Long-term effect of elevated CO_2 on spatial differentiation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in Norway spruce canopy

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Abstract

Total *in vitro* activity of RuBPCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme was assayed spectrophotometrically by the continuous measurement of 3-phosphoglycerate-dependent NADH oxidation in a coupled enzyme system. RuBPCO activities were found in the ranges 1.01–2.76 and 1.23–3.10 $\mu mol(CO_2)$ m^{-2} s^{-1} in current Norway spruce needles growing in ambient (AC) and elevated (EC) CO_2 concentration, respectively. RuBPCO activity in AC needles from the upper layer (U) was 11–15 % higher compared to those from the middle (M) layer, and even 44–56 % higher compared to the lower (L) layer of spruce crown. Over the vegetation season, we observed a highly significant decrease of RuBPCO activity in the EC-U needles from 3.10 (July) to 1.60 (October) $\mu mol(CO_2)$ m^{-2} s^{-1} as a consequence of downward feedback regulation. Moreover, this down-regulation was not caused by a non-specific decrease in total leaf nitrogen content.

Additional key words: down-regulation; nitrogen; photosynthesis; *Picea abies*; sun and shade needles; vertical profile.

Introduction

RuBPCO (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) enzyme is the most widespread and perhaps the most remarkable protein on the Earth because it is the only one which provides a significant link between the pools of inorganic and organic carbon in the biosphere. This enzyme catalyses carboxylation of D-ribulose-1,5-bisphosphate (RuBP), the first step of the Calvin cycle in competition with oxygenation of RuBP that leads to the photorespiratory pathway. Catalytic effectiveness of RuBPCO is low both in terms of its catalytic rate ($k_{cat} = 2$–12 s^{-1}) and of its substrate concentration [$k_{cat}/K_M(CO_2) = 5$–40×10^7 mol^{-1} m^3 s^{-1}] (Roy and Andrews 2000). Therefore, photosynthetic organisms invest up to 50 % of soluble leaf protein (Eichelmann and Laisk 1999) in RuBPCO to support acceptable rates of photosynthesis. RuBPCO must be reversibly activated with CO_2, Mg^{2+}, and RuBPCO activase before catalysis can occur. Hence, carbon dioxide has two direct biochemical effects on plants. It acts both as the activator and the substrate of RuBPCO enzyme.

Continuous increase of atmospheric CO_2 concentration ([CO_2]) led to extensive research over the last decades. Elevated [CO_2] (EC) strongly affects photosynthesis and growth of many plants, especially C_3 plants which constitute more than 90 % of terrestrial species. Short-term exposure of higher plants to EC usually increases photosynthetic CO_2 uptake, since an increased CO_2 partial pressure stimulates carboxylation and suppresses oxygenation. Photosynthetic responses to long-term EC are generally more variable (reviewed *e.g.* by Eamus and Jarvis 1989, Urban 2003).

The theoretical models (Luo *et al.* 1994, Griffin and Seemann 1996) and many experimental results (Ceulemans and Mousseau 1994, Sage 1994, Urban and Marek 1999, Urban *et al.* 2003) demonstrated that an adjustment of photosynthesis under EC is controlled by the interrelationships between biochemical and morphological feedback mechanisms. Over the time scale of

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hours to days, increased contents of specific saccharides (*e.g.* glucose, sucrose) lead, *via* hexokinase-related signal, to the repression of RuBPCO gene expression (Griffin and Seemann 1996) and a subsequent decrease of its content and activity (van Oosten and Besford 1996, Drake *et al.* 1997, Moore *et al.* 1999). Moreover, local phosphorus deficiency corresponds to RuBPCO de-carboxylation owing to reduced activity of RuBPCO activase (Portis 1990). These biochemical adjustments, which are often termed acclimation or down-regulation, reduce photosynthetic capacity. Morphological adjustments, most often observed as increased mesophyll tissue growth (Vu *et al.* 1989), are indicated by changes in leaf mass per unit area and tend to increase photosynthetic capacity, *i.e.* up-regulation. The mutual relations among these two processes at a given [CO_2] determine the net photosynthetic rate (P_N).

