Simultaneous Gas-Exchange and Fluorescence Measurements with Ozone-Fumigated Spruce

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A method was developed for carrying out gas-exchange and chlorophyll-fluorescence measurements simultaneously during fumigation of spruce twigs with peroxidic photooxidants. It is thus now possible to investigate how a pollutant affects distinct sectors of the photosynthetic apparatus of the plant: whereas fluorescence reveals any changes in the primary light reaction, CO_2 gas-exchange measurements supply information about the biochemical reactions of the Calvin cycle. Results of short-time fumigation with 750 ppb ozone are presented here. Gas-exchange and fluorescence data are affected strongly in early summer, but not in autumn. The assimilation rate decreases significantly: primarily as a result of Rubisco activity and possibly because of direct inhibition of the electron-transport chain as well. Closure of the stomata leads to further reduction in the assimilation rate. Though no damage becomes visible on the needles, the perturbance of the photosynthetic apparatus caused by ozone fumigation is not reversible within 24 h.

Introduction

There have been a number of hypotheses as to the cause of the "new-type" forest decline that has been observed since the beginning of the 1970s in various regions of Central Europe and North America. It seems certain that there is no single cause of all the symptoms, although they have occurred at almost the same time, and that different meteorological and geological conditions in the affected stands play a major role in causing the symptoms (Nießlein and Voss, 1985; Papke *et al.*, 1987). In some regions (high altitudes of subalpine mountains, alps) the participation of peroxidic substances, above all ozone (O_3), peroxyacetyl nitrate (PAN) and hydrogen peroxide (H_2O_2), in the "new-type" forest decline is considered most probable, at least as a predisposing factor for damage that weakens vitality of the trees and lowers their resistance toward other stressors (Nießlein and Voss, 1985; Elstner and Osswald, 1984).

In order to estimate the impact of these substances on the physiology of conifers, single twigs of cloned spruce trees were fumigated with peroxidic compounds that have been detected in polluted forest air, and the response of the photosynthetic and stomatal apparatus was investigated. To accomplish this, gas-exchange and chlorophyll-fluorescence measurements were carried out simultaneously during the fumigation. This combination of measurements makes it possible to observe distinct sectors of the photosynthetic apparatus of the plant in detail and thereby find out where and how a pollutant affects the photosynthetic reactions. Whereas chlorophyll fluorescence is an indicator for changes in the primary light reaction (photon capture, charge separation, electron transport), the CO_2 gas-exchange measurements supply information about the biochemical reactions of the Calvin cycle.

The experiments presented here were carried out with five-year-old cloned spruce trees, which were grown outdoors. Because of the unnatural laboratory environment, each fumigation experiment was restricted to a maximum duration of one day. In order to induce measurable effects in such

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a short time, it was necessary to use peroxide concentrations higher than those actually encountered in forest stands. Nevertheless, these experiments are an important and rapid tool for investigating the effects of gaseous pollutants on photosynthesis and also for providing information complementary to that obtained from long-term fumigation experiments with pollutants at atmospheric concentrations.

We were interested, however, not only in studying the effects of these pollutants but also in comparing the complementary measuring methods of chlorophyll fluorescence and gas exchange. This concept in itself is not new (Wong and Woo, 1986; Genty *et al.,* 1989; Berry and Weis, 1987), but it has not previously been applied to steady-state measurements with whole plants under nearly ecophysiological conditions. In addition, we applied characteristic parameters evaluated on the basis of a photosynthesis model to interpret the effects of fumigation. Simultaneously measuring gas-exchange and fluorescence during fumigation in order to investigate the effects of air pollutants on coniferous trees is also new.

In this article the measurement techniques and the processing of the data are described in detail and illustrated by the ozone fumigation. A later paper will deal with the application of this concept to comparative studies of the effects of several peroxides (O_3, H_2O_2, peroxyacyl nitrates).

Materials and Methods

Four-year-old spruce trees (*Picea abies* Karst.) of one clone (Bayer. Landesanstalt für forstliche Saat- und Pflanzenzucht, Teisendorf, Germany) were planted in a special soil with a standard nutrient level (Fruhstorfer Erde T plus 2 kg/m^3 Plantacote 4M long-term fertilizer) in plastic pots (22 cm in diameter) and grown outdoors for one year before the experiments were carried out. During winter they stood close together; in early spring some new soil was added, and the plants were placed *ca.* 1 m apart in rows on a gravel bed. An automatic watering system was used in summer. The trees exhibited deep-green needles and well established mycorrhiza. The formation of the new needles was largely complete before the start of the experiments. For each fumigation and photosynthesis measurement, two trees were taken into the laboratory in their pots, one to be fumigated and the other, treated similarly, to serve as a non-fumigated control. Single new, but well-established twigs about 15 cm long at the tip of similar branches of the second whorl, one from each tree, were enclosed in separate plexiglass-topped gas-exchange cuvettes ($18 \times 15 \times 12$ cm^3). The rest of each tree was protected from high light intensity and warming during the measurement.

To fumigate the twig, the ozone was added to the gas stream entering the cuvette. The pollutant concentration was followed by diverting a small, continuous sample of the gas close to the twig to an ozone detector (Dasibi 1009-CP, UV absorption).

Before, during, and after fumigation, CO_2/H_2O gas exchange and chlorophyll fluorescence were measured simultaneously.

The equipment used for the gas-exchange measurements was similar to the Walz minicuvette system, which has already been described in detail (Knoppik *et al.,* 1986). Measurements were made of transpiration and net photosynthesis rates of each of the enclosed twigs, the ambient CO_2 partial pressure in the cuvette (adjustable between 50 and 2500 μbar), the twig temperature (held constant at 25 °C) at the non-illuminated side of the twig (by means of a very small thermocouple), and the incoming light flux (Li-Cor quantum flux sensor, $\approx 1100\ \mu Em^{-2}\,s^{-1}$). The two lamps used for illumination (Osram HQI-T 2000 W/D) were mounted about 1.50 m above the gas-exchange cuvettes. The dewpoint of the air entering the cuvette was fixed at 8 °C and the wind speed parallel with the twig at about 2 ms^{-1}.

The calculation of the intercellular CO_2 partial pressure $p^c{}_i$ and the stomatal conductance g^c, by means of the measured parameters *A, E,* and the temperature, is described in von Caemmerer and Farquhar (1981) and Sharkey *et al.* (1982); for further details see the Appendix. Other characteristic parameters (maximum carboxylation rate, $V^c{}_m$; maximum assimilation rate, A_m; CO_2-compensation point, Γ^c; rate of dissimilative respiration in light, D^l) are calculated from CO_2-response curves as described in Ziegler-Jöns (1989) on the basis of the photosynthesis model of Farquhar and von Caemmerer (von Caemmerer and Farquhar, 1981; Farquhar and von Caemmerer, 1982). As in Wong and Woo (1986) these calculations were extended to evaluate a total electron-transport rate for carboxylation and oxygenation reactions of ribulose-1,5-bisphosphate (RuBP):

$$j_{GE} = (A + D^l) \cdot (4.5 + 10.5\,(\Gamma^*/p^c_i))/(1 - (\Gamma^*/p^c_i)).$$

The numerator represents the electrons used for carboxylation and respiration, respectively; the denominator takes into account the fact that 0.5 mol CO_2 is set free per mole of RuBP in respirative oxygenation. For Γ^*, the compensation point in the absence of dissimilative respiration, 31 µbar was used; D^l, derived from the CO_2-response curves, was used to calculate j_{GE} in the case of fumigations at constant CO_2 levels. For the fumigation in autumn, when no CO_2 responses were carried out, a mean value of 0.5 µmol $m^{-2} s^{-1}$ was assumed (resulting in an uncertainty in j_{GE} of less than 2%).

