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Apoplastic Ascorbate Does not Prevent the Oxidation of Fluorescent Amphiphilic Dyes by Ambient and Elevated Concentrations of Ozone in Leaves [1]

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Cut leaves of spinach were infiltrated with solutions containing oxidation-sensitive fluorescent dyes. Excess solution was removed from the intercellular space by centrifugation. Fluorescence of the dyes D494 and D283 started to decrease immediately after the onset of fumigation with ozone at concentrations similar to or not much higher than ambient concentrations in air on sunny days. Only part of ozone entering the leaves was intercepted by the dye. The major part was degraded by unspecified reactions. Photosynthesis was not inhibited while the introduced dye was oxidized by ozone, showing that open stomata facilitated gas exchange after the introduction of dye into the leaf interior. Feeding of ascorbate to the leaves via the petiole failed to affect the ozone-dependent decrease in fluorescence emission from the dye. Likewise, infiltration of leaves by solutions containing dye and 10 mM ascorbate did not produce significant protection of the dye against oxidation by ozone. However, such protection was observed in vitro, when solutions of dye and ascorbate were bubbled with air containing ozone. Although there is little doubt that apoplastic ascorbate occupies a central position in the antioxidative defense of leaf tissue, we are surprised to find that it is much less effective than expected to decrease the oxidation of fluorescent lipophilic probes which had been introduced into the leaf interior. The data suggest that ascorbate is not a primary reductant of ozone in the apoplast. With a microscope-mounted CCD-camera connected to the gas exchange equipment we obtained spatial information on the fluorescence signal and present first results on an heterogeneous distribution of ozone action.

Key words: Ascorbate — Fluorescent dyes — Leaves — Ozone — Spinach.

Ozone has been implicated as a causative factor in the decline of forests which is of public concern in the United States, Asia and a number of European countries. Different plant species differ in their sensitivity to ozone (Guderian 1985, Prinz and Krause 1989, Luwe and Heber 1995). Known effects of the prolonged exposure to elevated concentrations of ozone are reduced growth, decreased photosynthesis and respiration, changes in the protein pattern and fatty acid composition etc. (for reviews, see Guderian 1985, Saxe 1991, Sandermann et al. 1996). However, in view of the chemical aggressiveness of ozone, detrimental effects are not surprising. The question to be answered is not whether ozone can damage plants, but is rather at which concentration, and for how long, it can safely be detoxified by plants.

The main port of entry of ozone into leaves are the stomata. Uptake through the cuticle is less than about 0.01% of that through open stomata (Kerstiens and Lendzian 1989). Once ozone has entered the intercellular space, it is rapidly absorbed by the surrounding tissue so that its concentration in the air space inside the leaves is close to zero (Laisk et al. 1989). Uptake is driven by the concentration gradient between ozone in the surrounding air and in the intercellular space. Once dissolved inside the leaf, ozone reacts with oxidizable components first of the apoplast and then, if not intercepted in the apoplast, of the plasmalemma and the cytoplasm. Alternatively, it forms oxygen radicals which are similarly reactive as ozone itself (Heath 1987).

Previous work has revealed that apoplastic ascorbate acts as a detoxifying agent (Castillo and Greppin 1988, Polle et al. 1990, Foyer et al. 1994) either by intercepting ozone directly or by reducing other solutes such as apoplastic phenolic acids or membrane-localized tocopherol, which had been oxidized by ozone. However, ascorbate prevents oxidation of phenolics by cell wall bound peroxidases in vivo only to some extent (Córdoba-Pedregosa et al. 1996). Ascorbate is regenerated by reduction of its oxidation products presumably inside the cytoplasm (Luwe et al. 1993, Luwe and Heber 1995, Rautenkranz et al. 1994, Foyer and Lelandais 1995), but evidence has also been presented of reduction via membrane-bound Cyt *b* at the outer side of the plasma membrane (Horemans et al. 1994, Asard et al. 1995). There is the question of how effective apoplastic ascorbate is in intercepting ozone before it can damage the plasmalemma or cytoplasmic components. The rate constant of the reaction of ascorbate with ozone is $4.8 \times 10^7\ M^{-1}\ s^{-1}$ at pH 6 to 7 (Kanofsky and Sima 1995).

We have introduced an oxidation-sensitive fluorescent

Abbreviations: D283, 3,3′-diethylthiacarbocyanine iodide; D291, 4-(4-didecylamino-styryl)-*N*-methylpyridinium iodide; D494, 3,3′-dihexadecylthiacarbocyanine perchlorate.

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dye into the apoplast, or cell wall region, of leaves and measured its oxidation by ozone in the absence and the presence of ascorbate. Decreasing dye fluorescence was plotted against time or against ozone uptake into the leaves, and monoexponential decay constants were calculated. We report the surprising result, that ascorbate failed to protect the dye against oxidation by ambient or above ambient concentrations of ozone in air as they are measured in Europe (often 30 to 50 nl per liter or 60 to 100 $\mu g\ m^{-3}$, but also up to 200 nl per liter or 400 $\mu g\ m^{-3}$, Nantke and Lindemann 1990) or in the United States (up to 400 nl per liter, Smith 1991). We also examined the two-dimensional distribution of the oxidizing events by using a leaf chamber mounted on a microscope and a chilled CCD-Camera. Calculation of dye fluorescence decay kinetics showed an heterogeneous distribution pattern which might correlate with stomatal patchiness (Laisk 1983, Terashima 1992, Siebke and Weis 1995) and, finally, with necrotic spots.

