PHYSIOLOGIA PLANTARUM 103: 451–460. 1998
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ISSN 0031-9317

Interactive effects of growth-limiting N supply and elevated atmospheric CO_2 concentration on growth and carbon balance of *Plantago major*

Jeroen den Hertog, Ineke Stulen, Freek Posthumus and Hendrik Poorter

den Hertog, J., Stulen, I., Posthumus, F. and Poorter, H. 1998. Interactive effects of growth-limiting N supply and elevated atmospheric CO_2 concentration on growth and carbon balance of *Plantago major*. – Physiol. Plant. 103: 451–460.

To assess the interactions between concentration of atmospheric CO_2 and N supply, the response of *Plantago major* ssp. *pleiosperma* Pilger to a doubling of the ambient CO_2 concentration of 350 μl l^{-1} was investigated in a range of exponential rates of N addition. The relative growth rate (RGR) as a function of the internal plant nitrogen concentration (N_i), was increased by elevated CO_2 at optimal and intermediate N_i. The rate of photosynthesis, expressed per unit leaf area and plotted versus N_i, was increased by 20–30% at elevated CO_2 for N_i above 30 mg N g^{-1} dry weight. However, the rate of photosynthesis, expressed on a leaf dry matter basis and plotted versus N_i, was not affected by the CO_2 concentration. The allocation of dry matter between shoot and root was not affected by the CO_2 concentration at any of the N addition rates. This is in good agreement with theoretical models, based on a balance between the rate of photosynthesis of the shoot and the acquisition of N by the roots.
The concentration of total nonstructural carbohydrates (TNC) was increased at elevated CO_2 and at N limitation, resulting in a shift in the partitioning of photosynthates from structural to nonstructural and, in terms of carbon balance, unproductive dry matter. The increase in concentration of TNC led to a decrease in both specific leaf area (SLA) and N_i at all levels of nutrient supply, and was the cause of the increased rate of photosynthesis per unit leaf area. Correction of the relationship between RGR and N_i for the accumulation of TNC made the effect of elevated CO_2 on the relationship between RGR and N_i disappear.
We conclude that the shift in the relationship between RGR and N_i was due to the accumulation of TNC and not due to differences in physiological variables such as photosynthesis and shoot and root respiration, changes in leaf morphology or allocation of dry matter.

Key words – Carbohydrates, dry matter allocation, elevated CO_2, exponential N supply, nitrogen limitation, photosynthesis, *Plantago major*, respiration, relative growth rate, root weight ratio.

J. den Hertog, I. Stulen (corresponding author, e-mail g.stulen@biol.rug.nl) and F. Posthumus, Dept of Plant Biology, Univ. of Groningen, P.O. Box 14, NL-9750 AA Haren, The Netherlands; H. Poorter, Dept of Plant Ecology and Evolutionary Biology, Univ. of Utrecht, P.O. Box 800.84, NL-3508 TB Utrecht, The Netherlands.

Introduction

In many plant species from natural vegetations, an increase in the atmospheric CO_2 concentration leads to an increased dry matter production (Poorter 1993). This pertains to experiments with optimal nutrient supply: a doubling of the ambient CO_2 concentration then leads to an increase in the rate of photosynthesis and, at least transiently to an increase in the relative growth rate (RGR; Bazzaz 1990, den Hertog et al. 1993,

Received 30 January, 1998; revised 21 April, 1998

Poorter 1993). In most natural systems optimal growth conditions hardly ever occur, so that the question arises, whether the positive effect of elevated CO_2 persists when environmental conditions other than CO_2 limit growth of plants.

Nitrogen is a prominent nutrient that is often in short supply. Reports on the interactive effects of elevated CO_2 and nitrogen limitation provided variable results (Cure and Acock 1986), ranging from similar effects of increased CO_2 concentrations on dry matter production at any nutrient level (Wong 1979, Sionit et al. 1981, Sionit 1983, Hocking and Meyer 1991, Norby and O'Neill 1991, Bazzaz and Miao 1993), to either a decreased (Patterson and Flint 1982, Goudriaan and De Ruiter 1983, Bazzaz and Miao 1993, Johnsen 1993) or an increased effect of elevated CO_2 at low nutrient supply (Goudriaan and De Ruiter 1983, Peet and Willits 1984, Bowler and Press 1993).

In order to understand these apparently contradictory results, one should realise that growth is affected by several interacting factors. Stulen and den Hertog (1993) conclude that the mode of imposing the nutrient limitation and the type of growth substrate, i.e. nutrient solution or soil, may be decisive for the plants response to elevated CO_2. In general, the nutrient status is better controlled in water than in soil culture; but even so, the nutrient supply may vary with the CO_2 treatment, due to more rapid depletion of the nutrients in the solution when plant size increases. Plant size is, in turn, influenced by the CO_2 supplied (Coleman et al. 1993, Stulen and den Hertog 1993, den Hertog et al. 1996, Stulen et al. 1998).

