Latent Oxidative Stress Responses of Ozone-Fumigated Cucumber Plants Are Enhanced by Simultaneous Cold Exposures

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Z. Naturforsch. **51 c,** 355–362 (1996); received January 26/February 23, 1996

Cucumis sativus (Cucumber), Automobile Exhaust, Latent Injury, Oxidative Stress, Ozone

Cucumber plants (*Cucumis sativus* L.) were grown under controlled conditions and fumigated with either O_3, diluted automobile exhaust or a combination of both. The ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) was estimated as a measure of PSII activity. Activities of the enzymes catalase, glutathione reductase and guaiacol-dependent peroxidase and contents of the antioxidants ascorbate and glutathione were assayed as potential indicators of oxidative stress. The behavior of catalase and of PSII are of particular diagnostic interest because they require continuous repair in light. Exposures of up to 13 days to moderate concentrations of the pollutant gases alone did not induce striking changes in any of the activities that were assayed. Also when the plants were subjected to an additional stress treatment by exposing them to 4 short cold treatments (2h each at 0–4 °C in light on days 12–15 after sowing) which induced marked declines of the F_v/F_m ratio, the chlorophyll content and the catalase activity, these cold-induced symptoms of photodamage were not significantly enhanced by the fumigation treatments. However, increases of the activities of glutathione reductase and peroxidase observed during a period of recovery following the cold-exposures were markedly higher in O_3-fumigated plants, as compared to plants grown in filtered air or fumigated with car exhaust alone. The results emphasize that effects of moderate pollutant exposures may be latent or delayed over long time periods and that defence responses can be enhanced when plants are exposed to additional, naturally occurring stress situations.

Introduction

Air pollution is regarded as a major current stress factor affecting vegetation. It may cause injuries and yield losses of agronomically important crop plants and also appears to be one of the factors contributing to the symptoms of novel forest decline (Heath, 1980). Oxides of nitrogen (NO_x) which are components of automobile exhaust, and ozone (O_3) are considered to play key roles among the complex pollutant actions on plants (Rowland *et al.*, 1985; Krupa and Manning, 1988; Schmieden and Wild, 1995). Reactive oxidants, such as O_3 and NO_x may exert direct or indirect effects on membranes and affect the antioxidative systems. Weakening of antioxidative defence systems might enhance the susceptibility of photosynthetic tissues to photooxidative damage in strong light (Asada, 1994). Two very sensitive and early indicators of photodamage are the photoinhibition of PS II which can be monitored by a decline of the ratio of variable to maximum Chl fluorescence F_v/F_m (Krause and Weis, 1991) and the photoinactivation of the enzyme catalase (Feierabend *et al.*, 1992; Streb *et al.*, 1993). The reaction center protein D1 of PS II as well as the enzyme catalase suffer from photodegradation which is in a dose-dependent manner related to the photon flux. Consequently both proteins must be continuously replaced; the apparent level of each protein reflects the current steady state equilibrium. Additional oxidants may either further enhance the rate of degradation or, alternatively, stress factors may impair the capacity for repair with the consequence that constant steady state levels can no longer be maintained for these proteins and rapid losses become apparent (Aro *et al.*, 1993; Hertwig *et al.*, 1992). In the present work we have, therefore, used PS II and catalase as sensitive test systems to determine whether the actions of O_3 or automobile exhaust were severe enough

Abbreviations: Chl, chlorophyll; PSII, photosystem II.

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to either enhance the destruction of these indicator proteins or to interfere with their repair in a way that apparent declines were induced. To further analyse oxidative stress responses, several parameters of the antioxidative defense system were assayed, in addition.