Strong vertical differences of photosynthetically active radiation (PAR), typical within close forest stands, lead to the changes of both structure and function of the assimilatory apparatus (Hättenschwiler 2001). Because PAR is the limiting factor for shade acclimated leaves, a substantial amount of photosynthetic resources must be invested in the synthesis and maintenance of light-harvesting complexes, while large amounts of electron transport components, ATP synthase, or the stromal CO_2 fixation enzymes are not so required (Anderson *et al.* 1988, Priwitzer *et al.* 1998). On the contrary, sun adapted leaves are characterized by a high content of the cytochrome *b/f* complex, plastoquinone, plastocyanin, ferredoxin, and ATP synthase which support fast rates of electron transport and photophosphorylation (Anderson *et al.* 1988, Špunda *et al.* 1998). Also, formation of upward vertical gradients of important macroelements, especially nitrogen, is typical for closed canopies (Hrdlička 1996). It tightly correlates with the different activities of important photosynthetic enzymes, including RuBPCO (Roy and Andrews 2000), and it subsequently leads to double values of maximal carboxylation rate, that reflect RuBPCO activity *in vivo*, for exposed comparing to shaded needles (Špunda *et al.* 1998, Šprtová and Marek 1999).

General objectives of this paper are (*1*) to determine spatial variability of RuBPCO activity within spruce canopy, and (*2*) to evaluate its change under the impact of EC.

Materials and methods

Plants and experiment design: The experiment was conducted in 2002 at the experimental site Bílý Kříž in Beskydy Mts. (Czech Republic, 49°30′N, 18°32′E, 908 m a.s.l.). Seventeen-year-old Norway spruce (*Picea abies* [L.] Karst.) trees have been grown since 1997 in two glass domes with ambient [350 µmol(CO_2) mol^{-1}; AC variant] or elevated [700 µmol(CO_2) mol^{-1}; EC variant] CO_2 concentration (for details see Urban *et al.* 2001). Both the total RuBPCO activity and N content were assessed in current needles taken from upper (U, 3rd whorl), middle (M, 6th whorl), and lower (L, 9th whorl) parts of tree crowns. Needles were sampled between 11:00 and 15:00 h at the following dates: 16th July (midday maximum temperature 13.8 °C, midday maximum irradiance 1 290 µmol m^{-2} s^{-1}), 6th August (23.6 °C, 1 100 µmol m^{-2} s^{-1}), 16th September (9.4 °C, 1 050 µmol m^{-2} s^{-1}), and 10th October (5.6 °C, 150 µmol m^{-2} s^{-1}). After determining needle fresh mass (*Sartorius*, Japan) and leaf area (*Li-3000A, Li-cor*, USA), samples ($n = 3$) for determining total leaf N content were dried to a constant mass at 104 °C, while samples ($n = 6$) for total RuBPCO activity assay were irradiated 5 min by saturating photosynthetically active radiation, PAR (>1 200 µmol m^{-2} s^{-1}). To avoid any drought effects by this pre-treatment, the incident PAR was passing through the thermal filter and the room temperature was kept between 20 and 23 °C. After that, needle samples were immediately frozen in liquid nitrogen, transferred to Dewar vessels, and transported to the laboratory.

RuBPCO extraction and assay: RuBPCO protein was extracted from the needles and assayed for total activity using the techniques of Besford (1984), slightly modified by Hrstka *et al.* (2002). Approximately 0.3 g of needle tissue was homogenized in a chilled mortar with 0.1 g inert sand, liquid nitrogen, and 10 cm^3 extraction buffer composed of: 50 mM HEPES, 25 mM KHCO$_3$, 5 mM MgCl$_2$, 0.2 mM Na$_2$EDTA, 5 mM dithiothreitol, and 0.1 g of insoluble polyvinylpolypyrrolidone, all at pH 8.0. The homogenate was filtered through iced sintered glass *R2* and the filtrate was assayed for total RuBPCO activity.

