Journal of Experimental Botany, Vol. 46, No. 293, pp. 1797–1802, December 1995

Journal of Experimental Botany

Chloroplast ultrastructure of sugar beet (*Beta vulgaris* L.) cultivated in normal and elevated CO_2 concentrations with two contrasted nitrogen supplies

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Received 21 November 1994; Accepted 14 August 1995

Abstract

Sugar beet (*Beta vulgaris* L., cultivar Celt) plants were grown under simulated field conditions in pots and supplied with adequate or deficient nitrogen (HN and LN, respectively) combined with two CO_2 concentrations, ambient (*c.* 350 μmol mol^{-1} CO_2—AC), or elevated CO_2 (*c.* 600 μmol mol^{-1} CO_2—HC). Chloroplast structure in mesophyll palisade cells of mature leaves (leaf number 19 in HN and 9 in LN), sampled at midday on 16 August 1993 was studied by transmission electron microscopy and quantified stereologically. The ultrastructure of palisade parenchyma chloroplasts was affected by the elevated CO_2 concentration and strikingly affected by nitrogen supply. Chloroplast diameter (cross-sectional length) was slightly, but not significantly, greater in HC than AC treatments within an N treatment, but was smaller in LN than HN; chloroplast cross-sectional area also increased with HC in both N treatments, but only significantly so in LN. Elevated CO_2 reduced the proportion of total thylakoids (significant at 5% and 0.1% in HN and LN, respectively) due to decreased granal thylakoids, but the proportion of inter-granal (stromal) thylakoid membranes was not affected compared to chloroplasts from plants grown with ambient CO_2. Chloroplast stroma increased as a proportion of chloroplast volume with elevated compared to ambient CO_2 with HN but not LN. Starch inclusions were not significantly different with elevated compared to ambient CO_2 at HN, but the proportion of starch increased considerably at elevated compared to ambient CO_2 at LN, indicating an over-production of assimilates. Plastoglobuli in chloroplasts increased with deficient N, but decreased with elevated CO_2. Larger chloroplasts with a greater proportion of stroma, but a smaller proportion of granal thylakoids, suggest increased CO_2 assimilating capacity and decreased light harvesting/PSII capacity with elevated CO_2.

Key words: Chloroplast, ultrastructure, elevated CO_2 concentration, nitrogen deficiency, sugar beet, *Beta vulgaris*.

Introduction

The rapid increase in the carbon dioxide concentration of the earth's atmosphere is unquestioned. It is expected that the concentration will have doubled by approximately the middle of the next century from the pre-industrial value of about 270 μmol CO_2 mol^{-1} air up to 600 μmol mol^{-1} (Watson *et al.*, 1990). Increasing CO_2 in this range substantially increases photosynthesis (Šantrůček, 1992), due to the characteristics of the CO_2 assimilating mechanism: ribulose bisphosphate (RuBP) is carboxylated by CO_2, in a reaction catalysed by RuBP carboxylase-oxygenase (Rubisco). The oxygenase function of the enzyme (O_2 reacting with RuBP), which competes with the carboxylase function and leads to photorespiratory CO_2 release, is much decreased by CO_2 so stimulating photosynthesis (Lawlor and Keys, 1993). However, there is considerable evidence that the stimulation of photosynthesis does not persist in plants grown for long periods at elevated CO_2. Photosynthetic rate decreases to that expected for plants grown in current CO_2 concentrations; this is often associated with loss of

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photosynthetic components, such as Rubisco, as a consequence of feedback regulation. Assimilates, accumulating because growth, storage and respiration do not use all the carbon assimilated, trigger mechanisms preventing the synthesis of—or increasing the breakdown of—the components, although the changes are not well documented and the mechanisms are poorly understood (Delgado *et al.*, 1994). Thus substantial changes in chloroplast biochemistry occur with elevated CO_2.