In controlled exposures with individual pollutant gases deleterious effects can be less pronounced than in combination and synergism with other pollutants; therefore, either very long exposure times or unrealistic high pollutant concentrations may be required and were frequently applied. Under ambient conditions stress symptoms can, however, be aggravated by synergisms between the different pollutants as well as by interactions with various natural stress factors, such as temperature, light, drought or other abiotic or pathogenic conditions. Therefore, we have selected chilling-sensitive cucumber plants for our experiments and have examined the influence of both separate and simultaneous treatments with moderate concentrations of O_3 and exhaust in combination with cold stress. This procedure allows to analyse whether the pollutants were either able to enhance the chilling damage or to impair the recovery of plants after such a stress treatment.

Materials and Methods

Plant material and growing conditions

Seeds of cucumber (*Cucumis sativus* L. cv. Corona) supplied by TS Seeds, Ambacht, Holland, were planted in vermiculite moistened with H_2O. After 5 days, the seedlings were supplied with a modified half-strength Knop's nutrient solution (Streb *et al.*, 1993). Plants were raised at 28°C and 270 μmol m^{-2} s^{-1} photosynthetic photon flux provided by an Osram Power-Star HQI-T 2000 W/D lamp. After six days of germination, the seedlings were transferred to hydroponic culture in the presence of a modified full strength Knop's nutrient solution. After 3 or 8 days of germination, plants were transferred to fumigation chambers and cultured under controlled conditions with a 16 h photoperiod at a day/night temperature of 22/20 °C, 60–65% relative humidity and 300 ppm CO_2 as described (Schaub *et al.*, 1990). Actinic light with a photosynthetic photon flux of 650 μmol m^{-2} s^{-1} was provided by Osram HQI-T 1000 W/D lamps. For clean air controls, contaminants were removed by a previously described combination of filtering steps (Schaub *et al.*, 1990). Defined concentrations of CO_2, automobile exhaust gas (monitored as NO_x), or O_3 were maintained by a computer-controlled monitoring and injection system, as described previously (Schaub *et al.*, 1990). The plants were exposed to the following different experimental conditions in the fumigation chambers: 1. filtered air; 2. exhaust: combustion engine automobile exhaust gas (produced under controlled conditions by a 1.8 l Volkswagen motor in the third range of a FTP-75-US-test-cycle operation mode; EPA, 1981) was applied with two daily exposures of 5 or 4 h duration between 08:00–13:00 and 17:00–21:00 (see Bahl and Kahl, 1995), each rising to a maximal concentration of 180 ppb NO_x (equivalent to a daily mean concentration of 30 ppb h^{-1}). The freshly prepared undiluted exhaust gas had an average content of 12.5% CO_2, 9840 ppm CO, 769 ppm hydrocarbons, 1743 ppm NO_x. The gas was diluted 1:12 and stored for 48 h before use. 3. Ozone: 90 ppb O_3 was applied by a 10 h fumigation between 12:00–22:00. 4. O_3 + exhaust: combination of the described exposures for O_3 and exhaust.

For the experiments shown in Figs 1–4 the plants were exposed for 2 h in the morning of day 12 after sowing to a low temperature of 0–4 °C at 520 μmol m^{-2} s^{-1} photosynthetic photon flux (without fumigation) and subsequently returned to the fumigation chambers. The 2 h cold treatments were repeated daily over the next three days and measurements performed immediately after the fourth cold treatment. After the last cold treatment, the fumigation treatments were continued for four additional days. The time period following the last cold treatment is designated as recovery (from chilling damage).

Preparation of cell-free extracts

For biochemical assays, discs of 35 mm diameter were cut from the primary leaves. For enzyme assays, leaf discs were extracted by grinding with mortar and pestle in 50 mM potassium phosphate buffer, pH 7.5. Homogenates were centrifuged for 5 min at 4 °C and 7800 g and the resulting supernatants used for enzyme assays. For the determination of antioxidants, leaf discs were ground with

1% (w/v) metaphosphoric acid and extracts were centrifuged for 20 min at 4 °C and 48000×*g*. The resulting supernatants were filtered through a Millipore 0.45 μm filter. Chlorophyll was extracted with 80% (v/v) acetone.