For fluorescence measurements the PAM fluorometer (F. Walz, Effeltrich, Germany) was used (for technical details see Schreiber, 1986). The geometric arrangement had been optimized in preceding experiments to achieve acceptably intense fluorescence signals and to avoid casting shadows from the optical fibre onto the twigs. In this arrangement, the optical fibre was passed gas-tight through the cuvette cover at an angle of about 45°, and the distance between the fibre end and the twig or needles was about 2 cm (see Fig. 1). It was proved that under these conditions light saturation (referring to the fluorescence signal F_m, see below) could be achieved with a non-destructive light flash.

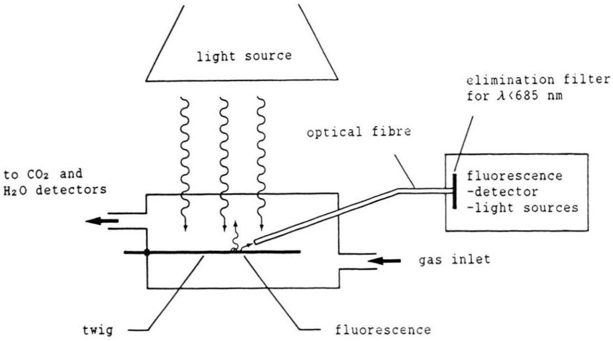

Fig. 1. Scheme of the experimental arrangement for simultaneous measurements of gas exchange and fluorescence at a spruce twig.

The following three fluorescence signals were measured during the experiments (see Fig. 2):

1) The steady-state fluorescence $F(t)$ during the normal illumination with the actinic light driving the photosynthesis (about 1100 $\mu Em^{-2} s^{-1}$).

2) The maximum fluorescence $F_m(t)$ during a short fluorescence-saturating light flash with high light intensity (approx. 3500 $\mu Em^{-2} s^{-1}$ for 1 s) applied by an additional light source through the fibre optics every 3 min.

3) The "dark-related" (not adapted!) fluorescence $F_o(t)$, for which the whole gas-exchange chamber was darkened every 5–15 min for less than 30 sec with an opaque black velvet cloth. We checked for any change in $F_o(t)$ on adding far-red light ($\lambda > 715$ nm), which should accelerate reoxidation of the PS II reaction centers, but none was found (compare Weis, 1988).

From fluorescence data the quantum-efficiency-correlated term $\Phi(t) = 1 - F_o(t)/F_m(t)$ (Genty *et al.,* 1989; Schreiber, 1986) can be calculated, as can the photochemical quench, q_P, with $q_P(t) = 1 - (F(t) - F_o(t))/(F_m(t) - F_o(t))$ (Schreiber *et al.,* 1986).

To a first approximation q_P represents the fraction of oxidized, "open" PS II reaction centers, and the product $\Phi \cdot q_P$ gives the probability that a light quantum absorbed by the antenna pigments is used for a charge separation in the PS II reaction center. With an absorption coefficient of the pigments α and the actinic light influx I, the product $j_{FL} = I \cdot \alpha \cdot \Phi \cdot q_P$ is a measure of (or is at least proportional to) the electron-transport rate at the beginning of the electron-transport chain (Wong and Woo, 1986). J_{FL}, derived from fluorescence data, describes the rate at the beginning of the electron-transport chain, whereas j_{GE}, calculated from gas-exchange data, represents the rate used for the ensuing carboxylation and respiration reactions of the Calvin cycle.

Two kinds of experiments are to be distinguished. In the first set, fumigation with ozone was carried out at constant CO_2 pressures of 350 µbar and 1250 µbar, and the fluorescence signals, the assimilation (A) and transpiration rates (E) were followed, as were the calculated values of the stomatal conductance (g^c) and the CO_2 partial pressure in the chloroplasts (p^c_i). The fumigation under enhanced CO_2 partial pressure was carried out for the purpose of separating effects on en-

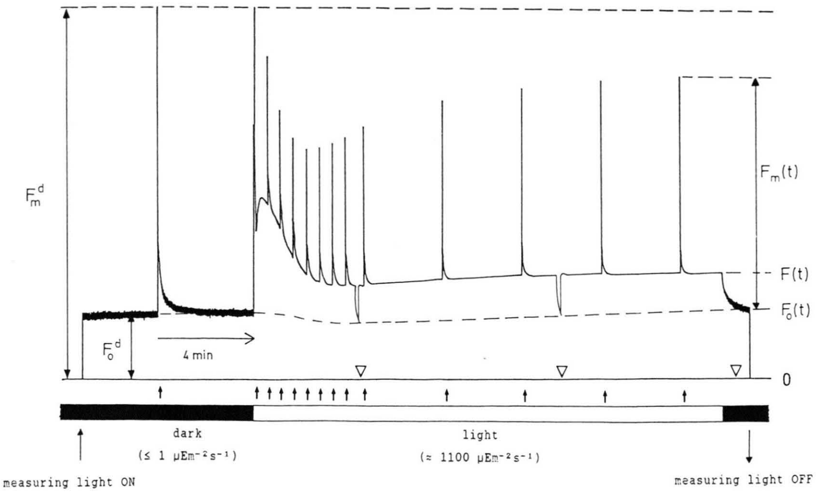

Fig. 2. Pattern of the course of a fluorescence experiment with definition of the terminology (↑ = saturating light flash; ▽ = measurement of F_o).

zyme activity from those on the stomatal apparatus. Fumigation experiments were carried out in early summer (June/July), when the young shoots were just completely developed but the axes still soft and flexible, and in autumn (end of October), when the axes of the young shoots had turned brown and wooden. In the second set of experiments (carried out in June and July, too) the dependence of the above-mentioned fluorescence signals, that of *A*, *E*, g^c, and p^c_i on the ambient CO_2 partial pressure and the alteration by fumigation of the CO_2-response curves thus obtained was determined.

The fumigation experiments at constant CO_2 partial pressure were carried out as follows: When assimilation and transpiration rates of the twigs had reached a constant level, the twig of one tree was fumigated for 3.5 h, from midday, with 750 ppb of ozone. This pollutant concentration is about ten times that commonly observed in elevated forest sites far away from emission sources during high-radiation fine-weather periods in spring and summer (UBA, 1983; GSF, 1988; Smidt, 1989). Though not physiologically relevant to forest stands, the ozone concentration applied is needed to induce measurable effects in short-time laboratory experiments.

Gas-exchange and fluorescence measurements continued for at least 1 h after the end of fumigation.

In order to find out how ozone fumigation affects CO_2-response curves ($A = f(p^c_i)$), a CO_2 response without fumigation was carried out in the morning of the first day of the experiment. This was followed by an ozone fumigation (3.5 h, 750 ppb) in the afternoon, when the twigs had reached a steady state at 350 µbar CO_2 partial pressure. Because previous experiments had shown that perturbations of the photosynthetic apparatus caused by ozone were hardly reversible within one day, the CO_2 response after fumigation was carried out in the morning of the following day.

It was not necessary to define the needle surfaces, because the same twig was used throughout each experiment: only the change in gas exchange and fluorescence on fumigation was investigated. Each measurement was carried out at least three times. All results are included in the calculations and discussion; the data shown in Fig. 3–8 represent individual measurements.

