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Original article

Photosynthesis, Rubisco activity and mitochondrial malate oxidation in pedunculate oak (*Quercus robur* L) seedlings grown under present and elevated atmospheric CO_2 concentrations

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Summary — Pedunculate oak seedlings were grown at 350 and 700 µL/L CO_2 in controlled chambers. After 130 days at elevated CO_2, the biomass of the whole plant did not significantly increase. Photosynthesis, Rubisco activity, mitochondrial malate oxidation, carbohydrates and nitrogen contents were examined in the fourth growth flush. At 700 µL/L CO_2, the leaf net photosynthetic rate was 220% higher than at 350 µL/L CO_2. The decreased activity of Rubisco was accompanied by an accumulation of sucrose and glucose. The decreased oxidative capacity of crude leaf mitochondria from elevated CO_2 plants was driven by the lower nitrogen and protein contents rather than by the higher carbohydrates contents in the leaves. Nevertheless, direct effects of elevated CO_2 on the respiratory biochemistry cannot be excluded.

CO_2 / Rubisco / carbohydrates / mitochondria / oak

Résumé — Photosynthèse, activité Rubisco et oxydation mitochondriale du malate chez des semis de chêne pédonculé (*Quercus robur* L) élevés à des concentrations en CO_2 atmosphérique actuelle et double. *Des germinations de chêne pédonculé ont été élevées sous 350 et 700 µL/L de CO_2 en chambres de culture. Après 130 jours de CO_2 élevé, la biomasse du plant entier n'a pas augmenté significativement. Les échanges foliaires de CO_2, l'activité Rubisco, l'oxydation mitochondriale du malate, les teneurs de sucres et d'azote ont été étudiées sur des feuilles de la quatrième vague de croissance. À 700 µL/L de CO_2, le taux de photosynthèse nette foliaire augmente de 220 % par rapport à celui à 350 µL/L de CO_2. La diminution de l'activité Rubisco est accompagnée d'une accumulation de saccharose et de glucose. La diminution de la capacité d'oxydation des mitochondries brutes de feuilles des plants sous CO_2 élevé est reliée plutôt à la diminution des teneurs en azote et protéines qu'à l'augmentation de la teneur en sucres dans les feuilles. Néanmoins, les effets directs de l'élévation de CO_2 sur la biochimie de la respiration ne sont pas exclus.*

CO_2 / Rubisco / sucres / mitochondries / chêne

INTRODUCTION

The present concentration of atmospheric CO_2 limits the photosynthesis of C_3 plants. Many studies have now well established that the increase in the atmospheric CO_2 concentration will induce higher photosynthetic rates in herbaceous C_3 plants and also in trees (Ceulemans and Mousseau, 1994). However, initial increases in photosynthesis are sometimes not maintained during long-term exposure to elevated CO_2, namely in studies with potted trees (Ceulemans and Mousseau, 1994; Gunderson and Wullschleger, 1994). A biochemical mechanism proposed for the acclimation of photosynthesis to elevated CO_2 is a diminution in the activity of Rubisco (Bowes, 1991), generally associated to a decrease of its amount (Tissue et al, 1994; Wilkins et al, 1994). Elevated CO_2 may affect the expression of Rubisco indirectly via carbohydrates accumulation (Webber et al, 1994).

A higher CO_2 concentration may also affect leaf respiration directly by as yet unknown modifications of the respiratory biochemistry (Amthor, 1991; Wullschleger et al, 1994) and indirectly through changes in growth rate and tissue composition (sugars, nitrogen) (Wullschleger et al, 1992a; Curtis et al, 1995). In trees, the CO_2 enrichment usually induced a reduction in leaf dark respiration (El Kohen et al, 1991; Wullschleger et al, 1992b; Reid and Strain, 1994; Teskey, 1995). However, the effects of a CO_2 enrichment on the mitochondrial respiratory chain have not been assessed in trees.

In this work, we studied the effects of a an enhanced concentration of CO_2 (700 µL/L) on the photosynthetic rate, the Rubisco activity and the mitochondrial oxidative and phosphorylative properties, in the leaves of oak (*Quercus robur* L) seedlings grown in a fertilized soil for 130 days. The modifications at the biochemical level will be discussed in relation with that found in the sugars and nitrogen concentrations of the leaves.

