Gas exchange acclimation to elevated CO_2 in upper-sunlit and lower-shaded canopy leaves in relation to nitrogen acquisition and partitioning in wheat grown in field chambers

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Abstract

Growth at elevated CO_2 often decreases photosynthetic capacity (acclimation) and leaf N concentrations. Lower-shaded canopy leaves may undergo both CO_2 and shade acclimation. The relationship of acclimatory responses of flag and lower-shaded canopy leaves of wheat (*Triticum aestivum* L.) to the N content, and possible factors affecting N gain and distribution within the plant were investigated in a wheat crop growing in field chambers set at ambient (360 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2, and with two amounts of N fertilizer (none and 70 kg ha^{-1} applied on 30 April). Photosynthesis, stomatal conductance and transpiration at a common measurement CO_2, chlorophyll and Rubisco levels of upper-sunlit (flag) and lower-shaded canopy leaves were significantly lower in elevated relative to ambient CO_2-grown plants. Both whole shoot N and leaf N per unit area decreased at elevated CO_2, and leaf N declined with canopy position. Acclimatory responses to elevated CO_2 were enhanced in N-deficient plants. With N supply, the acclimatory responses were less pronounced in lower canopy leaves relative to the flag leaf. Additional N did not increase the fraction of shoot N allocated to the flag and penultimate leaves. The decrease in photosynthetic capacity in both upper-sunlit and lower-shaded leaves in elevated CO_2 was associated with a decrease in N contents in above-ground organs and with lower N partitioning to leaves. A single relationship of N per unit leaf area to the transpiration rate accounted for a significant fraction of the variation among sun-lit and shaded leaves, growth CO_2 level and N supply. We conclude that reduced stomatal conductance and transpiration can decrease plant N, leading to acclimation to CO_2 enrichment.

Key-words - *Triticum aestivum* L., acclimation, chlorophyll, elevated CO_2, nitrogen, photosynthesis, Rubisco activity, stomatal conductance, transpiration.

Abbreviations – An, photosynthetic carbon assimilation; E, transpiration; g_s, stomatal conductance; Rubisco, ribulose-1, 5-bisphosphate carboxylase oxygenase.

Introduction

A reduction in the photosynthetic capacity of upper-sunlit leaves has often been observed in C_3 plants grown at elevated CO_2 (Drake *et al*., 1997; Nakano *et al*., 1997; Rogers and Humphries, 2000; Lee *et al*., 2001). The loss of photosynthetic capacity in elevated CO_2 has been attributed to a reduction in the amount and activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) (Drake *et al*., 1997; Rogers and Humphries, 2000), and is more pronounced in conditions where growth may become sink-limited or when plants are grown with a low N supply (Nakano *et al*., 1997; Rogers *et al*., 1998), suggesting that N availability plays an important role in maintenance of photosynthetic capacity. Also, the stomatal conductance (g_s) of sunlit leaves is severely reduced in elevated CO_2-grown plants (Drake *et al*., 1997; Lodge *et al*., 2001; Medlyn *et al*., 2001; Tezara *et al*., 2002). With few exceptions (Osborne et al., 1998; Adam et al., 2000), studies on photosynthetic acclimation to elevated CO_2 have focused on upper-sunlit leaves and little attention has been paid to the acclimatory responses of lower-shaded canopy leaves. In plants growing at elevated CO_2, leaves occupying lower positions will undergo both shade-acclimation, due to the development of upper canopy leaves, and CO_2-acclimation. As a consequence, the photosynthetic acclimation to elevated CO_2 appears to be more pronounced in the lower-shaded and older leaves of the canopy as compared to the uppermost and sunlit leaves (Osborne et al., 1998; Adam et al., 2000). Also, the acclimatory response of g_s to elevated CO_2 could differ among leaves occupying different positions within the canopy.

CO_2 enrichment often leads to decreased N concentration in leaves (Conroy and Hocking, 1993; Stitt and Krapp, 1999) and lower N uptake (Polley et al., 1999) but for reasons that are far from clear. The failure of nitrogen uptake to keep pace with the increased growth rate at elevated CO_2, or dilution of nitrogen by the accumulation of nonstructural carbohydrates (Stitt and Krapp, 1999) cannot provide a satisfactory explanation, particularly when the increases in carbohydrates and dry matter are small. Increased CO_2 leads to decreased stomatal conductance and lower water flow due to transpiration (Stitt and Krapp, 1999). This may decrease the mass flow of water in the soil to the roots and decrease the availability of mobile nutrients such as nitrate (Conroy and Hocking, 1993; Stitt and Krapp, 1999; McDonald et al., 2002), although it has been argued that this will only lead to nitrate becoming limited in low-fertility soils (Stitt and Krapp, 1999), and in any case other factors

responding to CO_2 enrichment can compensate for a low, transpiration-limited N supply (McDonald et al., 2002).

Nitrogen nutrition not only increases the amount of nitrogen in the whole canopy but also affects the distribution of N among the different leaves within the canopy, which is more uniform at high N nutrition (Del Pozo, 1994; Dreccer *et al*., 2000). Accordingly, N nutrition could mitigate CO_2-acclimation, particularly in lower-shaded leaves. Gradients in the leaf N content and photosynthetic capacity within the leaf canopy have been reported for several species (Charles-Edwards *et al*., 1987; Hirose *et al.,* 1989; Lemaire *et al.,* 1991; Evans, 1993; Lötscher *et al*., 2003; Yin *et al*., 2003), including wheat (Del Pozo, 1992; Dreccer *et al*., 2000). Upper-sunlit leaves usually have higher photosynthetic capacity than shaded ones from lower positions in the canopy, which correlates with the vertical distribution of leaf nitrogen per unit leaf area and with light within the canopy (Del Pozo and Dennett, 1999). It has been found that the difference in the transpiration rate among leaves is an important mediator in the response of plants to the vertical light gradient. Moreover, the allocation of resources to leaves in a canopy responds to the rate of transpiration, regardless of light intensity (Pons et al., 2001).

The aim of this study was to assess the acclimatory responses to elevated CO_2 of gas exchange in flag and lower canopy leaves of wheat growing in the field under ventilated plastic chambers with different levels of N supply, and to analyze the involvement in acclimation of nitrogen accretion and partitioning to above-ground plant parts and the possible relationships between N accumulation and transpiration.

Materials And Methods

Site and experimental setup

The experimental site was located at the IRNASA Muñovela Farm at Salamanca (41° N, 800 m.a.s.l.), Spain. The climate in Salamanca corresponds to a Mediterranean type; the long term average of the minimum temperatures of the coldest month (January) is 0.0 °C and of the maximum temperatures of the warmest month (July) it is 27.2 °C. Mean annual rainfall is 506 mm. The soil was a clay sand, alkaline (pH 7.7), with normal levels of P, K and Ca (22, 140 and 2800 ppm, respectively).

Spring wheat (*Triticum aestivum* L. cv. Alcalá) was sown at a rate of 180 kg ha^{-1} and 0.13 m between rows, on 11 February 2003. The crop was sown after turnip and no fertilizer was applied before sowing. The crop was watered weekly through a drip irrigation system and provided 198 mm between February and June, which is the average rainfall in the area during the experimental period. Weeds were controlled chemically.

Two chambers of 9.6 m long, 2.2 m wide and 1.7 m high at the ridge were put in place after crop emergence. The chambers were adapted from Rawson et al. (1995) and have been described in detail elsewhere (Pérez et al., 2005). One chamber was kept at ambient (383±32 360 μmol mol^{-1}) and the other at elevated (766±32 μmol mol^{-1}) CO_2 concentration during the light hours. Since previous work reported almost no effects of growth CO_2 level on leaf dark respiration (Jahnke and Krewitt, 2002), the lack of CO_2 enrichment during the night was considered irrelevant. Two levels of nitrogen supply were established by adding 70 kg ha^{-1} or none to the longitudinal halves of the chambers on 30 April 2003. Therefore, the treatments in this experiment involved growth CO_2 and nitrogen levels. Leaf gas exchange measurements and plant sampling were conducted in the middle module of each chamber, where temperature was close to ambient and homogeneous within the module. Fig. 1 shows the diurnal changes of air temperature, relative humidity, CO_2 concentration and irradiance.