In an analysis of plant growth responses to elevated CO_2, the effects on the accumulation of starch and soluble sugars have to be taken into account (Wong 1990, Kuehny et al. 1991, den Hertog et al. 1996, Stulen et al. 1998). Especially in experiments dealing with nutrient limitation and elevated CO_2 this may be an important factor, because of the considerable increase in sugars and starch (Bowler and Press 1996). A 50% increase in total nonstructural carbohydrate (TNC) was observed in *Plantago major* after transfer from a full to a diluted nutrient solution (Lambers et al. 1981) and in *Betula pendula* grown at limiting, exponential supply of nutrients (McDonald et al. 1986b).

In water culture, nutrient limitation can be imposed by varying the nutrient supply per unit time, by gradual depletion of the solution, or by exponential supply of nutrients as described by Ingestad and Lund (1979). With the last-mentioned technique, comparisons can be made between plants from different environmental conditions but with a common internal nutrient concentration (N_i). As regards exposure to different CO_2 levels, such comparisons have been made only for seedlings of *Betula pendula* (Petterson et al. 1993), but the report was restricted to a detailed growth analysis. However, rates of both photosynthesis (see Bazzaz 1990 for a review) and of shoot and root respiration (Poorter 1993) are often affected by the level of CO_2. To provide a complete carbon balance in relation to elevated CO_2 and nitrogen limitation, we measured these variables as well as production and allocation of dry matter and concentrations of carbon. Comparisons between growth variables as related to N_i of plants grown at ambient and elevated CO_2 have so far not been corrected for accumulated TNC. We have also computed our data on a structural dry matter basis, properly to evaluate the effect of elevated CO_2 on the relationship between growth variables and N_i.

In previous studies we analysed the growth of *P. major* at elevated atmospheric CO_2 concentration and at non-limiting supply of nitrogen (den Hertog and Stulen 1990, den Hertog et al. 1993, 1996). In the experiments presented here, the method of imposing nitrogen stress exponentially was adopted from Ingestad and Lund (1979) and used to analyse the growth responses and carbon balance of *P. major* L. as related to CO_2 concentration and N_i. We investigated how the different components of the carbon balance are affected by elevated CO_2, distinguishing effects on the rates of photosynthesis and shoot and root respiration from effects on plant morphology and dry matter allocation and from effects on TNC and total carbon concentrations.

Abbreviations – LWR, leaf weight ratio; N_i, internal plant nitrogen concentration; NP, nitrogen productivity; PNC, plant nitrogen concentration; RAR, relative addition rate; RGR, relative growth rate; R/S, root to shoot weight ratio; RWR, root to total plant weight ratio; SLA, specific leaf area; SWR, stem to total plant weight ratio; TNC, total nonstructural carbohydrates.

Materials and methods

Experimental design

Two climate room experiments were carried out with an inbred line of *Plantago major* L. ssp. *pleiosperma* Pilger, previously used in studies on CO_2 effects on plant growth (den Hertog et al. 1993, 1996). The plants were grown hydroponically and exposed to an atmospheric CO_2 concentration of 350 or 700 $\mu l\ l^{-1}$ in combination with different relative addition rates (RAR) of nitrate. In both experiments, growth rates, allocation of dry matter, and the concentrations of nitrogen and carbohydrate were determined. The results of the two experiments were similar and, therefore, only one of them is presented here. Also photosynthesis, respiration and total carbon were measured in this particular experiment.

Plant material

The plants were grown from seeds germinated in a greenhouse on a sterilised, commercial soil mixture (for details on germination and growth conditions see den Hertog et al. 1993, 1996). One week after sowing, the seedlings were transferred to a climate room kept at 350 $\mu l\ l^{-1}$ CO_2 (for conditions see below) and covered with one layer of cheesecloth until transfer to the nutrient solution. Beginning three weeks after sowing, the plants were grown for one week on an aerated and well stirred Hoagland solution, 1/16 the strength of that described by Smakman and Hofstra (1982). Four weeks after sowing, when the CO_2 and nitrate addition treatments were started (day 0), nitrate was supplied at two exponential, growth-limiting rates, 0.1 and 0.2 mol mol^{-1} day^{-1}, respectively. The other nutrients were supplied as in a 1/4 strength Hoagland solution, which was replenished once weekly. Nitrate was exponentially supplied as KNO_3, once daily, at the end of the light period. The quantity of KNO_3 to be given for each day was calculated as described by Ingestad and Lund (1979), with data on the mean fresh weight of 32 plants from a harvest at day 0, in combination with data on percentage dry matter and plant nitrogen concentration (N_i) from earlier experiments (den Hertog and Stulen 1993, den Hertog et al. 1993). Nitrate in the Hoagland solution was replaced by sulphate, but the concentrations of K_2SO_4 and $CaSO_4$ were reduced by 50% in order to prevent the formation of $CaSO_4$ crystals close to the solution surface. In addition, a treatment with a complete 1/4 strength Hoagland solution was included and will be referred to as the free access treatment. At the start of the experiment (day 0), plant density was 20 per 30-l tank. During the experiment thinning continued to a final density of 3 plants per 30-l tank in order to prevent mutual shading of the plants and depletion of the nutrient solution.