Materials and Methods

Preparation of leaves—Spinach (*Spinacia oleracea* L. cv. Polka) was grown in a greenhouse. Freshly cut leaves (0.3 to 0.8 g in fresh weight) were used for infiltration experiments with the oxidation-sensitive fluorescent dyes 3,3′-dihexadecyl-thiacarbocyanine perchlorate (D494), 3,3′-diethylthiacarbocyanine iodide (D283) and 4-(4-didecylaminostyryl)-*N*-methylpyridinium iodide (D291), which were obtained from Molecular Probes Inc., Eugene, Oregon, U.S.A. Leaves were placed inside a 60-ml syringe. They were then covered with a solution (35 ml), which contained 10 mM KCl, 3 to 10 μM dye (diluted from stock solution of 1 mM dye in DMSO) and different concentrations of ascorbate. Vacuum was applied three times by pulling and releasing the piston of the syringe for several seconds. While under vacuum, the syringe was shaken to release bubbles from the leaf surface. Releasing the vacuum filled the intercellular air space with the solution. After infiltration, the leaves were taken out and rinsed with water. They were then rolled, inserted into tubes and centrifuged for 10 min at 280 g to remove infiltration medium from the intercellular space. Recovered infiltration medium was colorless. After centrifugation, the petiole of the leaves was cut under water to facilitate water transport in the vascular system.

Measurements of gas exchange and dye fluorescence—In fumigation experiments with ozone, gas exchange, light scattering and fluorescence of the leaves were recorded simultaneously in a sandwich-type leaf cuvette (area 4.15 cm^2), which was connected through fiber optics to different light sources and detector systems. During the measurements the leaves were supplied with water through the petiole. Neutral ascorbate solution was prepared from sodium ascorbate. For feeding the ascorbate to the leaves, the water was replaced by ascorbate solution. Controls were fed with 10 mM KCl instead of ascorbate.

Fluorescence was excited by a weak beam of 540 nm (D494 and D283) or 485 nm light (D291) and measured at 577 nm by a photomultiplier via fiber optics (about 1 cm diameter). Fluorescence signals were detected both in transmission and reflection (angle about 15 degrees between excitation and emission fiber). Both methods yielded similar results but measurement of reflected light showed a better signal to noise ratio. Light scattering was measured by another photomultiplier as a change in transmission of 540 nm light through the leaf (Heber 1969). Photosynthesis was driven by red actinic light which did not penetrate the filters used to protect the photomultipliers. Filter combinations were Calflex C (Balzers, Liechtenstein) and RG 645 or RG 630 (Schott, Mainz, Germany) for the actinic light source and Calflex C, BG 18 (Schott), 9782 (Corning Glass Works, Corning, N.Y., U.S.A.) and IF 540 (Schott, half bandwidth 13.7 nm, for D494) or IF 485 (Schott, half bandwidth 10.6 nm, for D291) for fluorescence excitation. The photomultipliers were protected by a K 60 (Balzers) and two 577 nm interference filters (Sigma and Schott, half bandwidth 12.4 nm) for fluorescence detection, and by Calflex C, BG 18 (Schott), 9782 (Corning) and IF 540 (Schott) for transmission. The photon flux density of actinic light varied between 300 and 700 $\mu mol\ m^{-2}\ s^{-1}$. Fluorescence spectra were measured with an Aminco SPF-500 (American Instrument Company, Silver Springs, MD., U.S.A.).

Gas composition—Gas flow through the cuvette was regulated by Tylan or Brooks flow controllers and mass flow meters (Tylan Corporation, Eching, Germany; Brooks Instruments, B.V., Veenendaal, The Netherlands). Ozone was generated by the Ozomat COM (Anseros GmbH., Tübingen, Germany) from pure oxygen and measured prior to entry into the cuvette by the Ozomat Analysator MP. Usual concentrations varied between 200 and 800 $\mu g\ m^{-3}$ ($\cong$100 and 400 nl per liter). The rate of gas flow was between 440 and 500 ml min^{-1}. Air humidity was 60% and the CO_2 concentration 350 μl per liter. Absorption of ozone by the empty cuvette was about 5% of the ozone concentration. Transpiration and assimilation were measured by a H_2O/CO_2 analyzer (Li 6262; Licor Instruments, Lincoln, Nebraska, U.S.A.).

Imaging—For microscopical analysis, experiments were performed using a smaller water cooled sandwich type leaf cuvette (1.5 cm^2) which was mounted on an Zeiss inverse microscope (IM 35, Zeiss, Frankfurt, Germany). The cuvette was connected to the gas exchange system which has been described above. Gas flow was reduced to about 110 ml min^{-1}. Weak actinic light (30 to 80 $\mu mol\ m^{-2}\ s^{-1}$) was provided by the internal cone of an Xe-PAM unit (Walz, Effeltrich, Germany) with an additional RG 630 filter (Schott, Mainz, Germany) via fiber optics to the upper surface of the leaf. Fluorescence was excited via microscope optics by a stabilized mercury short arc DC lamp (Osram HBO 50W/3) powered by the L.O.T.-Oriel arc lamp power supply model 68806 (L.O.T.-Oriel GmbH, Darmstadt, Germany). The intensity was regulated by additional neutral gray filters. With the Zeiss Plan-Neofluar objective (1.25 × /0,035) the photon flux density was 225 $\mu mol\ m^{-2}\ s^{-1}$ over the whole cuvette area, when neutral gray filters were absent. The microscope was equipped with the filter combination H546 (Zeiss) and an additional special DT-Cyanfilter ($\lambda = 613$ nm; Balzers) in the emission beam to suppress Chl fluorescence. This filter combination has a transmission maximum at 603 nm with a half bandwidth of 28.5 nm for the fluorescence emission. For fluorescence detection we used a chilled integrating monochrome CCD-camera (Kappa CF 8/1DX, Kappa, Gleichen, Germany) which allowed adjustment of digitizing thresholds. It was mounted on a tube of the binocular. In addition, a photomultiplier was used without filters which was mounted on the opening of the binocular with removed eyepiece. The adjustment of the camera was done in such a way that transmitted actinic light, that passed through the filter set, formed a low background, which was taken as a separate image. For the calculation of monoexponential fluorescence decay it became necessary to know the full amplitude of the fluorescence signal, because totally bleached dye must not be below the threshold sensitivity of the camera. Part of the signal above background was due to scattered excita-

tion light or background fluorescence unrelated to dye fluorescence as shown by control leaves, which had not been infiltrated with dye. To avoid errors due to unspecific signals, we usually used moderate ozone concentrations (200 to 350 nl per liter) for fumigation up to 30 min. Then a high concentration of ozone (5,000 nl per liter) was applied for about 30 to 40 min. During this time all accessible dye was oxidized. Only a small fraction (about 20%) remained inaccessible for ozone. This image was taken as background for calculation. Integration time was about 40 s for this weak excitation. Images were usually recorded every 2 min. After recording of an image, the excitation beam was turned off for 70 s to avoid unnecessary dye bleaching by the excitation light. Dye bleaching was measured for each leaf prior to fumigation experiments under otherwise identical conditions for about 15 to 20 min. Interference of cuticle-bound dye was reduced by applying excitation light for about 5 min before bleaching experiments. This time was also used to adjust focus and camera settings. First order decay constants for light dependent dye bleaching and for fumigation experiments were calculated by a simplex algorithm using the background image as offset. Calculated decay constants were presented as a set of pseudocolored images.

