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Role of Ascorbate in Detoxifying Ozone in the Apoplast of Spinach (*Spinacia oleracea* L.) Leaves[1]

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Both reduced and oxidized ascorbate (AA and DHA) are present in the aqueous phase of the extracellular space, the apoplast, of spinach (*Spinacia oleracea* L.) leaves. Fumigation with 0.3 μL L^{-1} of ozone resulted in ozone uptake by the leaves close to 0.9 pmol cm^{-2} of leaf surface area s^{-1}. Apoplastic AA was slowly oxidized by ozone. The initial decrease of apoplastic AA was <0.1 pmol cm^{-2} s^{-1}. The apoplastic ratio of AA to (AA + DHA) decreased within 6 h of fumigation from 0.9 to 0.1. Initially, the concentration of (AA + DHA) did not change in the apoplast, but when fumigation was continued, DHA increased and AA remained at a very low constant level. After fumigation was discontinued, DHA decreased very slowly in the apoplast, reaching control level after 70 h. The data show that insufficient AA reached the apoplast from the cytosol to detoxify ozone in the apoplast when the ozone flux into the leaves was 0.9 pmol cm^{-2} s^{-1}. The transport of DHA back into the cytosol was slower than AA transport into the apoplast. No dehydroascorbate reductase activity could be detected in the apoplast of spinach leaves. In contrast to its extracellular redox state, the intracellular redox state of AA did not change appreciably during a 24-h fumigation period. However, intracellular glutathione became slowly oxidized. At the beginning of fumigation, 90% of the total glutathione was reduced. Only 10% was reduced after 24-h exposure of the leaves to 0.3 μL L^{-1} of ozone. Necrotic leaf damage started to become visible when fumigation was extended beyond a 24-h period. A close correlation between the extent of damage, on the one hand, and the AA content and the ascorbate redox state of whole leaves, on the other, was observed after 48 h of fumigation. Only the youngest leaves that contained high ascorbate concentrations did not exhibit necrotic leaf damage after 48 h.

Ozone, a chief constituent of photochemical air pollution, is a strong oxidant. In plants, decreased photosynthesis, foliar injury, reduction in shoot and root growth and in crop yields, and premature senescence are frequently observed ozone effects (Sakaki et al., 1983; Heagle, 1989; Langebartels et al., 1990; Sen Gupta et al., 1991; Ballach et al., 1992). Biochemical and physiological changes can be induced in plants by exposure to high or subacute levels of ozone (Tingey, 1974; Castillo and Greppin, 1986; Heath, 1987; Mehlhorn et al., 1987; Langebartels et al., 1990; Schmidt et al., 1990; Kyburz et al., 1991). Nevertheless, controversy exists as to whether ozone is involved in the forest decline that afflicts wide areas in Europe and Northern America, because it is not clear within which concentration range ozone can be safely detoxified by plants before irreversible damage is done. In polluted air, ozone is commonly found at concentrations of approximately 0.05 μL L^{-1}, with peak concentrations of about 0.2 μL L^{-1} in Germany (Nantke and Lindemann, 1990) and of 0.65 to 0.8 μL L^{-1} in North America (Smith, 1991).

After entering leaves through open stomata, ozone rapidly degrades into hydroxyl radicals and other reactive decomposition products (Grimes et al., 1983). Because stomata are the main barrier to entry, the ozone concentration in the intercellular air space of leaves is not far from zero (Laisk et al., 1989). This permits the simple calculation of ozone fluxes (F) into leaves according to a simplified version of Fick's law

$$F = c/R, \quad (1)$$

where c is the concentration of ozone in air and R is the sum of the boundary layer and stomatal resistances to flux. R can be calculated from transpiration measurements ($R_{O_3} = R_{H_2O} \times 1.6$ [Nobel, 1983]). The aqueous phase of the apoplastic compartment of the leaf is the site at which ozone and its highly toxic reactive intermediates must be detoxified if they are to be prevented from reacting with the plasma membrane and cytoplasmic components.

Recently, it has been shown that ascorbate, a well-known antioxidant in chloroplasts (Foyer and Halliwell, 1976; Halliwell, 1982), is also a constituent of the apoplast. Ascorbate peroxidase, which uses H_2O_2, a degradation product of ozone, as electron donor, has been reported to be present in the apoplast of *Sedum album* leaves (Castillo and Greppin, 1986) but was not found in the apoplast of spruce needles and spinach (*Spinacia oleracea*) leaves (Polle et al., 1990; Takahama et al., 1992). On fumigation with ozone, ascorbate levels were observed to increase in the apoplast (Castillo and Greppin, 1988).

In the present work, we show that AA acts as an ozone scavenger in the apoplast of spinach leaves. However, the transport of AA and DHA across the plasma membrane of leaf cells will be shown to be slower in spinach exposed to 0.3 μL L^{-1} of ozone in air than the influx of ozone into the leaves. The interrelation between ascorbate contents in leaves and the extent of ozone-induced leaf damage will also be discussed.