Growth conditions

The climate rooms were maintained at 20°C and 65% relative humidity with a photosynthetic photon flux density of 550 $\mu mol\ m^{-2}\ s^{-1}$ during 12 h a day. Light was provided by Philips HPI-T lamps (400 W, Eindhoven, The Netherlands) and 40 W incandescent bulbs in a 1:1 ratio. At the start of the CO_2 treatment the plants were randomly divided between two identical climate rooms as well as among the various nitrate treatments. The CO_2 concentration in the control room was kept at 350 $\mu l\ l^{-1}$, in the other CO_2 concentration was 700 $\mu l\ l^{-1}$, as measured by infrared gas analysers (ZFPCS and ZFP-DZ, Siemens, Karlsruhe, Germany). The CO_2 concentration at plant level was independently tracked by an infrared gas analyser (IRGA; ADC model 225 MK2, The Analytical Development Co., Hoddesdon, UK) and deviated by no more than 15 and 50 $\mu l\ l^{-1}$ from 350 and 700 $\mu l\ l^{-1}$, respectively. To avoid any climate room effect the plants and the CO_2 treatments were shifted weekly between the rooms.

Growth analysis

For all treatments, growth analysis was carried out according to Poorter (1989) in order to determine the period of steady state growth. Photosynthesis and shoot and root respiration as well as concentrations of total nonstructural carbohydrate (TNC), nitrogen and carbon were determined during the steady state growth interval that appears after a transient increase in RGR (den Hertog et al. 1993, 1996). Growth analyses lasted for up to four weeks from the start of the CO_2 treatment. The free access treatment was analysed from day 0, the start of the CO_2 and N treatments. Growth analyses for the 0.1 and 0.2 mol N (mol N)$^{-1}$ day^{-1} plants started on days 7 and 3, respectively, thus allowing for a period of adjustment to the limited N supply. Eight plants per harvest were sampled at 11.00 h, with 6 harvest days for the free access and 5 for the nitrogen-limited treatments and with double harvests on the first and last day of the growth analyses. Dry weights of leaves, stem and roots were determined after at least 24 h at 80°C. For plants used for measurements of shoot photosynthesis and respiration, leaf area was determined by photocopying the leaves and weighing the area copied. The ratios of leaf, stem and root dry weight to total plant dry weight (LWR, SWR and RWR) were determined for harvests on days 10, 23 and 17 for the free access, the 0.1 and the 0.2 mol N (mol N)$^{-1}$ day^{-1} treatments, respectively. The RGR was determined according to Poorter (1989), by fitting a third order polynomial through the mean RGR values for every day. The steady state RGR was derived from that part of the polynomial that had a slope close to zero.

Photosynthesis and respiration

The rates of photosynthesis and respiration of whole shoots of intact plants were measured during a 24-h cycle, on the days following the harvest days for which dry matter allocation was determined. The CO_2 concentration of in- and outcoming air was measured differentially in an open system with an IRGA (ADC model 225 MK3, The Analytical Development Co.). For 4 plants per treatment, photosynthetic rates were measured 5–9 times in the course of the light period. The rates of shoot respiration were determined 3–5 times during the dark period. Rates of photosynthesis were measured at an irradiance of 500 $\mu mol\ m^{-2}\ s^{-1}$, provided by Philips HPI-T lamps (400 W). Both the rates of photosynthesis and dark respiration were measured at 20 ± 1°C and 60–70% relative humidity. CO_2 concentrations were 326 ± 4 and 702 ± 19 $\mu l\ l^{-1}$ during measurements of photosynthesis and 347 ± 11 and

713 ± 26 µl l^{-1} during determinations of shoot respiration. In accordance with Poorter et al. (1990), little diurnal variation in the rates of photosynthesis and respiration were observed. Therefore, the measurements were averaged over the day and night periods, respectively.

Root respiration was polarographically measured at 10.00 h, on detached roots of 4 plants per treatment, placed in a well stirred solution similar to that used for plant culture (Lambers et al. 1993). Depletion of O_2 from the air-saturated solution was recorded, using a Clark-type O_2-electrode (Yellow Springs Instruments, OH, USA). Respiration was measured at 20.0 ± 0.5°C.

Chemical analyses

For the chemical analyses three samples, each consisting of two plants were made for every combination of N treatment and CO_2 concentration. All samples were divided into leaves, stems and roots, and subsamples were assayed in duplicate for N, C and TNC.

Total nitrogen concentrations were determined with a modified Kjeldahl method. The dried plant material was digested in a solution of Na-salicylate (0.2 *M*), which reduces free nitrate, in concentrated sulphuric acid. In addition, Na_2SO_4, $CuSO_4$ and Na_2SeO_3, in a ratio of 15:5:0.085 (w/w), were used as a catalyst. NH_4^+ concentrations were colorimetrically measured, in triplicate, with Nessler reagent (Merck, Darmstadt, Germany) at 410 nm.

The carbon concentrations were determined in duplicate, using an elemental analyser (model 1106, Carlo Erba Instrumentazione, Milano, Italy). For the determination of total carbohydrate concentrations, plant material was boiled in 3% HCl for 3 h in order to hydrolyse starch. In the supernatant the sugar concentration was colorimetrically determined, in triplicate, using anthrone reagent (Fales 1951), at 620 nm.