Measurements of apoplastic ascorbate—Intercellular washing fluid for the determination of apoplastic ascorbate was obtained by the infiltration and centrifugation procedures of Takahama and Oniki (1992) and Luwe et al. (1993). The redox state of ascorbate was measured spectrophotometrically (Takahama and Oniki 1992). Protoplasts were isolated from spinach leaves according to Brune et al. (1994). For fluorescence microscopy the inverse microscope (IM 35, Zeiss, Frankfurt, Germany) was used with the filter combination H546.

Calculation of rate constants in solution—Measurements of rate constants of the second order reaction of ozone with a dye possessing the same chromophore as D494 were made in solution using the method of Kanofsky and Sima (1995). Like D494, this dye, D283 (3,3′-diethylthiacarbocyanine iodide), was obtained from Molecular Probes. Because of its shorter lipophilic side chain (C_2) it was more soluble in aqueous solution and therefore more suited for the measurements than D494 (C_{16}). The reaction of ozone with potassium indigotrisulfonate in 10 mM phosphate (pH 3.8) served as a reference. Its rate constant is known ($k = 10^7$ $M^{-1} s^{-1}$; Bader and Hoigné 1981). Dimethylsulfoxide, which was introduced from stock solution together with D283 into the reaction medium, did not noticeably interfere with the oxidation of indigotrisulfonate by ozone at the concentrations used.

Results

Distribution of the dyes in Spinach laeves as revealed by fluorescence microscopy—The dyes D494 and D291 are, owing to their aliphatic side chains, amphiphilic. They have been used as markers for isolated membranes or artificial phospholipid bilayers (Barni and Savarino 1983, Huang and Haugland 1991). Whereas the side chains enter lipid matrices, the oxidation-sensitive cationic head of the dyes is oriented towards polar phases. After dye was introduced into the apoplast of leaves, fluorescence microscopy of leaf sections revealed fluorescence emission particularly from the stomata and cell walls (Fig. 1, 2). Cuticles also appeared stained as shown in the cross section of a leaf in Fig. 1. The lower part of Fig. 1 shows fluorescence intensity along a transection which is shown as a line in the upper part of Fig. 1. The main part of fluorescence originated from the leaf interior as shown by the area under the fluorescence curve. Less than 15% of the total fluorescence was emitted from the cuticle. Bleaching of the dye by high intensity 540 nm light, usually 225 μmol m^2 s^{-1}, but, with different focussing, up to 2,000 μmol m^2 s^{-1}, for several min prior to fumigation with ozone removed much of the fluorescence coming from the cuticle. Dye inside the leaf was much less sensitive to bleaching than dye absorbed to the cuticle and was measured after bleaching of external dye. In addition to the measuring light, the leaf was illuminated with red actinic light (RG 645 or RG 630).

There is the question of the location of dye inside the leaf. Protoplasts isolated from leaves which had been in-

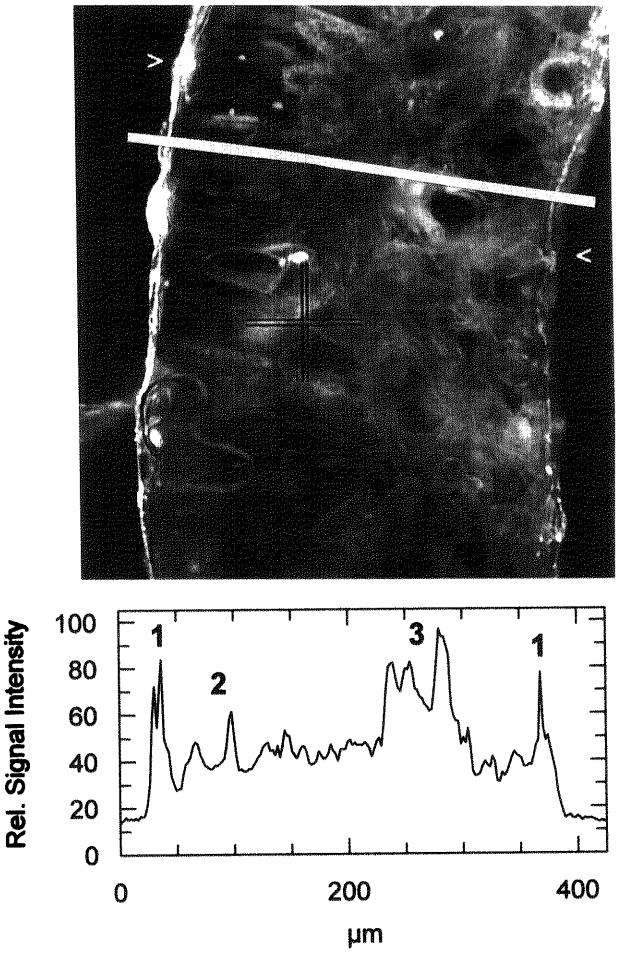

Fig. 1 Fluorescence of a cross section of a spinach leaf after introduction of D494. For infiltration, a solution of 10 μM D494 was used. Excitation by a beam of 540 nm light using a Zeiss IM 35 Microscope with the filter combination H546. Chl fluorescence was largely suppressed by a Dicyan filter of Balzers, Liechtenstein. Stomata are indicated by arrows. The lower part of the figure shows the fluorescence profile along the marked path. (1) Epidermis; (2) palisade parenchyme; (3) sponge parenchyme. Upper epidermis on left side of cross section.