Gas exchange measurements

The rates of photosynthesis (An), g_s and transpiration (E) measured at 360 and 700 μmol mol^{-1} CO_2 were determined in attached flag and lower canopy leaves using a portable open system infra-red gas analyzer (CIRAS-2, PP Systems, Hitchin, Herts., UK). Three to 8

hours after the start of the photoperiod, measurements were performed on 1.7 cm^2 leaf areas with a 300 ml min^{-1} air flow rate, at a photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1} and a leaf temperature of 25 ºC. Vapour pressure deficit (VPD) was maintained at 1.6 ± 0.23 kPa. Flag leaves were measured several times during development, while the 3 uppermost leaves in the canopy (flag, 5th and 4th) were measured at anthesis; little green area remained in leaves below these positions. For measurements, each plot was divided into four sampling sectors (replicates). The leaves of a replicate main shoot from each treatment, with treatments in random order, were measured before the next replicate, so that differences during the day could be included in the replicate effect in the analysis of variance.

The acclimatory or long-term response and the direct or short-term response of An to elevated CO_2 of leaves from different positions in the canopy were quantified as ratios of An according to Bunce (2001), and the net effect as the product of the acclimatory and the direct effects (Net effect = Acclimatory effect x Direct effect):

$$An(e,E)/An(a,A) = [An(e,E)/An(a,E)] \times [An(a,E)/An(a,A)] \quad (1)$$

where a and e refer to ambient (383 μmol mol^{-1}) and elevated (766 μmol mol^{-1}) growth conditions, respectively, and A and E refer to ambient (360 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) measurement conditions. Similar ratios were calculated for g_s.

After gas exchange measurements, the leaves of four main shoots from each sampling sector were rapidly transferred *in situ* to liquid nitrogen for the determination of chlorophyll content and Rubisco activity. Another seven shoots from each sector were harvested for leaf area, dry matter, and nitrogen determination.

Dry weight, green area and nitrogen content

The green area of leaves, last stem internode, rest of the stem and the ear were measured separately with an electronic planimeter (Li-3050A, Li-Cor, Lincoln, Nebraska, USA), dried at 60 ºC for 48 h and then weighed. After grinding in a mill and Kjeldhal digestion with H_2SO_4 using a Se catalyst, nitrogen was determined in these samples with a Bran

Luebbe (Hamburg, Germany) AAIII colorimetric continuous-flow analyzer, following the manufacturer's method.

Chlorophyll contents and Rubisco activity

The projected area of a subsample of frozen leaves from the various positions in the canopy was measured by image analysis, and then weighed and ground in liquid nitrogen. Chlorophyll was extracted with acetone (80%) and determined according to Arnon (1969), thus allowing the results to be expressed on a leaf area basis.

For Rubisco activity, samples of the frozen leaves were ground in a mortar with liquid nitrogen, extracted with 100 mM Bicine-NaOH (pH 7.8), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2% polyvinylpoly-pyrrolidone (PVPP) (w/v) and then centrifuged at 13000 g. A NADH oxidation- coupled spectrophotometric assay (Pérez et al., 2005) was used to determine Rubisco activity before (initial activity) and after (total activity) carbamylation of active sites; the activation state was estimated as initial activity, as a percentage of total activity.

Statistical analysis

Differences between treatments were determined through analysis of variance using a nested design according to Snedecor and Cochran (1967), with nitrogen as a stratum included in CO_2, and replicate samplings as a stratum included in that for nitrogen. For flag leaves there were four measurement dates and for the other leaves one measurement date (at anthesis), and hence date or leaf position were included in the analysis as a further stratum. Additional details about this analysis are described in this journal by Pérez et al. (2005). Regressions were performed with the GenStat 6.2 statistical package.

Results

Leaf gas exchange

Plants grown at elevated CO_2 displayed a highly significant ($P < 0.01$) reduction in An, g_s and E of flag leaves, measured either at 360 or 700 μmol mol^{-1}, relative to ambient CO_2-grown plants (Fig. 2). N supply increased An, but decreased g_s in plants grown at ambient CO_2. As development progressed from ear emergence towards grain maturity, An, g_s and E decreased significantly ($P < 0.05$) at both growth CO_2 levels (Fig. 2).

The rates of photosynthesis, transpiration and g_s, all measured at 700 μmol mol^{-1} at anthesis, decreased significantly ($P < 0.05$) with leaf position in elevated and ambient CO_2-grown plants (Tables 1 and 2). In general, growth in elevated CO_2 tended to decrease An, g_s and E, but only the effects on E reached statistical significance (Tables 1 and 2). N supply had no significant effect on g_s or E, but increased An significantly ($P < 0.05$, Table 2).

In flag leaves, the acclimatory responses to elevated CO_2 of An and g_s (measured at 700 μmol mol^{-1}) were similar with low and high N, but in lower canopy leaves they were reduced with N supply (Table 3), reaching values higher than 1 in the 4^{th} leaf, which indicates a positive, rather than a negative, acclimation to elevated CO_2. In ambient CO_2-grown plants, the increase in the measurement CO_2 from 360 to 700 μmol mol^{-1} CO_2 (the direct effect) stimulated An. Hence the net effects of elevated CO_2 were a moderate decrease in An in N-deficient plants, but an increase in An of lower canopy leaves with a high N supply (Table 3). Owing to the reduction in g_s when the measurement CO_2 increased from 360 to 700 μmol mol^{-1} CO_2 (the direct effect), except in the 5^{th} leaf in N-deficient plants, the net effect of elevated CO_2 was a reduction in g_s, which was stronger in N deficient plants (Table 3), except for an increase in gs in lower canopy leaves with a high N supply, similar to that observed for An.

Chlorophyll and N contents and Rubisco activity

The chlorophyll contents and Rubisco activities of flag and lower canopy leaves were significantly lower ($P < 0.05$) in elevated relative to ambient CO_2-grown plants (Table 2, Figs. 3 and 4). N-deficient plants had lower ($P < 0.05$) chlorophyll content and Rubisco activity at both growth CO_2 levels (Table 2, Figs. 3 and 4). Chlorophyll content and Rubisco

activity decreased with leaf position in the canopy at both growth CO_2 levels (Table 2, Fig. 4).

On a leaf area basis, elevated CO_2 decreased leaf N contents, except in 3rd leaves with a low N supply (Table 2, Fig. 5a). A higher N supply increased these contents in leaves above the 3rd one. The results on a dry-weight basis were similar, since specific leaf area was not affected by the growth CO_2 or N supply (data not shown). Leaf N decreased downwards in the canopy, although a high N supply combined with elevated CO_2 maintained similar N contents in 5th, 4th and 3rd leaves. The total nitrogen amount per shoot (Fig. 5b) decreased in elevated growth CO_2 and with nitrogen deficiency. Elevated CO_2 and nitrogen supply also affected the percent distribution of shoot nitrogen among organs (Fig. 5c). Thus, elevated CO_2 increased nitrogen allocation to the ear, stem, and lower (1st and 2nd) senescent leaves. Conversely, elevated growth CO_2 decreased the allocation of nitrogen to the flag and 5th leaves, while it did not affect allocation to the 4th and 3rd leaves. A higher nitrogen supply increased nitrogen allotment to the ear and last stem internode, decreased nitrogen allotment to the rest of the stem, and had no effect on nitrogen allotment to lower (1st and 2nd) senescent leaves. At elevated CO_2, N addition did not increase the allocation of N to flag leaves, as it did at ambient CO_2, and tended to decrease the allocation of N to 5th (penultimate) leaves.