Results and Discussion

Measurements at constant CO_2 partial pressure

a) 330 µbar CO_2

The results of fumigation at atmospheric CO_2 partial pressure (330 µbar) are presented first. Data were recorded before, during, and after fumigation with 750 ppb ozone that lasted 3.5 h.

Fig. 3 refers to a fumigation in early summer. The gas-exchange data *A* and g^c and the ozone concentration are plotted in Fig. 3a as a function of time. Fig. 3b shows the measured fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$ (in relative units); from these the fluorescence coefficients $q_P(t)$ and $\Phi(t)$ were calculated (see Fig. 3c). The electron-transport rates calculated from gas exchange, $j_{GE}(t)$, and from fluorescence data, $j_{FL}(t) = [I \cdot q_P \cdot \Phi](t)$, are then shown in Fig. 3d. The absorption coefficient α is assumed to be constant throughout the experiment; justification of this can be found in the Appendix. Because the electron-transport rates, as well as A and g^c, are normalized to the values at the beginning of the fumigation, α could be omitted. The light intensity I was nearly constant. On that graph (3d) the values for the intercellular CO_2 partial pressure are also plotted, as calculated from the gas-exchange data on the assumption of nearly homogeneous gas distribution in the needles. Whereas A, g^c, j_{GE}, and j_{FL} are presented in relative values, $p^c{}_i$, the ozone concentration and the fluorescence coefficients are plotted in absolute values.

After a lag at the beginning there is a decrease in all parameters, starting with the fluorescence values (except q_P) about 1 h after start of fumi-

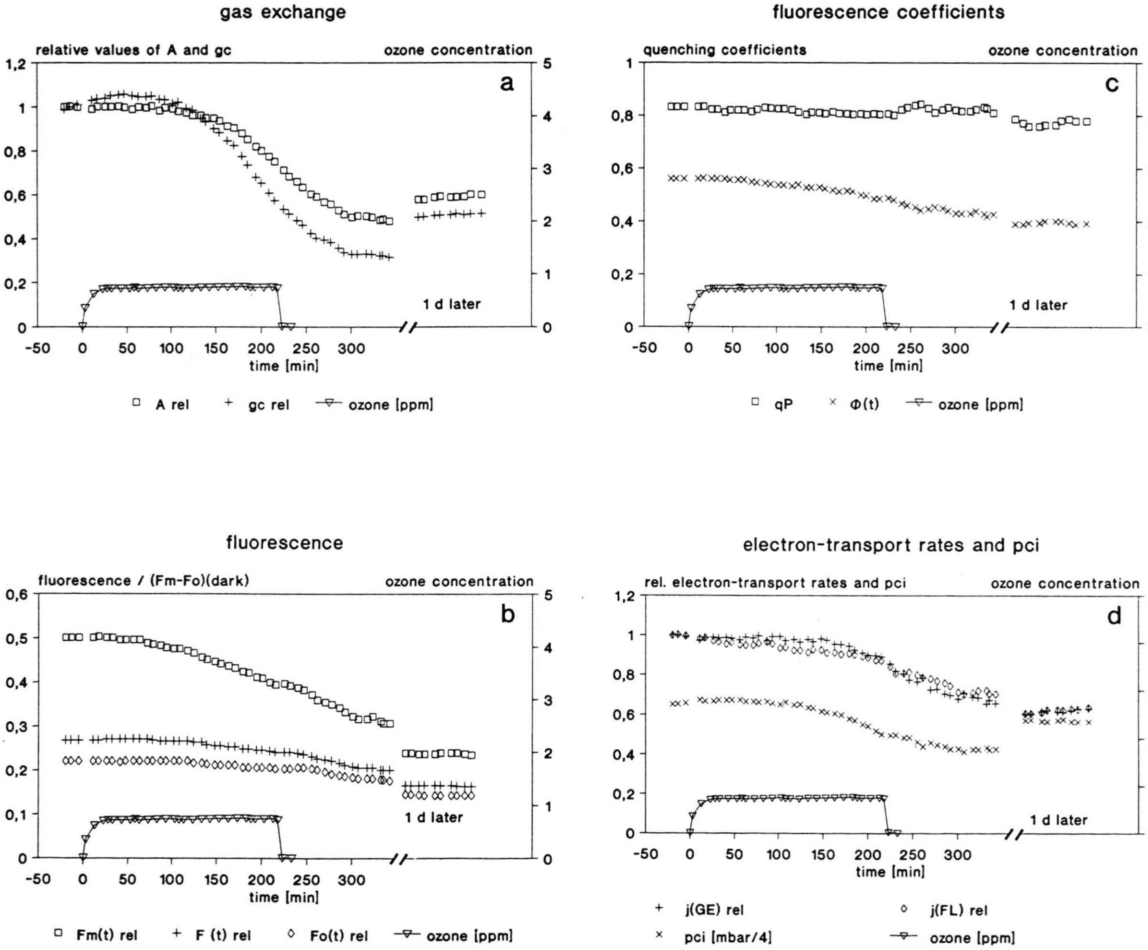

Fig. 3. Fumigation with ozone in early summer at 330 μbar CO_2 partial pressure. a) Net photosynthesis rate A, stomatal conductance g^c (relative to the values at the beginning of the fumigation), and ozone concentration; b) fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$, divided by $(F_m - F_o)_{dark}$; c) fluorescence coefficients $q_P(t)$ and $\Phi(t)$, calculated from the fluorescence data; d) relative electron-transport rates $j_{GE}(t)$, calculated from gas exchange, $j_{FL}(t)$, calculated from fluorescence, and intercellular CO_2 partial pressure $p^c{}_i$.

gation, followed by g^c and later A (about 1.5 h and 2 h after start of fumigation, respectively). Even after fumigation is over, the decrease continues for about 1 h before a steady state is reached, for A at 50% reduction, for Φ 20%. One experiment included measurements 24 h after fumigation (results shown in Fig. 3); there was little or no recovery. The corresponding control measurements, carried out the same way, but without ozone, reveal no significant changes in any of the parameters within the 8–10% experimental error (see Fig. 4).

The gas-exchange data exhibit a sharp decrease in the stomatal conductance g^c, the net photosynthesis rate A, and, to a lesser extent, the calculated intercellular CO_2 pressure $p^c{}_i$ (for a detailed discussion of the $p^c{}_i$ calculation see the Appendix). The faster and steeper decline in g^c than in A (50% decline for A, 65% for g^c) and the decrease in $p^c{}_i$ point to stomatal closure as a reason for the reduction of photosynthesis. A more detailed consideration of the calculated electron-transport rates, however, indicates Rubisco (= RuBP-carboxylase/oxygenase) damage as well. Closure of