Analytical methods

Enzyme activities, chlorophyll contents, and chlorophyll fluorescence (ratio of variable to maximal fluorescence F_v/F_m) were assayed according to previously described procedures (Streb *et al.*, 1993). Antioxidants were determined by HPLC analysis as described elsewhere (Kar *et al.*, 1993).

Statistical analysis

The results shown represent the mean of 3–4 independent extractions and estimations from two identical independent experiments. Representative values for standard error of the mean are indicated. Experiments with some modifications of the time and duration of the fumigation treatments were repeated several times and confirmed the results shown. For statistical analysis, Student's t-test was used (e.g. by applying the t-Test program of the Sigma Plot™ software package). Differences of treated plants were considered significant at the $P<0.05$ level.

Results

When young cucumber plants were fumigated for four days (starting 8 days after sowing) with automobile exhaust, O_3, or a combination of both, the primary leaves of treated plants (at the age of 12 days after sowing; see time zero of Figs 1–4) had lower fresh weights than those of plants kept in filtered air (Fig. 1a). For the O_3 treatment this difference was statistically significant. No marked differences were, however, observed for the chlorophyll (Chl) content (Fig. 1b), and for activities of catalase (Fig. 2a), glutathione reductase, guaiacol-dependent peroxidase (Fig. 3) or glycolate oxidase (Fig. 4a) on a fresh weight basis. Similarly, the ratio of variable to maximum fluorescence F_v/F_m was not affected. Also extended fumigation periods of up to 13 days were unable to induce striking differences of PS II or enzyme activities (Table I).

Table I. Chlorophyll content (mg g^{-1} fresh weight), ratio of variable to maximum fluorescence (F_v/F_m), and enzyme activities in primary leaves of 16-day-old cucumber plants after 13 days of continuous exposure to different fumigation treatments. Enzyme activities are μmol s^{-1} (catalase) or nmol s^{-1} (peroxidase) per g fresh weight.

Parameter	Filtered air	Automobile exhaust	O_3
F_v/F_m	0.70 ± 0.03	0.64 ± 0.11	0.74 ± 0.03
Chlorophyll	1.11 ± 0.02	1.53 ± 0.11	0.94 ± 0.09
Catalase	61.8 ± 6.8	80.6 ± 3.1	62.5 ± 5.1
Peroxidase	40.2 ± 0.9	54.3 ± 1.7	24.9 ± 0

In order to examine potential interactions of pollutants with an additional natural stress factor, we investigated whether symptoms of chilling

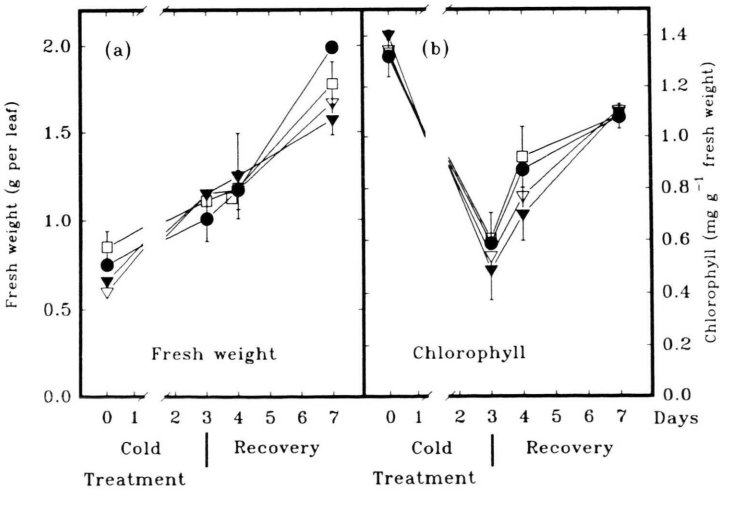

Fig. 1. Influence of cold treatments and fumigation with automobile exhaust and O_3 on the total fresh weight (a) and the Chl content (b) of primary leaves of cucumber during 7 days following a first 2-h cold exposure to 0–4 °C in light of 12-day-old plants. Cold exposures were repeated three times, once per day on days 1–3, and the plants then allowed to recover for an additional 4 days. Fumigation conditions: □ filtered air; ● automobile exhaust; ▼ automobile exhaust + O_3; ▽ O_3. At day 0, plants had been previously fumigated for 4 days.