In contrast, relatively little is known about changes in the ultrastructure of chloroplasts in response to growth of plants in elevated CO_2. Ehret and Joliffe (1985) grew bean plants in CO_2 concentrations 4-fold greater than current ambient and found that damage to chloroplasts related to an over-accumulation of starch. Pennanen *et al.* (1992), observed that growth of wheat, barley and rape (*Brassica napa*) in CO_2 concentration double the current ambient, accelerated chloroplast senescence. This was related to decreased proteins of the light harvesting complex of photosystem II, determined by immunogold labelling. It is important to establish if such changes commonly occur under relatively small increases of CO_2. Ultimately, they may be related to measurements of photosynthetic performance and biochemical composition.

Growth of leaves and the formation of the photosynthetic components is very dependent on nitrogen supply; a small supply of nitrogen restricts growth and yield and reduces photosynthetic competence associated with loss of components. Therefore, it is important to assess the effects of N supply on the response of photosynthesis and its acclimation to elevated CO_2. It is also expected that the combination of elevated CO_2 and deficient nitrogen supply would accelerate acclimation and senescence.

The aim of this study is, therefore, to compare, quantitatively, the ultrastructure of mesophyll cell chloroplasts from mature sugar beet (*Beta vulgaris* L.) leaves cultivated with ambient or elevated carbon dioxide concentrations (i.e. nominal 350 compared to 600 μmol CO_2 mol^{-1} air), and with normal or deficient N supply. Plants were grown in a controlled environment facility simulating field conditions (Lawlor *et al.*, 1993).

Materials and methods

Plant material and cultivation

Sugar beet (*Beta vulgaris* L.) seed of the monogerm cultivar Celt, was sown on 22 April 1993 in pots (15 dm^3) filled with sintered arcillite rooting medium (Terragreen, see Lawlor *et al.*, 1993), which has very limited plant nutrient content (allowing contrasted NO_3^- supply to be given) and good water-holding capacity. The pots were watered with deionized water and nutrients were provided as Long Ashton nutrient solution as described in Mitchell *et al.* (1993). Two different amounts of nitrogen fertilizer were applied as nitrate. The concentrations were 10 or 1 mol m^{-3} NO_3^- applied weekly in equal volumes of Long Ashton solution. Total N supply between sowing and harvest was 1.65 g (HN) and 0.165 g (LN) per pot. Plants were grown in a CO_2 and temperature controlled facility described by Lawlor *et al.*, (1993). The temperature in the system tracked the outside, ambient, air temperature throughout plant growth, simulating field conditions. Daylength was that experienced by crops grown in the field at that time of year. The photon flux from natural radiation plus that from quartz-halogen lamps, on the plants, integrated over time was approximately the same as in the field without the extremes experienced at highest and lowest natural radiation (Lawlor *et al.*, 1993).

Seeds were germinated at ambient CO_2 and ambient temperatures until emergence of the cotyledons had occurred in more than 90% of the seeds sown. Then the CO_2 concentrations were regulated to either 355 μmol mol^{-1} CO_2 (ambient CO_2, AC) or 600 μmol mol^{-1} CO_2 (high CO_2, HC), nominally: the concentrations achieved were within the range plus and minus 5% of these for 90% of the time. Plants grew under these conditions, with temperature tracking outside ambient temperature (measured in a Stevenson screen), until harvest.

Plant sampling

A recently expanded, mature leaf (no. 19 in HN and no. 9 in LN treatments) on each of three plants was sampled from plants grown in AC and HC treatments, respectively, on 16 August 1993 at about midday. Four samples of the central part of the leaf blade were removed and prepared as described. From one sample of each leaf, 8–12 individual chloroplasts from the palisade were sectioned and one photomicrograph evaluated from each, giving a total of 30 cross-sections per treatment.