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Abbreviations: AA, reduced ascorbate; DHA, oxidized ascorbate (=mono- plus di-dehydroascorbate); IWF, intercellular washing fluid.

MATERIALS AND METHODS

Plant Material

Experiments were carried out during the winter months with 5- to 7-week-old spinach plants (*Spinacia oleracea* L. cv hybrid 102, Yates) planted in 12-cm pots containing ordinary garden soil and grown in a greenhouse with additional illumination from halogen lamps (about 160 μE m^{-2} s^{-1}) in 10-h light and 14-h dark cycles. If not mentioned otherwise, only young leaves of a fresh weight between 0.2 and 0.35 g were used.

Exposure of Plants to Ozone

Fumigation experiments with potted plants were carried out in cylindrical Plexiglas chambers (24 cm high, 14 cm diameter). The light intensity was 240 μE m^{-2} s^{-1}. Experiments were carried out at room temperature (about 20°C). The gas flow was 2 L min^{-1}, and the flow velocity was 0.22 cm s^{-1} inside the chamber. The gas inlet was on the top of the chamber, and the outlet was at the same level as the leaves. The ozone concentration in the outgoing gas stream was 0.3 μL L^{-1}. Ozone was produced by an ozone generator (Ozomat COM; Anseros GmbH, Tübingen, Germany). The ozone concentration was monitored by an ozone analyzer (Ozomat MP; Anseros GmbH). Control experiments using ambient air were performed under the same conditions as fumigation experiments.

Preparation of IWF and of Leaf Extracts

Spinach leaves were harvested at different times before and after fumigation with ozone. After the leaves were washed with distilled water, they were vacuum infiltrated (−70 kPa) for 1 min with 50 mL of a 100 mM KCl solution (Takahama and Oniki, 1992). The leaves were blotted, rolled, and placed carefully into a syringe (50 × 10 mm), which was placed over a centrifuge tube. Solution uptake by infiltration was 55 ± 4% (n = 12) of the leaf fresh weight; 0.3 to 0.4 mL if IWF g^{-1} leaf (fresh weight) was obtained after centrifugation at 160g for 5 min at 4°C. IWF was kept on ice for only a short time. It was used immediately after dilution with 0.1 M sodium phosphate (pH 6.8) for the measurement of AA and DHA. For the calculation of apoplastic AA and DHA concentrations from values of AA and DHA measured in the IWF, we assumed the aqueous phase of the apoplast to be 10% of leaf fresh weight (Speer and Kaiser, 1991).

To determine AA, DHA, GSH, and GSSG contents in leaves after IWF isolation, the leaves were homogenized in 1 mL of cold 2% metaphosphoric acid (w/v). The homogenate was centrifuged at 4000g for 10 min (4°C), and the supernatant was used for the determinations.

Dehydroascorbate Reductase Assay

Dehydroascorbate reductase (GSH:dehydroascorbate oxidoreductase, EC 1.8.5.1.) was measured as described by Stahl et al. (1983) with following modifications. The assay contained 50 mM sodium phosphate buffer (pH 6.8), 0.4 mM DHA, 5 mM GSH, and 100 μL of IWF in a final volume of 1 mL. The A_{265} was recorded at 25°C.

Ascorbate and GSH Determination

All assays were performed at 25°C. AA and DHA were measured spectrophotometrically at 265 nm with a Shimadzu UV210 spectrophotometer as described by Takahama and Oniki (1992). The AA assay is based on the complete oxidation of AA to DHA by ascorbate oxidase (EC 1.10.3.3., from *Cucurbita* sp.). DHA was estimated by reducing DHA to AA by DTT. Reaction assays contained of 50 μL of IWF or 20 μL of leaf extract and 750 or 780 μL of 0.1 M sodium phosphate (pH 6.8), respectively. AA oxidation was started by addition of 1 μL of ascorbate oxidase (1 unit μL^{-1}). In a second assay DHA was reduced to AA by adding DTT to a final concentration of 0.1 mM. An extinction coefficient for AA at 265 nm of 14.3 mM^{-1} cm^{-1} was used. Ascorbate concentrations of the intracellular space were calculated by subtraction of the apoplastic ascorbate contents from the whole leaf ascorbate contents under the assumption that the aqueous apoplastic space is 10% of leaf fresh weight (Speer and Kaiser, 1991).