damage were enhanced or modified in fumigated plants. To this end, fumigated plants were exposed to low temperature in light and subsequently allowed to recover from chilling while the fumigation was continued. Cucumber is a chilling-sensitive plant. In order to avoid lethal effects of too extended cold treatments, plants were exposed for 2 h on four consecutive days to a low temperature between 0–4 °C in light, starting at the age of 12 days after sowing. After termination of the cold treatments recovery of the plants was observed for further four days with or without fumigation with pollutant gases. We have already previously described that short cold treatments induce marked declines of catalase activity and of the Chl content and some photoinhibition of PS II in cucumber leaves grown without fumigation and that these effects required the presence of light during the exposures to low temperature (Feierabend *et al.*, 1992). Our present question was whether these symptoms of cold-induced photodamage could be further enhanced by exposure of the plants to pollutants. Losses of Chl (Fig. 1b), catalase activity (Fig. 2a) and a decline of F_v/F_m indicating photoinhibition of PS II (Fig. 2b) were also in our present experiments observed both in plants kept in clean air and the presence of pollutants. The extent of the decline of F_v/F_m was not as large as that of Chl or catalase, because photoinhibition of PS II recovered more rapidly between the individual cold exposures. In all experiments that were performed, the decline of F_v/F_m, i.e. photoinhibition, was larger in plants fumigated with O_3 or exhaust than in controls kept in filtered air (Fig. 2b and data not shown), however, this difference was not significant at the $P<0.05$ level. The cold-induced declines of Chl and catalase were not markedly affected by the exposures to the pollutant gases, except for some delay in the recovery of catalase. Striking pollutant effects were, however, observed during the recovery period following the cold exposure. During recovery, increases of the antioxidative enzymes glutathione reductase and guaiacol-peroxidase were much higher in leaves fumigated with O_3 than in those kept in filtered air or in the presence of exhaust alone (Fig. 3). Glycolate oxidase, which together with catalase represents part of the peroxisomal photorespiratory pathway, is an example of a relatively non-responsive enzyme and thus served as an internal control. It was not influenced by the cold treatment. Also the fumigation treatments had no significant effects on the behavior of glycolate oxidase (Fig. 4a).

Reduced and oxidized forms of the antioxidants ascorbate and glutathione were also determined. Fumigation with O_3 or exhaust was not accompanied by significant and reproducible changes in the levels of these antioxidants. The cold treatments caused a decline of reduced ascorbate (Fig. 4b). This decline occurred in several experimental series but was not consistently accompanied by increases in dehydroascorbate (not shown) and appeared to reflect losses of total ascorbate. In clean air as well as under all conditions, the ratios of reduced to oxidized ascorbate declined from a