Electron microscopy and stereological analysis

The leaf samples were prepared by double fixation with glutaraldehyde and osmic acid, dehydrated in alcohol and embedded in Spurr's low viscosity resin. Thin sections were contrasted in solutions of uranyl acetate and lead citrate. The electron micrographs, one per chloroplast, were stereologically evaluated quantitatively, by measuring volume densities or relative partial volumes of chloroplast structures of median cross-sections of chloroplasts (selected on morphological and other evidence to be through the longest median axis of the chloroplast) using morphometric grids with regularly distributed points. Total chloroplast cross-sectional area and the proportion of the area occupied by particular chloroplast structures were measured using the point sampling method, as described by Kutík *et al.* (1993) following Kubínová (1991). The data within each N treatment were evaluated statistically to estimate significance of differences between CO_2 treatments by means of Student's *t*-test. This was done because of the different leaf number used for the study in the two N treatments due to the effects of N on plant growth. This is discussed further in the following section.

Results and discussion

Elevated CO_2 increased dry mass of sugar beet plants, which are considered to be 'indeterminate' in growth, by approximately 22% at final harvest in the HN-treated plants and increased the leaf area of those plants by approximately 28% at final harvest compared to ambient CO_2. The LN treatment decreased total dry matter production of plants grown in ambient and elevated CO_2 by

approximately 70% and decreased leaf area at the final harvest by approximately the same proportion (Demmers-Derks and Lawlor, unpublished). Thus the treatments applied throughout the growth of the crop substantially affected growth and productivity. There were no obvious differences between the treatments in anatomical structure of the sugar beet leaf laminae sampled for the EM study. The leaves were bifacial, with mesophyll differentiated into a multiple-layered palisade parenchyma adaxially and a multi-layered spongy parenchyma abaxially.

Some concerns over the evaluation of chloroplast structure must be mentioned. Chloroplast ultrastructure, even in apparently 'mature' leaves, is dependent on the age of the leaf and the plant (Kutík, 1985). During normal leaf development, volume densities of chloroplast structural parts as well as chloroplast dimensions change. Thus, leaves of the same age must be taken for evaluation. In our study, this was so within a nitrogen treatment, as CO_2 did not affect the rate of production of individual leaves and the temperature in the two CO_2 treatments was carefully controlled to be as similar as possible in order to avoid temperature causing differences which could be ascribed to the CO_2 treatments. However, there were differences in the rate of production and development of leaves caused by the nitrogen supply, hence the comparison of chloroplast structure from the two nitrogen treatments must be treated cautiously and statistical comparison is therefore avoided. Clearly, for a single N treatment, the changes observed in anatomy as a consequence of growing sugar beet under elevated CO_2, are related to the CO_2 treatment alone.

Differences between the sugar beet chloroplasts at the two nitrogen supplies and CO_2 concentrations were evaluated in palisade parenchyma cells only. Typical electron micrographs of the chloroplasts from the four treatments are presented in Plate 1. Means and statistical significance are given for the individual treatments of volume densities of chloroplast structures in Fig. 1 and the area and length of the chloroplast cross-sections in Table 1.

Differences between the sugar beet chloroplasts from the four treatments were mainly quantitative; no unique, qualitatively different features were observed for any treatment. Chloroplast cross-sectional area was slightly greater in HC-grown plants than in AC, but significant (5% probability) only at LN: chloroplast cross-sectional length was affected similarly but not significantly. However, chloroplasts from leaves grown with HN were slightly larger in both cross-sectional area and length than those grown in LN in both CO_2 concentrations. The shape of chloroplasts from the low nitrogen, high CO_2-grown plants was usually more rounded than those from plants grown with deficient nitrogen at normal CO_2 probably owing to a greater accumulation of starch in the chloroplasts.

Chloroplast ultrastructure was largely similar in leaves grown under high nitrogen at ambient CO_2 and elevated CO_2. A striking qualitative feature of the ultrastructure of chloroplasts of LN HC plants and present, but rarely seen, in chloroplasts from LN AC-grown plants, was the presence of clusters of electron dense particles, probably phytoferritin, which are formed by proteins binding non-usable ferrous ions. A typical feature of chloroplasts grown with deficient N, especially those grown in elevated CO_2, was low electron density of the chloroplast stroma, possibly reflecting the generally much smaller concentration of Rubisco protein and other proteins observed in N-deficient leaves (Lawlor *et al.*, 1989).