GSH and GSSG were assayed spectrophotometrically using the specific enzyme method described by Brehe and Burch (1976), which was modified as follows. For the estimation of the sum of GSH and GSSG, 0.7 mL of 125 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM NADPH and 6.3 mM EDTA were mixed with 0.1 mL of a solution containing 6 mM 5,5′-dithiobis-(2-nitro)-benzoic acid, 125 mM potassium phosphate, pH 7.5, and 6.3 mM EDTA, and with 0.195 mL of acid leaf extract diluted 10-fold in 0.28 M Na_2HPO_4. The acid extract was diluted just before starting the reaction with 5 μL of GSH reductase (EC 1.6.4.2., type III, from bakers' yeast; 0.1 unit μL^{-1}) to bring the pH to about 7.8. The change in A_{412} was followed for 200 s, and then a calibration was performed by several additions of GSH standard. Control rates in the absence of extract were subtracted. To measure GSSG, 1 mL of the 1:10 diluted acid extract was incubated for at least 1 h at 25°C with 40 μL of 2-vinylpyridine. Then, GSSG was assayed in the same way as described above. GSH contents of the extracts were calculated as the difference between total GSH and GSSG (reported as GSH equivalents). The detection limit for GSH and GSSG was about 20 nmol g^{-1} fresh weight.

Ascorbate oxidase and GSH reductase were obtained from Sigma Chemical Co.

RESULTS

Ascorbate Levels in Spinach Leaves as a Function of Leaf Age

Figure 1A shows that ascorbate contents in spinach leaves of different fresh weights varied fairly widely. Because younger leaves were smaller than older ones, increasing fresh weights corresponded to increasing leaf age. When the data from Figure 1A were expressed on a concentration basis (average AA concentration in the aqueous phase of the leaves), ascorbate levels were, in general, higher in the younger than in the older leaves (Fig. 1B). The redox state of cellular ascorbate did not change with leaf age. AA was always largely reduced in leaves that had not been exposed to ozone. However, in the apoplast of older leaves, AA/(AA + DHA) was often found to be slightly lower than in younger

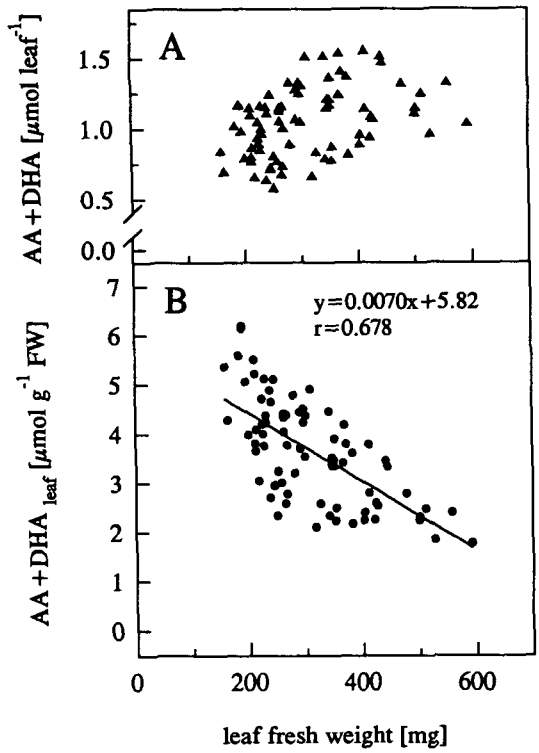

Figure 1. Relationship between the fresh weight (FW) of spinach leaves as an indicator of leaf age and the amount of reduced and oxidized ascorbate (AA + DHA) in the leaves (A) and ascorbate (AA + DHA) concentration (B). Each point represents one measurement.

leaves. For this reason, only leaves weighing between 200 and 350 mg were taken for the analysis of IWF after fumigation of plants with ozone.

Contents and Redox State of Ascorbate in the Apoplast of Spinach Leaves before and after Fumigation with 0.3 μL L^{-1} of Ozone

AA and DHA concentrations in the IWF prepared from spinach control leaves varied widely between 20 and 100 nmol cm^{-3} of AA and 2 and 12 nmol cm^{-3} of DHA. Assuming the aqueous apoplastic space to be 10% of leaf fresh weight (Speer and Kaiser, 1991), we calculated AA concentrations in the apoplast to be 440 ± 210 nmol cm^{-3}, whereas the DHA concentration in spinach apoplasts was 63 ± 18 nmol cm^{-3} (Fig. 2); 87 ± 4% of apoplastic total ascorbate was in the reduced state in control leaves. The average intracellular AA concentration (3.93 ± 0.88 μmol g^{-1} fresh weight) was far higher than the apoplastic AA concentration. Intracellular ascorbate in the reduced form measured 99.8 ± 0.3%.

It will be noted that SD values of ascorbate measurements in the apoplast were usually high. They approached 50% in the apoplast of different individual leaves. For apoplastic ions, similarly large SD values were reported by Speer and Kaiser (1991). However, SDs of determinations of the ascorbate redox state were much lower than those of concentration measurements. AA and DHA were measured in the same extracts. SDs of ascorbate determinations in leaf extracts were reasonably low.