Data analysis

When appropriate, the relationships between growth characteristics or chemical composition and N_i were determined by a linear regression analysis. The slope and intercept with the y-axis of the regression lines of plants grown at ambient and elevated CO_2 were compared by a *t*-test. The data were pooled when the regression statistics were not significantly different ($P > 0.05$). The slope of the linear regression line for pooled data was tested for deviation from zero. N_i and variables that were not linearly related to N_i, such as SLA and the rate of photosynthesis on a leaf area basis, were compared among N treatments and CO_2 concentrations by means of a two-way analysis of variance (ANOVA). The rates of photosynthesis on a dry weight basis for the two CO_2 concentrations could not be statistically distinguished. Therefore, the data for the two CO_2 concentrations were pooled and the rate of photosynthesis was fitted by a rectangular hyperbola, following Ågren and Ingestad (1987).

Results

In the free access treatment, where nitrate was always in excess for growth, steady state growth was not observed for any of the two CO_2 concentrations. Based on earlier experiments with *P. major* (den Hertog et al. 1993, 1996), data on growth and carbon balance were collected on day 10, when the transient stimulation of the RGR by exposure of the plants to elevated CO_2 had ended. At the N-limiting RARs of 0.1 and 0.2 mol N (mol N)$^{-1}$ day^{-1}, a steady state of the RGR was reached after about 14 and 10 days, respectively; in both cases it lasted for at least two weeks. In some cases the RGR at limiting N supply was lower than the RAR (Fig. 1A). N_i within a given nitrogen supply treatment was always lower (ANOVA, $P < 0.001$) at elevated than at normal CO_2. However, the relationship between RGR and N_i was not affected by the CO_2 concentration, as indicated by the absence of a significant difference between the regression lines for the respective CO_2 treatments.

Over the whole range of N_i, the SLA was lower in plants exposed to elevated than in those at normal CO_2 although a linear regression analysis did not show any significant difference between the two CO_2 treatments, due to the non-linear trend in the data (Fig. 1B). A two-way analysis of variance, with N supply and CO_2 concentration as independent variables, confirmed this difference in SLA between the two CO_2 concentrations ($P < 0.001$). The distribution of dry matter between leaves, stems and roots, as related to N_i, was not affected by the atmospheric CO_2 concentration (Fig. 1C). The LWR increased linearly with increasing N_i, whereas the RWR decreased. The SWR increased significantly with rising N_i, but this effect was small as compared to the changes in LWR and RWR.

When expressed on a leaf weight basis and plotted as a function of N_i, the rate of photosynthesis was not affected by the atmospheric CO_2 concentration (Fig. 2A). When the rate of photosynthesis was expressed on a leaf area basis, a 20–30% increase at elevated CO_2 was observed for N_i above 30 mg N (g^{-1} dry weight) (Fig. 2B, $P < 0.01$). The rates of both the shoot and root respiration increased with increasing N_i, but the concentration of atmospheric CO_2 did not influence this relationship (Fig. 2C,D).

Doubling the ambient atmospheric CO_2 concentration increased the TNC concentration in the leaves of *P. major* by 30% at high levels of N_i and over 50% at low N_i (Fig. 1D). TNC concentrations increased with decreasing N_i in both CO_2 treatments. The TNC concentration in the roots was also affected by elevated

CO_2 ($P < 0.05$), although this never resulted in more than a 10% increase at low N_i (Fig. 1D) and was absent at higher N levels. Neither the CO_2 concentration nor the N supply rate did affect stem carbohydrate concentrations (Fig. 1D). In the leaves, the carbon concentration at high N_i was higher at elevated than at ambient CO_2. This was not the case at intermediate or low N_i (Fig. 3A). No effect of CO_2 was observed on the carbon concentration in stems and roots of *P. major* (Fig. 3B,C). In the latter two parts of the plant, a larger N supply caused a reduction of the C concentration of less than 5% over the whole range of N_i observed.

As a result of the decreased differences in N_i among the respective CO_2 concentrations, correction of the measured variables for the accumulation of TNC resulted in an even closer relationship between RGR and N_i for plants grown at ambient or elevated CO_2 (Fig. 4A). This correction greatly affected the impact of CO_2 on N_i and the SLA (Fig. 4B), but introduced only a slight shift in the pattern of allocation of dry matter (Fig. 4C). The allocation of structural dry matter to the leaves was decreased at elevated CO_2 and low N_i. This was counterbalanced by an increased allocation of dry matter to stem and roots, but the RWR was still not significantly affected by exposure of plants to different concentrations of atmospheric CO_2.