Significant relationships of An measured at 700 μmol mol^{-1} CO_2 with chlorophyll contents and total Rubisco activities were observed, with common regressions for leaves in different canopy positions and growth CO_2 and N supplies (Fig. 6). In turn, chlorophyll and total Rubisco activity were related to N contents per unit leaf area ($R^2 = 0.64$, $P<0.01$ and $R^2= 0.77$, $P<0.01$, respectively; Fig. 6). We then looked at the relationship between leaf N content on an area basis and the transpiration rate measured at the growth CO_2 concentration and high irradiance at anthesis. A positive relationship was found that accounted for 51 % of the variation in the data for all leaves, growth CO_2 and N supplies (Fig. 7). Obviously, an estimate of accumulated transpiration of the leaf canopy, at the prevailing CO_2 level and irradiance at the different leaf positions, would be a more realistic parameter for use in an analysis of the relationship between nitrogen uptake or content and transpiration. With transpiration rates at the growth irradiance for the different leaves in the canopy, calculated with a multilayer canopy model (Humphries and Long 1995), the relationship with leaf N was improved (63 % of variance accounted for).

Discussion

Elevated CO_2 caused a down-regulation or decline of photosynthetic capacity, in agreement with many previous reports (Stitt, 1991; Sage, 1994; Drake et al., 1997; Stitt and Krapp, 1999; Martínez-Carrasco et al., 2005). Acclimation occurred both in upper-sunlit (flag) and in lower-shaded canopy leaves, among which a steep gradient in photosynthetic capacity was found at both growth CO_2 levels. As expected, the degree of CO_2-acclimation was more pronounced in N-stressed plants (Table 3). In addition, a noteworthy interaction of CO_2 and N was observed; thus, in plants with an adequate N supply the acclimatory responses to elevated CO_2 were less pronounced in lower canopy leaves relative to the flag leaf (Table 3), in disagreement with previous reports (Osborne *et al*., 1998; Adam *et al*., 2000). In N-stressed plants, however, we found a greater acclimation in lower canopy leaves than in the flag leaf. The relationships of An to the chlorophyll concentration and Rubisco activity across all CO_2 – nitrogen combinations in all canopy leaves (Fig. 6) suggest that the loss of photosynthetic capacity in elevated CO_2-grown plants, as well as the gradient in An within the leaf canopy, were due to decreased resource availability. Chlorophyll concentration and Rubisco activity per unit leaf area of flag and lower canopy leaves were increased with N supply (Fig. 4), and they were significantly related to leaf N contents (Fig. 6), as observed in earlier studies (Evans, 1993; Osborne *et al*., 1998); this suggests that a loss of N was the cause of photosynthetic acclimation. Moreover, the lower acclimation to elevated CO_2 in lower-shaded than upper-sunlit leaves with the high-N supply was associated with a smaller decrease in the N contents of leaves in lower positions in the stem as compared to plants grown in ambient CO_2 (Fig. 5a). Even though N addition increased shoot N, under elevated CO_2 it did not increase the fraction of this N allocated to leaves, as it did under ambient CO_2; this is consistent with previous studies (Makino et al., 1997; Pérez et al., 2005). Therefore, N supply did not prevent photosynthetic acclimation, particularly in upper-sunlit leaves. Although photosynthetic acclimation to elevated CO_2 has been attributed to an accumulation of carbohydrates that represses Rubisco gene expression (Krapp et al., 1993; Drake *et al*., 1997), other studies have indicated that soluble carbohydrates are unlikely to be responsible for acclimation (Stitt and Krapp, 1999, Pérez et al., 2005) and have suggested a temporal shift in leaf ontogeny (Ludewig and Sonnewald, 2000), or nitrogen limitation (Nakano et al., 1997; Stitt and Krapp, 1999; Pérez et al., 2005). Also, the accumulation of carbohydrate at

elevated CO_2 did not explain the reduction in leaf nitrogen concentration per unit leaf area and in nitrogen uptake usually observed under elevated CO_2 (Nakano et al; 1997; Polley et al., 1999; Lee et al., 2001; McDonald et al., 2002; Gloser et al., 2002). Alternative explanations for low N contents leading to acclimation of leaf photosynthesis to elevated CO_2 are required.

At elevated CO_2, leaf stomatal conductance was severely reduced (Fig. 2; Table 1), as reported in other studies (Drake et al., 1997; Lodge et al., 2001; Medlyn et al., 2001; Tezara et al., 2002). As a consequence, the transpiration rate of leaves was also reduced (Fig. 2; Table 1) (Senock et al., 1996; Pospisilova and Catsky, 1999) and so was total shoot N (Fig. 5b). The nitrogen content of leaves in different canopy positions, growth CO_2 levels, and N supplies showed a positive relationship with the transpiration rate (Fig. 7). Positive relationships between plant N status and transpiration have been observed previously (Conroy and Hocking, 1993; Polley et al., 1999; McDonald et al., 2002). Moreover, at low vapour pressure deficits the positive effect of elevated CO_2 on plant growth and photosynthesis disappeared and soluble protein contents decreased, accompanied by reduced transpiration (De Luis et al., 2002). Transpiration can facilitate N uptake by enhancing mass flow to the vicinity of roots, such that decreased N levels in plants exposed to elevated CO_2 may be attributable, at least in part, to decreases in transpiration (Conroy and Hocking, 1993; McDonald et al., 2002). Evidence from our experiments supports this conclusion.

An involvement of cytokinins in the delivery of xylem compounds to leaves in proportion to their transpiration rate has also been proposed (Pons et al., 2001). Thus, although more work needs to be done to clarify the role of cytokinins, and possibly other plant hormones in regulating photosynthetic acclimation to rising CO_2 (Yong et al., 2000), a link is hypothesized between transpiration, cytokinins and plant N contents and allocation, which can account for the observed photosynthetic down-regulation under elevated growth CO_2.

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References

Adam, N.R., Wall, G.W., Kimball, B.A., Pinter, P.J., LaMorte, R.L., Hunsaker, D.J., Adamsem, F.J., Thompson, T., Matthias, A.D., Leavitt, S.W., Webber, A., 2000. Acclimation response of spring wheat in a free-air CO_2 enrichment (FACE) atmosphere with the variable soil nitrogen regimes. 1. Leaf position and phenology determine acclimation response. Photosynth. Res. 66, 65-77.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24, 1-15.

Bunce, J.A.., 2001. Direct and acclimatory responses of stomatal conductance to elevated carbon dioxide in four herbaceous crop species in the field. Global Change Biol. 7, 323-331.

Charles-Edwards, D.A., Stutzel, H., Ferraris, R., Beech, D.F., 1987. An analysis of spatial variation in the nitrogen content of leaves from different horizons within a canopy. Ann. Bot. 60, 421-6.

Conroy, J., Hocking, P., 1993. Nitrogen nutrition of C_3 plants at elevated atmospheric CO_2 concentrations. Physiol. Plant. 89, 570-576.

De Luis, I., Irigoyen, J.J., Sánchez-Díaz, M., 2002. Low vapour pressure deficit reduces the beneficial effect of elevated CO_2 on growth of N_2-fixing alfalfa plants. Physiol. Plant. 116, 497–502.

Del Pozo, A., 1992. *Carbon/nitrogen relation in cereals and legumes*. Ph.D. Thesis, University of Reading, 290 pp.

Del Pozo, A., 1994. Leaf area index and relative irradiance within the canopy of wheat and faba bean. Proc 3rd European Society for Agronomy, Abano-Padova, Italy. pp: 108-109.

Del Pozo, A., Dennett, M.D., 1999. Analysis of the distribution of irradiance, leaf nitrogen and photosynthesis within the canopy of *Vicia faba* L. at two contrasting densities. *Austr. J. Agric. Res.* 50, 183-189.