Total RuBPCO activity was assayed spectrophotometrically by the continuous measurement of 3-phosphoglycerate-dependent NADH oxidation in a coupled enzyme system based on the method of Lilley and Walker (1974). Reaction mixtures in cuvettes of 1.0 cm path length consisted of 30 mm^3 sample extract and 940 mm^3 assay solution containing 50 mM HEPES (pH 8.0), 25 mM KHCO$_3$, 20 mM MgCl$_2$, 0.2 mM Na$_2$EDTA, 5 mM dithiothreitol, 3.5 mM ATP, 0.35 mM NADH, 3.5 mM phosphocreatine, 67 nkat glyceraldehyde-3-phosphate dehydrogenase, 111 nkat 3-phosphoglyceric phosphokinase, and 178 nkat creatine phosphokinase. After 15 min of complete activation, 30 mm^3 of 13 mM RuBP was added to the sample/assay solution and changes in A$_{340}$ were measured at 25 °C using a *Helios γ* (*Spectronic Unicam*, UK) spectrophotometer.

Leaf nitrogen content was determined after combustion by a *CNS-2000* analyser (*LECO Corp.*).

Statistics: Because of small number of repetitions ($n = 6$), normal like distribution of the data sets was presumed. Scheffe test (ANOVA) was used to evaluate

the statistically significant differences in RuBPCO activity among CO_2 treatments, canopy positions, and

Results

A spectrophotometric method described by Lilley and Walker (1974) is frequently used method for routine RuBPCO activity measurement. We modified this method for Norway spruce needles. The sensitivity of the used technique expressed as a slope of the calibration curve was 0.0039, and the linearity expressed as r^2 was 0.9967 for RuBPCO concentration interval of 0.1–2.0 kg m^{-3}. We compared RuBPCO activities in various extracts from Norway spruce needles with activities of RuBPCO standards for the concentration interval of 0.1–2.0 kg m^{-3}. The RuBPCO content in extracts from Norway spruce needles ranged between 0.2–0.3 kg m^{-3} making this method convenient for Norway spruce RuBPCO activity assay.

Total RuBPCO activities of Norway spruce ranged between 1.01–2.76 $\mu mol(CO_2)$ m^{-2} s^{-1} in AC needles and 1.23–3.10 $\mu mol(CO_2)$ m^{-2} s^{-1} in EC needles. We observed statistically significant differences of RuBPCO activity within the canopy profile. Thus RuBPCO activity in AC needles from the U layer was higher by 11–15 % as compared with those from the M layer and even by 44–56 % ($p<0.01$) to L layer of spruce crown (Table 1). These findings correspond to the results of the P_N measurements. Šprtová and Marek (1999) reported difference of photon-saturated P_N by 35–42 % between sun exposed and shade needles.

Table 1. Percentage distribution of RuBPCO activity in current Norway spruce needles. The trees were growing in ambient [AC; 365 $\mu mol(CO_2)$ mol^{-1}] and elevated [EC; 700 $\mu mol(CO_2)$ mol^{-1}] CO_2 concentration. The needles were collected from the upper (U), middle (M), and lower (L) layers of the crowns over the vegetation season (July–October). The same capital letters indicate highly significant differences ($p<0.01$), small letters significant differences (p<0.05), between AC and EC needles.