Discussion

RGR and N_i

Exponential addition of growth-limiting nitrogen doses led to a steady state growth of *P. major* at both 350 and 700 µl l^{-1} CO_2. The measured RGR was mostly lower than expected on the basis of the RAR (Fig. 1A), a discrepancy that could be caused by the mode of application of nitrate. A dosage of daily nitrate was given at the end of the light period, whereas nitrogen additions were made more often in the system described by Ingestad and Lund (1979). When supplied once daily, plants may not take up nitrogen during part of the day, and this may cause a decrease of the RGR. However, also McDonald et al. (1986a) and Petterson et al. (1993), who used the same type of growth units as Ingestad and Lund (1979), often found RGR values lower than RAR values. It should be noted that both

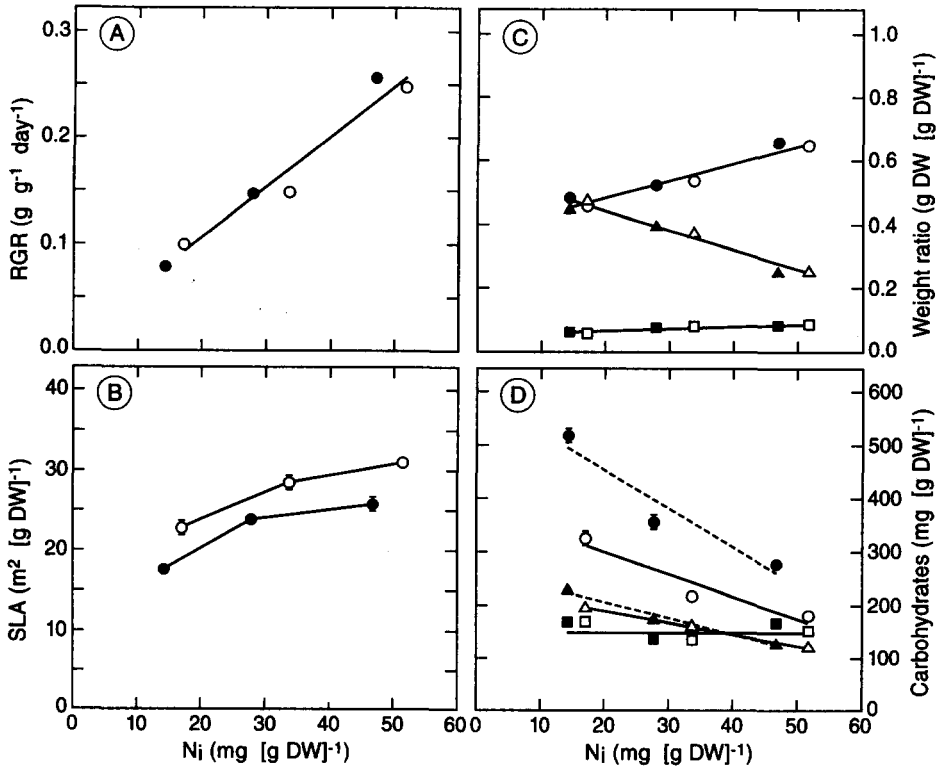

Fig. 1. Relative growth rate, RGR (A); specific leaf area, SLA (B, n = 4); leaf, stem and root weight ratios, LWR, SWR and RWR (C, n = 8); and of nonstructural carbohydrate concentration in leaves, stems and roots (D, n = 3) as functions of plant nitrogen concentration (N_i, n = 3) in *Plantago major* grown at 350 (open symbols, solid lines) or 700 µl l^{-1} CO_2 (filled symbols, dotted line). Linear regression lines in A, C and D were pooled for the two treatments when the slope between the two CO_2 treatments was not significantly different (solid lines), but presented separately when the the slope between the two CO_2 treatments was significantly different (t-test, $P < 0.05$); 350 µl l^{-1} CO_2 (solid line), 700 µl l^{-1} CO_2 (dotted line). ○,●, Whole plant (A) or leaves (B–D); □,■, stem (C, D); △,▲, roots (C, D). SE bars (B–D) shown when larger than symbols.