Drake, B.G., González-Meler, M.A., Long, S.P., 1997. More efficient plants: a consequence of rising atmospheric CO_2? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 609-639.

Dreccer, M.F., van Oijen, M., Schapendonk, A.H.C.M., Pot, C.S., Rabbinge, R., 2000. Dynamics of vertical leaf nitrogen distribution in a vegetative wheat canopy. Impact on canopy photosynthesis. Ann. Bot. 86, 821-831.

Evans, J.R., 1993. Photosynthetic acclimation and nitrogen partitioning within a lucerne canopy. I. Canopy characteristics. Austr. J. Plant Physiol. 20, 55-67.

Gloser, V., Frehner, M., Lüscher, A., Nösberger, J., Hartwig, U.A., 2002. Does the response of perennial ryegrass to elevated CO_2 concentration depend on the form of the supplied nitrogen? Biol. Plant. 45, 51-58.

Hirose, T., Werger, M.J.A., van Rheenan, J.W.A., 1989. Canopy development and leaf nitrogen distribution in a stand of *Carex acutiformis*. Ecology 70, 1610-8.

Humphries, S.W., Long, S.P., 1995. WIMOVAC: a software package for modelling the dynamics of plant leaf and canopy photosynthesis. CABIOS 11, 361-371.

Jahnke, S., Krewitt, M., 2002. Atmospheric CO_2 concentration may directly affect leaf respiration measurement in tobacco, but not respiration itself. Plant Cell Environ. 25, 641-651.

Krapp, A., Hofmann, B., Schäfer, C., Stitt, M., 1993. Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the "sink regulation" of photosynthesis? Plant J. 3, 817-828.

Lee, T.D., Tjoelker, M.G., Ellsworth, D.S., Reich, P.B., 2001. Leaf gas exchange responses of 13 prairie grassland species to elevated CO_2 and increased nitrogen supply. New Phytol. 150, 405-418.

Lemaire, G., Onillon, B., Gosse, G., Chartier, M., Allirand, J.M., 1991. Nitrogen distribution within a lucerne canopy during regrowth: relation with light distribution. Ann. Bot. 68, 483-8.

Lodge, R.J., Dijkstra, P., Drake, B.G., Morison, J.I.L., 2001. Stomatal acclimation to increased CO_2 concentration in a Florida scrub oak species *Quercus myrtifolia* Willd. Plant Cell Environ. 24, 77-88.

Lötscher, M., Stroh, K., Schnyder, H., 2003. Vertical leaf nitrogen distribution in relation to nitrogen status in grassland plants. Ann. Bot. 92, 679-688.

Ludewig, F., Sonnewald, U., 2000. High CO_2-mediated down-regulation of photosynthetic gene transcripts is caused by accelerated leaf senescence rather than sugar accumulation. FEBS Lett. 49, 19-24.

Makino, A., Harada, M., Sato, T., Nakano, H., Mae, T., 1997. Growth and N allocation in rice plants under CO_2 enrichment. Plant Physiol. 115, 199-203.

Martínez-Carrasco, R., Pérez, P., Morcuende, R., 2005. Interactive effects of elevated CO_2, temperature and nitrogen on photosynthesis of wheat grown under temperature gradient tunnels. Environ. Exp. Bot. 54, 49-59.

McDonald, E.P., Erickson, J.E., Kruger, E.L., 2002. Can decreased transpiration limit plant nitrogen acquisition in elevated CO_2? Funct. Plant Biol. 29, 1115-1120.

Medlyn, B.E., Barton, C.V.M., Broadmeadow, M.S.J., Ceulmans, R., De Angelis, P., Forestreuter, M., Freeman, M., Jackson, S.B., Kellomäki, S., Laitat, E., Rey, A., Roberntz, P., Sigurdsson, B.D., Strassemayer, J., Wang, K., Curtis, P.S., Jarvis, P.G., 2001. Stomatal conductance of forest species after long-term exposure to elevated CO_2 concentration: a synthesis. New Phytol. 149, 247-264.

Nakano, H., Makino, A., Mae, T., 1997. The effect of elevated partial pressures of CO_2 on the relationship between photosynthetic capacity and N content in rice leaves. Plant Physiol. 115, 191-198.

Osborne, C., LaRoche, J., García, R., Kimball, B.A., Wall, G.W., Pinter, P.J., LaMorte, R.L., Hendrey, G.R., Long, S.P., 1998. Does leaf position within a canopy affect acclimation of photosynthesis to elevated CO_2? Plant Physiol. 177, 1037-1045.

Pérez, P., Morcuende, R., Martín del Molino, I., Martínez-Carrasco, R., 2005. Diurnal changes of Rubisco in response to elevated CO_2, temperature and nitrogen in wheat grown under temperature gradient tunnels. Environ. Exp. Bot. 53, 13-27.

Polley, H.W., Johnson, H.B., Tischler, C.R., Torbert, H.A., 1999. Links between transpiration and plant nitrogen: variation with atmospheric CO_2 concentration and nitrogen availability. Int. J. Plant Sci. 160, 535-542.

Pons, T., Jordi, W., Kuiper, D., 2001. Acclimation of plants to light gradient in leaf canopies: evidence for a possible role for cytokinins transported in the transpiration stream. J. Exp. Bot. 52, 1536-1574.

Pospisilova, J., Catsky, J, 1999. Development of water stress under increased atmospheric CO_2 concentration. Biol. Plant. 42, 1-24.

Rawson, H.M., Gifford, R.M., Condon, B.N., 1995. Temperature gradient chambers for research on global environment change. I. Portable chambers for research on short-stature vegetation. Plant Cell Environ. 18, 1048-1054.

Rogers, A., Humphries, S., 2000. A mechanistic evaluation of photosynthetic acclimation at elevated CO_2. Global Change Biol. 6, 1005-1011.

Rogers, A., Fischer, B., Bryant, J., Frehner, M., Blum, H., Raines, C., Long ,S.P., 1998. Acclimation of photosynthesis to elevated CO_2 under low-nitrogen nutrition is affected by the capacity for assimilate utilization. Perennial ryegrass under free air CO_2 enrichment. Plant Physiol. 118, 683-689.

Sage, R.F. 1994. Acclimation of photosynthesis to increasing atmospheric CO_2: The gas exchange perspective. Photosynth. Res. 39, 351-368.

Senock, R.S., Ham, J.M., Loughin, B.A., Kimball, B.A., Hunsaker, D.J., Pinter, P.J., Wall, G.W., García, R.L., LaMorte, R.L., 1996. Sap flow in wheat under free-air CO_2 enrichment. Plant Cell Environ. 19, 147-158.

Snedecor, G., Cochran, W., 1967. *Statistical Methods*. The Iowa State University Press, Ames, Iowa, USA. 593 pp.

Stitt, M., 1991. Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant Cell Environ. 14, 741-762

Stitt, M., Krapp, A., 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ. 22, 583-621.

Tezara, W., Mitchell, V., Driscoll, S.P., Lawlor, D.W., 2002. Effects of water deficit and its interaction with CO_2 supply on the biochemistry and physiology of photosynthesis in sunflower. J. Exp. Bot. 53, 1781-1791.

Yin, X., Lantinga, E., Schapendonk, A.H.C.M., Zhong, X., 2003. Some quantitative relationships between leaf area index and canopy nitrogen content and distribution. Ann. Bot. 91, 893 - 903.

Yong, J.W.H., Wong, S.C., Letham, D.S., Hocart, C.H., Farquhar, G.D., 2000. Effects of elevated [CO_2] and nitrogen nutrition on cytokinins in the xylem sap and leaves of cotton. Plant Physiol. 124, 767–780.