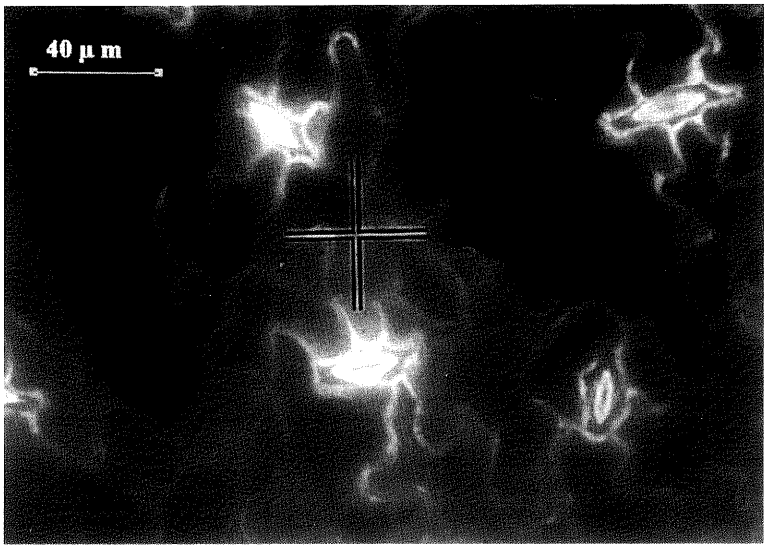

Fig. 2 Fluorescence emission from the lower surface of a spinach leaf infiltrated with 5 μM D283. Note the brightly fluorescent stomata. Measuring conditions as in Fig. 1.

filtrated with D494 were not fluorescent indicating that the dye had not been taken up into the cells. No fluorescence could be observed originating from their outer membranes. When incubated in dye solution, the protoplasts failed to become fluorescent. Very probably, the dye which had been infiltrated into the leaves was mainly absorbed to cell wall structures.

Irreversible quenching of dye fluorescence by ozone—Fig. 3 shows simultaneous recordings of D494 fluorescence emitted by a spinach leaf at 577 nm and of light scattering by the same leaf. Scattering was measured by the decrease in the transmission of a 540 nm measuring beam. Illumination of the leaf increased light scattering indicating energization of the thylakoid system (Heber 1969). Fluorescence decreased with identical kinetics as light scattering increased because of weakened fluorescence excitation. After light scattering and fluorescence had stabilized, 220 nl per liter ozone were added to the air stream which passed over the leaf. The response of light scattering to fumigation with ozone was negligible, but D494 fluorescence decreased rapidly. Increasing the ozone concentration decreased the fluorescence further until it was largely quenched. Quenching of dye fluorescence was irreversible. Full quenching was not observed, apparently because about 20% of the dye was inaccessible to ozone. No shift in emission wavelength could be observed due to binding of dye to cellular binding sites (data not shown).

Fluorescence quenching of D494, D283 and D291 indicates dye oxidation by ozone—Observations similar in principle to those made with D494 were also made with D283 (see Fig. 5) and D291 (data not shown). However, as the fluorescence of D291 and D283 was by about a factor of 10 less sensitive to ozone than D494, higher concentrations of ozone had to be used to obtain comparable fluorescence quenching. Also, during the first minute of fumigation, D291 fluorescence increased slightly before quenching became extensive. Because D291 has the ability of forming nonfluorescent aggregates (Haugland 1992), this might be due to reduced self-quenching by dimers or complexes which dissociate and become fluorescent as they are partly oxidized. This complication makes the thiocarbocyanine dyes D494 and D283 better suited to monitor effects of ozone on leaf tissue than the aminostyryl-dye D291.

Dihydrorhodamine 6G (Molecular Probes Inc.) is a nonfluorescent dye which is known as a marker for oxygen radicals or hydrogen peroxide in living cells (Haugland 1992). By oxidation, it is converted to a fluorescent dye. After dihydrorhodamine 6G was introduced into leaves, fluorescence was increased during fumigation with ozone (data not shown). From this, we conclude that quenching of the fluorescence of D494, D283 and D291 indicates dye oxidation rather than another reaction.

Oxidation of dye during ozone influx into a leaf—Influx of ozone through open stomata obeys the flux equation

$$\Phi = (c_o - c_i)/R,$$

where Φ is the rate of flux in nmol cm^{-2} s^{-1}, c_o and c_i are the concentrations of ozone in air (c_o) and in the intercellular space (c_i) in nmol cm^{-3} and R includes the boundary layer and stomatal resistances in s cm^{-1}. Since c_i can be taken to be zero (Laisk et al. 1989) and c_o is known, only R is missing for a calculation of flux. It has been obtained from measured transpiration considering the different diffu-