	July	August	September	October
AC-U	100	100	100	100 C
AC-M	85 a	88	87	89 D
AC-L	52 A	56 B	45	44
EC-U	100	100	100	100 C
EC-M	89 a	92	87	89 D
EC-L	69 A	71 B	59	76

RuBPCO activity of EC-U needles was higher by 8–13 % as compared with those from the M part. The difference of activities between U and L needles was 24–31 % ($p<0.01$), *i.e.* it was significantly smaller than in AC needles (Table 1). The reason of this phenomenon is a sharp decrease of RuBPCO activity in the EC-U needles as a consequence of down-regulation during the vegetation season (Fig. 1B). Thereby, the difference in activities between upper and lower EC needles was only 23 %,
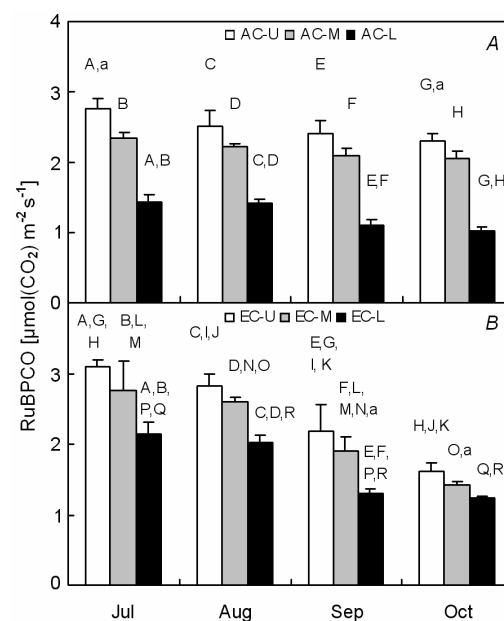
months. All statistical tests were performed using *STATISTICA* software.

Fig. 1. RuBPCO activities of Norway spruce current needles treated by (*A*) ambient (AC) or (*B*) elevated (EC) CO_2 concentration. Needles were sampled over the vegetation season (July–October) from upper (U), middle (M), and lower (L) layers of the tree crowns. *Columns* represent mean values, *bars* represent standard deviations. *The same capital letters* indicate highly significant difference ($p<0.01$), *small letters* indicate significant differences ($p<0.05$); $n = 6$.

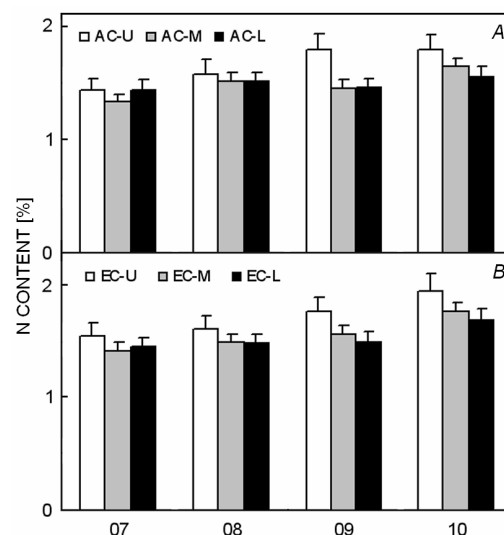Fig. 2. Total leaf nitrogen content of Norway spruce trees treated by (*A*) ambient (AC) and (*B*) elevated (EC) CO_2 concentration. Needles were collected from the same positions of upper (U), middle (M), and lower (L) layers of the tree crowns as those collected for RuBPCO activity analyses. *Columns* represent mean values, *bars* represent standard deviations. Statistical significances are discussed in the text. $n = 3$.

while it was 56 % in AC needles at the end of vegetation season (Fig. 1*B*; Table 1).

RuBPCO activity decreased over the vegetation season in both AC and EC treatments (Fig. 1). The slope of this decrease was similar for all AC sub-variants (tg $\alpha = -0.15$), while it was significantly higher in both EC-U (-0.51) and EC-L (-0.35) needles. In July and August, the RuBPCO activities were higher by 11–15 % (statistically significant; $p<0.05$) in EC-U and EC-M needles as compared with AC-U and AC-M needles, *i.e.* CO_2-stimulation effect. However in September and October, RuBPCO activities were lower by 10–30 % ($p<0.01$) in EC-U and EC-M needles as compared to AC needles, *i.e.* CO_2-downregulation effect. On the contrary, RuBPCO activity in shade adapted needles was stimulated by EC treatment over the whole year from 50 %

(summer; $p<0.01$) to 20 % (autumn; $p>0.05$).