Table 1. Mean values (± S.E.) of rate of photosynthesis (An, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mmol m^{-2} s^{-1}) and transpiration rate (E, mmol m^{-2} s^{-1}) measured at 700 μmol mol^{-1} CO_2 for flag, 5th and 4th leaves of wheat grown in field chambers either at elevated (700 μmol mol^{-1}) or ambient (360 μmol mol^{-1}) CO_2, and at low or high nitrogen supply. Measurements were taken at anthesis (27 May). PPFD and temperature were 1500 μmol m^{-2} s^{-1} and 25 ºC, respectively.

		Elevated CO_2		Ambient CO_2	
Parameter	Leaf	Low N	High N	Low N	High N
An	Flag	14.7 ± 3.2	20.4 ± 5.3	28.1 ± 7.9	39.5 ± 3.2
	5th	6.3 ± 3.2	18.1 ± 7.2	13.3 ± 6.9	21.6 ± 5.9
	4th	5.1 ± 1.7	9.8 ± 2.2	11.3 ± 6.0	6.6 ± 3.4
g_s	Flag	113 ± 37	122 ± 45	317 ± 144	278 ± 40
	5th	48 ± 24	150 ± 71	181 ± 103	161 ± 55
	4th	59 ± 19	110 ± 32	148 ± 78	35 ± 16
E	Flag	1.5 ± 0.3	1.4 ± 0.2	3.7 ±1.2	4.5 ± 0.5
	5th	0.6 ± 0.3	1.6 ± 0.7	1.9 ± 1.1	2.8 ± 0.7
	4th	0.9 ± 0.2	1.4 ± 0.1	1.7 ± 0.9	0.9 ± 0.4

TABLE 2. Analysis of variance (F-values) for rate of photosynthesis (An), leaf stomatal conductance (g_s), and transpiration rate (E) measured at 700 μmol mol^{-1} CO_2, chlorophyll (Chl) and leaf nitrogen (N) concentration, nitrogen amount per plant part (N part), and initial (IRbco) and total (TRbco) Rubisco activities of flag, 5^{th} and 4^{th} leaves of wheat grown in field chambers either at elevated (700 μmol mol^{-1}) or ambient (360 μmol mol^{-1}) CO_2, and low or high nitrogen supply. Measurements were taken at anthesis. Numbers in bold type represent significant effects ($P < 0.05$).

	An	g_s	E	Chl	IRbco	TRbco	N	N part
CO_2 (C)	1.86	1.44	**5.80**	**6.12**	**5.31**	3.64	3.39	2.54
Nitrogen (N)	**4.34**	0.38	1.10	**65.5**	**9.5**	**59.7**	**44.3**	**42.1**
Leaf /organ (L)	**11.83**	**3.99**	**5.67**	**3.83**	**13.5**	**38.7**	**267.9**	**330.8**
CN	0.17	0.49	0.04	3.20	1.28	**4.32**	3.96	0.99
CL	2.21	2.35	**3.54**	0.79	1.73	**4.58**	**38.9**	**15.1**
NL	1.12	0.52	0.70	0.35	0.86	2.78	**16.7**	**18.0**
CNL	0.55	0.03	0.67	0.04	0.66	0.26	2.72	0.47

TABLE 3. Acclimatory [P(e,E)/P(a,E)], direct [P(a,E)/P(a,A)] and net effects [P(e,E)/P(a,A)] of elevated CO_2 on rate of photosynthesis (An) and stomatal conductance (g_s) for flag, 5th and 4th leaves of wheat grown in field chambers either at elevated (700 μmol mol^{-1}) or ambient (360 μmol mol^{-1}) CO_2, and at low or high nitrogen supply. Measurements were taken at anthesis (27 May). P is An or g_s. Values are means of four replicate plants. See Materials and Methods for more explanations.

Nitrogen	Leaf	Acclimatory		Direct		Net effect	
supply	number	An	g_s	An	g_s	An	g_s
Low	Flag	0.52	0.36	1.47	0.76	0.77	0.27
	5th	0.47	0.26	1.84	1.12	0.87	0.29
	4th	0.45	0.40	2.17	0.69	0.97	0.27
High	Flag	0.52	0.44	1.70	0.81	0.88	0.35
	5th	0.84	0.93	1.75	0.85	1.47	0.79
	4th	1.49	3.16	2.11	0.74	3.14	2.33

Figure captions

Fig. 1. Mean daily courses of air CO_2 concentration (a), temperature (b), and humidity (d) in field chambers set at either ambient (360 μmol mol $^{-1}$, open symbols) or elevated (700 μmol mol $^{-1}$, closed symbols) CO_2. The irradiance outside (open symbols) and inside (closed symbols) the chambers is shown in (c) and the temperature and humidity outside the chambers (▲) are shown in (b) and (d).

Fig. 2. Change with time in the rate of photosynthesis (a, d), stomatal conductance (b, e), and transpiration (c, f) measured at 350 (a, b,c) and 700 (d, e, f) μmol mol^{-1} CO_2, of flag leaves of wheat grown at elevated CO_2, high N (■); elevated CO_2, low N (●); ambient CO_2, high N (□); and ambient CO_2, low N (○). Values are means of four replicate plants. PPFD and leaf temperature were 1500 μmol m^{-2} s^{-1} and 25 °C, respectively. Vertical bars represent standard errors of means.

Fig. 3. Change with time in chlorophyll concentration (a), and initial (b) and total (c) Rubisco activities of flag leaves of wheat grown in field chambers either at elevated CO_2, high N (■); elevated CO_2, low N (●); ambient CO_2, high N (□); and ambient CO_2, low N (○). Values are mean of four replicates. Vertical bars represent standard errors of means.

Fig. 4. Mean values at anthesis of chlorophyll concentration (a, b), and initial (c, d) and total (e, f) Rubisco activities for flag, 5th and 4th leaves of wheat grown in field chambers either at elevated (shaded bars) or ambient (open bars) CO_2, and low (a, c, e) or high (b, d, f) nitrogen supply. Values are means of four replicates. Horizontal bars represent standard errors of means.

Fig. 5. Nitrogen content per unit leaf area (a), total nitrogen per shoot (b) and distribution of nitrogen between plant organs (c) at anthesis of wheat grown in field chambers either at ambient (□,▩) or elevated (▨,■) CO_2, and low (□,▨) or high (▩,■) nitrogen supply. A, ambient CO_2; E, elevated CO_2; last internode, last stem internode. Values are means of four replicates. Vertical bars represent standard errors of means.

FIG. 6. Relationships of rate of photosynthesis (An) measured at 700 μmol mol^{-1} CO_2 to chlorophyll concentration (y = 48.943x - 2.5329 R^2 = 0.40) (a) and to total Rubisco activity (y = 0.5126x + 2.6817 R^2 = 0.51) (b); and relationships of chlorophyll concentration (y = 2.866x + 0.0106 R2 = 0.64) (c) and total Rubisco activity (y = 337.81x - 18.115 R2 = 0.77) (d) to leaf N concentration for flag, 5^{th} and 4^{th} leaves at anthesis. Wheat was grown in field chambers at elevated CO_2, high N (■); elevated CO_2, low N (●); ambient CO_2, high N (□); and ambient CO_2, low N (○). For measurements of photosynthesis, PPFD and temperature were 1500 μmol m^{-2} s^{-1} and 25 °C, respectively.

Fig. 7. Relationship between leaf N content and rate of transpiration per unit leaf area measured at the growth CO_2 concentration for flag, 5^{th} and 4^{th} leaves at anthesis. Wheat was grown in field chambers at elevated CO_2, high N (■); elevated CO_2, low N (●); ambient CO_2, high N (□); and ambient CO_2, low N (○). The regression line was y = 0.0199x + 0.0964 (R^2 = 0.51). For measurements of transpiration, PPFD and temperature were 1500 μmol m^{-2} s^{-1} and 25 °C, respectively.