Figure 2A shows the time course of changes of AA and DHA in the apoplast during fumigation with 0.3 μL L^{-1} of ozone for 24 h. During the first 6 h of ozone treatment, AA decreased from 420 to about 50 nmol cm^{-3}. This corresponds to a rate of AA loss in the apoplast of about 60 nmol cm^{-3} h^{-1}. Because 1 cm^3 of apoplast corresponds to about 10 g leaf fresh weight with 375 cm^2 of leaf surface area, the rate of AA loss was, on a unit leaf area basis, 0.045 pmol cm^{-2} s^{-1}.

For comparison, rates of ozone uptake by leaves fumigated with 0.3 μL L^{-1} of ozone were measured. Ozone concentrations were monitored both at the inlet and at the outlet of the fumigation chamber. Uptake rate calculated from the difference and the gas flow rate was corrected for the ozone degradation on the surfaces of tubes, chamber, and pot. Under our experimental conditions an ozone uptake of 119 ± 4 nmol g^{-1} fresh weight h^{-1} (n = 3) was observed. Because 1 g leaf fresh weight corresponds to 37.5 ± 2.4 cm^2 of leaf area (n = 22), the ozone uptake was on a leaf surface area basis 0.88 ± 0.03 pmol cm^{-2} s^{-1}. The sum of the boundary layer and stomatal flux resistances for water, R_{H_2O}, was calculated to be 9.5 s cm^{-1} (Nobel, 1983). The slow gas flow

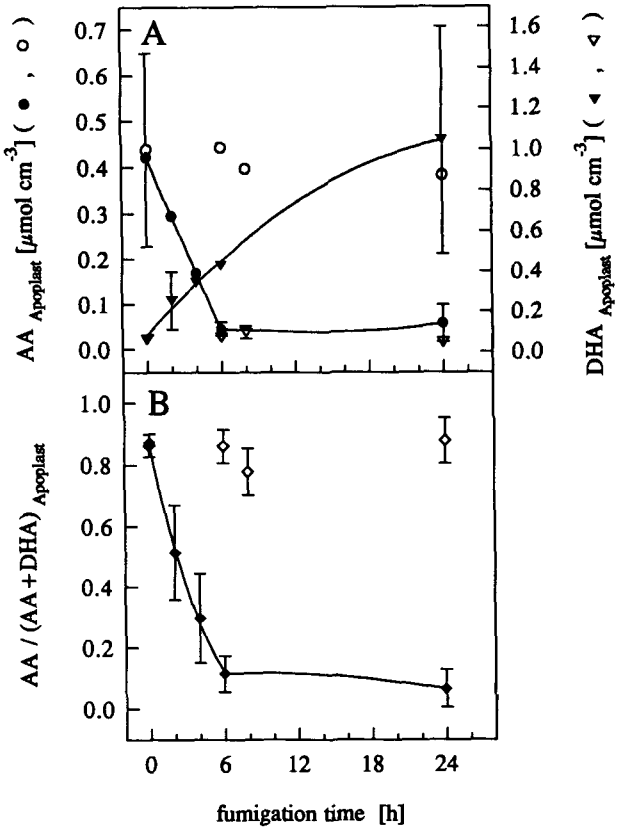

Figure 2. Changes in the contents of AA and DHA (A) and of the redox state of ascorbate [AA/(AA + DHA), B] in the apoplast of leaves during fumigation of spinach plants with 0.3 μL L^{-1} of ozone at room temperature (n = 8–12). Open symbols, Untreated controls; error bars, SD.

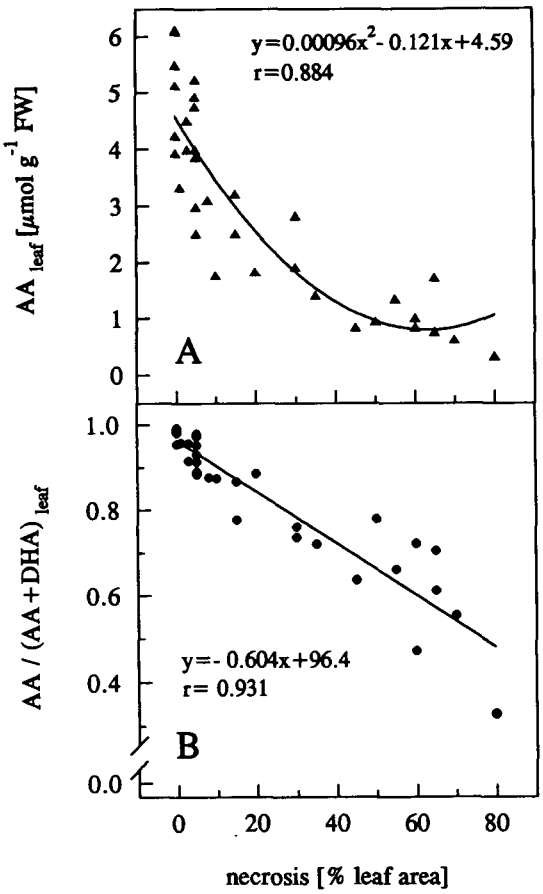

Figure 3. Relationship between leaf damage (percentage of necrotic leaf area, estimated optically) and AA concentration (A) and ascorbate redox state [AA/(AA + DHA), B] in spinach leaves. Spinach plants had been fumigated for 48 h with 0.3 μL L^{-1} of ozone at room temperature. Each point represents one measurement.