The decrease of RuBPCO activity with canopy depth was not correlated with leaf N content. Nitrogen content in AC needles from the L part of spruce crowns was lower than in needles from the U part of the crown only in September and October by 19 % ($p<0.05$) and 8 % ($p>0.05$), respectively (Fig. 2*A*). However, RuBPCO activity in AC-L needles was 44–56 % lower compared to AC-U needles from July till October (Table 1). Nitrogen content in EC-L needles was reduced relative to needles from the EC-U needles from July till October by 8–13 % (Fig. 2*B*). By contrast, RuBPCO activity in EC-L needles was 24–31 % lower than in EC-U needles (Table 1).

Nitrogen content in EC needles was not significantly higher ($p>0.05$) than in AC needles.

Discussion

RuBPCO activity may be modulated in response to changes in irradiance, CO_2 or O_2 supply through the reversible carbamylation of lysine 201 of the large catalytic subunits (Miziorko and Lorimer 1983). In addition to the basic mechanism of activity modulation are several other mechanisms of regulation: (*1*) the irradiance-dependent activation by RuBPCO activase, (*2*) the inhibition by 2-carboxy-D-arabinitol-1-phosphate (CA1P), and (*3*) modulation by stromal metabolites (Parry *et al.* 1997). The relative contribution of each mechanism of regulation varies with time and species (Servaites *et al.* 1986, Salvucci and Anderson 1987). Therefore, it is very questionable to compare activities obtained by various authors and experiments. For example, Tissue *et al.* (1993) presented RuBPCO activities of *Pinus taeda* in the range of 1.3–9.8 $\mu mol(CO_2)$ m^{-2} s^{-1}, while Myers *et al.* (1999) reported that activities of *Pinus taeda* were 10.1–22.2 $\mu mol(CO_2)$ m^{-2} s^{-1}. In our experiments, Norway spruce activities ranged between 1.0–3.1 $\mu mol(CO_2)$ m^{-2} s^{-1}, whereas Urban and Marek (1999) presented activities of 4.0–7.2 $\mu mol(CO_2)$ m^{-2} s^{-1} in solitaire spruce trees from the same territory.

The effect of carbamylation on RuBPCO activity *in vivo* may be determined by the extent to which the activity, measured immediately in extracts prepared rapidly in the cold, is less than the activity measured following incubation with saturating concentrations of CO_2 and Mg^{2+} to carbamylate lysine 201 in vacant catalytic sites. These measurements are called initial and total activities, respectively. The term total activity is a misnomer, since it does not include active sites blocked by inhibitors, for example CA1P, which are not removed by incubation with CO_2 and Mg^{2+} (Parry *et al.* 1997). Total activity measurements may therefore underestimate the potential activity of fully activated, non-inhibited RuBPCO. Besides, the great problem in RuBPCO activity assay *in vitro* is a low solubility of RuBPCO enzyme. Rogers *et al.* (2001) showed that only 33 % of RuBPCO got into supernatant (or into filtration) during the extraction from

the leaf. That is another reason why values V_{Cmax} that express RuBPCO activity *in vivo* are higher than values of RuBPCO activity obtained *in vitro*; *e.g.* Urban *et al.* (2003) presented from the same site V_{Cmax} 28–40 $\mu mol(CO_2)$ m^{-2} s^{-1} for sun exposed and 10–17 $\mu mol(CO_2)$ m^{-2} s^{-1} for shade needles. However, until now no other method to determine the maximum catalytic potential of RuBPCO *in vitro* has been identified.