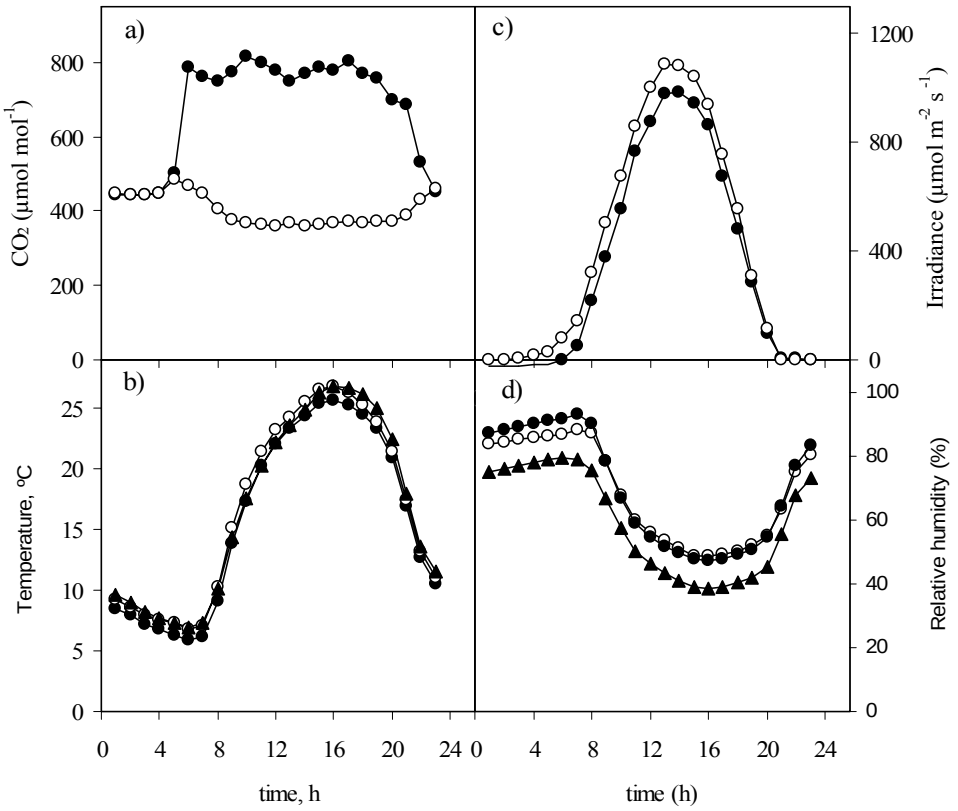
a)
c)
b)
d)
CO2 (µmol mol-1)
Irradiance (µmol m-2 s-1)
Temperature, °C
Relative humidity (%)
time, h
time (h)
0
200
400
600
800
1200
5
10
15
20
25
40
60
80
100
4
8
12
16
24

Fig. 1

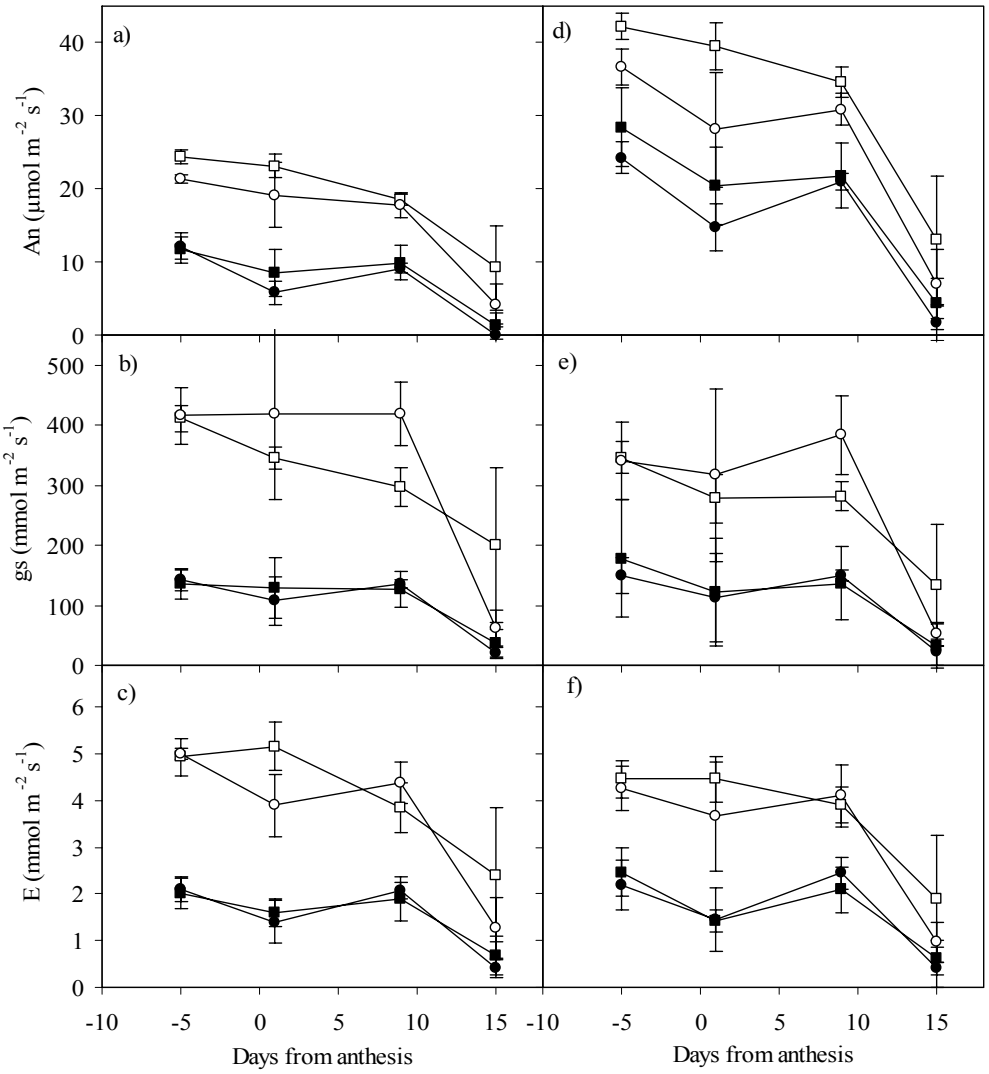
a)
d)
b)
e)
c)
f)
An (µmol m -2 s-1)
gs (mmol m -2 s-1)
E (mmol m -2 s-1)
Days from anthesis
Days from anthesis