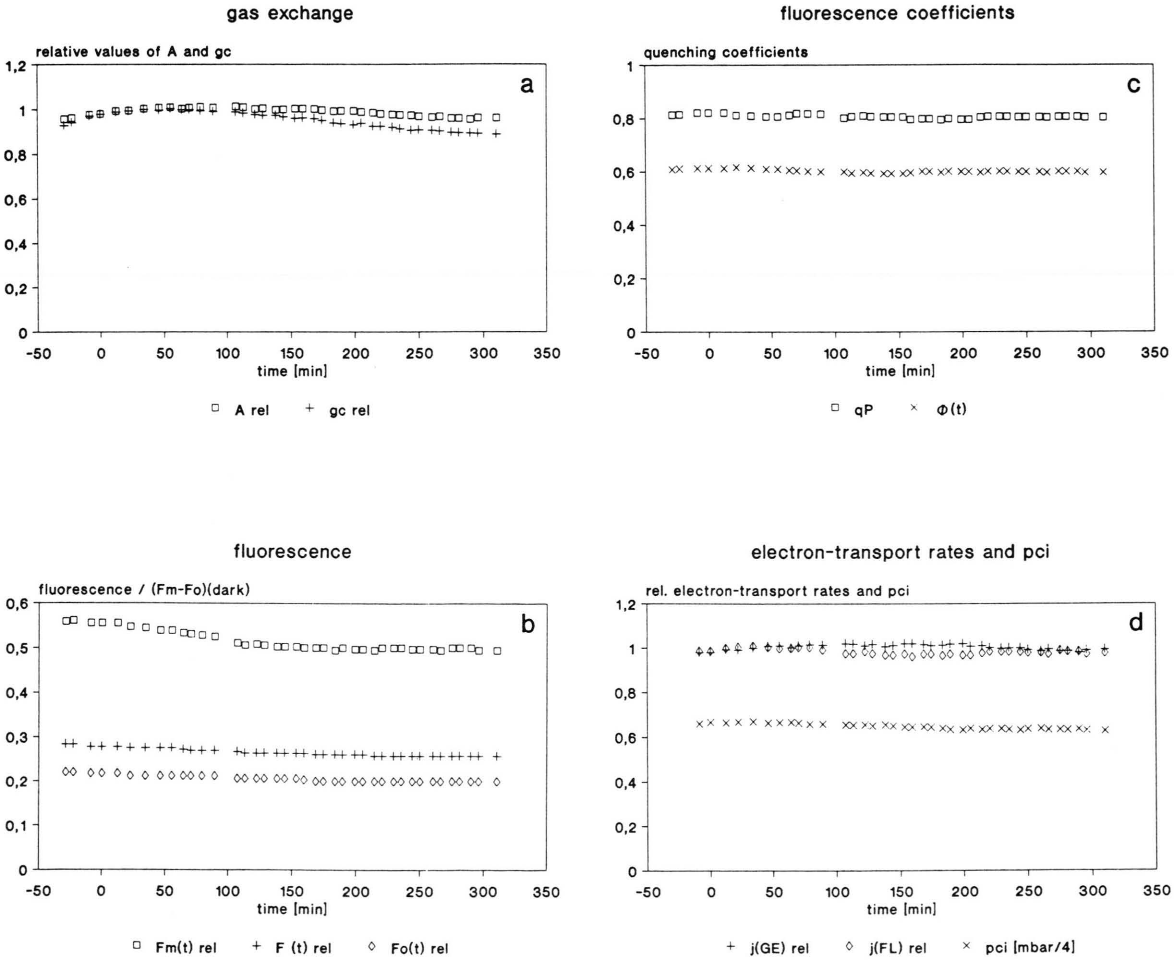

Fig. 4. Control measurement in early summer at 330 μbar CO_2 partial pressure. a) Net photosynthesis rate A and stomatal conductance g^c (relative to the values at the beginning of the experiment); b) fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$, divided by $(F_m-F_o)_{dark}$; c) fluorescence coefficients $q_P(t)$ and $\Phi(t)$, calculated from the fluorescence data; d) relative electron-transport rates $j_{GE}(t)$, calculated from gas exchange, $j_{FL}(t)$, calculated from fluorescence, and intercellular CO_2 partial pressure $p^c{}_i$.

the stomata alone would reduce the CO_2 supply and result in a reduced carboxylation rate and therefore in a reduction of the electron-transport rate. As the oxygen partial pressure (210 mbar) is about 1000 times as high as the CO_2 partial pressure, there will be no simultaneous O_2 shortage. Part of the Rubisco will be diverted away from carboxylation in favour of higher oxygenation rates. Since about 15% more electrons are needed for the oxygenation reaction than for carboxylation (the theoretical ratio is 5.25:4.5; the experimental 5.19:4.62 (Sharkey, 1986)), j_{GE}, the electron-transport rate used for Rubisco reactions, should hardly be affected. The measured j_{GE}, however, decreases in parallel with A and $p^c{}_i$. This would be expected if the Rubisco activity itself decreases. On the other hand one would not expect a $p^c{}_i$ decrease in that case. In the experiment, the $p^c{}_i$ decline (−32%) turns out to be similar to that of j_{GE} and significantly less than that of A (−50%). Thus, both Rubisco deactivation and stomatal closure seem to occur, the two probably being linked regulatorily.

The time lag observed between fumigation start and the change in the measured parameters can be explained by various mechanisms. Several authors (Botkin *et al.*, 1971; Gross, 1987; Führer *et al.*, 1993) report similar observations when fumigating conifers with ozone and measuring gas exchange. There seems to be a physiological threshold dose for ozone, above which damage to the photosynthetic apparatus occurs. Below this threshold, protection mechanisms or metabolic repair are capable of compensating for deleterious effects of the strong oxidant. The ascorbic acid content of the cell wall seems to play a major role in prevention of oxidative damage (Chameides, 1989). Other possible causes of the delay in measurable effects are the replacement of damaged enzymes from a Rubisco pool or a need for injuries to the stomatal apparatus to accumulate before damages become obvious.

Additional information can be derived from the fluorescence data. The decrease in all measured fluorescence signals (F, F_o, F_m) indicates an increased dissipative deactivation of excited antenna pigments. It should be noted that, contrary to expectation, in most cases fluorescence intensity and photosynthetic activity run parallel rather than antiparallel. The decrease of Φ is interpreted as weakened cooperation between the antenna pigments, resulting in lowered exciton transfer to the reaction center. As q_P remains unchanged, the fraction of open reaction centers itself seems to be constant, at least to a first approximation. There is therefore no hint of any backup of electrons in the electron-transport chain.

J_{FL} decreases in parallel with j_{GE}, indicating that the fraction of electrons used for other reactions is no larger than that for carboxylation or oxygenation. Electron supply and demand seem to be well balanced, even under fumigation.

The results of a similar fumigation experiment carried out in October are shown in Fig. 5 in the same configuration as in Fig. 3. In contrast to the fumigation in early summer, there is within experimental error no change in the parameters investigated. In general, the parameters behave very similarly to those in the corresponding control measurements (graphs not shown, but nearly identical with those from early summer; see Fig. 4). As ambient CO_2 partial pressure and ozone concentration were exactly the same as in summer (Fig. 3), the different response should be due to different physiological status of the spruce trees in fall and early summer.

b) 1250 μbar CO_2

The results of a fumigation experiment (carried out in early summer) with an ambient atmosphere enriched with CO_2 (1250 μbar) are shown in Fig. 6. Within the first hour there is a fast but limited drop in A (−15%) and g^c (−20%), paralleled by a drop in all measured fluorescence signals (F, F_o, F_m), the exciton-transfer probability Φ, and the two electron-transport rates j_{GE} and j_{FL}, whereas $p^c{}_i$ remains constant. This can be explained by a decrease in Rubisco activity or an effect on the pigments that results in a reduced electron supply.