Differences in RuBPCO activity between sun exposed and shaded needles correlate to a degree of the canopy closure, *i.e.* distribution of PAR within the canopy. Šprtová and Marek (1999) reported from the same research site that shaded needles received only 11 % PAR compared with the exposed needles. Assimilation capacity of shaded needles was lower by 11–44 % as well as maximal P_N by 35–42 %, carboxylation efficiency by 35–45 %, CO_2 concentration at the active site of RuBPCO by up to 27 % compared to the sun exposed needles (Priwitzer *et al.* 1998). These findings correspond well with our results (Table 1). High activity of RuBPCO enzyme in sun needles enables not only more efficient carboxylation but due to photorespiration also protects plants from excess of photons (Muraoka *et al.* 2000).

Fig. 1 documents a decrease of RuBPCO activity over the vegetation season in both AC and EC treatments. The slope of this decrease was significantly higher in EC, particularly in EC-U, compared to AC needles. Hence RuBPCO activity in EC-U needles decreased more sharply due to down-regulation, so the difference in activities between U and L EC needles became smaller at the end of the season (Fig. 1*B*). The second reason is that, as compared with the sun exposed needles, the shaded ones have a larger part in a free assimilation capacity (Priwitzer *et al.* 1998), calculated as the ratio between the potential and actual P_N. This part of the free capacity is decreasing with increasing C_a according to higher diffusion of CO_2 to RuBPCO active sites. Impact of double $[CO_2]$ resulted in the decrease of the free assimilation capacity by 41 and 52 % for sun and shade acclimated

needles, respectively, compared to the situation of natural AC (Urban *et al.* 2003). Thus, the contribution of shaded needles on the whole tree to P_N may be increased under the long-term impact of EC.

Šprtová and Marek (1999) estimated a lower ratio of J_{max}/V_{Cmax} up to 18 % for shade acclimated needles compared to sun exposed ones in spruce trees. This typical manifestation rather indicates that lower P_N in shaded plants come from lower RuBPCO activity than from RuBP regeneration capacity (Zhang *et al.* 1995, Hättenschwiler 2001). Foliage acclimated to high irradiance often displays structural changes such as an increase in number of mesophyll cells per unit leaf area (Pearcy and Sims 1994). The greater internal leaf area of sun acclimated needles facilitates CO$_2$ diffusion to the chloroplast places which leads to the lower internal resistances and to higher value of J_{max}/V_{Cmax} ratio (Šprtová and Marek 1999).

Lloyd *et al.* (1995) and Hrdlička (1996) established that within monospecific stands N tends to be distributed in proportion to absorbed radiant energy and the concentration of this element decreases with depth in the canopy. These authors assumed that the lower RuBPCO activity in shade needles is a consequence of lower N content. We did not confirm this hypothesis in our experiments. Nitrogen content in needles from the L part of the crown of Norway spruce grown in AC was lower in

comparison with AC-U needles only in September and October, by 19 and 8 %, respectively (Fig. 2*A*). On the contrary, RuBPCO activity in AC needles was 44–56 % lower in the L layer compared to the U layer of the crown (Table 1). In EC, nitrogen content was 8–13 % higher in the U compared to the L part of the crown from July till October (Fig. 2*B*), while RuBPCO activity differed only by 24–31 % (Table 1). Our results show that RuBPCO activity decreased much more with crown depth than with N content. Thus down-regulation of RuBPCO activity must be more strongly influenced by other factors than by N content. Similarly Tissue *et al.* (1999), Griffin *et al.* (2000), and Rogers and Ellsworth (2002) concluded that a diminution of total leaf N content is not a cause, but a consequence of RuBPCO activity decrease.

In summary, total RuBPCO activity is very variable in dependence on plant species and many endogenous and exogenous factors. For that reason it is necessary to use this quantity very carefully. However, in our experiments the total RuBPCO activity was enhanced in current needles of Norway spruce trees grown at EC. The difference between sun exposed and shaded needles was more pronounced in AC (44-56 %) than EC (24–31 %). Down-regulation of RuBPCO activity was manifested by greater decrease of its activity in EC exposed needles compared with AC exposed needles. This down-regulation was not caused by a non-specific decrease in total leaf N content.

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