through a large chamber was apparently responsible for appreciable boundary layer resistance. Ozone decomposition on the leaf surface, the cuticle, was neglected because ozone uptake through the cuticle of leaves is <0.01% of the ozone uptake through stomata (Kerstiens and Lendzian, 1989).

The difference between ozone influx and the rate of the decrease of apoplastic ascorbate shows that ozone oxidized not only apoplastic AA. After apoplastic AA had decreased, the remaining low apoplastic AA concentration of 50 nmol cm^{-3} did not change appreciably during the next 18 h of fumigation. However, there was a steady increase in DHA up to a concentration of >1 μmol cm^{-3}. The initial rate of DHA increase was almost 100 nmol cm^{-3} h^{-1} or 0.074 pmol cm^{-2} s^{-1} on a leaf area basis. After 6 h of fumigation the rate of DHA increase was about 35 nmol cm^{-3} h^{-1} or 0.026 pmol cm^{-2} s^{-1}. The AA/(AA + DHA) ratio in the apoplast decreased from about 0.87 to 0.11 after 6 h of fumigation and further to 0.06 after 24 h of fumigation (Fig. 2B). Regeneration of AA in the apoplast by enzymic reduction of DHA can be excluded because no dehydroascorbate reductase activity in the IWF of ozone-treated (6 or 24 h) or control leaves could be detected. In control experiments, no significant changes in apoplastic ascorbate contents and in the redox state of apoplastic ascorbate could be observed during a 24-h period (Fig. 2). During a 24-h period of fumigation with ozone, no appreciable changes in AA and DHA concentrations and none in the ratio AA/(AA + DHA) could be observed in the intracellular space of spinach leaves (data not shown). Also, there were no visible leaf lesions (necrosis or chlorosis) during this period.

Ascorbate Degradation and Foliar Injury after 48 h of Fumigation with 0.3 μL L^{-1} of Ozone

After 48 h of ozone fumigation, considerable leaf damage was observed primarily in the older leaves. In the youngest leaves (with high ascorbate concentrations, see Fig. 1B) only very little (up to 5% of the leaf area) leaf injury developed within 48 h of fumigation. In older leaves, usually 20 to 80% of the leaf area exhibited necrotic damage. These leaves were slowly drying out. Figure 3A shows the correlation between AA contents in leaves and the estimated extent of leaf injury after 48 h of fumigation with 0.3 μL L^{-1} of ozone. In young leaves, the average AA concentration was about 5 μmol g^{-1}. This value had decreased to 0.5 to 1.5 μmol g^{-1} fresh weight in leaves with 40 to 80% necrotic damage. The ratio AA/(AA + DHA) decreased in the leaves from >0.95 (no visible damage) to about 0.5 (extensively damaged leaves, see Fig. 3B).

Oxidation of GSH during Fumigation with 0.3 μL L^{-1} of Ozone

Neither reduced nor oxidized GSH could be detected in IWF from spinach leaves. The average GSH plus GSSG concentration of untreated leaves was 0.36 ± 0.02 μmol g^{-1} fresh weight; 84% of the GSH was in the reduced form.

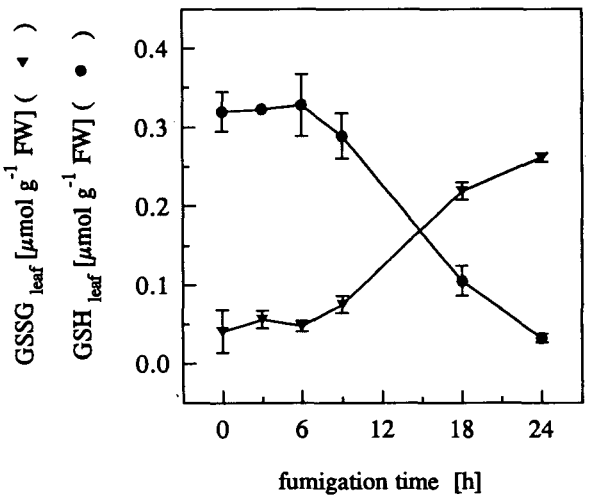

Figure 4. Effect of fumigation with 0.3 μL L^{-1} of ozone on GSH and GSSG contents of spinach leaves. Error bars represent SD (n = 3). FW, Fresh weight.