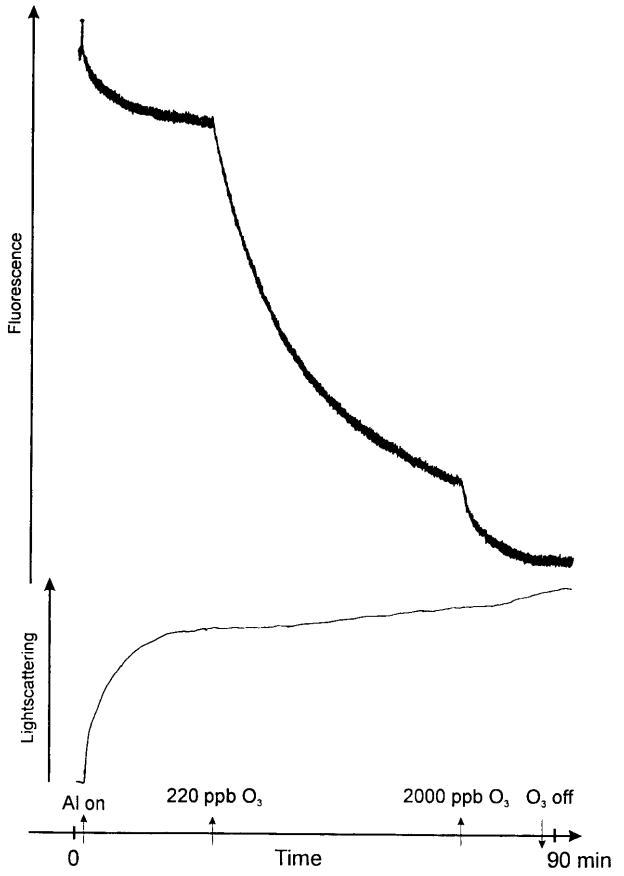

Fig. 3 Simultaneous recording of scattering of 540 nm light by a spinach leaf and of D494 fluorescence emission from the leaf as affected by ozone. The leaf contained D494 (infiltration with a solution containing 3.6 μM D494). Fluorescence of D494 was excited by the same 540 nm beam which also served to record light scattering. Fluorescence detection at 577 nm. (AL) indicates illumination with red actinic light (600 μmol m^{-2} s^{-1}). Fumigation first with 220 nl per liter and then about 2,000 nl per liter ozone in air as indicated.

sion coefficients of H_2O and O_3 (Nobel 1983). From Φ and known exposure times, amounts of ozone taken up by a leaf can be calculated and compared to quenching of dye fluorescence. When fluorescence quenching was plotted against uptake of ozone, the curve showed a largely monoexponential decline of dye fluorescence (Fig. 4). Complicated kinetics were absent indicating that oxidation of dye absorbed by the cuticle was a minor contribution to the total loss of fluorescence which was dominated by the oxidation of dye introduced into the apoplastic compartment of the leaf interior. This was confirmed by a set of experiments in which the cuticle was stained by floating the spinach leaf in the dye solution in the 60-ml syringe for 1 min without application of vacuum. These leaves were much less, by a factor of about 10, fluorescent than leaves which had been infiltrated with dye solution. Also, when in-

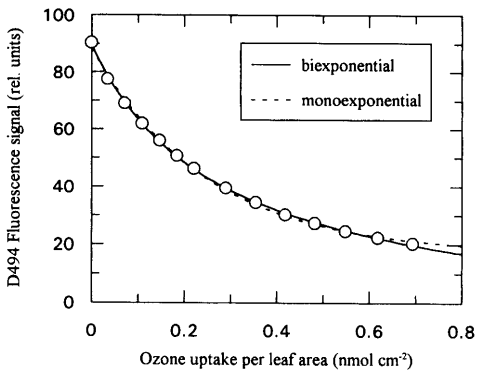

Fig. 4 Loss of D494 fluorescence as observed in the experiment of Fig. 3 versus calculated ozone influx into the leaf during fumigation with 220 nl per liter O_3. The data points were fitted by a monoexponential and a biexponential fit (both with offset). From the rate constant of the monoexponential fit the amount of ozone necessary to reduce the the fluorescence signal by 2/3 was calculated to be 0.24 nmol cm^{-2}.

filtrated leaves, whose stomata had been closed by feeding of 0.1 mM ABA via the petiole, where fumigated with ozone, there was almost no ozone-dependent decay of dye fluorescence (Fig. 5). This shows that the ozone-dependent signal had its main origin inside the leaf.

Dye oxidation consumes only part of the ozone entering leaves—The fresh weight of spinach leaves grown in the greenhouse was about 25 mg cm^{-2}. Infiltration of spinach leaves with a dye-containing solution increased weight by

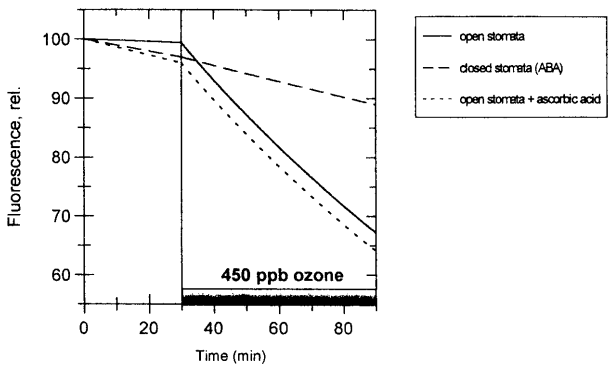

Fig. 5 Loss of D283 fluorescence from spinach leaves before and after fumigation with ozone (about 450 nl per liter) under different conditions. Drawn line: leaf with open stomata. Dotted line (long dots): leaf, whose stomata had been closed by feeding 100 μM ABA via the petiole before starting the experiment. Dotted line (short dots): leaf with open stomata, which had been fed 10 mM of ascorbate via the petiole for 1 h before starting the experiment. Feeding of ABA or ascorbate was continued during the experiment. Each line represents the mean of two independent experiments. The difference in the slopes of the lines before starting fumigation with ozone is due to differences in bleaching.

about 50 to 60%. This corresponds to the uptake of solution into the leaves of about 12.5 to 15 μl cm^{-2} leaf area. The infiltration medium released from the leaves during centrifugation was colorless. Thus, all dye remained absorbed in the leaf interior. In the experiment of Fig. 3, the dye concentration in the infiltration medium was 3.6 μM. The amount of dye available for oxidation by ozone was therefore about 0.05 nmol cm^{-2}. A comparison of ozone influx and dye oxidation (Fig. 4) shows that only part of the ozone entering into the leaf (about 20% in the beginning, totally less than 10%) was consumed in the oxidation of dye. The greater part was degraded in competitive reactions. Observations very similar to those made with D494 were also made with D291 and D283.

Dye oxidation as a function of ozone concentration in air—A spinach leaf was first exposed to 55 nl per liter ozone, and than to 125 nl per liter ozone for 5 min each, and finally to 290 nl per liter ozone. The additions caused immediate increases in the rate of fluorescence quenching, similar to that shown in Fig. 3. To account for the monoexponential loss of fluorescence during the experiment, declining slopes were corrected so as to give initial rates of fluorescence quenching. Fig. 6 shows that the corrected initial rates indicated dye oxidation which was almost proportional to the concentration of ozone in air. A similar result was obtained, when rates of fluorescence quenching were plotted against ozone uptake with leaves in different experiments. However, scattering of data was larger in this case.