After 1 h, however, there is a stabilization of A and most of the other parameters (except g^c and $p^c{}_i$), as if Rubisco or pigment damage is no longer continuing or some repair mechanism is compensating for further damage. Apart from that, stomata are closing drastically after their brief recovery, so that there is a continuous reduction in the intercellular CO_2 partial pressure. About 1.5 h after fumigation ends, g^c and $p^c{}_i$ stabilize at 20% and 25% of their initial values, respectively. As ex-

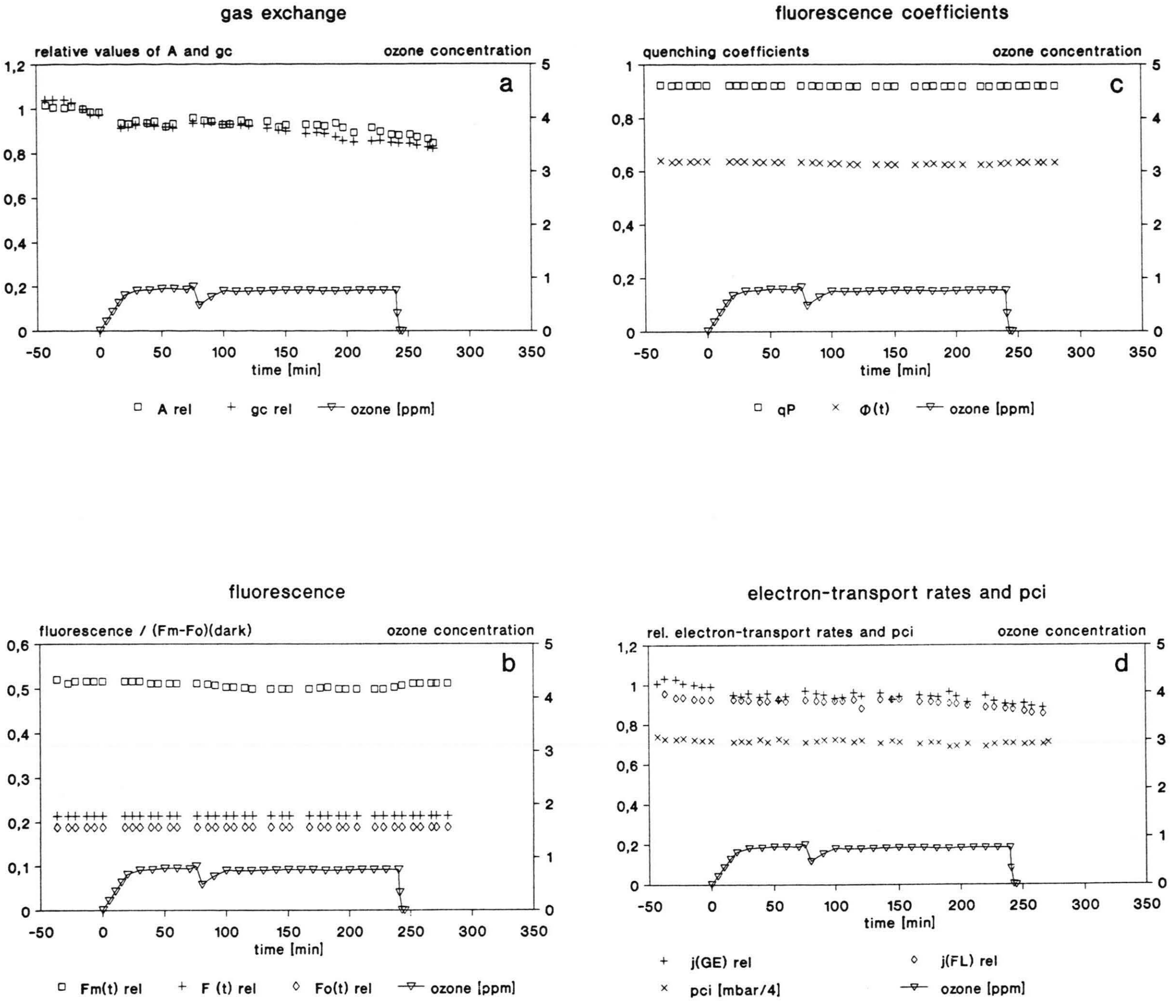

Fig. 5. Fumigation with ozone in autumn at 330 µbar CO_2 partial pressure. a) Net photosynthesis rate A, stomatal conductance g^c (relative to the values at the beginning of the fumigation), and ozone concentration; b) fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$, divided by $(F_m-F_o)_{dark}$; c) fluorescence coefficients $q_P(t)$ and $\Phi(t)$, calculated from the fluorescence data; d) relative electron-transport rates $j_{GE}(t)$, calculated from gas exchange, $j_{FL}(t)$, calculated from fluorescence, and intercellular CO_2 partial pressure $p^c{}_i$.

pected, A is not influenced as long as $p^c{}_i$ exceeds 250 µbar; this behaviour is similar to that of $p^c{}_i$ at atmospheric CO_2 levels.

The rapid decline in the fluorescence signals and Φ, but not in q_P, and the stabilization after 1 h again indicate an enhanced energy dissipation of the trapped photons. After fumigation, fluorescence tends to recover slightly.

During the whole experiment the two calculated electron-transport rates j_{GE} and j_{FL} change in parallel, in agreement with the general trend, thus showing no electron loss to reactions other than oxygenation and carboxylation. The small recovery of j_{GE} after fumigation is not understood and could not be clearly reproduced. It has to be added that in the case of enhanced CO_2 pressure even the corresponding controls exhibit some slight changes, as shown in Fig. 7: A, the F-values, Φ, and the relative electron-transport rates decrease continuously, being reduced by about 10% (F_m 20%) over the 5 h-experiment; $p^c{}_i$ increases by about 10%.

Though the fumigation under enhanced CO_2 partial pressure was carried out with the intention