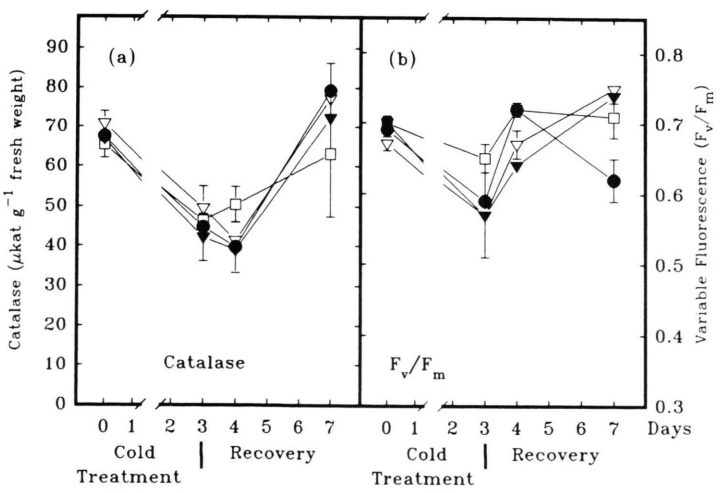

Fig. 2. Influence of cold treatments and fumigation with automobile exhaust and O_3 on the activity of catalase (a) and the ratio of variable to maximum fluorescence F_v/F_m (b) in primary leaves of cucumber during 7 days following a first 2-h cold exposure to 0–4 °C in light of 12-day-old plants. Cold exposures were repeated three times, once per day on days 1–3, and the plants then allowed to recover for an additional 4 days. Fumigation conditions: □ filtered air; ● automobile exhaust; ▼ automobile exhaust $+O_3$; ▽ O_3. At day 0, plants had been previously fumigated for 4 days.

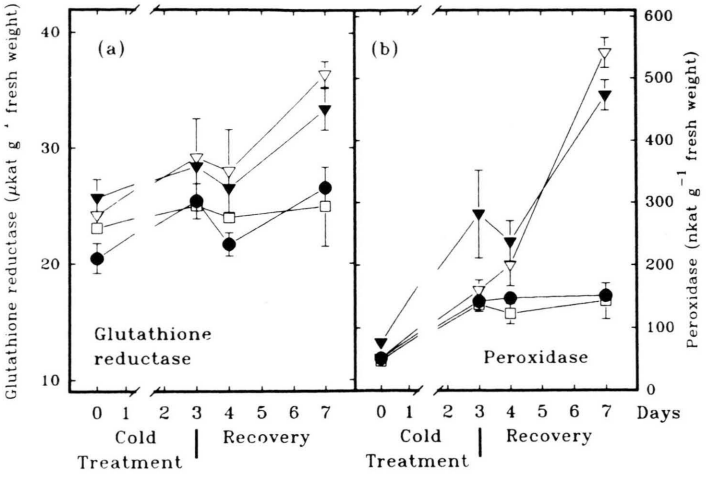

Fig. 3. Influence of cold treatments and fumigation with automobile exhaust and O_3 on the activities of glutathione reductase (a) and guaiacol-peroxidase (b) in primary leaves of cucumber during 7 days following a first 2-h cold exposure to 0–4 °C in light of 12-day-old plants. Cold exposures were repeated three times, once per day on days 1–3, and the plants then allowed to recover for an additional 4 days. Fumigation conditions: □ filtered air; ● automobile exhaust; ▼ automobile exhaust + O_3; ▽ O_3. At day 0, plants had been previously fumigated for 4 days. The difference of peroxidase activities after 7 days treatment in the presence or absence of O_3 is significant at the $P<0.001$ level.

range between 1.6–2.7 before the cold treatments to a range of 1.2–1.7 at the end of the recovery period. The GSH content was slowly decreasing during our experimental treatments. Its oxidized form (GSSG) appeared to increase slightly in treated plants during the cold exposures and transiently declined during the first day of recovery after the cold exposures (Figs. 4c and d). The ratios of reduced to oxidized glutathione were between 5–6 before the cold-treatment and between