Figure 4 shows that fumigation caused considerable oxidation after a lag phase of about 6 h. After 24 h of ozone fumigation, 89% of the GSH was oxidized (GSSG), but the total pool of GSH plus GSSG was not much altered. It should be noticed that intracellular ascorbate was still kept in the reduced state, whereas GSH was slowly oxidized. The oxidation of GSH in ozone-fumigated leaves of poplar, *Phaseolus vulgaris*, and *S. album* was reported by Sen Gupta et al. (1991), Guri (1983), and Castillo and Greppin (1988).

Changes in the Apoplastic Levels of AA and DHA and in the Apoplastic Redox State of Ascorbate after 6 h of Fumigation with 0.3 μL L^{-1} of Ozone

Figure 5 shows changes in AA and DHA contents in the apoplast of spinach leaves during 6 h of fumigation with ozone and after fumigation was discontinued. After its increase during fumigation, the DHA concentration started to decrease when fumigation was terminated. As pointed out before, dehydroascorbate reductase activity was not detectable in the apoplast. DHA levels reached levels of control plants after 70 h of recovery from fumigation. During the first 2 h of recovery the rate of the decrease in DHA was about 40 nmol cm^{-3} h^{-1} or 0.030 pmol cm^{-2} s^{-1} on a leaf surface area basis. It is surprising that AA levels in the apoplast of fumigated leaves did not recover after fumigation was terminated. Apoplastic AA still remained at the low level of about 60 nmol cm^{-3}. This observation is in apparent contradiction to the data recorded in Figure 2, where apoplastic DHA levels continued to increase when the period of fumigation was extended beyond 6 h, indicating that under the conditions of the experiment ascorbate was imported from the cytosol into the apoplast where it was oxidized. It is tentatively concluded that ascorbate transport is stimulated when ascorbate is oxidized. In the absence of apoplastic oxidation, no ascorbate transport into the apoplast could be observed (Fig. 5).

Because intracellular ascorbate contents (AA and DHA) and the intracellular ascorbate redox state remained largely constant during the experiment both in fumigated and in control leaves (Table I), the concentration gradient of total ascorbate from the intracellular space (average concentration) to the apoplast was calculated to increase from about 8 (control) to about 23 (70 h after fumigation, see Fig. 6). Nevertheless, there were no significant differences in total ascorbate contents of whole leaves between treated and control plants in this recovery experiment (Fig. 6). Neither visible leaf injury nor growth inhibition of the fumigated leaves could be observed.

DISCUSSION

After ozone has entered the intercellular air space, detoxification in the apoplast is the only possibility to spare the plasmalemma and adjoining cytoplasm from destructive reactions. Membrane lipids of the plasmalemma contain unsaturated fatty acids. Their double bonds are attacked by ozone. Urbach et al. (1989) calculated that the major part of ozone, which manages to reach the plasmalemma after crossing the apoplast by diffusion, is broken down inside the plasma-