Distribution pattern of dye oxidation—The monoexponential decay of dye fluorescence under the influence of ozone shown in Fig. 4 does not necessarily indicate a homogeneous reaction, but may be the resultant of several reactions. In Fig. 7A dye fluorescence is shown from the lower side of a leaf segment with the dimensions 5 mm × 3.7 mm. Image analysis was performed of dye bleaching from this segment first under the influence of measuring light only (Fig. 7B). Pseudocolors show the distribution of monoexponential decay constants of fluorescence. Different colors indicate heterogeneity of reactions. No large differences in reaction constants are apparent in this control experiment, and observed rates were low compaired with those in the fumigation experiment (Fig. 7C). In this case, extreme heterogeneity became apparent when the reaction with ozone was measured. It should be noted that the color pattern shown in Fig. 7B and C indicates different decay constants, not different signal amplitudes. The extremes of individual life times were 33 h for black (rel. rate = 1) and 7.8 min for the red color (rel. rate = 255) in Fig. 7B and C.

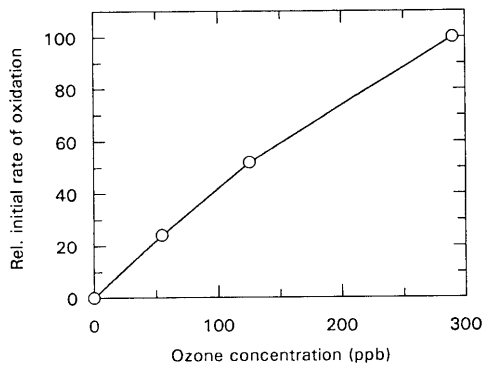

Fig. 6 Rate of loss of D494 fluorescence emitted from a spinach leaf versus ozone concentration in air. For calculation of the data from a spinach leaf experiment with increasing concentrations of ozone, see text. The leaf had been infiltrated with a solution containing 10 μM D494.

Photosynthesis during fumigation with ozone—Within periods of fumigation with ozone (up to 400 ppb) of 30 to 60 min, photosynthesis remained essentially unaffected. This was true both for light-saturated and light-limited photosynthesis. The small decline in light-saturated photosynthesis occasionally observed during prolonged fumigation at elevated ozone levels could be accounted for by stomatal closure (as indicated by decreased transpiratory water loss from the leaves), not by ozone-dependent damage to the photosynthetic apparatus.

Failure to observe appreciable protection by ascorbate of dye oxidation by ozone in the leaf apoplast—Apoplastic ascorbate has been proposed to be an efficient reductant of ozone (Castillo and Greppin 1988, Chameides 1989, Polle et al. 1990, Foyer et al. 1994). Immediately after cutting leaves from spinach plants, apoplastic ascorbate was largely in the reduced state (Luwe et al. 1993, Luwe and Heber 1995). In the light, slow oxidation of this ascorbate was observed in detached leaves within a few hours even in the absence of fumigation with ozone (Takahama and Oniki 1992). However, feeding ascorbate solution to detached leaves through the petiole maintained an appreciable level of ascorbate in the apoplast while dehydroascorbate accumulated slowly. After feeding was discontinued, reduced ascorbate was slowly oxidized. Detached leaves that were infiltrated with dye solution showed a similar uptake of ascorbate into the apoplast as control leaves when they were fed through the petiole. Fumigation with ozone of a spinach leaf which received ascorbate (10 mM) through the petiole for 16 min failed to decrease dye oxidation significantly (Fig. 8). The inset of Fig. 8 shows a control experiment with the hydrophilic fluorescent dye pyranine which was fed to a leaf through the petiole at a concentration of 500 μM. Pyranine fluorescence from the leaf lamina was observed within 1 min after beginning of feeding (time zero in the inset) indicating that the transpiration stream was capable of transporting the hydrophilic solute rapidly into the leaf. Similar transport would be expected for ascorbate. Even after fumigation experiments with 300 ppb ozone for 40 min, and simultaneous feeding of 10 mM ascorbate through the petiole, we still measured between 1

to 2 mM ascorbate in the apoplast of the fumigated area of the leaf.

When dye was introduced into the leaf by infiltration together with 10 mM ascorbate, fumigation after removal of the infiltration solution by centrifugation also failed to reveal appreciable protection of the dye against oxidation (Fig. 5) even though the leaf was simultaneously fed with 10 mM ascorbate for at least 1 h before and during measurement.

Protection by ascorbate of dye oxidation by ozone in aqueous solution—The apparent failure of ascorbate to protect oxidation-sensitive dyes, which had been introduced into the apoplast of leaves, against oxidation by ozone was unexpected. Attempts were therefore made to determine the rate constant of the reaction between dye and ozone. For this, the method of Kanofsky and Sima (1995) was used. Air containing ozone (about 37 ppm) was bubbled through a porous membrane (to reduce bubble size) into 10 mM phosphate buffer (pH 3.8) which contained 18 μM potassium indigotrisulfonate with and without different concentrations (between 1 and 100 μM) of D283, which contains the same chromophore as D494 but is more soluble in aqueous media. Oxidation of indigotrisulfonate was monitored at 600 nm. In the presence of D283, indigotrisulfonate was less rapidly oxidized by ozone than in its absence. The extent of protection of indigotrisulfonate oxidation by D283 suggested a rate constant of the reaction between ozone and D283 which is larger by a factor of about 2 than the rate constant of the reaction of indigotrisulfonate with ozone (which is about $10^7\ M^{-1}\ s^{-1}$). The rate constant of the reaction of ascorbate with ozone in neutral or slightly acidic solution has been reported to be $4.8 \times 10^7\ M^{-1}\ s^{-1}$ (Kanofsky and Sima 1995). Thus, rate constants of the reactions between ozone and either D283 or ascorbate as shown by this approach do not appear to be very different.