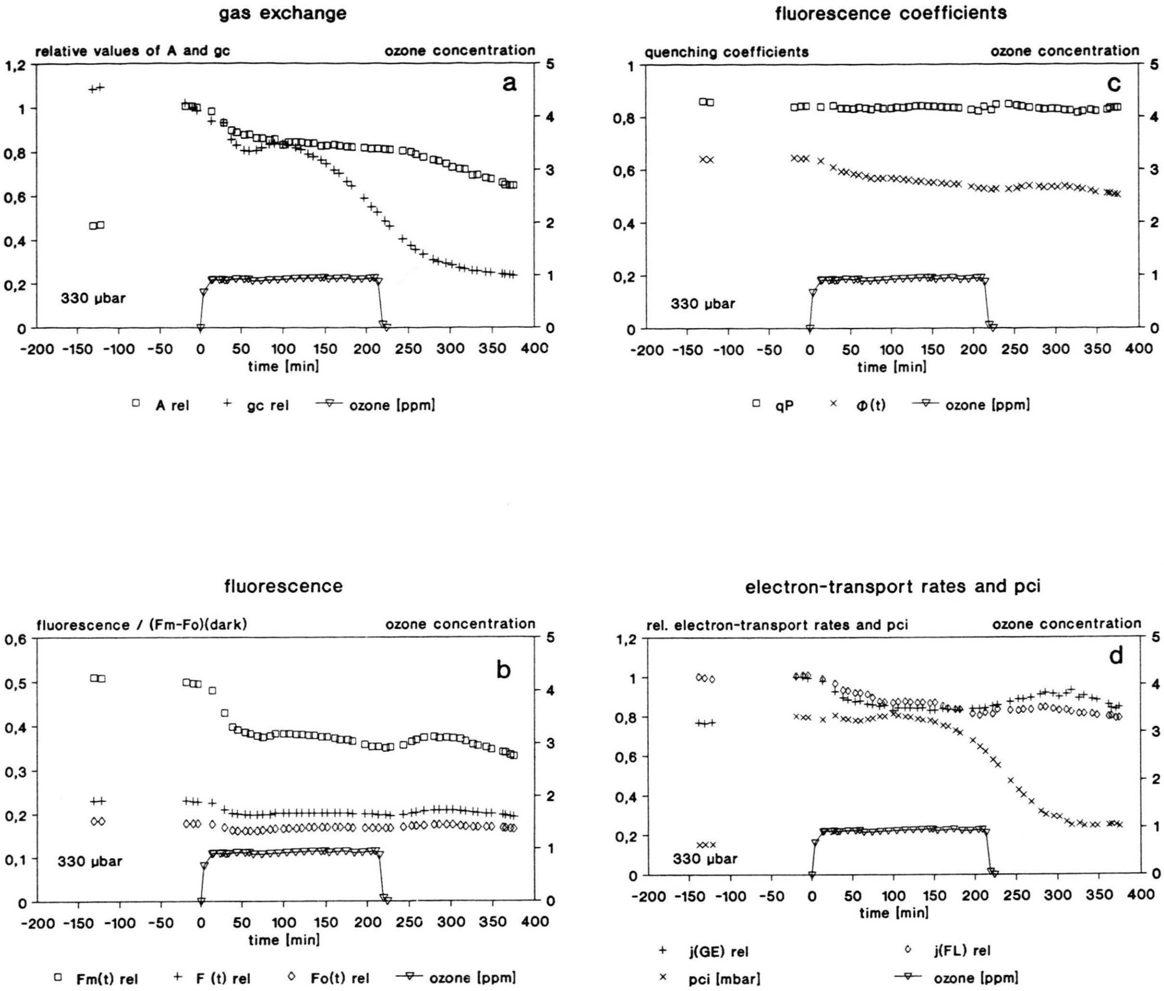

Fig. 6. Fumigation with ozone in early summer at 1250 µbar CO_2 partial pressure. a) Net photosynthesis rate A, stomatal conductance g^c (relative to the values at the beginning of the fumigation), and ozone concentration; b) fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$, divided by $(F_m - F_o)_{dark}$; c) fluorescence coefficients $q_P(t)$ and $\Phi(t)$, calculated from the fluorescence data; d) relative electron-transport rates $j_{GE}(t)$, calculated from gas exchange, $j_{FL}(t)$, calculated from fluorescence, and intercellular CO_2 partial pressure $p^c{}_i$. Also shown here are the corresponding data at 330 µbar CO_2 before increasing the CO_2 partial pressure.

of separating stomatal from enzyme damage, the results indicate that the effects of ozone under the two CO_2 regimes differ in sequence, in extent and perhaps in mechanism.

CO_2 responses

By means of CO_2-response curves, the influence of fumigation at atmospheric CO_2 partial pressure on Rubisco activity can be separated from the influence on the stomata. The results are plotted in Fig. 8 as a function of $p^c{}_i$, before fumigation on the left and after on the right. The upper graphs (Fig. 8a) show the course of the net photosynthesis rates and the two electron-transport rates (j_{GE}, j_{FL}) calculated from the measured gas exchange and fluorescence. All these data are given relative to the starting values at 330 µbar ambient CO_2 partial pressure before fumigation. In the middle (Fig. 8b), the measured fluorescence sig-

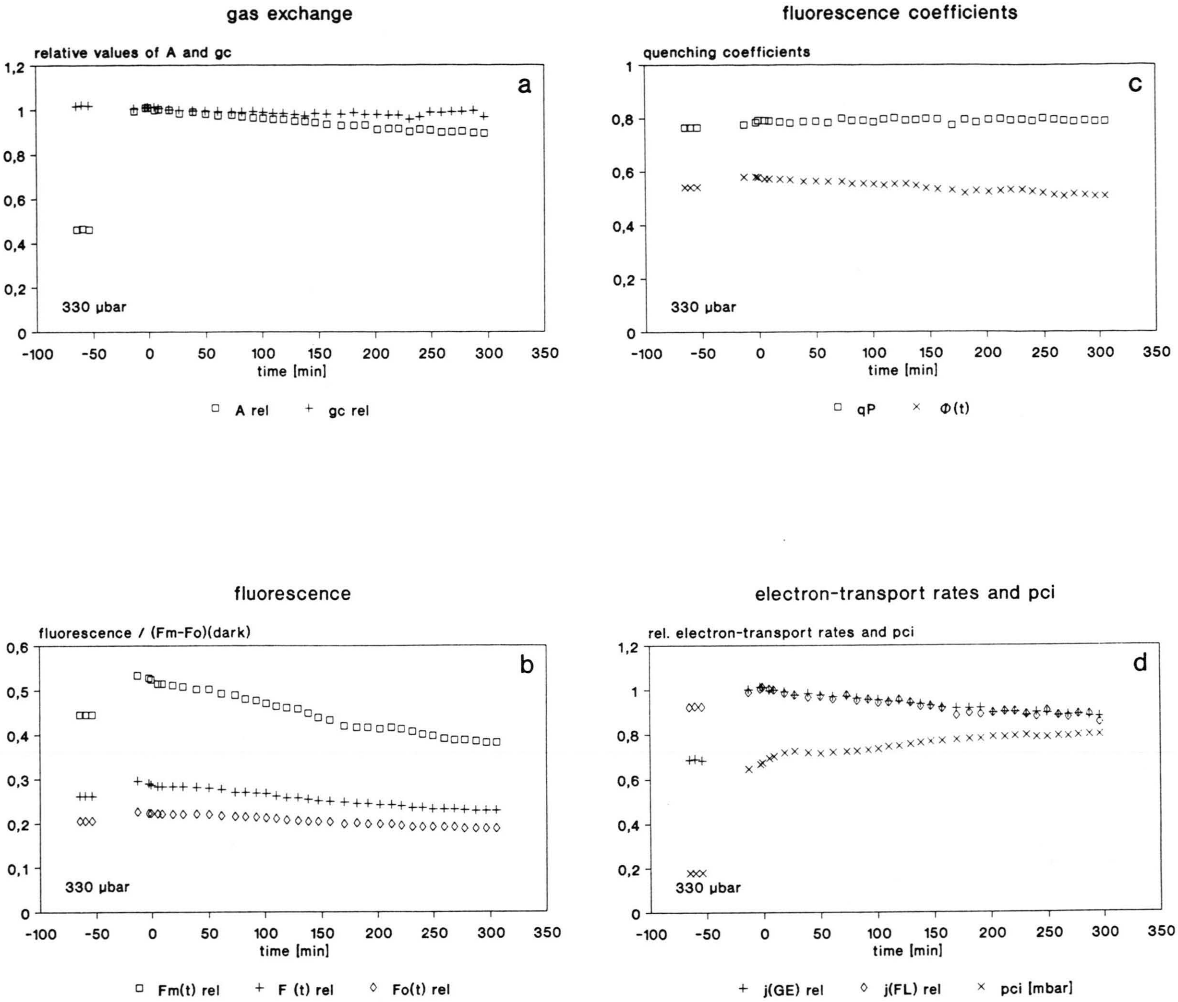

Fig. 7. Control measurement in early summer at 1250 µbar CO_2 partial pressure. a) Net photosynthesis rate A, stomatal conductance g^c (relative to the values at the beginning of the experiment); b) fluorescence data $F_m(t)$, $F(t)$, and $F_o(t)$, divided by $(F_m-F_o)_{dark}$; c) fluorescence coefficients $q_P(t)$ and $\Phi(t)$, calculated from the fluorescence data; d) relative electron-transport rates $j_{GE}(t)$, calculated from gas exchange, $j_{FL}(t)$, calculated from fluorescence, and intercellular CO_2 partial pressure $p^c{}_i$. Also shown here are the corresponding data at 330 µbar CO_2 before increasing the CO_2 partial pressure.

nals are plotted in relative units; in the lower graphs (Fig. 8c) the calculated fluorescence coefficients are shown in absolute values.