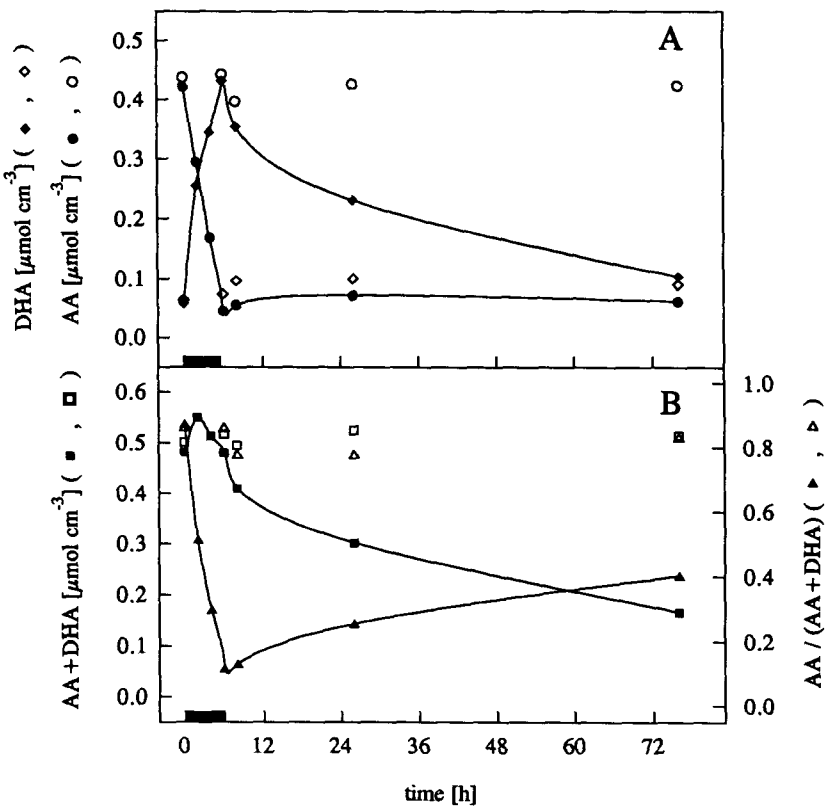

Figure 5. Changes in the content of AA and DHA (A) and of AA plus DHA and the redox ratio AA/(AA + DHA) (B) in the apoplast of control leaves (open symbols) and of ozone-treated spinach leaves (filled symbols). Spinach plants were fumigated for 6 h with 0.3 μL L^{-1} of ozone at room temperature (black bar) and were then left to recover for a period of almost 70 h (10-/14-h light/dark cycles) (n = 7–12). For SD, see Figure 2 and Table I.

Table I. *Effect of ozone on content and redox state of ascorbate in whole leaves, in the apoplast, and in the intracellular space of spinach leaves*

Ascorbate contents (AA, DHA, AA + DHA) and redox states of ascorbate [AA/(AA + DHA)] in spinach leaves, the apoplast, and the intracellular space of control leaves (C), ozone-treated leaves (6 h with 0.3 μL L^{-1}, F), and ozone-treated leaves (6 h with 0.3 μL L^{-1}) after a 70-h period of recovery (R). Data are mean values of 8 to 12 leaves from at least two different experiments. Values in parentheses indicate SD.

Treatment	AA	DHA	AA + DHA	Redox State
		$\mu mol\ cm^{-3}$		
Whole leaf				
C	3.57 (0.80)	0.013 (0.011)	3.59 (0.80)	0.997 (0.003)
F	3.30 (0.76)	0.052 (0.036)	3.35 (0.76)	0.984 (0.011)
R	3.46 (0.48)	0.016 (0.008)	3.48 (0.49)	0.996 (0.002)
Apoplast				
C	0.42 (0.21)	0.058 (0.022)	0.48 (0.225)	0.873 (0.036)
F	0.045 (0.015)	0.43 (0.26)	0.48 (0.27)	0.115 (0.056)
R	0.062 (0.023)	0.104 (0.048)	0.166 (0.051)	0.403 (0.184)
Intracellular space				
C	3.92 (0.875)	0.008 (0.011)	3.93 (0.88)	0.998 (0.003)
F	3.66 (0.845)	0.009 (0.026)	3.67 (0.84)	0.997 (0.007)
R	3.84 (0.54)	0.006 (0.010)	3.85 (0.54)	0.998 (0.003)

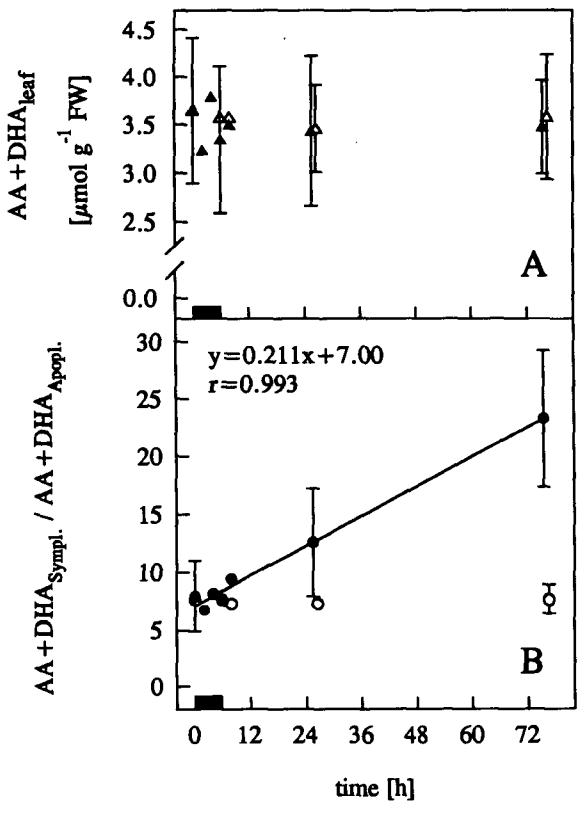

Figure 6. Changes in the content of (AA + DHA) in spinach leaves (A) and in the concentration gradient of (AA + DHA) from the intracellular space of the tissue (average concentration) to the apoplast (B) during and after fumigation with ozone. Open symbols, Control leaves; filled symbols, ozone-treated leaves. Plants were fumigated for 6 h with 0.3 μL L^{-1} of ozone at room temperature (black bar) and then left to recover for a period of about 70 h (10-/14-h light/dark cycles) (n = 7–12). Error bars represent SD.

lemma, damaging this biomembrane. Only a small percentage enters the cytosol, continuing a path of destruction. Radicals derived from ozone, e.g. hydroxyl radicals (Grimes et al., 1983), also wreak havoc. Detoxification is based on the principle of competition. Ozone- and radical-scavenging reactions must be fast enough to outcompete destructive reactions. The scavengers must be regenerated so that detoxification can continue in a flux situation. If regeneration is not possible in the apoplast, oxidized products of detoxification must be exported into the cytoplasm and reduced there for subsequent import of active scavengers.