Fig. 2

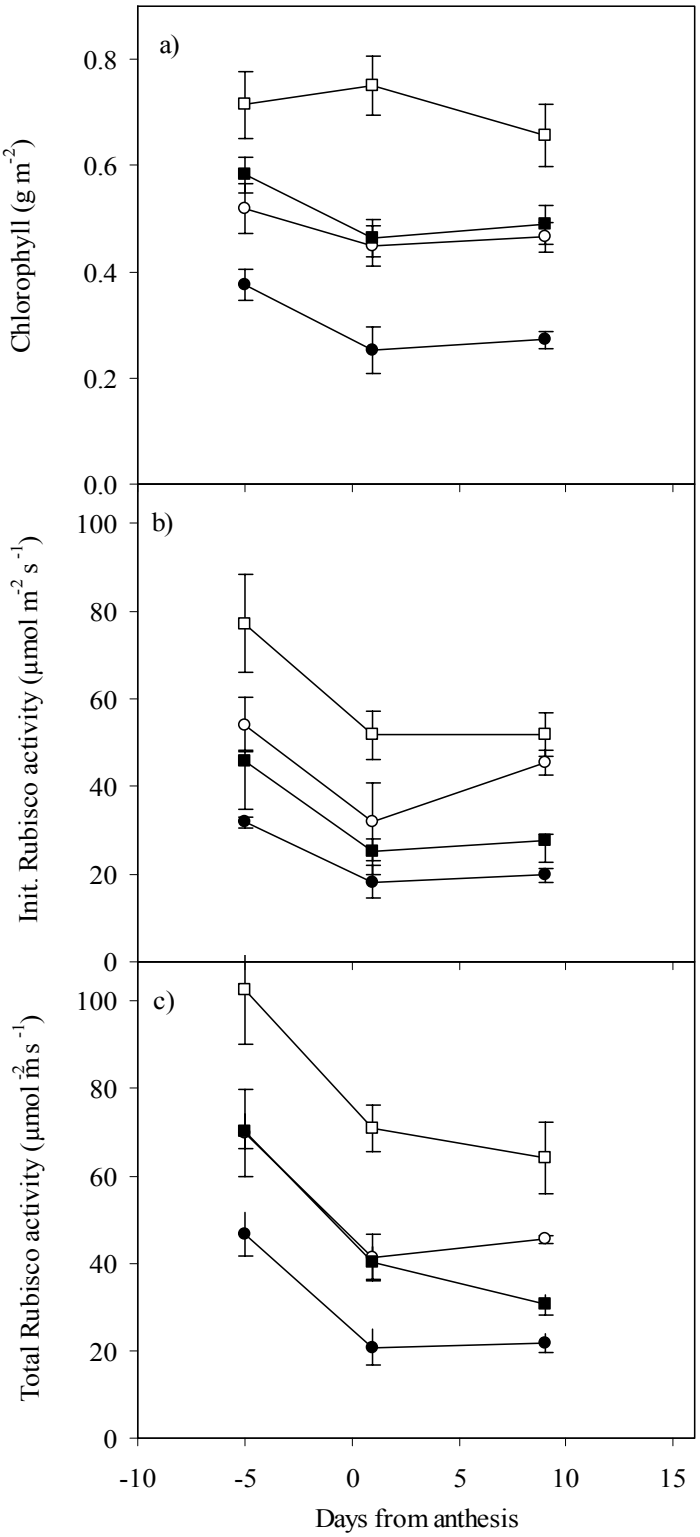
a)
b)
c)
Chlorophyll (g m-2)
Init. Rubisco activity (µmol m-2 s-1)
Total Rubisco activity (µmol m-2 s-1)
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
100
-10
-5
0
5
10
15
Days from anthesis

Fig. 3

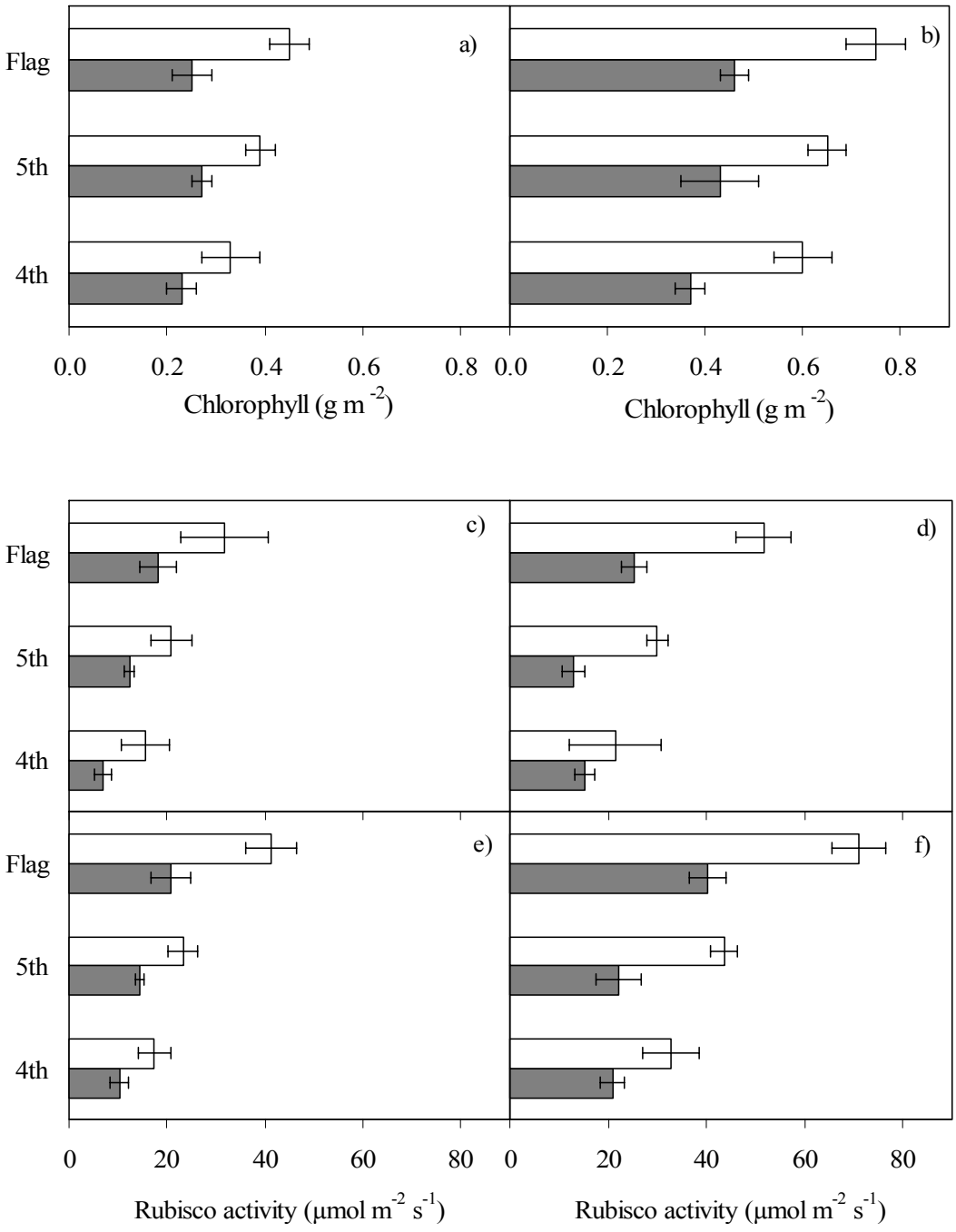
a)
b)
c)
d)
e)
f)
Flag
5th
4th
0.0
0.2
0.4
0.6
0.8
Chlorophyll (g m -2)
0
20
40
60
80
Rubisco activity (µmol m-2 s-1)