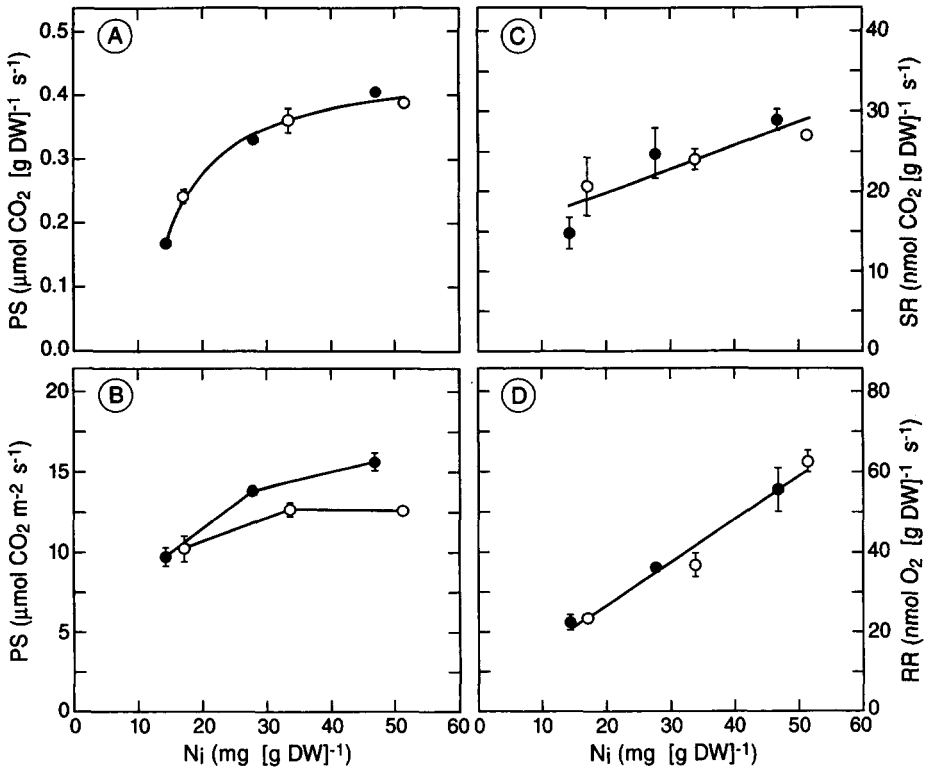

Fig. 2. Shoot photosynthesis (n = 4) on the basis of dry weight (A) or leaf area (B), respiration of shoot (C, n = 4) and root (D, n = 4) all as functions of plant nitrogen concentration (N_i, n = 3) in *Plantago major* grown at 350 (open symbols) or 700 µl l^{-1} CO_2 (filled symbols). A rectangular hyperbola (B) or linear regression lines (C,D) were fitted through the mean values of all combinations of CO_2 and N concentrations. SE bars shown when larger than symbol.

RGR and allocation of dry matter were constant for a period of at least two weeks in our experiments with *P. major*.

At all three levels of nitrogen availability, an increase of the atmospheric CO_2 concentration caused a decrease of N_i (Fig. 1A). However, the effect was too small to cause a significant difference between the regression lines of RGR versus N_i, although, at least at high and intermediate N supply, RGR of *P. major* plants was slightly higher in plants grown at elevated CO_2 at a given N_i. For seedlings of *B. pendula*, grown at limiting, exponential addition rates of nutrients, a similar decrease of N_i at elevated CO_2 was noted and caused an increase of the RGR for any particular N_i (Petterson et al. 1993).

An explanation for the small change in the relationship between RGR and N_i of plants grown at ambient and elevated CO_2 can be found along various lines. Do physiological variables, such as rates of photosynthesis and respiration contribute to the observed response? Are leaf morphology and allocation of dry matter decisive? Or is the accumulation of TNC the key factor?

Photosynthesis and respiration

To explain the effects of elevated CO_2 on the RGR or total biomass production, attention usually focuses on the rate of photosynthesis and possible acclimatory responses (Bowes 1991). In the short term, a repression of photorespiration and an increase of the internal CO_2 concentration in the leaf will cause an increase in the rate of net carbon fixation per unit leaf area. In the longer term this effect is often counteracted by down-regulation of photosynthesis (Bowes 1991). A decrease in activity and in quantity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) as well as a decrease of mRNA encoding Rubisco activase and chlorophyll-binding proteins contribute to this acclimation to elevated CO_2 (Bowes 1991, Besford 1993, Van Oosten et al. 1994). But even after such acclimation has taken place, the rate of photosynthesis per unit leaf area is often increased by 20–30% in plants exposed to a doubling of the ambient CO_2 concentration (Cure and Acock 1986, Bowes 1991). In the present experiments, an increase in the rate of photosynthesis per unit leaf area was observed in *P. major*, for N_i above 30 mg N

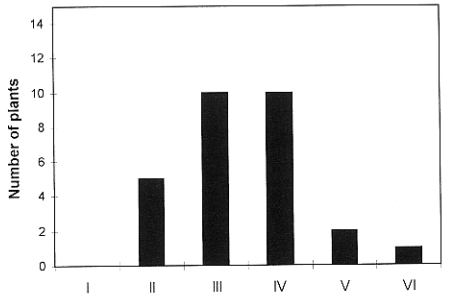

Fig. 1. The distribution of non-acclimated freezing tolerances (NA-freezing tolerance) of the segregating S1 genotypes (n = 25) and parental lines in predeterminated freezing tolerance categories. I = −2.0 to −2.5°C; II = −2.6 to −3.0°C; III = −3.1 to −3.5°C; IV = −3.6 to −4.0°C; V = −4.1 to −4.5°C; VI = −4.6 to −5.0°C. The NA-freezing tolerance of parental lines were as follows: *S. commersonii* −4.64°C (category VI), *S. tuberosum* (spv11) −2.95°C (category II) and the somatic hybrid (sh9a) −3.82°C (category IV).

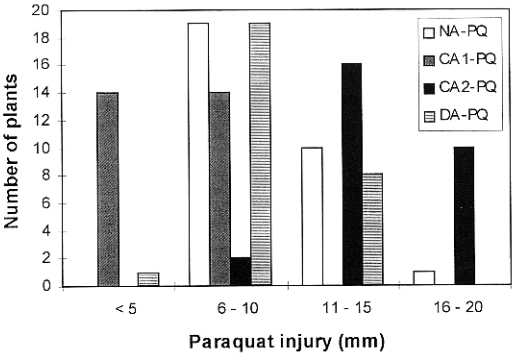

Fig. 2. The distribution of paraquat (PQ) tolerance of the segregating S1 genotypes (n = 25) at different acclimation conditions. NA-PQ, PQ tolerance of non-acclimated plants; CA1–PQ, PQ tolerance of cold acclimated plants tested at 10°C; CA2-PQ, PQ tolerance of plants tested at 20°C; DA-PQ, PQ tolerance of deacclimated plants. For details see Materials and methods.

age test were confirmed by freezing tests of whole plants and freezing injury was estimated by visual scoring. A freezing treatment of 1 h at −3.0°C distinguished the most freezing-sensitive genotypes showing various degrees of freezing injuries. Visual scoring was found to be well correlated with LT_{50} values (data not shown).