Ascorbate is known to be an effective radical scavenger (Nakano and Asada, 1981; Halliwell, 1982; Gillham and Dodge, 1986). In the apoplast of spinach leaves, it is oxidized in the presence of phenolics by hydrogen peroxide (Takahama and Oniki, 1992). Ozone also reacts rapidly with ascorbate, faster than with the double bonds of fatty acids (bimolecular velocity constants k for the reaction with ascorbate 210,000 $M^{-1}\ s^{-1}$, with fumarate 60,000 $M^{-1}\ s^{-1}$, and with oleate 68,000 $M^{-1}\ s^{-1}$ [Urbach et al., 1989]). Nevertheless, in the spinach experiments performed in this investigation, only part of the ozone was intercepted by apoplastic ascorbate. There is a striking discrepancy between the ozone influx into the leaves of 0.9 pmol $cm^{-2}\ s^{-1}$ and the ascorbate oxidation in the apoplast during oxidative stress. In the beginning of fumigation with 0.3 μL L^{-1} of ozone, the decrease in the concentration of apoplastic AA was 0.045 pmol $cm^{-2}\ s^{-1}$ and the increase in DHA was 0.074 pmol $cm^{-2}\ s^{-1}$. After 6 h of fumigation the increase in apoplastic DHA was about 0.026 pmol $cm^{-2}\ s^{-1}$, and the DHA flux out of the apoplast (as found after fumigation was discontinued) was 0.03 pmol $cm^{-2}\ s^{-1}$. Apparently, only part of the ozone entering the leaves was detoxified by AA in the apoplast.

It is surprising that the rate of ascorbate flux from the cytosol to the apoplast appeared to be linked to oxidative stress. When fumigation was discontinued, ascorbate export into the apoplast was no longer measurable. Conversely,

when ozone levels were increased, the ascorbate flux across the plasmalemma increased (data from preliminary experiments, not shown). In the work of Castillo and Greppin (1988), AA was oxidized in the apoplasts of *S. album* L. leaves during 2 h of exposure to 0.4 μL L^{-1} of ozone. In contrast to our observations, AA increased in the IWF after fumigation was terminated, reaching a level after 6 h of about 2-fold higher than before fumigation; 24 h after fumigation initial levels of ascorbate and the initial redox state were reestablished (Castillo and Greppin, 1988).

So far, both our data and data reported in the literature indicate that oxidized ascorbate cannot be reduced efficiently in the apoplast. Neither dehydroascorbate reductase nor GSH reductase activity could be detected in the apoplast (Castillo and Greppin, 1988; Polle et al., 1990). DHA must be exported into the cytosol where it is reduced to ascorbate, which can then enter the apoplast.

The discrepancy between ozone fluxes into the leaves and ascorbate transport across the plasmalemma into the apoplast shows that ozone and its reactive degradation products not only react with apoplastic ascorbate but, to a greater extent, with other cellular constituents. Some of these reactions are likely to have taken place in the apoplast, but others caused cellular damage as shown by GSH oxidation in the tissue and, later, by leaf necrosis. The concentration of 0.3 μL L^{-1} of ozone was chosen to be as close as possible to peak stress situations encountered under field conditions, simultaneously producing observable ozone effects within a reasonable period of observation. Nevertheless, this concentration is unphysiologically high, at least for European conditions but not necessarily always for conditions found in the United States (Smith, 1991). Two points must be emphasized:

(a) The observed ascorbate flux across the plasmalemma was slower by a factor of about 10 than the ozone flux across the stomata, although the chosen ozone concentration was higher by only a factor of about 2 compared to peak concentrations of ozone observed in Europe. It may be comparable to ozone fluxes into leaves at commonly observed ozone concentrations in polluted air. This illustrates the danger of leaf damage by ozone, if ascorbate were the only antioxidant of the apoplast.

(b) Damage was extensive after about 2 d of fumigation with 0.3 μL L^{-1} of ozone, showing that neither ascorbate nor other apoplastic scavengers of ozone and of ozone-generated radicals could cope with the oxidative stress caused by ozone. These observations must be viewed in the light of field conditions where, although ozone concentrations are lower than in our experiments, exposure times are, as a rule, much longer. It would be premature to draw conclusions from our spinach data with regard to the role ozone plays in forest decline. Nevertheless, if in forest trees ascorbate transport across the plasmalemma is not much faster than in spinach, ozone detoxification must rely on other apoplastic protectants for ozone not to become damaging and not to contribute to observed forest decline.

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