In the following discussion all curves after fumigation are to be compared with the corresponding curves before fumigation. Though all parameters except q_P are significantly reduced after fumigation, the overall shape of the curves is nearly unchanged. All parameters are lowered by nearly the same factor, with minor deviations at very high CO_2 levels. This behaviour points to non-stomatal primary damage, especially as the modelling indicates a dramatic decrease in the maximum Rubisco activity, $V^c{}_m$ (see Table I), similar to that of A and j_{GE}. J_{FL} shows a little less relative reduction at low $p^c{}_i$, which may mean that oxygenation, with electron demand about 15% higher than carboxylation, is favoured under these conditions (see above).

As already mentioned, the reduction in A (less obvious in j_{GE}) at the highest $p^c{}_i$ values is slightly smaller than at the other CO_2 levels. The reason

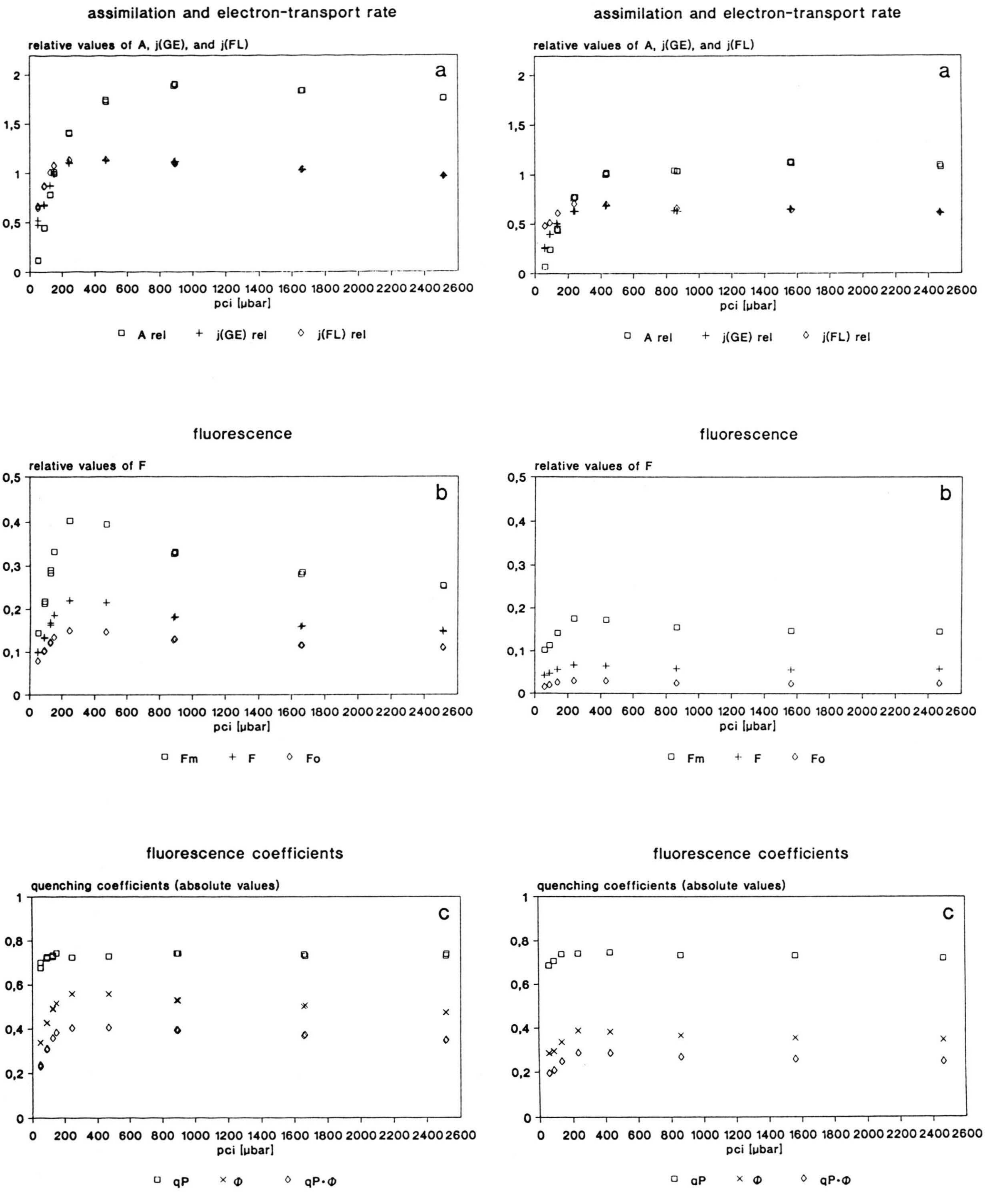

Fig. 8. CO_2-response curves before ozone fumigation (on the left) and after (on the right). a) Course of the net photosynthesis rate A and the two electron-transport rates j_{GE} and j_{FL}, all data relative to the values at 330 µbar CO_2 partial pressure before fumigation; b) course of the measured fluorescence signals F_o, F, and F_m, in relative units; c) course of the calculated fluorescence coefficients q_P, Φ, and $q_P \cdot \Phi$, in absolute values.

Table I. Alteration in the most important photosynthetic parameters by fumigation with ozone given as per cent of the values before fumigation (± maximum deviation).

	δV^c_m	δJ_m [A_m]	δD^l	$\delta\Gamma^c$
Fumigated twig (750 ppb ozone)	−40 (± 12)	−55 (± 12)	+ 120 (± 60)	+ 20 (± 10)
Corresponding control	−5 (± 4)	−6 (± 4)	−1 (± 10)	+ 1 (± 7)

is a decrease in A at the highest p^c_i that is greater before than after fumigation. This must be seen in light of the reduction in the calculated theoretical maximum net photosynthesis rate A_m (see Table I; this parameter is derived from the model by means of V^c_m and the highest measured $A(p^c_i)$). The decrease in A_m by about 50% after fumigation is significantly greater than the measured decrease in A at high p^c_i. This indicates that some additional limiting process at very high photosynthetic activity is only indirectly influenced by fumigation. After fumigation the photosynthetic activity is decreased, so that this additional effect is no longer limiting. There are strong hints that phosphate translocation at the cell membrane is responsible for limitation under conditions of high photosynthetic activity (Walker and Sivak, 1986; Walker and Osmond, 1986). Insufficient *P*-translocation is hold responsible for accumulation of intermediary products, which results first in a pH change and then in a decrease in Rubisco activity (Sharkey *et al.,* 1986). This is supported by investigations showing a decrease in Rubisco activity at high photosynthesis rates in general (Sage *et al.,* 1988).

The modelling leads to additional parameters: D^l, the respiration in light, is strongly increased after fumigation. This indicates a higher energy demand possibly due to metabolic changes, recovery, and repair of secondary damage. The slight increase in Γ^c, the CO_2-compensation point, after fumigation corresponds to the increase in D^l (see above).

Additional information can again be derived from the fluorescence measurements. After fumigation the signals are smaller and exhibit a less distinct maximum. As all *F*-signals change in a similar way, the effects on the calculated coefficients and j_{FL} are smaller, q_P remaining unchanged even at high p^c_i. The signals and the coefficients reach their maxima at $p^c_i \approx 300$ μbar as j_{GE} does, whereas the measured net photosynthesis rate reaches its maximum at about 800 μbar. This shows the close link between fluorescence and electron transport.