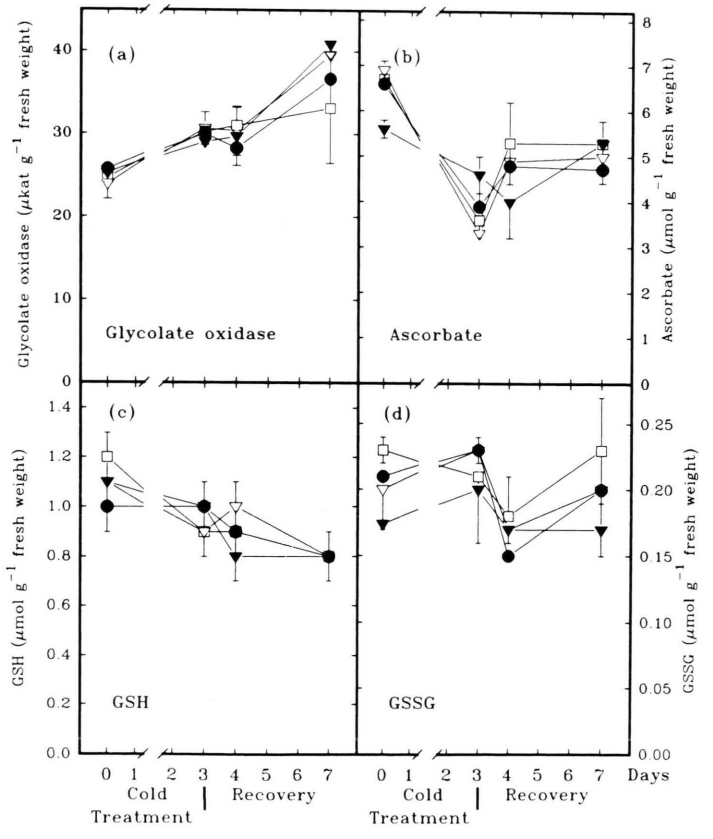

Fig. 4. Influence of cold treatments and fumigation with automobile exhaust and O_3 on the activity of glycolate oxidase (a) and the contents of reduced ascorbic acid (b), reduced glutathione (c) and oxidized glutathione (d) in primary leaves of cucumber during 7 days following a first 2-h cold exposure to 0–4 °C in light of 12-day-old plants. Cold exposures were repeated three times, once per day on days 1–3, and the plants then allowed to recover for an additional 4 days. Fumigation conditions: □ filtered air; ● automobile exhaust; ▼ automobile exhaust + 0_3; ▽ O_3. At day 0, plants had been previously fumigated for 4 days.

4–5 at the end of the recovery period. While the contents and the ratios of reduced to oxidized forms of ascorbate or glutathione were to some extent affected by the cold-treatments they were not markedly changed by the pollutant treatments. In particular, the O_3-stimulation of glutathione reductase and peroxidase was not reflected by any comparable changes of the antioxidants during the recovery period.

Discussion

Various stress conditions can induce marked photoinactivation of both PS II and of the enzyme catalase within hours of exposure (Streb *et al.*, 1993). Therefore, stress injuries are frequently much more pronounced in light than in darkness (Wild, 1988). Photoinhibition of PS II and of catalase represent sensitive early stress symptoms because catalase as well as the D1 reaction center protein of PS II have a rapid turnover in light. Stress conditions can either enhance the rate of photodegradation or impair the capacity of translation with the result that the rate of breakdown may, finally, exceed that of repair (Hertwig *et al.*, 1992; Aro *et al.*, 1993; Streb *et al.*, 1993). Exposure times of several days to realistic concentrations of O_3 or automobile exhaust, or a combination of both, were, however, unable to induce any apparent photoinhibition in the first leaves of cucumber plants. The inefficiency of the fumigation treatments applied indicates that their action on treated plants in light was much less severe, relative to conventional stress exposures, such as low temperature (below 10 °C), heat shock (above 40 °C) or salt (above 0.1 M NaCl; Feierabend *et al.*, 1992; Streb *et al.*, 1993). Similarly, in other species, moderate O_3 treatments did not immediately impair the development of the photosynthetic apparatus but induced premature senescence of the leaves (Nie *et al.*, 1993; Fangmeier *et al.*, 1994). Such observations suggest that pollutant effects may be delayed or latent. With respect to photoinhibition, Godde and Buchwald (1992) have, for instance, observed that the turnover of the D1 protein of PS II was greatly enhanced in O_3-fumigated spruce needles but the photosynthetic activity was hardly affected. As long as the plants can compensate for increased breakdown by enhanced repair no apparent photoinhibition will be observed. Only in combination with a cold treatment was the repair capacity apparently exceeded.