MATERIALS AND METHODS

Plant material and growth conditions

Acorns of pedunculate oak (*Quercus robur* L) were collected beneath a single tree in Richardménil (Meurthe et Moselle, France). Fifteen germinated acorns were planted together in a 7 L pot filled with a peat-clay-black soil mixture (4C, De Baat). A total of 26 pots were placed in two growth chambers with 14 h light (600 $\mu mol.m^{-2}.s^{-1}$), 21 °C/16 °C day/night air temperatures, 70%/90% day/night air humidities. The CO_2 concentrations of the charcoal-filtered air were 350 µL/Land 700 µL/L (day and night, respectively). Seedlings were watered at field capacity every 3 days to compensate for evapotranspiration. Under these conditions, seedlings flushed every 4 weeks. Just after the second flush was fully expanded, 30 g of Osmocote Plus (Sierra Chemical Company, Milpitas, USA), with NPK 15/10/12 + oligoelements were added to each pot.

Analyses

All physiological measurements were made after 10 h of light, on the just fully expanded fourth flush, after 130 days of growth.

Net photosynthetic rate (A, $\mu mol.m^{-2}.s^{-1}$) was measured on the third leaf from the top of four to seven seedlings, using a portable photosynthesis system (LI-6200, Li-Cor, Inc) with a 4 L cuvette. A was measured under the two CO_2 growth concentrations.

For enzymatic analyses, 50 mg of fresh leaf matter were sampled from each leaf used for photosynthesis measurements. The samples from the seven different seedlings were bulked. The desalted extract for Rubisco and protein assays was obtained as in Gérant et al (1988) with modifications. Two extracts were made per treatment. Carboxylase activity of Rubisco (EC 4.1.1.39) was assayed in a coupled system (Lilley and Walker, 1974). The reaction was started by adding 0.5 mM RubP after a 15 min incubation period

of the desalted extract in the reaction mixture (Van Oosten et al, 1992) (total activity). The two extracts were assayed twice.

Crude mitochondria were obtained from 20 g of fresh leaf without mid-rib, taken from 10 to 15 seedlings. The extraction method was modified from Gérard and Dizengremel (1988). The homogenate was filtered through a 22 µm nylon net. The soluble protein content was determined in the filtrate using the Coomassie blue method (Bradford, 1976). The filtrate was submitted to differential centrifugation. The last pellet contained the crude mitochondria. Two mitochondrial extractions were made per treatment. The crude mitochondria were assayed for malate oxidation by monitoring the oxygen uptake on a polarograph at 25 °C. The reaction medium was that of Gérard and Dizengremel (1988). The oxidation of malate (30 mM) was measured in the presence of glutamate (2 mM) and nicotinamide adenine dinucleotide (NAD) (400 µM). Adenosine diphosphate (ADP) (80 µM) was added to couple phosphorylation with the oxidation of malate. Respiratory control (RC) was calculated as the ratio of the oxidation rate in the phosphorylating state to the nonphosphorylating state. Phosphorylating efficiency (ADP/O) was calculated as the ratio of the fixed amount of ADP added to the quantity of oxygen atoms consumed for the phosphorylation of ADP. Potassium cyanide (KCN) (800 µM) and salicylhydroxamic acid (SHAM) (750 µM) were used as inhibitors of the cytochrome and alternative pathways, respectively. At least two malate oxidation measurements were made per crude mitochondrial pellet.

Shoots and roots were harvested for the determination of biomass, starch, glucose, sucrose and nitrogen contents. The measurements were made on a dry powder pooled from dry powders of the seedlings used for photosynthesis and Rubisco measurements and of the seedlings used for mitochondria extraction. Total nitrogen was measured using a carbon nitrogen autoanalyser (Carlo Erba Instruments NA-1500). Soluble sugars and starch were separated according to Haissig and Dickson (1979). Starch (pellet) and sucrose (supernatant) were then assayed as described by Alaoui-Sossé et al (1994). An aliquot of the methanol/water phase was evaporated to dryness and sugars were dissolved in water. Glucose was assayed in this fraction using commercial glucose oxidase and peroxidase enzymes as for the determination of starch-derived glucose.