At low p^c_i, photosynthesis is limited by CO_2 supply. Though part of the absorbed energy (electrons) is used for oxygenation reactions, the pigments dissipate a larger part to avoid photoinhibition or photochemical damage, so that the *F*-signals are lower. Additionally, the link between the pigments is lowered (low Φ), and more reaction centers are reduced ("closed"; low q_P), which indicates a backup of electrons in the chain.

At higher CO_2 levels the oxygenation reaction is repressed in favour of carboxylation. As the latter demands fewer electrons, fewer charge separations are needed, and again more energy is dissipated. This effect is enhanced before fumigation, because the photosynthetic activity decreases at high CO_2 levels (see above). In comparing j_{GE} and j_{FL}, one must bear in mind that j_{FL} is set equal to j_{GE} at the maximum before fumigation, as j_{FL} is not determined absolutely. J_{FL} again parallels to j_{GE} under nearly all conditions, indicating that the fraction of electrons used for oxygenation and carboxylation is always the same. Only if the CO_2 partial pressure is very low are a large number of electrons used for reactions other than those that are Rubisco-related; this is especially true after fumigation. This result can be interpreted as an additional means of protecting the pigments from photoinhibitory damage. One possible alternative electron path could be the enhanced reduction of O_2 (Mehler reaction) and the subsequent detoxification of O_2^-.

Conclusions

- Spruce is sensitive to ozone at the beginning of the vegetation period, but much less so at the end.

- Within 24 h there is hardly any measurable recovery of photosynthesis and fluorescence.
- At an atmospheric CO_2 level a reduction in the maximum Rubisco activity as well as a closure of the stomata occurs. While the results of the fumigation experiments at constant CO_2 partial pressure do not allow a decision as to which is the primary reaction, the comparison of CO_2-response curves before and after fumigation suggests that Rubisco is damaged first.
- Fumigation at a high CO_2 level leads to damage in a different sequence and to a different extent.
- Not only could fluorescence measurements be interpreted properly at all CO_2 levels, but they also constitute an independent tool for interpreting the fumigation experiments. With their help regulatory feedback mechanisms like decreased exciton transfer between antenna pigments during and after fumigation are detected, whereas the average fraction of reduced reaction centers is kept constant except at extremely low CO_2 partial pressure.
- The electron-transport rates calculated from gas exchange ("Rubisco-related") and fluorescence ("pigment-related") always run parallel during and after fumigation, indicating that there is no change in the fraction of electrons needed for reactions other than those of the Calvin cycle.
- After fumigation, respiration is drastically enhanced, so that the cells are provided with additional energy and metabolic products.

Appendix

In the previous discussion we assumed a homogeneous distribution of the intercellular CO_2 partial pressure in the needles, $p^c{}_i$. The results of Raschke *et al.* (1989) indicate that this is not necessarily correct for flat leaves. Though the shape of needles and the arrangement of their stomata, in lines instead of clusters, are significantly different from those of flat leaves, thereby favouring our assumption, we can derive further proof by examining our experimental observations.

Ozone fumigation at 330 μbar ambient CO_2 partial pressure causes a rapid and extreme decrease in stomatal conductance (see Fig. 3). A heterogeneous rather than homogeneous $p^c{}_i$ distribution would require the assumption of a heterogeneous aperture of the stomata and insufficient lateral CO_2 diffusion inside the needles; otherwise, $p^c{}_i$ would equilibrate. The most heterogeneous $p^c{}_i$ distribution would occur if the stomata could assume only two different positions, open or closed, and there were no lateral diffusion. Under such conditions a decrease in transpiration by 50% would be due to a closure of just half of the open stomata. Beneath open stomata $p^c{}_i$ will remain unchanged, whereas it would drop to near the compensation point beneath closed stomata, *i.e.* about 42 μbar according to von Caemmerer and Farquhar (1981) and Farquhar and von Caemmerer (1982). From this microscopic CO_2 distribution a mean $p^c{}_i$ can be calculated:

$$p^c{}_i(t,\text{het}) = p^c{}_i(t=0) \cdot g^c(t)/g^c(t=0) + (1 - g^c(t)/g^c(t=0)) \cdot 42\ \mu\text{bar},$$

and thus a mean relative $j_{GE}(p^c{}_i)$. In Fig. 9 the mean values during the ozone fumigation are plotted along with the corresponding curves for homogeneous $p^c{}_i$ distribution (already plotted in Fig. 3) and the relative electron-transport rate based on the fluorescence data.

A decrease in g^c (see Fig. 3), implying a stomatal closure, and significant changes in the plotted parameters j and $p^c{}_i$, especially differences in the corresponding parameter sets (*e.g.* $p^c{}_i$(hom), $p^c{}_i$(het)), become obvious at the latest after 3 h of fumigation. Whereas $j_{GE}(p^c{}_i(\text{hom}))$ behaves similarly to j_{FL}, the decrease in $j_{GE}(p^c{}_i(\text{het}))$ is less

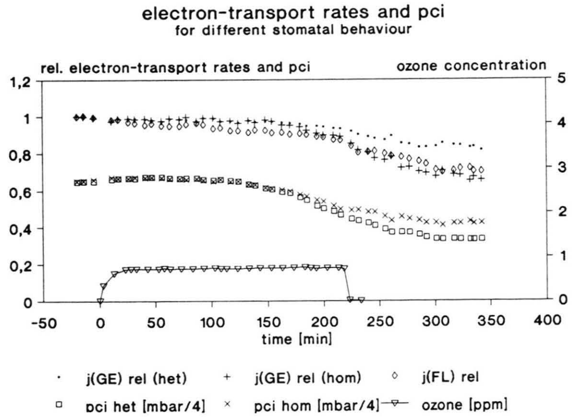

Fig. 9. Course of the electron-transport rates j_{GE} and j_{FL} (relative to the initial values), and intercellular CO_2 partial pressure $p^c{}_i$, calculated for homogeneous (hom) and heterogeneous (het) behaviour of the stomata during fumigation with ozone at 330 μbar CO_2 partial pressure.

pronounced. This implies that for heterogeneous $p^c{}_i$ the consumption of electrons in the Calvin cycle is decreased by an amount less than the amount of electrons represented by j_{FL} at the beginning of the electron-transport chain. This can only be explained if electrons that are used for reactions other than those of the Calvin cycle before fumigation are used for carboxylation/oxygenation during and after severe fumigation. According to the difference in the two j_{GE} in Fig. 9, this should be true for at least 20% of all electrons. Not only is this in itself very unlikely, but ozone is thought to reduce the Calvin-cycle activity, as is also reflected in the decrease in $V^c{}_m$ (see Table I). Thus a heterogeneous $p^c{}_i$ distribution leads to contradictions, while the assumption of a homogeneous $p^c{}_i$ distribution does not. However, simultaneous closure of the stomata is not necessarily required, as long as sufficient lateral diffusion is guaranteed.

A second common assumption made in calculating j_{FL}, the constancy of the light-absorption coefficient of the needle pigments, α, even under severe fumigation, was supported by the experimental observations as well. If there were any change in α due to fumigation, one should expect a reduction. This would cause an additional decrease in j_{FL} on fumigation over that in the curve shown in Fig. 9. In that case, though, j_{FL} would be even smaller than j_{GE}(hom), the lowest gas-exchange-derived electron-transport rate that can be calculated. The likelihood that j_{GE} could be greater than j_{FL} was already shown in the Discussion to be very small (see above). In addition, insensitivity of α towards changes caused by stress is supported by experiments in which plants were submitted to severe drought: a significant decrease in α could only be detected when the colour of the leaves changed drastically from green to brown (Drenkard *et al.,* unpublished results).

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