With abundant N, elevated CO_2 compared to ambient CO_2 decreased the proportion of total thylakoids due to decreased granal thylakoids (significant at 5%), but did not affect intergranal thylakoids. There was no significant difference in starch inclusions, but the proportion of plastoglobuli decreased. The proportion of stroma in the chloroplast increased (significant at 1%) with elevated CO_2 at high nitrogen. At low nitrogen the decrease in the proportion of thylakoids with elevated CO_2 was pronounced: granal thylakoids decreased greatly (significant at 0.1%) so although there was no effect on intergranal thylakoids, total thylakoids decreased substantially ($P=$ 0.1%). In contrast to the high nitrogen treatment, starch increased 8-fold when the leaves were grown in elevated CO_2 ($P=0.1\%$) and very deficient N. There was also a small, non-significant, decrease in the proportion of the chloroplast cross-section occupied by plastoglobuli and in the proportion of stroma. The observed effect of the low nitrogen treatment on increasing the volume density of plastoglobuli compared to HN, suggests greater accumulation of lipid than protein, as expected if protein synthesis is inhibited by lack of N or chloroplast senescence is accelerated with nitrogen deficiency, agreeing with observations made by Kutík *et al.* (1993) in wheat. The importance of adequate nitrogen nutrition for the formation of normal chloroplast structure is well established (Lawlor *et al.*, 1987, 1989).

Accumulation of starch in the LN HC treatment is attributable to production of photosynthates in excess of the sink capacity to utilize them; this agrees with results by Ehret and Joliffe (1985). The tendency to accumulate starch in nitrogen-deficient plants, particularly at low temperature, is seen in the study of wheat by Lawlor *et al.* (1987). The effects of elevated CO_2 with abundant nitrogen supply were not significant and suggest that, with adequate nutrition and water, sugar beet provides an adequate sink for assimilate even at elevated CO_2. However, it is not known if the rate of CO_2 fixation in elevated CO_2 was larger than in ambient CO_2 or whether regulatory processes decreased the rate of photosynthesis of the plants grown in elevated CO_2 matched those of plants grown in ambient CO_2. Future work will examine these questions.

