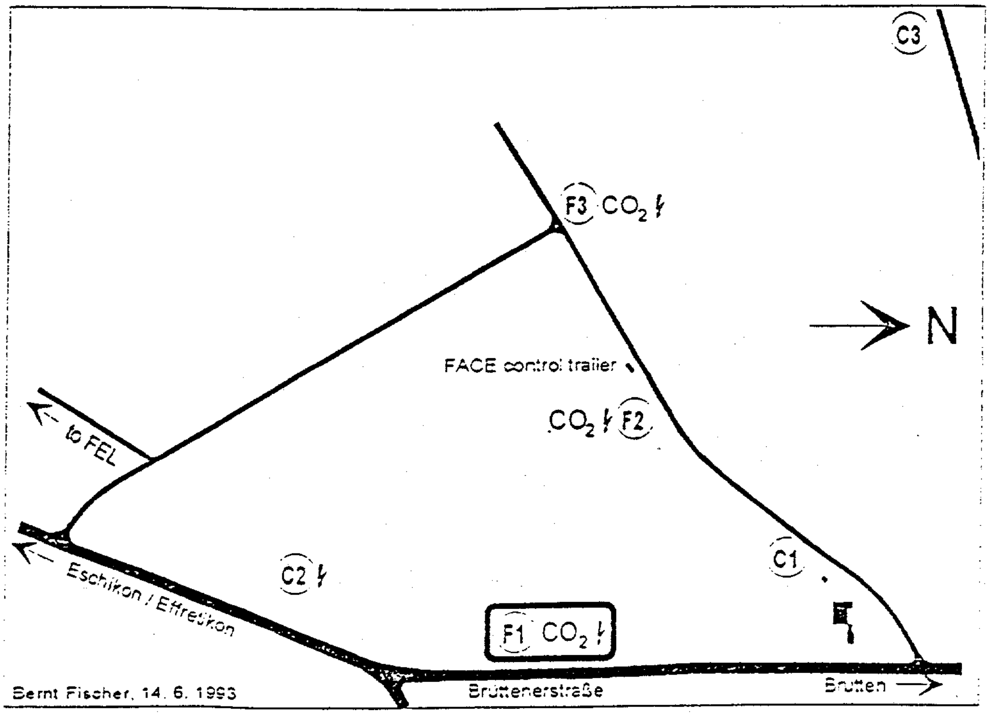
APPENDIX. 1. - Micrometeorological data for the period of measurements.

Date	Av. day air temp. - °C	Av. night air temp.- °C	Av. day + night - °C	Max. air temp. - °C	Min. air temp. - °C	% R.H.	Global rad. - MJ/m^2	Past 7 days precip.- mm
30/5 (A/Ci)	12.9	9.4	11.6	15.1	7.2	70.2	13.1	36.8
1/6 (A/Ci)	21.7	15.3	19.3	28.4	11	39	25.8	22.4
6/6 (A/Ci)	11.9	11.5	11.7	15.1	4.7	51.8	11	25.8
12/6 (A/Ci)	12	10.6	11.5	13.7	8.9	53.4	6.4	48.9
14/6 (A/Ci)	16.9	11.9	15.1	20.6	9	38.1	22.9	44.5
17/6 (A/Ci)	19.4	12	16.7	23.7	9.5	23	27.2	15.2
21/6 (A/Ci)	19.1	14.7	17.4	22.7	12.1	47.4	18.8	15.4
23/6 (Diu.)	21.7	17	19.9	25.4	13.7	35.1	27.1	15.4
25/6 (Diu.)	24.8	16.8	21.6	28.8	14.6	31.4	24.9	15.4
27/6 (A/Ci)	23	18.3	21.2	28.9	16.1	55.8	20.6	7.6
3/7 (A/Ci)	Data	Not	Taken					2.0
6/7 (A/Ci)	24	16.9	20.8	26.4	13.7	45.7	19.4	0.1
9/7 (A/Ci)	19.6	14.9	17.8	23.3	12.8	40	25.4	3.6
12/7 (A/Ci)	23.4	17.1	21	28.7	13.4	37.5	26.5	4.6

APPENDIX. 2.

Map of ETH - Eschikon FACE site showing location of the 3 ambient and 3 control experimental rings.



Bernt Fischer, 14. 6. 1993

APPENDIX 3 - Canopies of *Lolium perenne* growing at high CO_2 in a) High nitrogen and b) low nitrogen. Note large differences in canopy density.

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