Paraquat tolerance of non-acclimated plants

Similar to freezing tolerance of non-acclimated plants (NA-freezing tolerance), the S1 population was observed to segregate in its paraquat tolerance of non-acclimated plants (NA-PQ; Fig. 2). No correlation (R = 0.02) was found between NA-PQ and NA-freezing tolerance when 25 plants of the S1 population were tested simultaneously for these two traits. Most of the genotypes (n = 19) had as high tolerance to PQ as scmm (7.75 mm diameter) or sh9a (8.00 mm diameter), even though scmm and sh9a varied significantly in NA-freezing tolerance, $LT_{50} = -4.6$°C and $LT_{50} = -3.8$°C, respectively.

There was a significant variation between the genotypes in their PQ tolerance. The diameter of the necrotic area in the most PQ-sensitive genotype was 15.2 ± 0.1 mm (2041) when measured 72 h after PQ application, whereas in the most tolerant one it was only 8.0 ± 0.9 mm (2019; Tab. 2). Since morphological differences of the leaf surface may affect the uptake of PQ from the leaf surface, the results were confirmed by dipping shoots of the most sensitive and tolerant genotypes into 0.1 m*M* PQ solution for 16 h. PQ sensitivity and tolerance was confirmed by the ratio of variable to maximum fluorescence (F_v/F_m). In sensitive genotypes

Tab. 1. Selection for the most freezing-tolerant and -sensitive plants. The LT_{50} values of parental lines scmm (*S. commersonii*) and spv11 (*S. tuberosum*) are also included. For comparison, the somatic hybrid, sh9a, is included as a representative of an intermediate plant. The number of independent experiments for LT_{50} is indicated in parentheses. The freezing tolerance of non-acclimated plants (NA-freezing tolerance) and acclimation capacity (AC) are calculated from the average LT_{50} values of independent experiments. In each experiment, the calculated LT_{50} value is an average of three replications ± SE.

Genotype	NA-freezing tolerance	AC
Most tolerant		
scmm	−4.6 ± 0.2 (5)	2.1 ± 0.2 (2)
1020	−4.4 ± 0.5 (2)	1.3 ± 0.5 (2)
2019	−4.3 ± 0.2 (4)	2.1 (1)
Intermediate		
sh9a	−3.8 ± 0.2 (7)	0.9 ± 0.4 (2)
Most sensitive		
2051	−3.0 ± 0.3 (4)	1.0 (1)
2022	−3.0 ± 0.1 (3)	1.9 ± 0.2 (2)
spv11	−3.0 ± 0.6 (6)	0.8 ± 0.7 (2)
2045	−2.9 ± 0.1 (3)	1.3 ± 0.2 (2)

Tab. 2. Classification of genotypes by paraquat (PQ) tolerance in non-acclimated plants (NA-PQ). PQ tolerance is calculated as an average diameter of a necrotic lesion (mm) in 3 to 6 individual leaves after 72 h of PQ application. The number of independent experiments is indicated in parentheses. The mean value of each experiment consists of 4 to 6 independent measurements ± SE.

Genotype	NA-PQ
Most resistant	
scmm	7.8 ± 1.2 (2)
sh9a	8.0 ± 0.9 (1)
2019	8.0 ± 0.9 (3)
1020	8.8 ± 0.2 (2)
Most sensitive	
2051	12.3 ± 0.3 (2)
2037	13.8 ± 2.2 (2)
2057	15.1 ± 0.2 (2)
2041	15.2 ± 0.1 (3)

experiments the stimulation of root respiration was correlated with an increased availability and/or flux of carbohydrates to the roots during the same period. In the present experiments, the data on respiration were obtained during the phase when the RGR was approximately constant and elevated CO_2 had no effect on carbon partitioning to the roots (Stulen and den Hertog 1993, Lambers et al. 1995, 1996, Fonseca et al. 1997).

We conclude that the shift induced by elevated CO_2 in the relationship between RGR and N_i was not, in the current experiments, caused by differences in photosynthesis or shoot and root respiration.

Leaf morphology and allocation of dry matter

The generally observed decrease of the SLA at elevated CO_2 (Bazzaz 1990, den Hertog et al. 1993, Petterson et al. 1993, Poorter 1993) occurred at all levels of N supply (Fig. 1B). SLA increased with increasing N_i. In some cases the response to the CO_2 concentration has been related to differences in leaf thickness and number of cell layers in the leaf (Thomas and Harvey 1983). However, for *P. major* as for most other species grown at different atmospheric CO_2 concentrations, CO_2 effects on SLA could be attributed to accumulation of TNC (Figs 1D and 4B). This response to both elevated CO_2 and nitrogen limitation was also observed in *Betula pendula* in the study by Petterson et al. (1993).