Attempts were also made to measure more directly protective effects of ascorbate on dye oxidation in aqueous solution. Instead of acidic phosphate buffer, different concentrations of ascorbate in 10 mM phosphate (pH 6.2) were used and oxidation of D283 (25 μM; but it should be noted that the molecular structure of the dye suggests at least two reaction sites per molecule) was measured as a change in 511 nm absorption. Corrections had to be made for dye oxidation by oxygen in the absence of ozone. Ascorbate at 250 μM decreased dye oxidation by ozone by about 35 to 40% and 1 mM ascorbate by 70 to 85%. This approach sug-

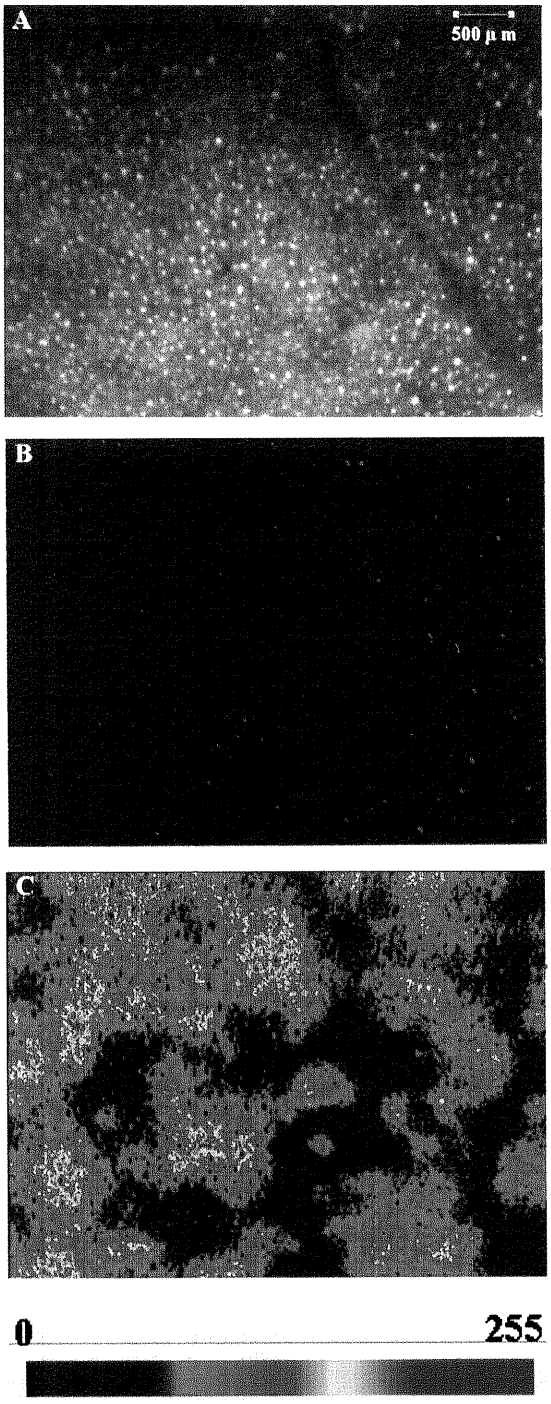

Fig. 7 Distribution of the dye fluorescence (A) and rate constants of the loss of fluorescence under the influence of measuring light only (B) or of ozone (C). Measurement were performed by a new microscope-based imaging system (description see Materials and Methods). Pictures were taken every 2 min with an integration time of 39.6 s. No additional red actinic light was provided. Stomatal resistance was about 13 s cm^{-1} for water vapor loss. (A) Fluorescence signal of a spinach leaf which had been infiltrated with a solution containing 5 μM D494 after bleaching of cuticle bound dye. (B) Calculated monoexponential decay constants for light dependent dye bleaching under measurement conditions ($PFD_{540\ nm} = 225\ \mu mol\ m^{-2}\ s^{-1}$). (C) Calculated monoexponential decay constants for dye bleaching in the presence of 250 ppb O_3. For (B) and (C), pictures were stained with pseudocolors according to relative decay constants (black = 1; red = 255) which correspond to apparent lifetimes between 33 h (black) and 7.8 min (red). The size of the leaf section is 5 mm × 3.7 mm.

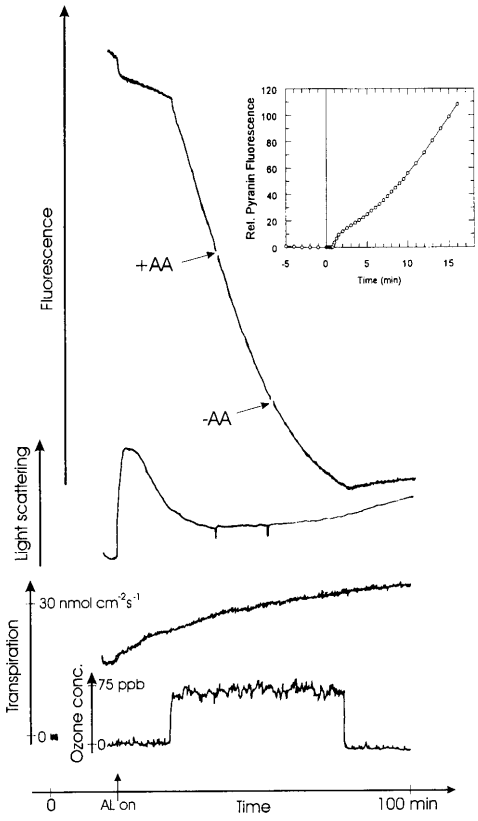

Fig. 8 Simultaneous recording of D494 fluorescence, light scattering and transpiratory water loss during exposure of a spinach leaf to 65 ppb ozone in air. The leaf had been infiltrated with a solution containing 10 μM D494. Its petiole was initially in water. After 15 minutes exposure of the leaf to ozone, water was exchanged for 10 mM ascorbate (+AA), so that ascorbate could be transported with the transpirational stream to the leaf blade. After 16 minutes in ascorbate solution, water replaced the ascorbate solution while fumigation was continued (−AA). (AL) indicates illumination with red actinic light (600 μmol m^{-2} s^{-1}). The inset shows the uptake of 500 μM pyranine in 10 mM KCl through the petiole of a detached spinach leaf as revealed by pyranine fluorescence from the leaf lamina. The optical arrangement was identical to that which was used to record D494 fluorescence. Pyranine fluorescence was excited using a UG11 filter of Schott and detected at 509 nm.

gests that the reaction of ozone with dye may actually be one order of magnitude faster than the reaction with ascorbate.