Fig. 4

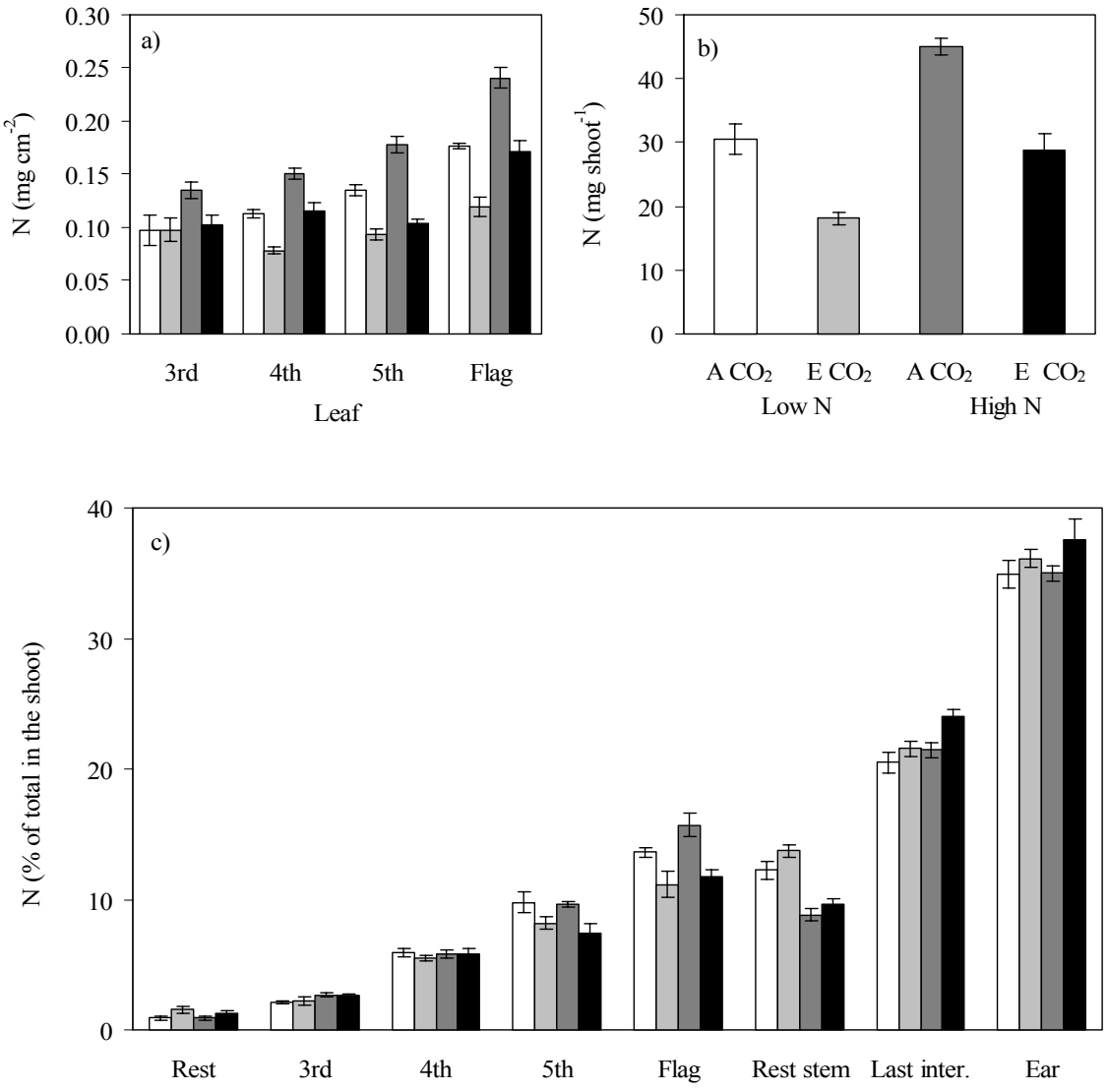
a)
N (mg cm-2)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3rd
4th
5th
Flag
Leaf
b)
N (mg shoot-1)
50
40
30
20
10
0
A CO2
E CO2
A CO2
E CO2
Low N
High N
c)
N (% of total in the shoot)
40
30
20
10
0
Rest
3rd
4th
5th
Flag
Rest stem
Last inter.
Ear

Fig. 5

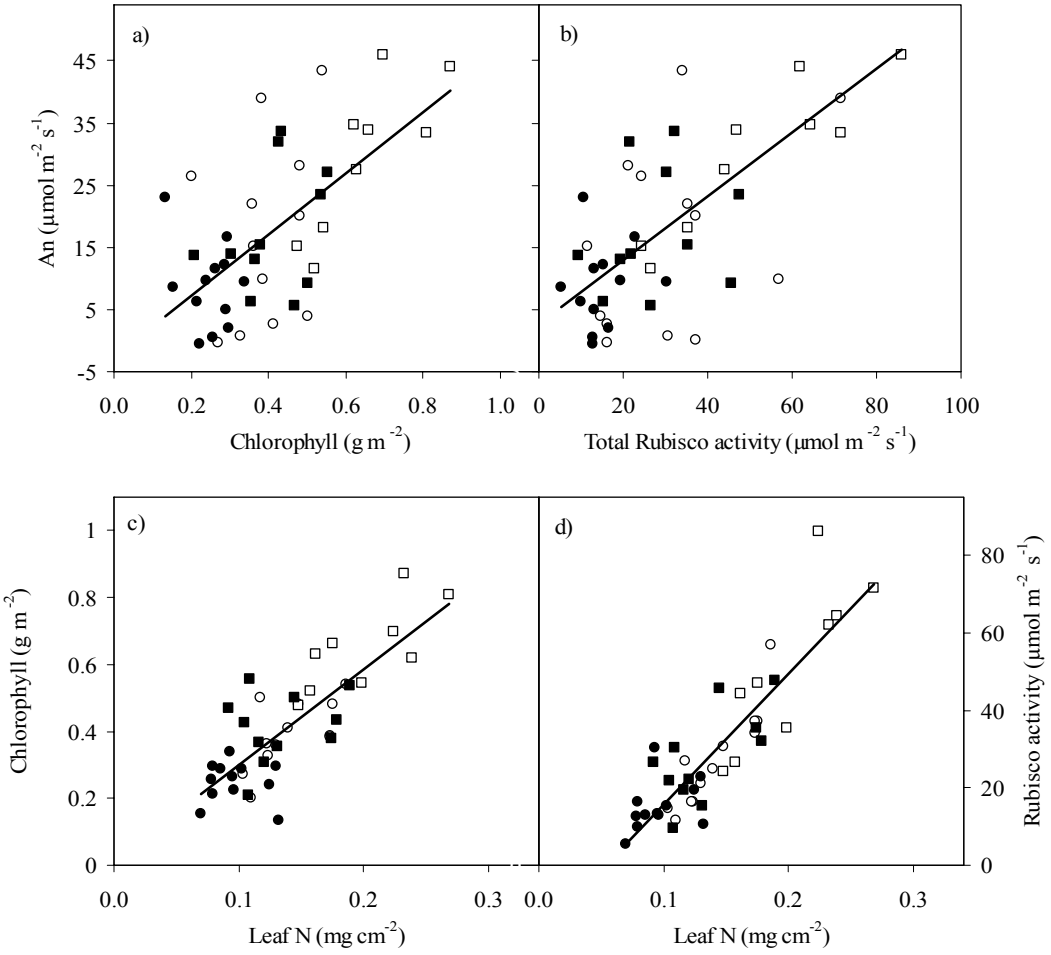
a)
b)
c)
d)
An (µmol m-2 s-1)
Chlorophyll (g m-2)
Total Rubisco activity (µmol m-2 s-1)
Leaf N (mg cm-2)
Rubisco activity (µmol m-2 s-1)

FIG. 6

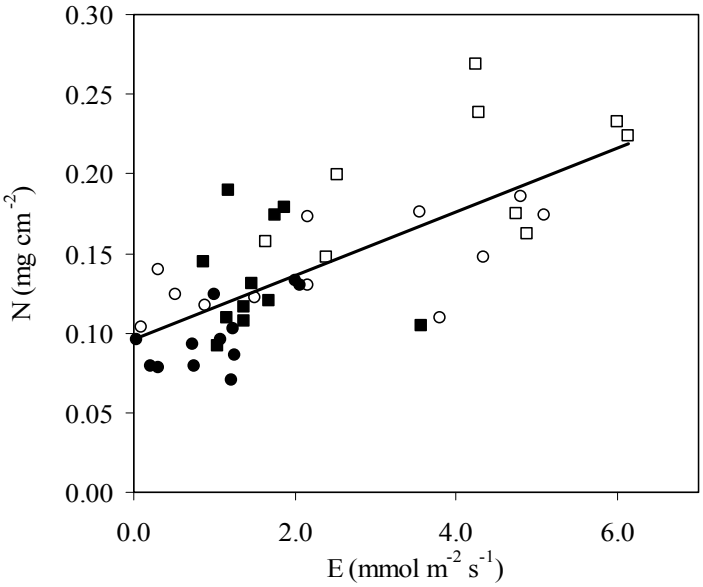

Fig. 7