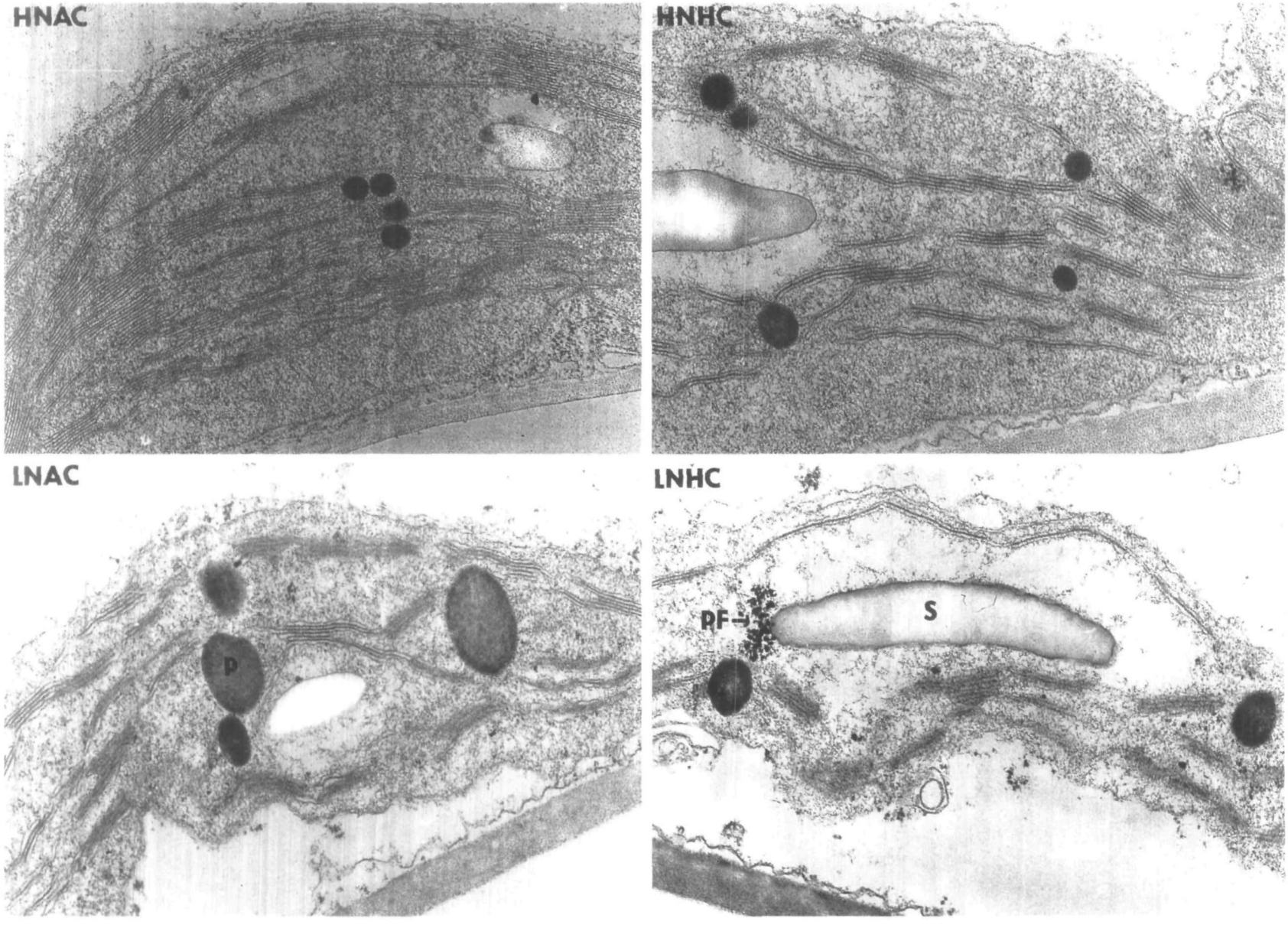

Plate 1. Transmission electron micrographs of chloroplast cross-sections from sugar beet mesophyll cells at a magnification of 34 000 ×. The electron micrographs are chosen to conform as closely as possible to the mean values of volume densities of chloroplast structural components given in Table 1 and Fig. 1. The treatments were high nitrogen with ambient CO_2 (HN AC), high nitrogen with elevated CO_2 (HN HC), deficient nitrogen at ambient CO_2 (LN AC) and deficient nitrogen and elevated CO_2 (LN HC). Note the large plastoglobuli (P), especially in LN AC, and large starch inclusions (S) and (presumably) phytoferritin grains (PF) in LN HC.

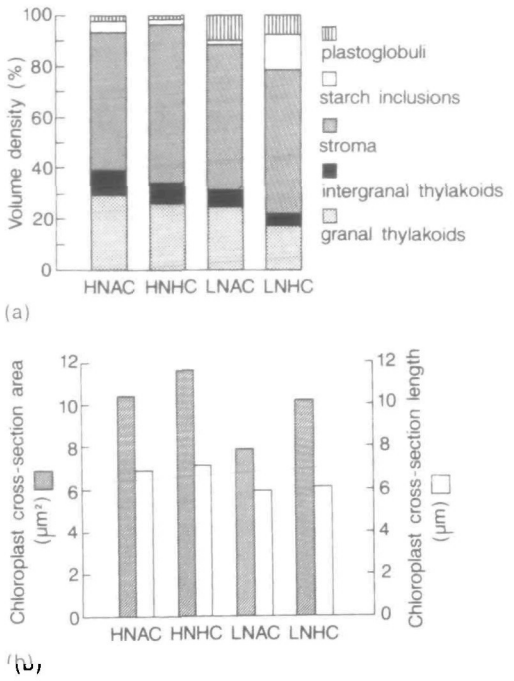

Fig. 1. (a) Volume densities of chloroplast structures determined by means of point counting on chloroplast cross-sections. (b) The area and length of chloroplast cross-sections determined on material grown in treatments HN AC, HN HC, LN AC, and LN HC as described in Plate 1 and Materials and methods.