RESULTS AND DISCUSSION

Plant biomass

After 130 days of growth, biomass of pedunculate oak seedlings grown at elevated CO_2 (700 µL/L) (13.6 ± 2.2 mg dry weight [DW]) was higher but not significantly different from that of seedlings grown at ambient CO_2 (8.6 ± 1.1 mg DW). The shoot/root biomass ratio under high CO_2 (2.1 ± 0.3) and ambient CO_2 (2.6 ± 0.4) were not significantly different.

Table I. Net photosynthetic rate, total activity of Rubisco per unit area and capacity of mitochondria to oxidize malate in the phosphorylating state (per unit nitrogen and per unit soluble proteins) in leaves of pedunculate oak seedlings grown under ambient and elevated CO_2 concentrations.

CO_2	*A*	*Total Rubisco activity*	*Mitochondrial oxidation rate of malate in phosphorylating state*	
µL/L	*µmol $CO_2.m^{-2}.s^{-1}$*	*nkat.dm^{-2}*	*nmol $O_2.min^{-1}.g^{-1}$ N*	*nmol $O_2.min^{-1}.g^{-1}$ soluble proteins*
350	1.15 ± 0.17	216	443	151
700	3.68 ± 0.65	99	247	105

For net photosynthesis, data represent the mean ± SE of four or five seedlings. For Rubisco activity and for mitochondrial oxidation rate, data represent the mean of two extracts with at least two replicates per extract.

Net photosynthetis and Rubisco activity in relation to carbohydrate contents

After 130 days of growth, net photosynthetic rate (μmol CO_2 . m^{-2} . s^{-1}) of the fourth flush of oak seedlings grown at high CO_2 was 220% higher than that of the seedlings grown under ambient CO_2 (table I). The total leaf area of this flush was not significantly higher under 700 μl/L CO_2 (4.4 ± 1.0 dm^2) than under 350 μl/L CO_2 (3.7 ± 1.0 dm^2). The fourth flush leaves of the CO_2-enriched plants had a higher dry mass and also a higher dry mass per unit area (table II). Rubisco activity was lower at 700 μl/L than at 350 μL/L CO_2 (table I). Hence, the fixation of CO_2 by Rubisco was not a limiting factor for photosynthesis at elevated CO_2.

In the CO_2-enriched leaves, the decrease in Rubisco activity was accompanied by the accumulation of sucrose and glucose per unit area (table II). It is likely that these accumulated sugars may repress the expression of Rubisco, resulting in a lower activity of the enzyme. Indeed, Sheen (1990) demonstrated that, in photosynthetic cells, these sugars control the expression of the nuclear-encoded gene of the small subunit of Rubisco (*rbcS*). Moreover, abundant biochemical and molecular evidences indicate that glucose acts as a regulatory signal for feedback control of photosynthetic genes in higher plants (Sheen, 1994). In addition, under elevated CO_2, a decreased activity of Rubisco associated with a decreased amount of the enzyme was already reported for spruce (Van Oosten et al, 1992) and wild cherry (Wilkins et al, 1994).

Malate oxidation by crude leaf mitochondria in relation to carbohydrates and nitrogen contents

In nongrowing organs, like fully expanded leaves, respiration is restricted to the maintenance of the existing tissues. It is controlled by the supply of substrates to the mitochondria and by the demand for energy and carbon skeletons which will be used mainly in the synthesis of amino acids and proteins. In our experiment, the crude leaf mitochondria were able to couple the oxidation of malate to the phosphorylation of ADP in adenosine triphosphate (ATP) (fig 1). However, the values of the ADP/O ratio and of the respiratory control were lower compared to theoretical values (Bonner, 1967; Laties, 1974). The values of ADP/O

Table II. Leaf mass per unit area, glucose and sucrose content per unit area and glucose, sucrose, starch, nitrogen and soluble proteins content per unit dry weight in leaves of pedunculate oak seedlings grown under ambient and elevated CO_2 concentrations.