The only significant responses of fumigated plants were strong increases in glutathione reductase and guaiacol-peroxidase activities, which were solely seen when O_3-fumigation was combined with cold treatments, while fumigation with exhaust gas had no effect. Although the increased activities of the two enzymes appeared to reflect a plant response to enhanced oxidative stress, they were not accompanied by any significant changes of the total amounts of the antioxidants ascorbate and glutathione nor of the proportion of their oxidized forms in O_3-fumigated and cold-treated cucumber leaves. Glutathione reductase represents an important constituent of the antioxidative defence system (Asada, 1994) and its enhanced expression is accompanied by increased tolerance of transgenic tobacco to photooxidative stress (Aono *et al.*, 1993). Glutathione reductase activity was increased also in spinach leaves within a few days of fumigation with low O_3 concentrations without visible injuries and even in the absence of any additional strain (Tanaka *et al.*, 1988). Pumpkin leaves grown in polluted air had higher levels of glutathione reductase and also of reduced ascorbic acid and glutathione, as compared to controls grown in filtered air (Ranieri *et al.*, 1993).

Increases of guaiacol-peroxidase activity represent very general stress symptoms which also accompany, for instance, mechanical wounding or senescence (Birecka *et al.*, 1979). Nonspecific peroxidase activity has been frequently assayed as a potential biomarker in plants exposed to O_3 or NO_2. However, conflicting results have been reported. While O_3-induced increases of its activity were observed in several plants, particularly in herbaceous plants and under the influence of high doses which also caused visible leaf injuries (Dass and Weaver, 1972; Curtis *et al.*, 1976; Endress *et al.*, 1980; Patton and Garraway, 1986; Castillo *et al.*, 1987; Manes *et al.*, 1990; Fangmeier *et al.*, 1994), other authors did not find considerable changes of peroxidase activity, e.g. in spruce needles (Klumpp *et al.*, 1989; Manderscheid and Jäger, 1990). Potential reasons for such discrepancies may be that apoplastic peroxidases appear to be preferentially stimulated by O_3 exposures (Castillo *et al.*, 1987; Ikemeyer *et al.*, 1993) but are easily missed in total leaf extracts because they represent

only a low percentage of the total activity (Polle *et al.*, 1990). Alternatively, the occurrence of enhanced peroxidase activity or its magnitude may differ according to potential interactions with other stress conditions to which the plants were exposed or not, and which cannot be rigorously controlled in experiments where plants were grown under ambient conditions.

The results of our present investigation emphasize that even moderate O_3-exposures which do not produce immediate visible injuries can, nevertheless, have latent or delayed effects which become apparent in cucumber when combined with chilling injury, mainly during the recovery period following cold treatments. Although no substantial ozone-induced damage was yet recognized in our experiments, the enhanced peroxidase activity appears to represent an alarm signal, indicating that defence reactions were activated. However, fumigation with automobile exhaust did not enhance O_3 effects in cucumber leaves in our present investigation despite previous work reporting that O_3 injuries were significantly aggravated when other plants had been also exposed to NO_2 (Mehlhorn and Wellburn, 1987; Runeckles and Palmer, 1987). Additional examples for delayed pollutant effects are observations that O_3 exposures during summer affect hardiness and freezing tolerance of trees in winter (Barnes and Davison, 1988; Lucas *et al.*, 1988; Fincher *et al.*, 1989).

Acknowledgements

The present work was funded by the Bundesministerium für Forschung und Technologie, Bonn (project no. 0339229A). We are grateful to Prof. G. Hohenberg, Darmstadt, for providing the exhaust gas. Technical assistance by Barbara Kramer und Christel van Oijen is greatly appreciated.

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