Table 1. *Volume densities of chloroplast structures (in per cent of total chloroplast volume) and chloroplast cross-sectional area and length (μm^2 and μm, respectively) and the statistical significance of the differences between chloroplasts from leaves grown in ambient and elevated CO_2 concentrations (AC and HC, respectively) and with two nitrogen treatments, abundant nitrogen (HN) and deficient nitrogen (LN)*

The significance is based on Student's *t*-test ($P = 5\%^*$; $1\%^{**}$; $0.1\%^{***}$; NS not significant at 5% or below) and applies to the AC–HC comparison within each N treatment only as comparison between N treatments is subject to uncertainties related to the equivalence of the leaves sampled (see text).

	HN AC	HN HC	LN AC	LN HC
Granal thylakoids	30.0	26.3^*	25.2	17.7^{***}
Inter-granal thylakoids	9.1	7.5^{NS}	5.5	3.9^{NS}
Total thylakoids	39.1	33.8^*	30.6	21.5^{***}
Starch inclusions	4.2	2.6^{NS}	1.7	14.3^{***}
Plastoglobuli	2.2	1.2^*	9.9	7.7^{NS}
Stroma	54.6	62.4^{**}	57.7	56.6^{NS}
Chloroplast area (μm^2)	10.4	11.6^{NS}	7.9	10.2^*
Chloroplast length (μm)	6.9	7.1^{NS}	5.9	6.1^{NS}

Sugar beet is a plant without distinct growth stages during the period for which it is normally grown commercially (and as in this study), and is regarded as indeterminate, i.e. as sink limited not source limited with respect to carbon assimilates under current CO_2 conditions (Lawlor and Mitchell, 1991). However, our study indicates that even with adequate nitrogen (and other nutrients) changes do occur in chloroplast ultrastructure as a result of growing plants in elevated CO_2, but they are not associated, on the basis of this sampling, with the build-up of starch inclusions. Potentially, the most significant aspect is in the decreased proportion of granal thylakoids in chloroplasts from leaves grown in elevated CO_2 with both N treatments. This will be offset, in part, by the small (generally non-significant) increase in chloroplast size (cross-sectional area and length). Elevated CO_2 may alter the light harvesting and energy transducing capacity of sugar beet under conditions where the supply of CO_2 as a substrate for Rubisco is potentially much increased. In the ultrastructural study of chloroplasts grown in elevated CO_2, Pennanen *et al.* (1992) showed that components of the light-harvesting complex of PSII decrease: perhaps the loss of granal thylakoids and of components of PSII in the separate studies reflects 'down-regulation' of both the amount and functional capacity of light-harvesting and energy transduction in chloroplasts. Whether this is related to the small increase in proportion of stroma in the slightly larger chloroplasts with adequate N can not be established from this study. Speculating, the increase in the stroma might reflect a greater capacity of the Calvin cycle reactions of CO_2 fixation, which under elevated CO_2 requires less energy than at normal CO_2 (due to much decreased photorespiration). In low nitrogen, by contrast, nitrogen limitation might prevent any increase in stromal components in LN HC plants. This is also reflected in the inability of sink organs to grow adequately, in starch accumulation and the pronounced decrease in granal thylakoids. Such speculation will be tested in future experiments.

Acknowledgements

The opportunity to work with the electron microscope in the State Institute of Public Health, Prague, in the laboratory headed by Dr Dagmar Hulínska and kind advice from Ing. Miloslav Doubek are gratefully acknowledged by JK, as is a European Community grant to LN. IACR receives grant-aided support from the Biotechnology and Biological Science Research Council of the United Kingdom. The work is in collaboration with IACR-Brooms Barn (Professor T Thomas).

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