Discussion

The introduction of oxidation-sensitive fluorescent dyes such as D494, D283 or D291 into the apoplast of leaves opens the way to assess by fluorescence measurements oxidative stresses acting on the cell walls of the leaf interior. D494 does not penetrate biomembranes. It is a highly sensitive probe. For instance, ozone-induced fluorescence loss can be reliably measured at ozone concentrations even below 30 nl per liter (60 μg m^{-3}). After bleaching of dye to eliminate the cuticular contribution to fluorescence, 60 to 100 nl per liter ozone are required for reliable measurements of fluorescence quenching in the leaf interior with D494 and more than 300 nl per liter for the dyes D283 and D291. For comparison, 60 nl per liter were exceeded at the measuring station Schauinsland in the Black Forest in Germany during 141 d in 1991, and 90 nl per liter during 30 d (2 h averages; UBA 1994). Because D494 and D283 have the same chemical structure of their reactive chromophore, differences in sensitivity must be due to different distribution in the leaf. This needs to be worked out in further experiments.

The experiments described in the present communication touch the question of the effectiveness of apoplastic ascorbate in providing antioxidative protection to leaf tissue outside the cytoplasm. In the present work, 1 mM ascorbate was capable of decreasing the oxidation of micromolar concentrations of oxidation-sensitive dyes by ozone in aqueous solution, but not when dye was introduced into the apoplast of leaves. This apparent contradiction needs to be resolved. At the present state of knowledge, the most likely explanation for the failure of apoplastic ascorbate to provide appreciable protection to D494 oxidation by ozone at concentrations in air, which are not higher than ambient concentrations on sunny days, is spatial separation of dye and ascorbate inside the apoplast of the intact leaf. Microscopy has shown the main part of the introduced dye to be localized in the apoplast of cells bordering the substomatal chamber (Fig. 2). This is where entering ozone will first encounter oxidizeable solutes and be degraded by reduction. From our observations, we are forced to conclude that the concentration of ascorbate is lower there than in other parts of the leaf apoplast. Moreover, even the highly reactive dye D494 was not particularly protective when introduced into the apoplast of leaves as shown by the observation that it intercepted only part of the entering ozone (Fig. 4). Flux calculations indicated that the major part of entering ozone was degraded in other unspecified reactions.

Even in earlier work with spinach, where calculated ozone uptake was compared with oxidation of apoplastic ascorbate (Luwe et al. 1993), it had become apparent that only about 5 to 10% of the entering ozone was detoxified by ascorbate. This calculation was based on observations that transport of oxidized ascorbate across the plasmalemma into the cytoplasm of leaf cells and import of regenerated ascorbate back into the apoplast is slow. Actually, necrosis and chlorophyll bleaching were observed in leaves after fumigation of young beech plants with 100 to 150 nl per liter ozone for 4 to 6 weeks, although the leaves had maintained their apoplastic ascorbate largely in the reduced state (Luwe and Heber 1995). Apparently, apoplas-

tic ascorbate had not been effective enough to prevent damage to the leaf tissue. In this context, results from our new microscope based measuring system (Fig. 7) might give some explanation. Apparently there are areas were fast oxidation occurs whereas other areas are more or less unaffected. The patchiness pattern shown in Fig. 7C is reminiscent of the patchiness of Chl fluorescence (Siebke and Weis 1995) in leaves of several plant species. This patchiness is interpreted as resulting from heterogeneous stomatal opening (Laisk 1983, Terashima 1992). It suggests that oxidative stress by ozone is a localized phenomenon. Where stomata are widely open, ozone can enter rapidly. As the data of Fig. 7C show, it is degraded locally, presumably in the vicinity of its points of entrance. One may speculate if there is a relationship to the well-known phenomenon of appearance of necrotic spots after long term ozone treatment. Measurements of apoplastic ascorbate integrate over the whole apoplast. Horemans et al. (1994) and Asard et al. (1995) have proposed that oxidized ascorbate is rapidly reduced via Cyt *b* of the plasmalemma. In contrast, persistent oxidation of apoplastic ascorbate was observed in leaves of spinach and broad bean even after termination of fumigation with 150 nl ozone per liter (Luwe et al. 1993). This observation argues against fast regeneration of ascorbate at least in these plant species. Published rate constants of the reaction of ozone with other cellular reductants (cysteine 4.4 10^6; glutathione 2.5 $10^6\ M^{-1}\ s^{-1}$; Kanofsky and Sima 1995) indicate that the reaction of ozone with ascorbate is about 10 to 20 times faster than the reactions with cysteine and glutathione. Moreover, neither cysteine nor glutathione are constituents of the apoplast. Although there is little doubt that ascorbate occupies a key position in the antioxidative defense of cells, our observations show that it is not as effective as expected in preventing the unspecific oxidation of other cellular constituents by ozone. If, as the experiments clearly show, ozone cannot be efficiently detoxified by ascorbate in a fast and specific reaction in the apoplast of leaves, the main burden of antioxidative defense must be borne by apoplastic components different from ascorbate. Some preliminary experiments show that there might be a protection of D491 by α-tocopherol. Also, cellular repair reactions may need to receive increased attention if oxidative damage should really prove to be unavoidable. It is most remarkable, in this context, that we failed to observe decreased photosynthesis of spinach leaves while dye oxidation in the apoplast was going on at an ozone concentration in air as low as 65 ppb (Fig. 8), i.e. at a concentration which is not only reached but often exceeded during sunny days in summer.

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