Allocation of dry matter shifted from leaves to roots with decreasing levels of N_i (Fig. 1C), in accordance with large numbers of observations on N-limited plant growth (Brouwer 1962, Ingestad and Lund 1979, Freijsen and Veen 1990). Dry matter allocation was not affected by the CO_2 concentration (Fig. 1C), which is in contrast to earlier reports and reviews on the effect of elevated CO_2 on investment of dry matter in roots and shoots. On the basis of many reports available at that time, Enoch (1990) concluded that RWR generally increased at elevated CO_2. Later reports and reviews have elaborated on possible interactions between CO_2 concentration and plant nutrition. In particular in studies using solid substrates, in which nutrient availability is harder to control than in studies using nutrient solution, biomass investment between shoot, root and fine roots and plant nitrogen concentration (PNC) can be changed at elevated CO_2 (Stulen and den Hertog 1993). Under these conditions the decrease in PNC may be explained via an effect on mass flow of nitrate to the root, caused by a reduction in the rate of transpiration at elevated CO_2, which results in a decrease in nitrate uptake by the root (Lambers et al. 1996, Stulen et al. 1998). However, provided that plants are grown with ample supply of water and nutrients, and are not pot-bound, RWR does not respond to prolonged exposure to elevated CO_2 (Arp 1991, Chu et al. 1992, den Hertog et al. 1993, 1996, Stulen and den Hertog 1993). Only plants with large extra sinks for carbohydrates, such as N_2-fixing legumes, plants producing tubers, or trees with growing stems, respond to elevated CO_2 with an increase in RWR or SWR, respectively (Stulen and den Hertog 1993).

The control of dry matter allocation between root and shoot has been modelled in relation to the efficiency of nitrogen uptake and use. Ågren and Ingestad (1987) related the control of RWR to the nitrogen productivity (NP), the amount of dry matter produced per unit of N per plant and time, and the rate of photosynthesis. NP can be calculated as the slope of the relationship between RGR and N_i (Fig. 1A). For *P. major*, the slopes at 350 and 700 ml l^{-1} CO_2 were not significantly different. As for the relationship between the rate of photosynthesis and N_i, no distinction could be made between plants grown at ambient or those grown at elevated CO_2. According to Ågren and Ingestad (1987), this implies that the atmospheric CO_2 concentration does not affect RWR. A similar conclusion can be drawn, based on growth models derived by Hilbert (1990) and Hilbert et al. (1991). In their models elevated CO_2 does not affect RWR over a large range of leaf N_i either. In the present experiments, in which nitrate limitation was imposed in a controlled way, the response of *P. major* to elevated CO_2 with respect to the dry matter allocation is thus in good agreement with these theoretical models, which are based on a balance between the rate of photosynthesis of the shoot and the acquisition of N by the roots.

Accumulation of TNC

The answer to the question which component of the carbon balance caused the small change in the relationship between RGR and N_i by elevated CO_2 may be found in the effect of the CO_2 concentration on the accumulation of TNC. Increased levels of TNC diluted N_i and caused a change in the plots of RGR versus N_i. When the RGR was plotted versus N_i on a structural dry weight basis (Fig. 4A), the atmospheric CO_2 concentration no longer affected the RGR at any level of N stress. Recalculation of the data on *Betula pendula* presented by Petterson et al. (1993) on a structural dry weight basis revealed similar effects of the atmospheric CO_2 concentration in *B. pendula*, but the RGR remained higher at elevated CO_2 at all levels of N_i. The presence of a large sink, the stem, may be responsible for the persisting effect of elevated CO_2 on the RGR in this species. Sink strength may play a crucial role in determining the long-term growth response of a species to elevated CO_2 (Stulen et al. 1998).

Changes in the chemical composition of the plant induced by environmental conditions, such as elevated CO_2, may be reflected in altered carbon concentrations (Poorter et al. 1992). Changes in carbon concentration

upon transfer to elevated CO_2 were absent or small in *P. major* (Fig. 3). A shift in the carbon balance of *P. major*, due to effects of elevated CO_2 on carbon concentrations, did not occur. The most important change in chemical composition, due to increased CO_2 concentrations, was the increase in TNC concentration. The relative concentration of carbon in carbohydrates is 40%, close to the value for the various organs in *P. major*. Therefore, changes in the TNC concentration are not likely to induce major shifts in total carbon concentration. There is no indication of another chemical component being responsible for the observed change in carbon concentration in the leaves (cf. Poorter et al. 1992).

In conclusion, the most striking effect in *P. major* plants exposed to elevated CO_2 for periods exceeding the initial period of growth stimulation was an increased TNC concentration in the leaves. This led to a decrease of both the SLA and N_i. The shift in the relation between RGR and N_i upon elevation of the CO_2 concentration and the increased rate of photosynthesis per unit leaf area are a reflection of this change in carbohydrate level. The accumulation of TNC, therefore, is the key factor in explaining the change in the relationship between RGR and N_i for plants grown at ambient and elevated CO_2.

Acknowledgments – We thank Profs P. J. C. Kuiper and H. Lambers for critically reading earlier versions of the manuscript and Mr Dick Visser for drawing the figures. Jeroen den Hertog was financially supported by the Dutch Organisation for Scientific Research (NWO).

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Edited by A. Kylin