CO_2	Leaf mass per area	Glucose		Sucrose		Starch	Total nitrogen	Soluble proteins
μL/L	$mg.dm^{-2}$	$mg.dm^{-2}$	$mg.g^{-1}$	$mg.dm^{-2}$	$mg.g^{-1}$	$mg.g^{-1}$	$mg.g^{-1}$	$mg.g^{-1}$
350	313[a] ± 28	0.43	1.4	4.9	15.5	56.6	26.3	77.0
700	481[b] ± 60	0.74	1.5	13.0	27.0	121.9	22.1	52.3

For leaf mass per area, data represent the mean ± SD of seven seedlings. [ab] Values followed by the same letter are not significantly different from each other at the 0.05 level of probability (*F*-test). For glucose, sucrose, starch, total nitrogen and soluble proteins, data represent the values obtained on the common powder.

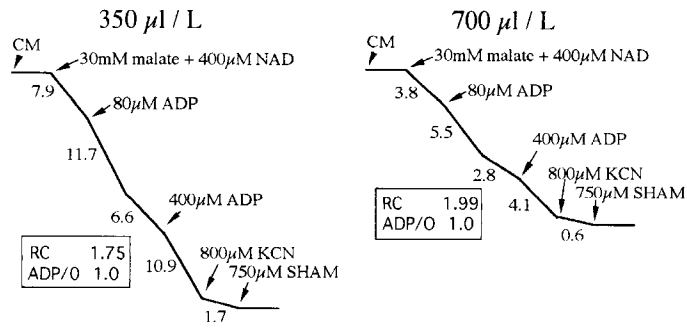

Fig 1. Oxygen consumption rate by crude mitochondria oxidizing malate, extracted from leaves of pedunculate oak seedlings grown under 350 and 700 µl/L CO_2. Numbers along the traces are nmol O_2. $min^{-1}.g^{-1}$ dry weight (DW) and are mean values of two extracts with at least two replicates per extract. CM: crude mitochondria; RC: respiratory control; ADP/O: phosphorylating efficiency; SHAM: salicylhydroxamic acid; ADP: adenosine diphosphate; NAD: nicotinamide adenine dinucleotide; KCN: potassium cyanide.

and RC were not modified by elevated CO_2 (fig 1). The crude mitochondria extracted from oaks grown at 700 µl/L CO_2 had a lower capacity per unit of DW to oxidize malate, in both nonphosphorylating and phosphorylating states, than the mitochondria from ambient CO_2 grown oaks (fig 1). This lowered capacity in the elevated CO_2 grown seedlings was accompanied by a greater amount of glucose, sucrose and starch per unit of DW (table II). These sugars are a source of tricarboxylic acids such as malate. Their accumulation may lead to a higher supply of substrates to mitochondria and leaf mitochondria of the elevated CO_2 grown seedlings may have a higher capacity to oxidize malate. In our experiment, the lowered oxidative capacity of mitochondria, in CO_2-enriched oak seedling leaves was rather independent of the higher contents of carbohydrates. On the other hand, the lowered capacity of oxidation was associated with a lower amount of nitrogen and soluble proteins per unit dry weight (table II). In CO_2-enriched plants, lower respiration rates than in ambient CO_2 grown plants, are often associated with lower nitrogen contents (Ryan, 1991; Wullschleger et al, 1992a). It is likely that the response of the leaf mitochondria from elevated CO_2 grown oaks was driven by the lower nitrogen and protein contents rather than by the higher starch and sucrose contents in the leaves. However, the decreased capacity to oxidize malate remained even when data were expressed on a nitrogen basis and on a soluble proteins basis (table I). This suggests that the inhibition of the respiratory processes with increasing CO_2 may not only result from long-term changes in the composition of the leaves (indirect effects). In trees, a direct effect of high CO_2 on respiration was observed in *Castanea sativa* Mill (El Kohen et al, 1991) and in *Pinus taeda* L (Teskey, 1995). These direct effects, reviewed by Amthor (1991), have been suggested to involve changes in the intercellular pH and/or in membrane properties, and more likely inhibitions of respiratory enzymes by carbamylation.

After 130 days, the increase in CO_2 was beneficial for net photosynthesis of pedunculate oak seedlings. However, at the biochemical level, the activity of Rubisco was lowered at elevated CO_2. That was accompanied by an accumulation of sucrose and glucose. Concerning respiration, the long-term inhibition at high CO_2 levels may result from both changes in leaf composition and from direct effects of CO_2 on the respiratory biochemistry (Ziska and Bunce, 1994). Considering our results, direct effects of elevated CO_2 on the respiratory processes in oak leaves need to be tested.

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